Identification of novel G1 to S phase regulators in *Drosophila*

A thesis submitted for the degree of Doctor of Philosophy

by

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'Success is the ability to go from failure to failure without losing your enthusiasm'

- Sir Winston Churchill (1874-1965)

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Abstract

During development, cell proliferation must be coordinated with tissue patterning. Critical to the control of proliferation during development is the regulated activity of a family of Cyclin-dependent serine-threonine kinases (Cdks), that act to phosphorylate substrates required for the G2/M and G1/S transitions. Cyclin E is a rate limiting G1 cyclin, required for the activity of Cdk2 during the G1 to S phase transition. Regulation of *cyclin E* is critical for normal development, although how *cyclin E* is regulated in response to growth or developmental signals, and the downstream targets of Cyclin E/Cdk2 involved in triggering S phase entry, remain largely unknown. The work described here has focussed on identifying genes involved in the regulation *cyclin E* transcription or function during *Drosophila* development.

The identification of genes required for Cyclin E-mediated entry into S phase was facilitated by the availability of a hypomorphic *cyclin E* allele, $DmcycE^{JP}$. This allele is homozygous viable, but gives rise to adults with rough eyes due to insufficient cells entering S phase during eye development. The $DmcycE^{JP}$ rough eye phenotype is sensitive to levels of G1/S regulators, as halving the gene dosage of *Retinoblastoma* (*RBF*), *E2F* or *DP* modified the $DmcycE^{JP}$ eye phenotype by altering the number of S phases during eye development as expected. Surprisingly, reducing the dose of the G2 to M regulators, *cyclin A* or *string*, enhanced the $DmcycE^{JP}$ phenotype without affecting the number of S phases, but by reducing the number of mitoses. This finding suggests a novel role for Cyclin E in the regulation of Cyclin A and String during eye development.

To identify novel dominant modifiers of the $DmcycE^{JP}$ rough eye phenotype, a genetic interaction screen using the mutagens X-irradiation and ethyl methanesulphonate (EMS) was undertaken. Stocks were generated of 246 modifers of $DmcycE^{JP}$, with characterisation of the 79 homozygous lethal second chromosome suppressors forming the basis of the work described in this thesis. While 36 single alleles were identified, 10 second chromosome suppressor complementation groups had more than one allele. Cytological positions were determined for seven of these 10 groups, three of which are allelic to previously identified mutations, three have candidate gene(s) assigned that have not been tested, and one is likely to be novel. All complementation groups tested suppress the $DmcycE^{JP}$ by increasing the number of S phases during eye development, indicating that they encode negative regulators of the G1 to S phase transition. Two suppressor groups were characterised in detail, $Su(DmcycE^{JP})2\text{-}2$, which is likely to be allelic to the previously identified gene *phyllopod*, and $Su(DmcycE^{JP})2\text{-}1$. Analysis of the $Su(DmcycE^{JP})2\text{-}1$ and $Su(DmcycE^{JP})2\text{-}2$ homozygous mutant phenotypes revealed cell cycle defects, implicating these gene products in the regulation of proliferation during development. In addition to characterising second chromosome suppressors, one enhancer of $DmcycE^{JP}$, that corresponds to the first known allele of the *Drosophila* homologue of Cdk2, Cdc2c, was examined. Characterisation of this *cdc2c* allele, $cdc2c^{JS}$, has implicated this kinase in the G1 to S phase transition, consistent with its specific interaction with Cyclin E.

This work has formed the basis for the identification of regulators of G1 to S phase transition. Further analysis of the mutants obtained in the genetic interaction screen will potentially reveal novel mechanisms for the control of proliferation during development.

Statement

This work contains no material which has been accepted for the award of any other degree or diploma in any university or tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is given in the text.

I give my consent for my thesis, when deposited in the University library, being made available for loan and photocopying.

Julie Secombe, May 1999

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Chapter 1: Introduction

1-1 Regulation of the cell cycle

The coordination of cell proliferation with morphogenesis is important during the development of metazoans, as misregulation can lead to cell death or tumourigenesis (reviewed by Elledge *et al.*, 1996). Fundamental to the control of proliferation are a group of highly conserved cell cycle control proteins that directly regulate cell cycle progression. The presence and activity of these cell cycle regulators must therefore be tightly regulated during development. Although the mechanisms that control progression through the cell cycle are relatively well understood, how they are integrated with developmental cues resulting in promotion or restriction of proliferation is far from clear.

The eukaryotic cell cycle can be divided into four phases, S phase, G2, M phase and G1. During S phase (DNA synthesis) chromosomes are replicated, doubling the DNA content of the cell. During M phase (mitosis), nuclear division occurs with the replicated chromosomes separating into two daughter cells with equal genetic content. G1 and G2 represent 'gap' phases separating the completion of mitosis and S phase, and S phase and the subsequent mitosis, respectively. The G2 to M phase and the G1 to S phase transitions are major control points of the cell cycle, ensuring that mitosis does not occur before the completion of DNA replication and *vice versa*. The G1 to S phase transition is also an important regulatory point of the cell cycle as it is at this point that extracellular signals such as growth factors, can exert their effect. The stereotypical G1-S-G2-M cell cycle is not the only type to occur during metazoan development. Variations of this type of cycle include the meiotic cycle in which the genetic content of a cell is halved by two consecutive mitoses, and endoreplicative cycles in which S and gap phases occur with no intervening mitosis. Thus, not only is cell division tightly controlled, but also the type of cell cycle that occurs is regulated during development. This introductory chapter will focus on the regulation of the G1 to S phase transition, specifically the role of *cyclin E*, during development.

1-2 Cdks and Cyclins

Progression through the cell cycle phases requires the regulated activity of one of a family of Cyclin-dependent serine-threonine kinases (Cdk) (reviewed by Reed, 1996). In general, the abundance of Cdk proteins does not vary through the cell cycle, although their activity is tightly regulated. Critical to the activation of a Cdk is the binding of a regulatory Cyclin subunit. In addition, Cyclin/Cdk activity is regulated by phosphorylation of the Cdk

subunit, by binding of small inhibitory proteins, and regulated proteolysis of the Cyclin (reviewed by Morgan, 1995).

In budding and fission yeasts, control of the cell cycle depends on a single Cdk, *CDC28* and cdc2 respectively, at both the G2 to M phase and the G1 to S phase transitions (reviewed by Reed, 1996). In mammalian cells there are a family of related Cdks, and essential cell cycle roles have been demonstrated for Cdk1 in the G2 to M phase transition (Th'ng *et al.*, 1990), and Cdk2 in the G1 to S phase transition (Heuval and Harlow, 1993; Tsai *et al.*, 1993). Cyclins were initially characterised as proteins that varied dramatically in abundance through the cell cycle (Evans *et al.*, 1983), although this is not the case for all Cyclins. In general, Cyclins are characterised by a sequence motif of about 100 amino acids called the cyclin box, which is sufficient for association with Cdk proteins (Lees and Harlow, 1993; Horton and Templeton, 1997). The structural changes brought about by the binding of a Cyclin to a Cdk have been investigated by X-ray crystallography using human Cyclin A and Cdk2 (DeBondt *et al.*, 1993; Jeffrey *et al.*, 1995; reviewed by Morgan, 1996). Monomeric Cdk remains inactive due to an α-helical region (αL12) that constrains the ATP-binding site and a 'T-loop' domain that blocks the catalytic cleft. The binding of Cyclin to the Cdk shifts the inhibitory T-loop domain and 'melts' the αL12 helical region, allowing substrates to bind to and be phosphorylated by the Cdk. Different Cyclin/Cdk complexes are present and required for progression into and through different stages of the cell cycle. For example, in mammalian cells Cyclin A and/or Cyclin B complex with Cdk1 for the G2 to M phase transition, Cyclin D1, D2 or D3/Cdk4 or 6 and Cyclin E/Cdk2 are required for the G1 to S phase transition and Cyclin A/Cdk2 is required for progression through S phase (reviewed by Reed, 1996).

1-3 The G1 to S phase transition

1-3.1 Cyclins D, E and A

In mammalian cells, Cyclins D, E and A/Cdk complexes play different, essential and rate-limiting roles in entry into or progression through S phase (reviewed by Reed 1996; Desdouets *et al.* 1995). Depletion of Cyclin D1, E or A in cell culture by antibody injection or anti-sense plasmid results in cells arresting in G1, demonstrating that these proteins are essential for entry into S phase (Girard *et al.*, 1991; Pagano *et al.*, 1992; Baldin *et al.*, 1993; Quelle *et al.*, 1993; Ohtsubo *et al.*, 1995). Conversely, overexpression of Cyclins D1, D2, E or A promotes entry of G1 cells into S phase, indicating that any of these cyclins can be rate-

limiting for the G1 to S phase transition (Quelle *et al.*, 1993; Ohtubo and Roberts, 1993; Resnitzky *et al.*, 1994). Cyclins D, E and A appear to have different roles during G1 and S phase. While depletion of Cyclin D1 or E during G1 prevents entry into S phase, depletion close to the initiation of DNA replication does not (Baldin *et al.*, 1993; Ohtsubo *et al.*, 1995). In contrast, depletion of Cyclin A during S phase is able to inhibit DNA replication (Baldin *et al.*, 1993). Thus while Cyclins D1 and E act during G1, Cyclin A acts during DNA replication. Consistent with a role for Cyclin A in progression through S phase, Cyclin A has been reported to localise to replicating foci of DNA during S phase (Cardosa *et al.*, 1993). Cyclins D1 and E also have different roles during G1, as co-expression of both of these proteins results in a greater acceleration of the G1 phase than expression of either cyclin alone (Retnitzky and Reed, 1995; Connell-Crowley *et al.*, 1997). In addition, expression of *cyclin D* is stimulated by external growth signals, whereas the role for extracellular signals in the regulation of *cyclin E* is less clear (reviewed by Sherr, 1995). Cyclins D and E are therefore likely to regulate different, perhaps overlapping, pathways during G1 that lead to the initiation of DNA replication. Consistent with Cyclins D, E and A acting at different times during G1 and S phase, these Cyclin/Cdk complexes also appear to have different substrates (reviewed by Reed, 1996; see section 1-5).

Drosophila Cyclin E (DmcycE) interacts with the *Drosophila* homologue of the mammalian Cdk2 protein, Cdc2c (Lehner and O'Farrell, 1990b; Stern *et al.*, 1993), and is necessary and sufficient for the G1 to S phase transition (Knoblich *et al.*, 1994; Richardson *et al.*, 1995). In mammalian cells, an essential role has been shown for Cdk2 in the G1 to S phase transition (Heuval and Harlow, 1993; Tsai *et al.*, 1993). An essential role in the G1 to S phase transition has not yet been demonstrated for *Drosophila* Cdc2c due to the absence of a specific *cdc2c* mutation. Homologues of mammalian Cyclin D and its associated kinase, Cdk4(6), have also been identified in *Drosophila* (Finley *et al.*, 1996; Sauer *et al.*, 1996). Expression of *cyclin D* anticipates the pattern of cell division during embryogenesis and eye development, consistent with observations from mammalian systems. However, no mutations in *Drosophila* Cyclin D or Cdk4(6) exist, thus a role for these genes in the G1 to S phase transition has not been demonstrated.

While in mammalian cells there is evidence that Cyclin A/Cdk2 is required for entry into or progression through S phase (reviewed by Desdouets *et al.*, 1995), an S phase role for Cyclin A in *Drosophila* is less clear. *Drosophila* Cyclin A interacts specifically with the mitotic Cdk, Cdk1 and not with the G1/S Cdk,Cdc2c (Sauer *et al.*, 1995; Stern *et al.*, 1993), and *cyclin A* mutants arrest in G2 phase during embryogenesis (Lehner *et al.*, 1991). *Cyclin A*

mutant embryos then enter ectopic endoreplicative Cyclin E-mediated S phases, indicating that Cyclin A is not normally required for S phase in *Drosophila*. There is, however, evidence that Cyclin A can function in the G1 to S phase transition, as ectopic expression of *cyclin A* can induce G1 cells to enter S phase (Sprenger *et al.*, 1997; Dong *et al.*, 1997; Thomas *et al.*, 1997). Cyclin A is normally prevented from acting in G1 by the Roughex (Rux) protein, for which there is no known homologue in mammalian systems. By a mechanism that does not require a direct association between Rux and Cyclin A, Rux causes the relocation of Cyclin A to the nucleus, where is it degraded, thus downregulating Cyclin A activity during G1 (Sprenger *et al.*, 1997; Thomas *et al.*, 1997). Rux is phosphorylated and inactivated at the G1 to S phase transition by Cyclin E/Cdc2c, allowing accumulation of Cyclin A (discussed in more detail in section 1-8.2).

1-4 Regulation of Cyclin/Cdk activity

1-4.1 Phosphorylation

In addition to Cdk activation by the binding of a Cyclin, Cyclin/Cdk activity is also regulated by phosphorylation, the binding of inhibitory proteins and proteolysis of the Cyclin subunit (reviewed by Sherr and Roberts, 1995; Lew and Kornbluth, 1996; Krek, 1998). Cyclin B/Cdk1 activity is required for the G2 to mitosis (M phase) transition. The activity of this complex is (in part) regulated by the phosphorylation of Cdk1 tyr15 and thr14 by the Wee1 kinase, that prevents Cyclin B/Cdk1 kinase activity (Featherstone and Russell, 1991; Lundgren *et al.*, 1991). Removal of these inhibitory phosphates on Cdk1 by Cdc25 (String in *Drosophila*) can be a rate-limiting step for entry into mitosis (Edgar and O'Farrell, 1989, 1990). In mammalian cells, a Cdc25 homologue Cdc25A has been implicated in dephosphorylating and activating Cyclin E/Cdk2, Cyclin D/Cdk4(6) and Cyclin A/Cdk2 complexes in the G1 to S phase transition (Jinno *et al.*, 1994; Hoffman *et al.*, 1994; Steiner *et al.*, 1995). The existence and importance of this control in the activation of *Drosophila* Cyclin E, Cyclin D or Cyclin A complexes is not known. Both G2 Cyclin and G1 Cyclin/Cdk complexes also require the Cdk component to be phosphorylated on Thr160/161 by Cdk activating kinase (CAK), which is itself composed of a Cyclin and a Cdk (Poon *et al.*, 1993; Solomon *et al.*, 1993).

1-4.2 Proteolysis

In both yeast and mammalian cells, ubiquitin/proteosome-mediated proteolysis has been shown to play a critical part in progression through the cell cycle (reviewed by Krek,

1998). The G2 Cyclins, Cyclins A and B have a 'destruction box' motif that is essential for these proteins to be degraded at metaphase, allowing the completion of mitosis (reviewed by Glotzer, 1995). Ubiquitin-mediated proteolysis is also important in the regulation of the G1 to S phase transition (reviewed by Krek, 1998). In human cells, Cyclin E is degraded by the ubiquitin/proteosome pathway (Won and Reed, 1996; Clurman *et al.*, 1996), which requires a sequence in the C-terminus of the protein, LTPP, termed the destruction sequence (Won and Reed, 1996). Phosphorylation of the Thr residue in this motif by the associated Cdk2 is required for targeting of human Cyclin E to ubiquitin-dependent degradation. The LTPP destruction sequence is conserved in *Xenopus*, mouse and *Drosophila* Cyclin E, although the functional significance of this motif has not been demonstrated in these systems.

1-4.3 Inhibitors

Cdk inhibitory proteins, CKIs, also appear to play a major role in the regulation of Cdk activity in the G1 to S phase transition (reviewed by Sherr and Roberts, 1995). There are two families of CKIs, the INK4 family and the p21 family. The INK4 family of inhibitors includes p15^{INK4b}, p16^{INK4a}, p18^{INK4c}, and p19^{INK4d}, that are characterised by multiple ankyrin repeat motifs (reviewed in Sherr and Roberts, 1995; Harper and Elledge, 1996). This family of inhibitors bind specifically to Cdk4 or 6 and prevent their association with Cyclin D, thus inhibiting this complex from acting to initiate entry into S phase. Consistent with this, the G1 arrest induced by overexpression of p16^{INK4a} in cultured cells is dependent on the presence of pRb, the only essential target of Cyclin D/Cdk4(6) activity (Lucas *et al.*, 1997; see section 1-5.1).

p21^{CIP1}, p27^{KIP1} and p57^{KIP2} form the p21 family of inhibitors that bind to and inhibit the kinase activity of Cyclin D/Cdk4(6), Cyclin E/Cdk2 and Cyclin A/Cdk2 complexes (reviewed by Sherr and Roberts, 1995). Consistent with p21 family proteins inhibiting G1 Cyclin/Cdk activity, overexpression of any of these proteins results in cultured cells arresting in G1 of the cell cycle (El-Deiry *et al.*, 1993; Gu *et al.*, 1993; Harper *et al.*, 1993; Toyoshima and Hunter, 1994; Matsuoka *et al.*, 1995). It has been reported that p21 family proteins associate with both inactive and active G1 Cyclin/Cdk complexes (Zhang *et al.*, 1994; Harper *et al.*, 1995). To explain this, the number of molecules of p21 proteins was proposed to dictate whether the Cyclin/Cdk complex was active. Complexes having only one molecule of a p21 inhibitor bound were proposed to be active, with additional p21 molecules being required to inhibit kinase activity. Contradicting this model, it has been shown recently that one molecule of p21^{CIP1} or p27^{KIP1} is sufficient for Cyclin-dependent kinase

(Hengst *et al.*, 1998). Whether p21^{CIP1} results in cells arresting in G1 therefore depends on the fraction of Cyclin/Cdk complexes bound to p21^{CIP1} within each cell.

p21^{CIP1} also interacts with proliferating cell nuclear antigen (PCNA), a subunit of DNA polymerase-δ, which is required for DNA replication and repair (Zhang *et al.*, 1993). p21^{CIP1} is able to inhibit the ability of PCNA to activate the DNA replication activity of DNA pol-δ *in vitro*, independent of any interaction between p21^{CIP1} and G1 Cyclin/Cdk complexes (Zhang *et al.*, 1994; Flores-Rozas *et al.*, 1994; Waga *et al.*, 1994). PCNA is also required for nucleotide excision repair of DNA, although this process is not affected by the binding of p21^{CIP1} (Li *et al.*, 1994; Shivji *et al.*, 1994). Consistent with p21^{CIP1} affecting DNA replication and not DNA repair pathways, the p53 tumour suppressor acts to arrest cells in G1 in response to DNA damage by inducing the expression of p21^{CIP1} (El-Deiry *et al.*, 1993). However, this is not the only means by which p53 leads to G1 arrest, as p21^{CIP1} knock out mice are only partially defective in the G1 DNA damage checkpoint (Deng *et al.*, 1995; Brugarolas *et al.*, 1995). p21^{CIP1} is therefore an important, but not essential, component of p53-mediated G1 arrest *in vivo*.

p21^{CIP1} has also been implicated in muscle cell differentiation in cell culture (Halevy *et al.*, 1995). The transcription factor MyoD activates the expression of p21^{CIP1} in addition to inducing the transcription of muscle specific genes. Although the induction of p21^{CIP1} was presumed to induce cell cycle arrest required for muscle differentiation, p21^{CIP1} knock out mice do not show any muscle differentiation defects (reviewed by Elledge *et al.*, 1996). p21^{CIP1} therefore does not play a critical role in muscle differentiation *in vivo*.

p27^{KIP1} is also involved in the G1 arrest induced by regulatory signals. The negative growth factor TGF-β induces G1 arrest in epithelial cells by inhibiting Cyclin D/Cdk4(6) and Cyclin E/Cdk2 activity (reviewed by Harper and Elledge, 1996; Alevizopoulos and Mermod, 1997). Interestingly, this occurs not by induction of p27^{KIP1} expression, but by promoting the expression of p15^{INK4b} (Hannon and Beach, 1994). P15^{INK4b} binds to Cdk4 and 6, leading to the dissociation of Cyclin D and releasing p27^{KIP1} bound to this complex. This 'free' p27^{KIP1} then binds to and inhibits Cyclin E/Cdk2 activity (Reynisdottir *et al.*, 1995). TGF-β therefore leads to G1 arrest by inhibiting both Cyclin D and Cyclin E-associated kinase activity. However this is not the only mechanism by which TGF-β induces cells to arrest in G1, as cells derived from p27^{KIP1} knock out mice are still sensitive to TGF-β (Nakayama *et al.*, 1996). TGF-β also results in downregulation of *cdc25A* transcription (Iavarone and Massague, 1997), reducing levels of this activator of Cyclin E/Cdk2 and Cyclin D/Cdk4 complexes. In addition, TGF-β leads to downregulation of *cdk4* transcription

(Ewen *et al.*, 1993). Thus these p27^{KIP1}-independent mechanisms are likely to contribute to TGF-β mediated cell cycle arrest.

One p21 family protein, Dacapo (Dap), has been identified in *Drosophila* (de Nooij *et al.*, 1996; Lane *et al.*, 1996). In addition to being expressed at low levels in proliferating cells, Dap is transiently expressed at high levels as cells exit from the mitotic cycle, indicating an involvement in growth arrest prior to terminal differentiation. Like mammalian p21 family proteins, Dap interacts with Cyclin E/Cdc2c *in vivo*, and is able to inhibit Cdc2c-associated kinase activity *in vitro*. It is not currently known if Dap also interacts with Cyclin D complexes. In addition, as *Drosophila* Cyclin A associates exclusively with Cdk1 and not Cdc2c (Stern *et al.*, 1993), Dap does not inhibit Cyclin A-associated kinase activity. Unlike mammalian inhibitors, which are not essential for viability, mutations in *dap* result in lethality (reviewed by Harper and Elledge 1996; de Nooij *et al.*, 1996; Lane *et al.*, 1996). Epidermal cells of embryos homozygous for mutations in *dap* undergo an additional cell cycle during embryogenesis, indicating that Dap is required for the establishment of the G1 arrest normally seen in these cells (de Nooij *et al.*, 1996; Lane *et al.*, 1996). In addition, ectopic expression of *dap* is sufficient to induce G1 arrest. Thus, while analysis of knockout phenotypes in mice has failed to demonstrate a role for p21 family proteins in exit from the mitotic cycle prior to differentiation, Dap is clearly required for timely exit from the cell cycle during *Drosophila* development.

1-5 Targets of G1 Cycin/Cdk activity

1-5.1 Retinoblastoma

Although the mechanisms that control Cdk activity are relatively well characterised, few targets of G1 Cyclin/Cdk complexes have been described (Figure 1.1). In mammalian cells, the most extensively characterised target of Cyclin/Cdk complexes are the retinoblastoma tumour suppressor (pRb) family of 'pocket proteins', pRb, p107 and p130 (Reviewed by Dyson, 1998; Adams and Kaelin, 1998). The phosphorylation state of pRb proteins varies through the cell cycle, and is correlated with activity (reviewed by Mittnacht, 1998). Dephosphorylated pRb proteins are present in quiescent cells and cells in early G1 while phosphorylated forms of these proteins are present from late in G1 until the end of mitosis. The growth inhibitory effects of the pRb family of proteins are associated with the hypophosphorylated forms, that interact with one of six E2F proteins and one of three DP proteins. The three pRb-related pocket proteins do not interact with all E2F/DP dimers

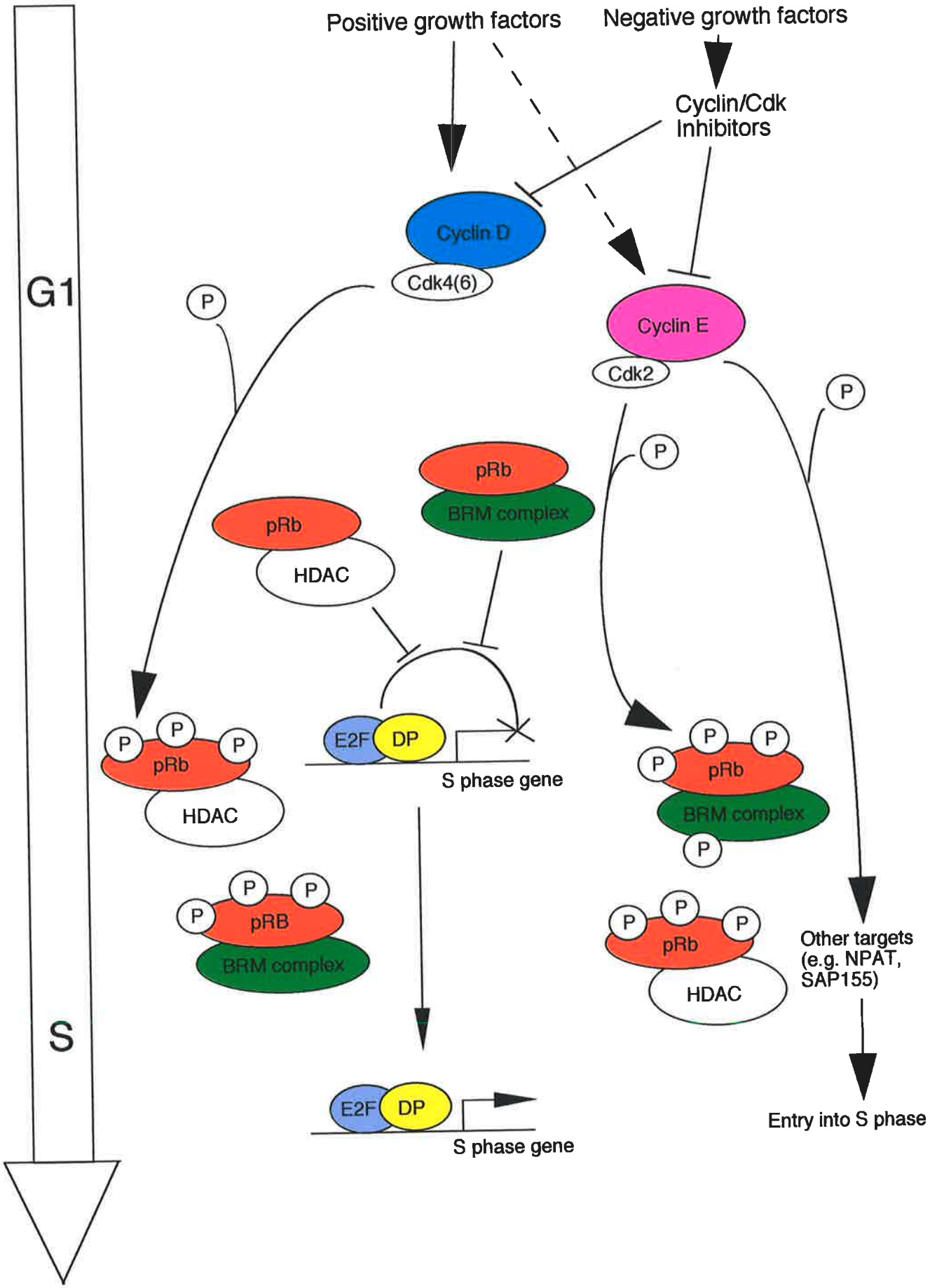
(referred to as E2F), with pRb associating with E2F1, E2F2 or E2F3 containing complexes, and p107 or p130 with E2F4 or E2F5 complexes (reviewed by Dyson, 1998). E2F/DP heterodimers are important in activation of genes required for entry into S phase, such as *cyclin E*, *cyclin A* and *cdk2* (Ohtani *et al.*, 1995; Schulze *et al.*, 1995; Botz *et al.*, 1996; Geng *et al.*, 1996; Huet *et al.*, 1996). E2F is also required for expression of genes important for DNA synthesis such as *PCNA*, *RNR2*, *Cdc6*, *dihydrofolate reductase*, *DNA polymerase* and *thymidine kinase* (Pearson *et al.*, 1991; Ogris *et al.*, 1993; Karlseder *et al.*, 1996; Ohtani *et al.*, 1998; Hateboer *et al.*, 1998). Hyperphosphorylated pRb proteins are unable to interact with E2F, allowing E2F-mediated activation of S phase specific genes. Thus, the phosphorylation state of pRb proteins regulates the G1 to S phase transition.

pRb has 15 consensus G1 Cyclin/Cdk phosphorylation sites that are conserved among a wide variety of organisms (Mittnacht, 1998). *In vitro*, human Cyclin D/Cdk4(6) and Cyclin E/Cdk2 are able to phosphorylate pRb at different sites, with neither being sufficient to phosphorylate all sites seen *in vivo*, indicating that both of these kinases are involved in the phosphorylation of pRb (Kitagawa *et al.*, 1996; Zarkowska *et al.*, 1997; Zarowska and Mittnacht, 1997). Indeed, by selectively inhibiting Cdk2 or 4(6) function, it has been shown that Cyclin D complexes are only able to partially phosphorylate pRb, and this phosphorylation is required for the action of Cyclin E/Cdk2 on pRb (Lundberg and Weinberg, 1998). There is also strong evidence that pRb is the only essential target of Cyclin D/Cdk complexes, as the G1 arrest induced by injection of anti-Cyclin D antibodies is dependent on wild-type pRb function (Tam *et al.*, 1994; Lukas *et al.*, 1995). In contrast, Cyclin E is required for entry into S phase in pRb deficient cells, indicating that Cyclin E/Cdk2 has additional essential targets in the G1 to S phase transition (Ohtsubo *et al.*, 1995). Although these additional targets are likely to include the pRb related proteins p107 and p130, Cyclin E is still required for the G1 to S phase transition after collective inactivation of all of these pocket protein using the SV40 DNA tumour virus large T antigen (Hoffman and Livingston, 1996). Overexpression of Cyclin A in cell culture also leads to phosphorylation of pRb, and Cyclin A/Cdk2 is able to phosphorylate pRb *in vitro* (Hinds *et al.*, 1992; reviewed in Weinberg, 1995). It remains unknown however, whether phosphorylation of pRb by Cyclin A/Cdk2 is important for pRb inactivation.

Drosophila homologues of the retinoblastoma protein (RBF), two E2F proteins (E2F1 and E2F2) and one DP (DP) protein have been identified (Duronio *et al.*, 1995; Du *et al.*, 1996; Royzman *et al.*, 1997; Duronio *et al.*, 1998). *Drosophila* RBF is equally similar to the mammalian pRb, p107 and p130 proteins, suggesting that this may represent the archetypal

Figure 1.1 Regulation of the G1 to S phase transition

Simplified schematic representation of the molecular events that occur during the G1 to S phase transition in mammalian cells. Positive growth factors induce the expression of *cyclin D*, leading to the phosphorylation of the only essential target of Cyclin D/Cdk4(6), pRb. This then allows E2F/DP-mediated transcription of genes required for S phase. External growth signals may also contribute to the expression of *cyclin E*, although this is not well defined (as indicated by dashed line). Cyclin E, in addition to contributing to the phosphorylation of pRb (and other related pocket proteins), also has other targets required for entry into S phase. These include BRG1 and BAF155 that are members of the human chromatin remodelling complex, the spliceosomal associated protein SAP155 and the novel protein NPAT. Cyclin A may also have a role in the phosphorylation and inactivation of pRb, although this is not shown. Both Cyclin D and Cyclin E/Cdk complexes are inhibited by the binding of small inhibitory proteins. See text for details.


Positive growth factors
Negative growth factors
Cyclin/Cdk
Inhibitors
Cyclin D
Cdk4(6)
Cyclin E
Cdk2
G1
S
P
pRb
HDAC
BRM complex
E2F
DP
S phase gene
pRB
Other targets
(e.g. NPAT,
SAP155)
Entry into S phase

member of the family (Du *et al.*, 1996). As in mammalian cells, RBF is phosphorylated by Cyclin E/Cdc2c (Du *et al.*, 1996), although whether Cyclin D-associated kinase activity contributes to the phosphorylation of RBF in *Drosophila* has not been determined. RBF is required to prevent E2F-dependent transcription of S phase genes, as embryos lacking maternal and zygotic *RBF* expression show ectopic expression of the E2F target genes *PCNA* and *RNR2* (Du and Dyson, 1999). Despite the ectopic expression of *PCNA* and *RNR2*, epidermal cells of *RBF* mutant embryos appear to transiently arrest in G1, although these cells subsequently enter ectopic S phases (Du and Dyson, 1999). Thus while the Cyclin E/Cdc2c inhibitor Dap is required for the initiation of the epidermal cell G1 arrest (de Nooij *et al.*, 1996; Lane *et al.*, 1996), RBF is required for the maintenance of this state.

The roles of E2F1 and DP during *Drosophila* development have also been characterised (Duronio *et al.*, 1995; Royzman *et al.*, 1997; Duronio *et al.*, 1998). E2F is required for the G1-S transcriptional program, as embryos homozygous for null mutations in *E2F1* or *DP* do not express E2F target genes such as *RNR2* and *PCNA*. Transcription of *cyclin E* is also affected in some tissues in *E2F1* or *DP* mutants. Interestingly, while expression of *RNR2*, *PCNA* and *cyclin E* are not detected in many tissues in *E2F1* or *DP* mutant embryos, S phases (as detected by the incorporation of the nucleotide analogue BrdU) are still observed (Royzman *et al.*, 1997; Duronio *et al.*, 1998). These S phases occur in the correct spatial pattern, although the BrdU labelling observed was less intense than wild-type, and S phases were prolonged. As Cyclin E has previously been shown to be essential for S phase during embryogenesis (Knoblich *et al*, 1994), there are presumably undetectable levels of *cyclin E* present, allowing S phases in *E2F1* and *DP* mutant embryos. Thus, while E2F is required for normal levels of *cyclin E* expression, clearly E2F-independent mechanisms also exist. Expression of E2F1 and DP is also sufficient to induce G1 cells to enter S phase, and this E2F1/DP-induced entry into S phase is dependent on Cyclin E (Duronio *et al.*, 1996). The converse of this, however, is not true as Cyclin E mediated entry into S phase does not require E2F function. This is not simply due to E2F acting upstream of *cyclin E*, as this would predict that mutations in *E2F1* or *DP* would have the same phenotype as mutations in *cyclin E*. To explain this, Duronio *et al.* (1998) propose that E2F has two functions in the G1 to S phase transition. The first of these is that E2F is required for the transcription of replication factors that are limiting for efficient DNA replication. Thus, by reducing E2F function, pre-replicative complexes would be limiting and fewer origins would fire, leading to weak BrdU labelling and a prolonged S phase. In addition, E2F regulates the expression of *cyclin E*, which leads to an all-or-nothing triggering of the G1 to S phase

transition. A recent study by Rozyman *et al.* (1999) has also indicated that E2F may have a more direct role in the establishment of pre-replicative complexes as female sterile alleles of *E2F1* and *DP* lack ORC2 (origin of replication complex subunit 2) localisation during chorion gene amplification.

1-5.2 Chromatin remodelling proteins

In mammalian cells, efficient repression of E2F-mediated transcription and G1 arrest by pRb requires the human homologues of SWI2-SNF2, hBRM and BRG1 (Dunaief *et al.*, 1994; Strober *et al.*, 1996; Trouche *et al.*, 1997). pRb is able to bind hBRM or BRG1 and E2F1 simultaneously (Trouche *et al.*, 1997). In addition, hBRM or BRG1 cooperate with pRb in transient transfection assays, indicating that these proteins are likely to act as co-repressors. hBRM and BRG1 are putative ATP-dependent helicases that, as part of a multimeric complex, affect gene transcription by altering chromatin structure (reviewed by Tamkun *et al.*, 1992). Analysis of BRM knock out mice has also implicated this protein in the negative regulation of the G1 to S phase transition (Reyes *et al.*, 1998). BRM-/- mice are viable, but are larger than their heterozygous sibs, and show an increased rate of proliferation. Embryonic fibroblasts derived from these animals are also deficient in their ability to arrest in G1 in response to confluency or DNA damage. When bound to DNA, E2F-pRb complexes also actively repress transcription of target genes by recruiting histone deacetylase (HDAC1) (Brehm *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998; Luo *et al.*, 1998). HDAC1 removes the highly charged acetyl group from histones, altering chromatin structure and preventing access by transcription factors. Whether both HDAC1 and SWI/SNF chromatin remodelling factors are bound to pRb simultaneously is not known. Taken together, these data provide clear evidence that pRb-mediated repression of E2F target genes requires chromatin remodelling.

Recently, it has been shown that BRG1 and the associated factor BAF155 can bind to, and be phosphorylated by, human Cyclin E/Cdk2 (Shanahan *et al.*, 1999; Figure 1.1). Interestingly, this occurs independently of the interaction between BRG1 and pRb, and predicts that BRG1 acts as a negative regulator of proliferation (Shanahan *et al.*, 1999). Consistent with this, overexpression of BRG1 is able to induce growth arrest and senescence in tissue culture cells. This growth arrest phenotype was abrogated by the co-expression of Cyclin E or Cyclin D1, indicating that these G1 Cyclin/Cdk complexes are able to phosphorylate BRG1. As a stable *in vitro* interaction could be detected between BRG1 and Cyclin E/Cdk2 and not Cyclin D/Cdk4(6), it is more likely that Cyclin E/Cdk2 acts to phosphorylate and inactivate BRG1 *in vivo*. Thus, Cyclin E/Cdk2 activity has been implicated

implicated in the regulation of proliferation by maintaining chromatin in a transcriptionally permissive state.

The involvement of SWI/SNF components in the regulation of the G1 to S phase transition is also likely to be conserved in *Drosophila* (Staehling-Hampton *et al.*, 1999). In a genetic interaction screen to isolate regulators of E2F activity, dominant modifiers of a rough eye phenotype caused by overexpression of *E2F1* and *DP* (*GMR-E2F/DP*) were generated. Among the enhancers were mutations in two members of the *Drosophila* SWI/SNF complex, *brahma* and *moira*, indicating that these proteins normally act as negative regulators of the G1 to S phase transition. Brahma also appears to act through RBF, as the enhancement of *GMR-E2F/DP* by halving the dosage of *brahma* was able to be abolished by the co-overexpression of *RBF*. Thus, although it has not been demonstrated that RBF and Brahma interact *in vivo*, the genetic interactions observed using the *GMR-E2F/DP* are consistent with studies from mammalian systems.

1-5.3 Targets of Cyclin E/Cdk2

As described previously (section 1-5.1), the only essential target of Cyclin D/Cdk activity is pRb, while Cyclin E/Cdk2 has other targets critical for entry into S phase. Specifically in *Drosophila*, one Cyclin E/Cdc2c target is Roughex, a negative regulator of Cyclin A (see sections1-3.1 and 1-8.2). Additional targets of Cyclin E/Cdk2 are presumed to include proteins required at origins of replication, although there is little direct evidence for this in metazoans. During G1, origins of DNA replication exist in a prereplicative state, in which the origin recognition complex (ORC), Cdc6, Cdc45 and minichromosome maintenance proteins (MCMs) are bound (reviewed by Dutta and Bell, 1997; Leatherwood, 1998). Interestingly, evidence from *Saccharomyces cerevisiae* suggests that the loading of Cdc45 onto the prereplicative complex is dependent on G1 Cyclin/Cdk activity, which is required for the firing of origins (Zou and Stillman, 1998). Cdc6, Cdc45 and MCM proteins are released from origins of replication after the initiation of DNA replication, while ORC remains bound through the cell cycle. Many of these prereplicative complex proteins are good candidates for Cyclin E/Cdk2 phosphorylation targets, leading directly to initiation of DNA replication. Consistent with Cyclin E-associated kinase activity regulating events at origins of replication, expression of *Drosophila cyclin E* in the developing salivary gland has been shown to regulate the association of MCM proteins with chromosomes (Su and O'Farrell, 1998).

In an attempt to identify targets of Cyclin E/Cdk2 activity in mammalian cells, two groups have recently isolated proteins that physically associate with Cyclin E/Cdk2 (Seghezzi *et al.*, 1998; Zhao *et al.*, 1998). Immunoprecipitation of Cyclin E-associated proteins coprecipitated the spliceosome-associated proteins SAP155, SAP145 and SAP114 (Seghezzi *et al.*, 1998). SAP155, SAP145 and SAP114 are subunits of the essential splicing factor SF3, a component of U2 snRNP. SAP155 also appears to be a target of Cyclin E/Cdk2 activity, as it has multiple consensus phosphorylation sites and serves as an excellent substrate for Cyclin E/Cdk2 *in vitro*. As no Cyclin A, Cyclin D, Cdk4 or 6 were able to be detected in SAP155 immunoprecipitates, SAP155 is likely to be a target specific to Cyclin E/Cdk2. The significance of the interaction observed between Cyclin E/Cdk2 and components of the pre-mRNA spicing machinery remains unclear. In *Saccharomyces cerevisiae*, mutations in *dbf3-1*, which encodes a protein of the U5 snRNP, result in defective DNA replication and arrest before or during S phase (Johnston and Thomas, 1982). Thus, there may be a highly conserved link between mRNA processing and cell cycle progression. The phosphorylation of SAP155 by Cyclin E/Cdk2 and the phenotype of yeast U5 snRNP mutants suggests that a subset of pre-mRNAs encoding cell cycle regulators undergo cell cycle regulated processing (Seghezzi *et al.*, 1998).

Novel targets of human Cyclin E/Cdk2 have also been identified by screening a λgt11 expression library with radio-labelled Cyclin E/Cdk2 complex (Zhao *et al.*, 1998). In addition to identifying proteins that have been previously shown to interact with Cyclin E/Cdk2 such as $p27^{KIP1}$, p107 and p130, novel proteins were also identified. One of these, NPAT (nuclear protein mapped at the AT locus) interacts with Cyclin E/Cdk2 *in vitro* and *in vivo*. Overexpression of NPAT in tissue culture cells is able to accelerate S phase entry in a manner similar to Cyclin D or E. This effect was enhanced by the co-expression of NPAT and Cyclin E, indicating that these proteins cooperate to induce entry into S phase. NPAT also appears to be a specific Cyclin E/Cdk2 target, as NPAT is unable to be phosphorylated by Cyclin D/Cdk4 or Cyclin A/Cdk2 *in vitro*, and co-expression of Cyclin D or A and NPAT has no cooperative effect on S phase entry. Phosphorylation of NPAT is therefore likely to be an *in vivo* rate-limiting step of Cyclin E-mediated S phase entry. Whether NPAT plays an essential role in the G1 to S phase transition, and the precise role this protein plays in S phase entry remain to be elucidated.

1-6 Co-ordination of proliferation and development

In addition to understanding the mechanisms that limit and promote progression through the cell cycle, it is also important to study control of proliferation in a developmental context. *Drosophila* development consists of several different types of replicative cell cycle (reviewed by Edgar, 1995) and provides a genetically manipulable system in which to study developmental control of proliferation. The remainder of this chapter will focus on the control of the G1 to S phase transition by Cyclin E during *Drosophila* development.

1-7 Cell cycle control during *Drosophila* development

After fertilisation, the first 13 nuclear divisions of *Drosophila* embryogenesis are rapid, synchronous, occur in a syncytium and consist of alternating S and M phases with no detectable G1 or G2 phases (reviewed by Foe *et al.*, 1993). These divisions are driven by maternal mRNA deposited during oogenesis (Edgar and Schubiger, 1986). The first 7 cycles occur very rapidly, and without detectable oscillation in Cyclin B or A/Cdk1 activity (reviewed by Foe *et al.*, 1993). Periodic degradation of Cyclin B is, however, required for these cycles to occur, as injection of an inhibitor of cyclin proteolysis prevented exit from mitosis during syncytial divisions (Su *et al.*, 1998a). Fluctuations in Cyclin B levels required for these rapid cycles is achieved by localised degradation of Cyclin B surrounding centrosomes or kinetochores. Cycles 7 to 13 become progressively longer, and Cyclin A and B levels show noticeable fluctuations. Cellularisation of the embryo occurs at cycle 14, and it is from this point that zygotic transcription is required for control of cycle progression (reviewed by Orr-Weaver, 1994). In cycles 14, 15 and 16, DNA synthesis and mitosis are separated by a G2 phase of variable length (Foe, 1989), but no G1 phase is detectable. These cycles are regulated by the product of the *string* gene, the *Drosophila* homologue of the Cdc25 phosphatase that is rate-limiting for the activation of Cyclin B/Cdk1 complexes (Edgar and O'Farrell, 1990; Edgar *et al.*, 1994).

After cycle 16, a majority of cells of the epidermis cease proliferation and enter into a G1 phase prior to differentiation. The exceptions to this G1 arrest are small sets of cells in the thoracic epidermis, known as the thoracic patches, that undergo an additional 17th cell cycle (Knoblich *et al.*, 1994). These thoracic patch cells appear to have a regulated G1 phase, since zygotic expression of *cyclin E* is required for these cells to enter S phase. Cells of the peripheral and central nervous system also continue to divide throughout embryogenesis. Neuroblasts of the central nervous system (CNS) delaminate from the ectoderm during embryonic stage 9 (see Figure 1.2 for embryonic stages) in three successive waves (SI, SII

and SIII) (Hartenstein *et al.*, 1987; Truman and Bate, 1988). SI, SII and SIII neuroblasts then divide a number of times before most cease division during stage 15, presumably in G1 phase of the cell cycle. Some neuroblasts such as the mushroom body neuroblasts, however, continue to divide throughout embryogenesis and after larval hatching (Truman and Bate, 1988; Ito and Hotta, 1992). Cells of the peripheral nervous system (PNS) delaminate from the ectoderm during the 15th and 16th cell cycles during embryonic stage 10. These cells divide 2-3 times before arresting, presumably in G1, during embryonic stage 12 (Bodmer *et al.*, 1989). During CNS and PNS divisions, S phase appears to follow immediately after mitosis, indicating that while these divisions have a detectable G2 phase, there is no obvious G1 phase (Edgar and O'Farrell, 1990).

Endoreplicative cycles also occur during *Drosophila* embryogenesis (Smith and Orr-Weaver, 1991). These cycles comprise alternating S and 'gap' phases with no M phase, producing polytenised tissues. During embryogenesis, endoreplication domains are observed after the 16th division, and are summarised in figure 1.3. Endoreplication cycles continue during larval development, with most larval tissues becoming polyploid.

1-7.1 *Drosophila* Cyclin E during embryogenesis

DmcycE encodes two proteins (type I and type II) that arise from differential splicing of the mRNA, producing proteins with unique amino-terminal and common carboxy-terminal regions. Whereas type II *cyclin E* is maternally deposited, type I *cyclin E* mRNA is expressed zygotically (Richardson *et al.*, 1993). The pattern of expression of *cyclin E* during embryogenesis is consistent with it playing a role in the G1 to S phase transition (Figure 1.4; Richardson *et al.*, 1993). Early in embryonic development, type II *cyclin E* mRNA is expressed ubiquitously throughout the whole embryo, consistent with these early divisions containing no G1 phase. Presumably, high levels of Cyclin E drive entry into S phase immediately after the completion of M phase. Type II *cyclin E* mRNA is degraded soon after cellularisation, although low levels of type II Cyclin E protein persist during cycles 14, 15 and 16 (Crack *et al.*, 1999). At G1 of cycle 17, mRNA levels of *cyclin E* are downregulated as cells of the epidermis stop dividing (Knoblich *et al.*, 1994). An exception to this downregulation of *cyclin E* expression are cells of the epidermal thoracic patch that undergo a 17th mitotic cycle (Knoblich *et al.*, 1994). Neural cells also continue to express *cyclin E*, with the distribution of type I Cyclin E mirroring the pattern of S phase cells as revealed by detection of bromodeoxyuridine (BrdU) incorporation. Cyclin E expression is also detected in endoreplicating tissues, in a pattern correlating with S phases. Oscillations in Cyclin E levels

Figure 1.2 Stages of *Drosophila* embryogenesis

This figure (and legend) is taken from 'The atlas of *Drosophila* development', V. Hartenstein, 1993, and is a useful reference for the of discussion embryonic stages.

All embryos are in lateral view (anterior to the left). (Red) Endoderm, midgut, (green) mesoderm, (purple) central nervous system, (blue) foregut, hindgut and (yellow) pole cells. Abbreviations used are (amg) anterior midgut rudiment, (br) brain, (cf) cephalic furrow, (cl) clypeolabrum, (df) dorsal fold, (dr) dorsal ridge, (es) oesophagus, (gb) germ band, (go) gonads, (hg) hindgut, (lb) labial bud, (md) mandibular bud, (mg) midgut, (mp) malpighian tubules, (mx) maxillary bud, (pc) pole cells, (pmg) posterior midgut rudiment, (pnb) procephalic neuroblasts, (pro) procephalon, (ps) posterior spiracle, (pv) proventriculus, (sg) salivary gland, (stp) stomodeal plate, (st) stomodeum, (tp) trachael pits, (vf) ventral furrow, (vnb) ventral neuroblasts, (vnd) ventral nerve chord.

STAGES OF EMBRYONIC DEVELOPMENT

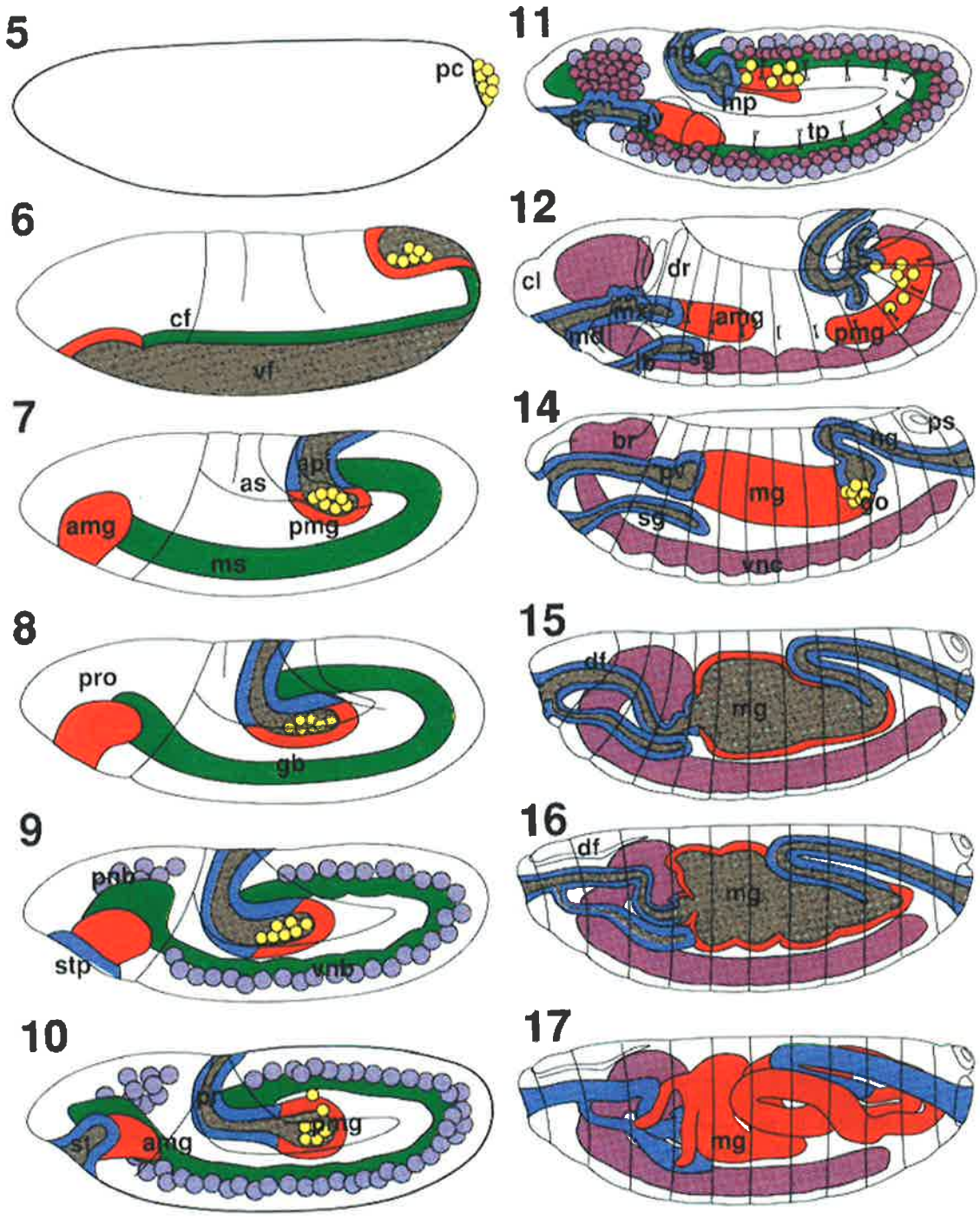
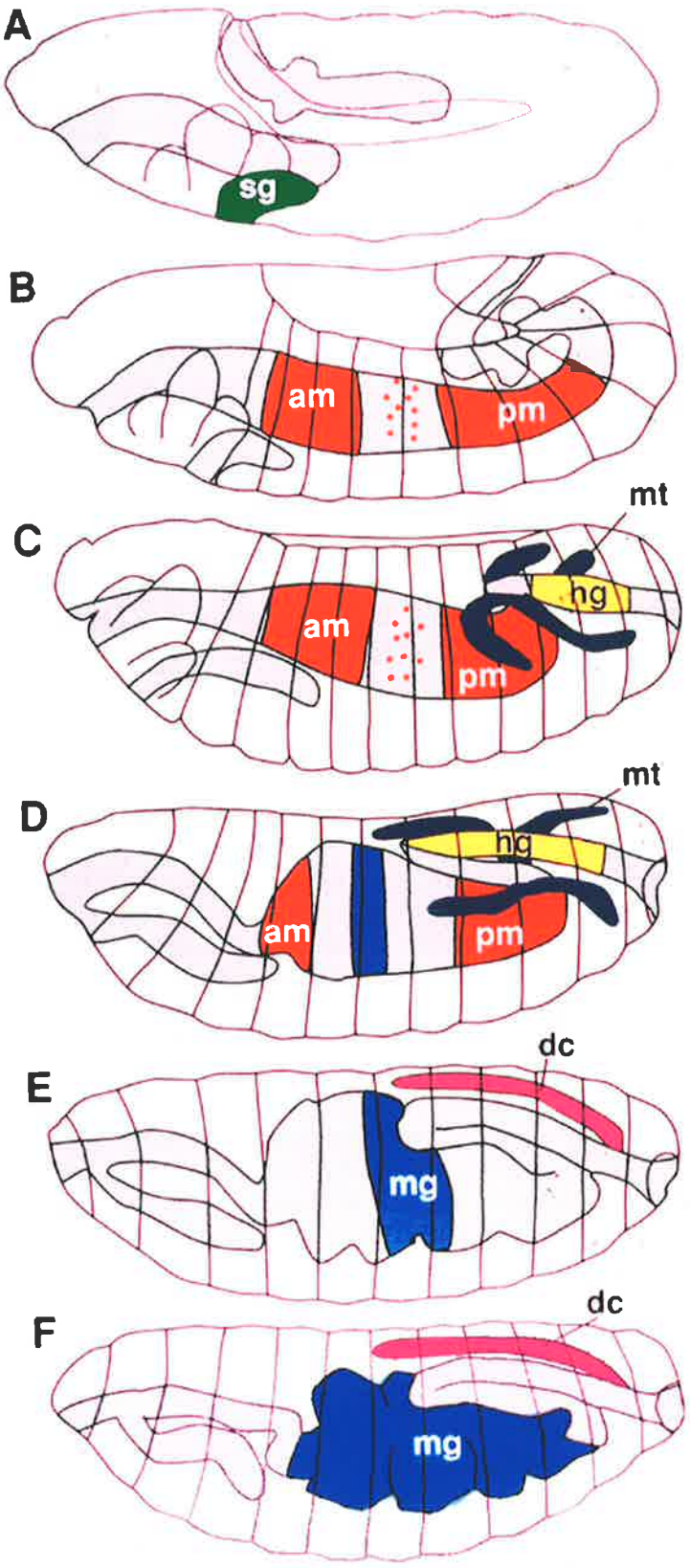


Figure 1.3 The spatial patterning of endoreplication during embryogenesis

This figure (and legend) are taken from Smith and Orr-Weaver, (1991).

The endoreplication domains are shown for the embryonic stages between the onset of polyploidisation and cuticle deposition. The developing gut and salivary gland are shown in light pink when these tissues are not observed to replicate. (A) As the germ band begins to retract (stage 11) the salivary gland (green) is the first tissue to which endoreplication is observed. (B) During germ band retraction (stage 12), the anterior and posterior midgut (red) begin to endoreplicate. The small red circles represent the large replicating nuclei seen between the two parts of the developing midgut. (C) During the dorsal closure stage of embryogenesis (stage 13) the hindgut (yellow) endoreplicates. Shortly thereafter, replication begins in the Malpighian tubules (dark blue). (D) At head involution (stage 14) replication persists in the developing midgut, hindgut and the Malpighian tubules. The replication in the midgut proceeds anteriorly in the anterior midgut and posteriorly in the posterior midgut, there is a stripe of replication near the middle of the sac-like midgut (bright blue). (E) The replication seen at the middle of the midgut is co-incident with the first constriction of the developing midgut (stage 15). As the constrictions appear, the stripe of labelling observed in the midgut expands anteriorly and posteriorly. Replication is next observed in a group of cells that extend along the posterior part of the dorsal side of the embryo (dark pink). (F) As the midgut becomes more convoluted (stage 16), replication is observed to extend through the entire midgut and replication persists in the dorsal cells.

Anterior is to the left, dorsal up. sg = salivary gland, am = anterior midgut, pm = posterior midgut, hg = hindgut, mt = Malpighian tubules, dc = dorsal cells.


A
sg
B
am
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D
mt
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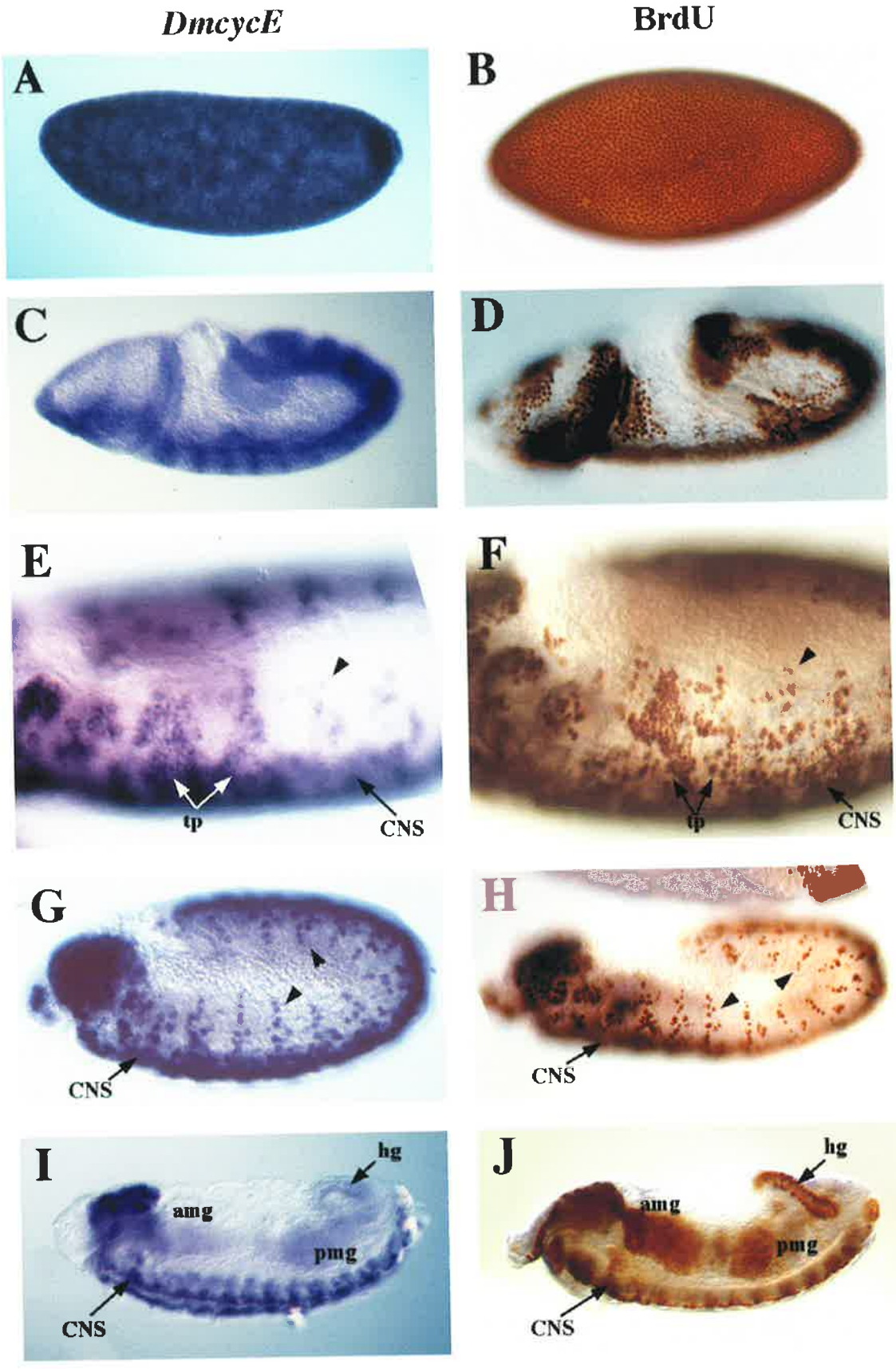
E
dc
mg
F
dc
mg

Figure 1.4 Comparison of *cyclin E* transcription and the pattern of S phases during embryogenesis

This figure is taken from Jones, (1997).

(A, C, E, G, I) show the distribution of *cyclin E* transcripts detected by whole mount in situ hybridisation using a digoxigenin labelled antisense RNA probe. (B, D, F, H, J) show BrdU labelled embryos to show the pattern of S phases. (E, F) are shown at a higher magnification that the other images.

(A, B) Stage 5 embryos showing type II *cyclin E* transcripts at high levels and synchronous S phases. (C, D) Stage 7 embryo showing type I *cyclin E* expression constitutively throughout the embryo at low levels. S phases at this stage do not correlate with *cyclin E* transcription. (E, F) Late stage 10 embryo showing type I *cyclin E* expression in cells of the central nervous system (CNS), peripheral nervous system (PNS; arrowhead) and thoracic patches (tp). S phases at this stage correlate with *cyclin E* transcription. (G, H) Stage 12 embryos showing that type I *cyclin E* transcripts correlate with the pattern of S phases observed in the CNS and PNS. (I, J) Stage 13 embryo showing the pattern of type I *cyclin E* transcription in the endoreplicating anterior midgut (amg), posterior midgut (pmg) and the hindgut (hg). *cyclin E* expression correlates with S phases in these tissues.


DmcycE
BrdU
A
B
C
D
E
tp
CNS
F
tp
CNS
G
CNS
H
CNS
I
hg
amg
pmg
CNS
J
hg
amg
pmg
CNS

have also been shown to be necessary for endo-cycles to occur, as continuous ectopic expression of *cyclin E* blocks re-replication in the larval salivary gland (Weiss *et al.*, 1998; Follette *et al.*, 1998).

Mammalian Cyclin E has been shown to be required for the G1 to S phase transition in cell culture (reviewed by Reed, 1996), but not in a whole animal. In *Drosophila*, *DmcycE* loss of function mutations cause G1 arrest at cycle 17 during embryogenesis, demonstrating that Cyclin E is required for entry into S phase (Knoblich *et al.*, 1994). Expression of *cyclin E* is also required for endoreplication, as these cycles do not occur in homozygous mutant embryos. In addition, Cyclin E is sufficient for the G1 to S phase transition, as heat shock induced ectopic expression of *cyclin E* in larval eye imaginal discs induces G1 arrested cells to enter S phase (Richardson *et al.*, 1995). The eye imaginal disc has a spatially defined pattern of cells in particular stages of the cell cycle (see section 1-8.2). Prior to their final division, cells in a band across the eye disc undergo a developmental arrest in G1 phase. Ectopic expression of *cyclin E* induced these G1 arrested cells to enter S phase. Heat shock induced ectopic expression of *cyclin E* after the final mitosis at cycle 16 during embryonic development is also able to induce terminally arrested G1 cells to enter S phase and undergo another complete cell cycle (Knoblich *et al.*, 1994). Thus, ectopic expression of *cyclin E* in temporarily G1 arrested eye imaginal disc cells and terminally G1 arrested embryonic cycle 17 cells were induced to enter and complete an additional cell cycle. Cyclin E is therefore rate-limiting for the G1 to S phase transition in both these developmental situations.

1-7.2 *cyclin E* expression is not always associated with proliferating cells

Although, in general, *cyclin E* expression correlates with S phases during embryogenesis, there are two cases in which *cyclin E* expression does not correlate with proliferation. During embryogenesis, Cyclin E is detectable in amnioserosa cells and in the pole (presumptive germ) cells for several hours after they cease proliferation (Richardson *et al.*, 1993; Crack *et al.*, 1999). Interestingly, Cyclin E in the amnioserosa persists without the presence of detectable *cyclin E* transcript (Richardson et al., 1993), implying that Cyclin E protein is stabilised in these cells. Amnioserosa cells are arrested in G2 (Foe *et al.*, 1993), where they are likely to be refractory to S phase induction by Cyclin E due to the block to re-replication (reviewed by Botchan, 1996; Diffley, 1996). Similarly, in the non-proliferating pole cells, Cyclin E is also maintained at high levels throughout embryogenesis, even though these cells remain dormant in G2 for at least 14 hours and do not recommence division until

the end of embryogenesis (Foe *et al.*, 1993; Su *et al.*, 1998b). In both of these cases, it is likely to be type II Cyclin E protein that persists.

There are also two cases during larval development in which the presence of type I Cyclin E does not correlate with proliferation. The first of these is the lamina of third instar larval optic lobes (Richardson *et al.*, 1995; see section 1-8.1). In the lamina, only a few S phase cells can be detected and cells do not appear to express Cyclin B or enter mitosis (Richardson *et al.*, 1995; Selleck *et al.*, 1992; Nakato *et al.*, 1995). All cells of the lamina, however, express Cyclin E. It is possible that these cells may be in G2 phase, or fail to enter S phase because of the presence of an inhibitory mechanism. The second case occurs during third larval instar wing disc development, where a zone of non-proliferation exists in the region that gives rise to sensory bristles of the adult wing margin (Johnston and Edgar, 1998). In this tissue, Cyclin E and the S phase transcription factor E2F are present in cells arrested in G1, but are inactive, suggesting that they may be regulated by inhibitors such as CKIs or RBF (de Nooij *et al.*, 1996; Lane *et al.*, 1996; Du *et al.*, 1996).

1-7.3 Developmental regulation of *cyclin E* expression during embryogenesis

Analysis of the pattern of *cyclin E* transcription in embryos homozygous for a mutation in the *string* gene has demonstrated that expression of *cyclin E* is regulated by developmental rather than cell cycle signals (Knoblich *et al.*, 1994; Jones, 1997). *string*$^-$ embryos show a cell cycle arrest in G2 of embryonic cycle 14, but developmental processes, such as gastrulation, still occur (Edgar and O'Farrell, 1989). *cyclin E* expression is observed in the correct spatial and temporal pattern in *string* mutant embryos, with *cyclin E* being appropriately downregulated in the epidermis, and present in cells of the CNS and PNS when they would normally be dividing. Promoter dissection analysis has defined many of the enhancers required for *cyclin E* expression during embryogenesis (Jones, 1997), although the trans-acting factors that regulate *cyclin E* transcription have not been determined.

1-8 Control of proliferation during larval development

After hatching, many larval tissues become polyploid, which is required for the larva to grow (Royzman *et al.*, 1997; Britton and Edgar, 1998). Exceptions to this are cells of the central nervous system, abdominal histoblast nests, germ cells and imaginal cells that give rise to adult structures. During the first larval instar, neuroblasts of the brain show a stereotypical pattern of proliferation, regulated by the products of the *anachromism* (*ana*) and *terribly reduced optic lobes* (*trol*) loci (Data, 1995; Caldwell and Datta, 1998). While

mutations in *ana* result in neuroblasts prematurely entering into S phase (Ebens *et al.*, 1993), mutations in *trol* result in a failure to activate proliferation in most neuroblasts (Datta, 1995). Genetic evidence suggests that *trol* acts to relieve Ana-mediated repression of neuroblast proliferation (Datta, 1995). While *trol* has not been cloned, it may encode a regulator of *cyclin E* transcription, as *trol* mutants show little neuroblast proliferation and dramatically reduced levels of *cyclin E* expression. Whether Trol is likely to be a direct or indirect regulator of *cyclin E* transcription during these division awaits the cloning of this gene. During the third larval instar, optic lobe development and differentiation of the eye imaginal disc exhibit two characterised examples of a developmentally regulated G1 to S phase transition. These examples are discussed in more detail below.

1-8.1 Larval brain lamina precursor cells

The third larval instar optic lobes have several proliferative regions, the outer proliferative centre (OPC), the lamina precursor cells (LPCs) and the inner proliferative centre (IPC) (Hofbauer and Campos-Ortega, 1990; Figure 1.5). Consistent with Cyclin E being required for S phase in these cells, Cyclin E is detected in the OPC, LPC and IPC proliferating domains of the optic lobe (Figure 1.5; Richardson *et al.*, 1995). During optic lobe development, cells of the OPC migrate posteriorly, entering a furrow to become LPCs before differentiating into lamina neurons (Selleck *et al.*, 1992). As cells enter the lamina furrow to become LPCs, cell cycle synchronisation occurs. Posterior-most cells entering the lamina furrow are in G2 or M phase, but once within the furrow, these cells undergo a developmentally regulated G1 arrest before completing the cell cycle and differentiating into neurons. Progression from G1 into S phase within the lamina furrow requires innervation by photoreceptor axons from the developing eye disc, as in the absence of this innervation, LPCs arrest in G1 and fail to differentiate.

The signalling molecule Hedgehog (Hh) is required for LPC to begin differentiation (Huang and Kunes, 1996). Hh is expressed in differentiating eye imaginal disc photoreceptor cells and is required for eye development (reviewed by Heberlein and Moses, 1995). Hh is also transmitted by innervating eye photoreceptor axons to the LPCs of the larval brain, where it directly or indirectly stimulates LPCs to enter S phase (Huang and Kunes, 1996). Innervation by axons lacking Hh results in LPCs arresting in G1, a phenotype similar to the absence of photoreceptor innervation. Consistent with Hh being the critical component of photoreceptor innervation, ectopic *hh* expression is sufficient to induce LPCs to enter S phase in mutants lacking photoreceptor axons. The Hh signalling pathway may therefore act to

regulate G1 to S phase transition regulators. Whether expression of *cyclin E* is important for this developmentally regulated entry into S phase and is therefore a target of Hh signalling, has not yet been determined.

1-8.2 Control of proliferation in the eye imaginal disc

The *Drosophila* adult eye is composed of a highly organised, repetitious array of approximately 800 ommatidia, each composed of 19 cells; 8 photoreceptor cells and 11 accessory cells. Differentiation of the eye imaginal disc single cell layer epithelium has been well characterised (reviewed by Wolff and Ready, 1993). Throughout the first and second larval instars, cells of the eye-antennal imaginal disc undergo rounds of cell division to increase the size of the disc several fold. During third larval instar development, differentiation begins, progressing from posterior to anterior across the disc in association with an indentation called the morphogenetic furrow (MF). As this wave of differentiation occurs, cells immediately anterior to and within the MF undergo a transient developmentally regulated G1 arrest (Wolff and Ready, 1993; Thomas *et al.*, 1994). For the purposes of this thesis, the MF is defined as these G1 arrested cells. Within the MF, cells are progressively sequestered for differentiation into ommatidia, beginning with R8 and followed by the R3, R4, R2 and R5 photoreceptors, which collectively form preclusters. Immediately posterior to the MF, non-photoreceptor cells enter a synchronous S phase from which the remainder of the cells required for eye development are derived. Correct regulation of this developmental G1 to S phase transition that occurs within and immediately posterior to the MF is essential for normal eye development (Thomas *et al.*, 1994; Richardson *et al.*, 1995; Secombe *et al.*, 1998).

The distribution of type I Cyclin E coincides with S phase cells during eye disc development, consistent with expression of *cyclin E* being rate limiting for S phase entry in this tissue (Richardson *et al.*, 1995; Figure 1.5). In addition, expression of type I or type II *cyclin E* is sufficient to induce most G1 cells in the MF to enter S phase (Richardson *et al.*, 1995; Crack *et al.*, 1999). These ectopic S phases disrupt pattern formation and lead to adults with disorganised (rough) eyes. Interestingly, overexpression of type I and type II Cyclin E have differing abilities to induce the G1 arrested cells within the MF into S phase (Crack *et al.*, 1999). While overexpression of type I *cyclin E* is able to drive anterior, but not posterior, G1 phase cells within the MF into S phase, ectopic expression of type II *cyclin E*, is able to drive all G1 cells within the MF into S phase. As type I and II differ in their N-termini, two models can be proposed to explain this difference in activity. An activator required for type I

Figure 1.5 Comparison of Cyclin E and S phases during brain optic lobe and eye imaginal disc development

These images were taken from Richardson *et al.* (1995).

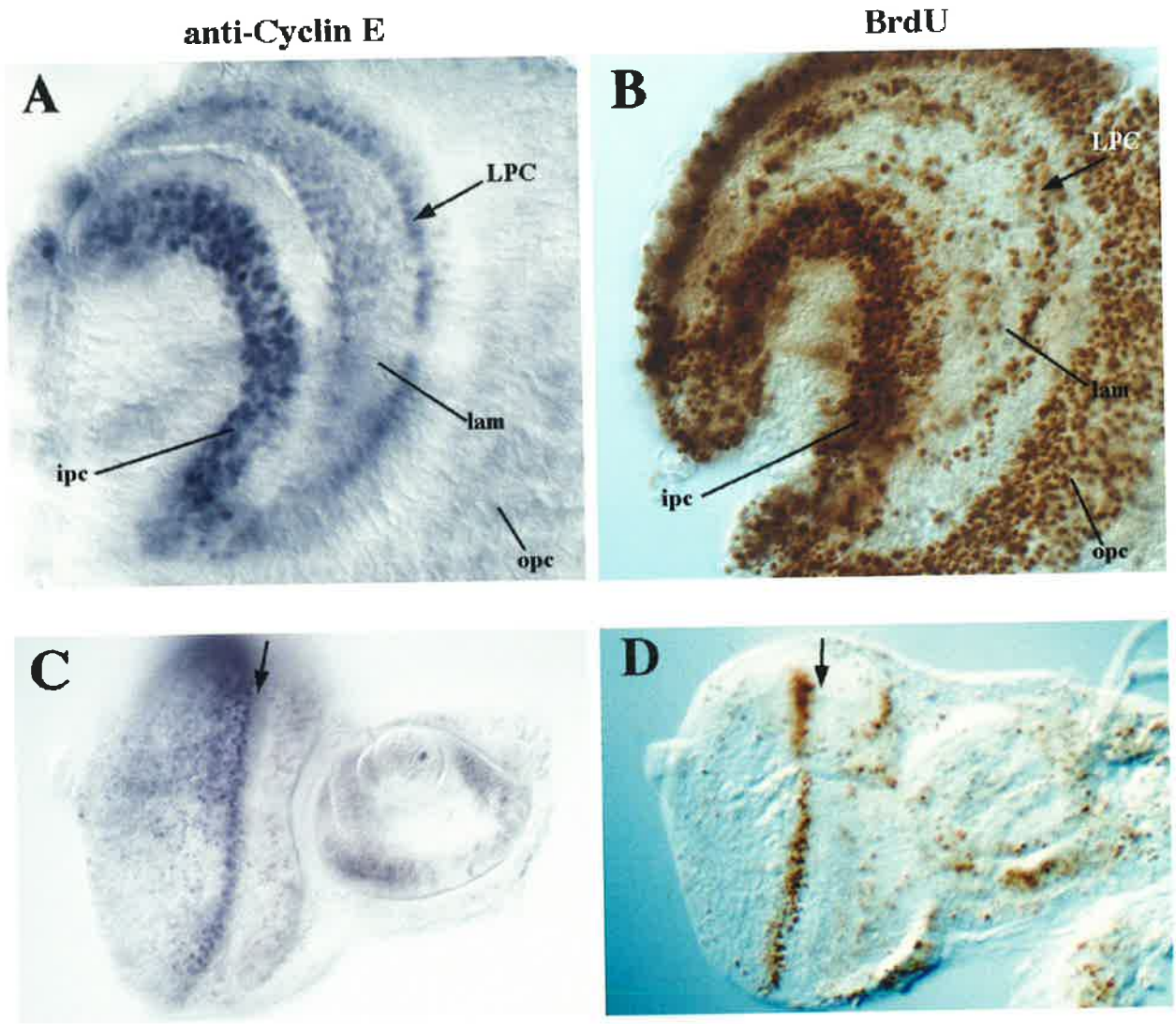
(A) Wild-type third instar larval optic lobe showing the distribution of Cyclin E protein in the inner proliferative centre (ipc), the lamina precursor cells (lpc), outer proliferative centre (out of focus in this image) and the lamina (lam).

(B) Wild-type third instar larval opic lobe showing S phases as detected by incorporation of BrdU. While Cyclin E correlates with proliferation in the ipc, opc and the lpcs, Cyclin E is detected in cells of the lamina that are not proliferating.

(C) Wild-type third instar larval eye-antennal imaginal disc showing the distribution of Cyclin E protein. Cyclin E is detected in asynchronously dividing cells anterior to the MF (arrow), and in the band of synchronous S phase cells immediately posterior to the MF. No Cyclin E is detectable in the G1 arrested cells within the MF.

(D) Wild-type third instar larval eye-antennal imaginal disc showing S phase cells, labelled by BrdU incorporation. The pattern of S phases seen correlates with expression of Cyclin E.

Anterior is to the right in all panels.


anti-Cyclin E
BrdU
A
LPC
lam
ipc
opc
B
LPC
lam
ipc
opc
C
D

Cyclin E activity may be absent from posterior MF cells. Alternatively, an inhibitor, present in the posterior region of the MF, may act via the N-terminal region of type I Cyclin E to inhibit its activity in these cells. While the p21 type CKI Dacapo is a candidate for this inhibitor, it appears to be expressed in only a subset of posterior MF cells, possibly precluster cells, and in differentiating cells posterior to the MF (de Nooij *et al.*, 1996; Lane *et al.*, 1996). Dacapo is therefore an unlikely candidate for this inhibitor.

Roughex (Rux) has been shown to play a critical role in the G1 arrest that occurs in the MF during eye development (Thomas *et al.*, 1994; Dong *et al.*, 1997). Cells within the MF do not arrest in G1 in *rux* mutants. Instead, all cells within the MF enter S phase, preventing normal development and leading to adults with rough eyes. In both embryonic and eye development Rux acts by negatively regulating Cyclin A-associated kinase activity by relocating cytoplasmic Cyclin A to the nucleus where it is degraded (Sprenger *et al.*, 1997; Thomas *et al.*, 1997). Conversely, Cyclin A is stabilised by Rca1 (Regulator of Cyclin A) during eye development (Dong *et al.*, 1997). Thus, Rux and Rca1 act antagonistically on Cyclin A to control its function. Cyclin E/Cdc2c has been shown to phosphorylate and inactivate Rux, leading to the accumulation of Cyclin A (Sprenger *et al.*, 1997; Thomas *et al.*, 1997). Cyclin A can therefore act to induce entry into S phase in *Drosophila*, but is normally inhibited by Rux until after S phase initiation.

A *Drosophila* TGF-β homologue, Decapentaplegic (Dpp), has been demonstrated to have a Rux-independent role in the regulation of the G1 arrest that occurs within the MF (Horsfield *et al.*, 1998). *dpp* is expressed in cells of the MF (Masucci *et al.*, 1990) and appears to be required to establish the G1 arrest seen in these cells, as cells within somatic clones of the Dpp receptor Thickveins (Tkv) that span the MF continue to cycle (Penton *et al.*, 1997; Horsfield *et al.*, 1998). Interestingly, although anterior MF cells within *tkv*$^{-}$ clones continue to cycle, these cells do arrest in G1 in the posterior half of the MF (Horsfield *et al.*, 1998). It is therefore likely that while anterior MF cells require Dpp signalling to enter G1, a Dpp-independent mechanism is required for maintenance of this state in posterior MF cells. The Dpp-independent mechanism that acts to induce G1 arrest in the posterior part of the MF (Horsfield *et al.*, 1998) may act upon the N terminus of type I Cyclin E in the posterior part of the MF, since ectopic expression of type I Cyclin E can overcome the G1 arrest in the anterior part of the MF, but not in the posterior part of the MF. This presumptive inhibitor also appears to be specific to type I Cyclin E, since ectopic expression of *Drosophila* Cyclin A or type II Cyclin E can induce all cells within the MF into S phase (Dong *et al.*, 1997). The Dpp-mediated G1 arrest of anterior MF cells appears to act downstream of Cyclin E protein

accumulation, as ectopic Dpp expression inhibits S phases without altering Cyclin E levels (Horsfield *et al.*, 1998). Analogous to the mechanism of G1 arrest by mammalian TGF-β, Dpp may be leading to the induction of a Cdk inhibitor. However, as described above, the *Drosophila* p21/p27 homologue, *dacapo*, is not expressed in all cells of the MF (de Nooij *et al.*, 1996; Lane *et al.*, 1996). The Dpp-mediated G1 arrest is therefore likely to occur by the induction of a novel inhibitor. A summary of the mechanisms known to act in the G1 arrest during eye disc development are presented in Figure 1.6.

1-9 Identification of an eye specific *cyclin E* mutation, *DmcycE^{JP}*

Numerous *P* element insertions have been localised to the *cyclin E* promoter region (Jones, 1997). One homozygous viable and fertile *P* insertion, the *P*[*w*$^{+}$*m-lac*] element allele *14.11G*, has been localised to ~14 kb 5' to the transcriptional start site of the type I *cyclin E* transcript (Secombe *et al.*, 1998). To generate small promoter deletions for *cyclin E* promoter analysis, the *DmcycE$^{14.11G}$ P* element was mobilised using Δ2-3 transposase. In addition to generating homozygous lethal revertants, presumably due to imprecise excision of the *P* element removing critical *cyclin E* regulatory regions, one *white*$^{-}$ revertant was homozygous viable, but adults had rough eyes when transheterozygous for *DmcycE* null alleles (Secombe *et al.*, 1998). Genomic Southern analysis and DNA sequence analysis of *P* element rescue plasmids demonstrated that this allele, *DmcycE^{JP}*, is an internal deletion of the *DmcycE$^{14.11G}$ P* element (Secombe *et al.*, 1998; Figure 1.7). This internal deletion of ~4 kb removes the *P* element *white* gene while maintaining the *amp^{R}* and *lacZ* genes and the *P* element ends (Figure 1.7).

Dosage sensitive genetic interactions screens using rough eye phenotypes have been used extensively in *Drosophila* to identify components of regulatory pathways (for example Simon *et al.*, 1991; Raftery *et al.*, 1995; Dong *et al.*, 1997; Staehling-Hampton *et al.*, 1999). Thus, the *DmcycE^{JP}* rough eye phenotype may provide an opportunity to identify components of Cyclin E-mediated entry into S phase by identifying genes that, when halved in dosage, enhance or suppress this phenotype. Characterisation of the basis for the *DmcycE^{JP}* rough eye phenotype and genetic interactions with this allele form the basis of the work described in this thesis.

Figure 1.6 Regulation of the G1 arrest that occurs in the MF during eye development

Schematic representation of the MF region of an eye imaginal disc during third larval instar development. Within the MF, mechanisms exist to prevent the inappropriate expression and/or activation of Cyclin E and Cyclin A-associated kinase activity. Cyclin A is prevented from accumulating by Rux, which is inactivated by Cyclin E/Cdc2c, allowing Cyclin A/Cdk1 to act after the initiation of DNA replication. Cyclin E is kept inactive in the anterior half of the MF by a Dpp-dependent mechanism that acts downstream of Cyclin E protein accumulation, and a Dpp-independent mechanism in the posterior half of the MF. See text for details.

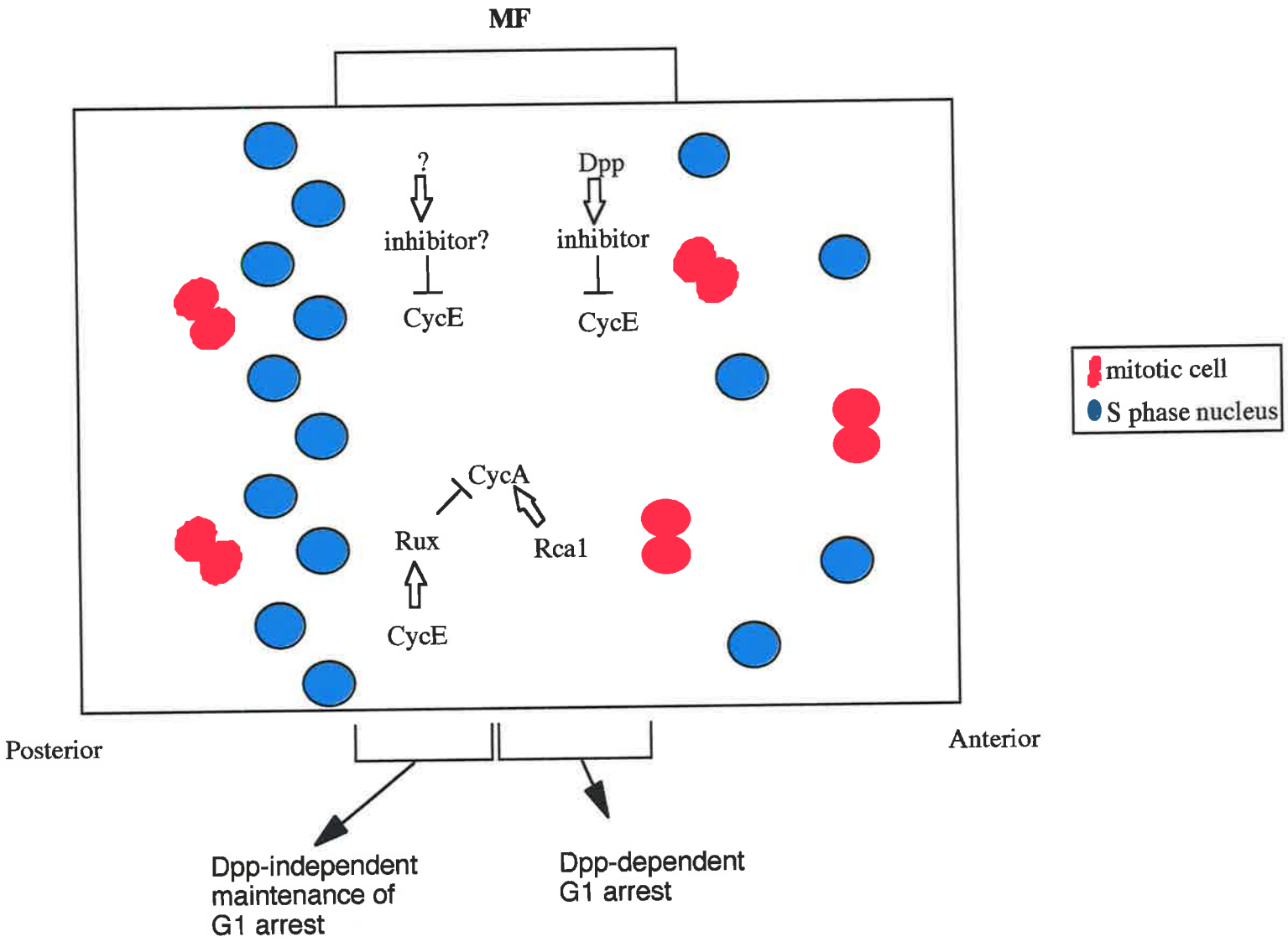
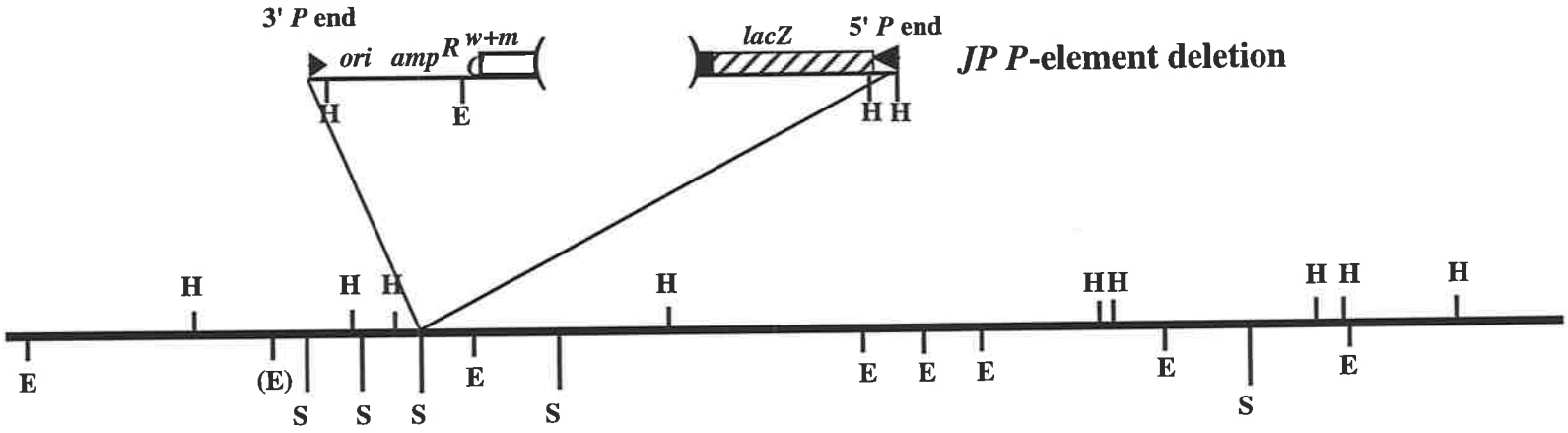

MF
?
inhibitor?
CycE
Dpp
inhibitor
CycE
CycA
Rux
Rca1
CycE
mitotic cell
S phase nucleus
Posterior
Anterior
Dpp-independent
maintenance of
G1 arrest
Dpp-dependent
G1 arrest

Figure 1.7 The *14.11G* and *JP P* elements are inserted in the same position upstream of the *DmcycE* type I transcript

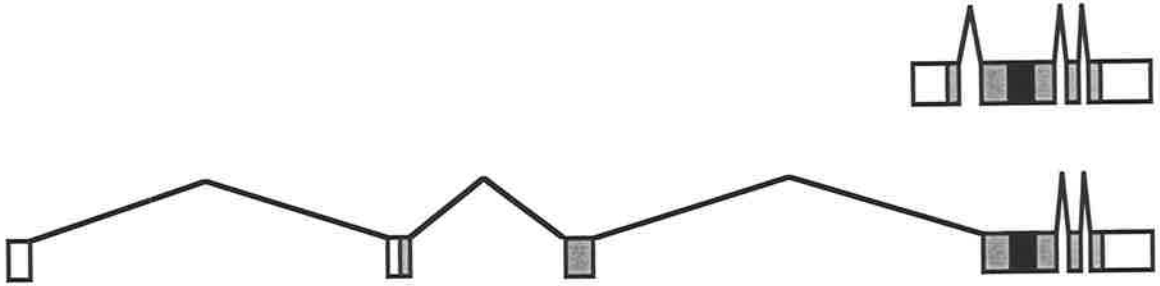
Map of the *DmcycE* genomic region showing the location of the *14.11G P* element, which is inserted ~14kb upstream of the start of the *DmcycE* type I transcript (Secombe *et al.*, 1998). *JP* was identified as a w^- revertant of *14.11G* using Δ2-3 transposase. Southern analysis and *P* element rescue demonstrated that *JP* has a deletion of ~4kb within the *14.11G P* element, removing most of the mini-w^+ (w^{+mC}) gene, but maintaining the *lacZ* and amp^R genes, and *P* element ends.

Light shaded boxes within the *cyclin E* type I and type II transcripts indicate coding regions, and the black box indicates the region coding for the cyclin box.

Adapted from Jones *et al.*, in preparation.



E=*Eco*RI
H=*Hin*dIII
S=*Sal*I



***DmcycE* Type I transcript**
(zygotic)

***DmcycE* Type II transcript**
(maternal)

1-10 Aims and approaches of this thesis

The research described in this thesis was undertaken with the aim of identifying genes involved in the regulation of *cyclin E* transcription and function. Chapter 3 describes the characterisation of a homozygous viable hypomorphic allele of *cyclin E*, $DmcycE^{JP}$, that results in adults with a rough eye phenotype. Results described in Chapter 3 demonstrate that $DmcycE^{JP}$ is a sensitised system that can be used to examine genetic interactions by altering the dosage of cell cycle regulatory genes.

To identify novel components of Cyclin E-mediated entry into S phase, two different approaches were used. Firstly, as detailed in Chapter 4, deficiencies were used to identify chromosomal regions that, when heterozygous in a $DmcycE^{JP}$ background, modified the $DmcycE^{JP}$ phenotype. To generate specific alleles of enhancers and suppressors of the $DmcycE^{JP}$ rough eye phenotype, a random mutagenesis was undertaken using the mutagens ethyl methylsulphonate and X-irradiation. A description of the mutagenesis and characterisation of suppressor mutations mapping to the second chromosome is described in Chapter 5.

Three modifiers generated in this 'fly screen' were characterised in detail. Chapter 6 describes an enhancer of $DmcycE^{JP}$ that is the first described allele of *cdc2c*, and provides evidence that this Cyclin E regulated kinase is required for the G1 to S phase transition. Chapters 7 and 8 focus on two second chromosome suppressors isolated in the mutagenesis, $Su(DmcycE^{JP})2\text{-}2$ and $Su(DmcycE^{JP})2\text{-}1$ respectively, and Chapter 9 summarises the results obtained in this thesis.

Chapter 2: Materials and Methods

2-1 Abbreviations

Abbreviations used are as described in "Instructions to authors", Biochem. J (1978) 169, 1-27. In addition;

AP	Alkaline phosphatase
APS	ammonium persulphate
BCIG	5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal)
BCIP	5 Bromo-4-chloro-3-indolyl phosphate
bisacrylamide	N,N'-methylene-bisacrylamide
Blotto	5% skim milk powder in PBT
BrdU	5 Bromo 2' deoxyuridine
BSA	bovine serum albumin
CIP	alkaline calf intestinal phosphatase
DAB	3, 3' Diaminobenzidine
DIC	differential interference contrast
EMS	ethyl methanesulphonate
HRP	Horse radish peroxidase
IPTG	Isopropyl β-D-thiogalactopryanoside
kb	number of kilobase pairs
kDa	number of kilo daltons
NBT	4-Nitro blue tetrozolium chloride
PAGE	polyacylamide gel electrophoresis
PBS	phosphate buffered saline
r.p.m.	revolutions per minute
SEM	Scanning electron micrograph
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N-tetramethylenediamine

2-2 Materials

2-2.1 Enzymes

Enzymes were obtained from the following sources:

Restriction endonucleases	Pharmacia, New England Biolabs, Boehringer Mannheim.
T4 DNA ligase and Alkakine calf intestinal phosphatase	Boehringer Mannheim
RNase and Lysozyme	Sigma

2-2.2 Antibodies

Primary Antibodies

Antibody	dilution	Source
α-cyclin E 8B10 ascites fluid (mouse)	1/1000 in blotto	Dr H. E. Richardson.
α-cyclin E rat polyclonal antibody	1/1000 in blotto	Dr H. E. Richardson.
α-BrdU (mouse monoclonal)	1/50 in 2% goat serum/PBT	Becton Dickinson
α-DIG conjugated to Alkaline Phosphatase (sheep Fab fragment)	1/2000 in PBT	Boehringer Mannheim
α–lacZ (rabbit polyclonal)	1/500 in blotto	lab supply
MPM2 anti mitotic protein (mouse monoclonal)	1/10 in 5%BSA/PBT	DAKO corporation
22C10 (mouse monoclonal)	1/10 in blotto	N. Patel
α-Engrailed	1/5 in 5%BSA/PBT	Developmental Biology Hybridoma Bank

Secondary and Tertiary Antibodies

Antibody	Dilution	Source
α–mouse-HRP	1/400 in blotto	Jackson labs
biotinylated α-mouse (donkey)	1/200 in blotto	Jackson labs
Streptavidin-HRP (Vectastain ABC)	1/200 in PBT	Amersham
Streptavidin-FITC	1/200 in PBT	Jackson Labs

2-2.3 Radionucleotides

α ^{32}P-dATP (4000 Ci/mmole) Bresatec

2-2.4 Antibiotics

Ampicillin: Sigma.

2-2.5 Molecular weight standards

(i) DNA

DNA was digested with *Bst* EII and *Sal* I to produce fragments of (in kb) 14.14, 7.24, 4.82, 4.32, 3.68, 3.13, 2.74, 2.32, 1.93, 1.37, 1.26, 0.70, 0.45, 0.22 and 0.11

(ii) Protein

High molecular weight markers (GIBCO BRL) sizes (in kDa) 205, 116, 97, 66, 45 and 29

2-2.6 Oligonucleotides

(i) Primers used for Sequencing

T3 5'-d(ATTAACCCTCACTAAAGGGA)-3'

T7 5'-d(TAATACGACTCACTATAGGG)-3'

(ii) Primers used for amplifying and sequencing *cdc2c^{JS}* allele.

The position of the oligonucleotides within the *cdc2c* cDNA are given in brackets and *Eco*RI restriction enzyme sites used for cloning are underlined.

cdc2c julie1 (44-62) 5'-d(GGAATTCGCCAACATCGATCTCAAGG)

3' cdc2c (1122-1138) 5'-d(GGAATTCTGATCGTGTATTTATCG)

cdc2c int1 (385-400) 5'-d(GGAATTCCCCCTCAGTTGATAAAG)

cdc2c rev1 (455-473) 5'-d(GGAATTCCTTGAGATCGCGATGCAGG)

(iii) Primers used for analysis of *DmcycE*^{JP} revertants

1411G	5'-d(CGTTTTAGTACTGCGCTC)-3'
PzDD	5'-d(CTTCACTCGTTTACTCC)-3'

2-2.7 Cloning vectors and clones

pBluescript KS+	Stratagene
UbcD2 cDNA	Dr. Stefan Jentsch

2-2.8 Buffers and solutions

Agarose gel loading buffer:	50% (w/v) glycerol, 50 mM EDTA, 0.1% (w/v) bromophenol blue
Ringers solution:	0.65 % NaCl, 0.014% KCl, 0.02% $NaHCO_3$, 0.012% $CaCl_2$, 0.001% NaH_2PO_4
PBS	7.5mM Na_2HPO_4, 2.5 mM NaH_2PO_4, 145mM NaCl
PBT	1 x PBS, 0.1% Tween 20 or Triton X-100
Protein gel transfer buffer:	48mM tris-base, 39mM glycine, 0.037% (w/v) SDS, 20% methanol
Protein gel loading buffer:	10% glycerol, 2% SDS, 5% b-mercaptoethanol, 0.05% bromophenol blue12.5% 0.5M Tris-HCl pH6.8
Protein gel running buffer:	1.5% Tris-base, 7.2% Glycine, 0.5% SDS
TAE	40mM Tris-acetate, 20mM sodium acetate, 1mM EDTA, pH 8.2

2-2.9 Bacterial strains

E. coli DH5α: F'/endA1 *hsd*R17 ($r_k^- m_k^+$) *sup*E44 *thi*1 *rec*A1 *gyr*A (Nal^r) *rel*A1 D(*lacIZYA-arg*F)U169 deoR (f80 *dlac*D(*lacZ*)*M15*)

2-2.10 Bacterial media

All buffers and media were prepared with distilled and deionised water and sterilised by autoclaving, excepts heat labile reagents, which were filter sterilised. Antibiotics were added from sterile stock solutions after the media had been autoclaved.

L-Broth:	1% (w/v) amine A, 0.5% yeast extract, 1% NaCl, pH 7.0.
SOC:	2% bactotyptone, 0.5% yeast extract, 10mM NaCl, 2.5 mM KCl,10mM $MgCl_2$, 10 mM $MgSO_4$, 20mM glucose.

Plates: L-Broth with 1.5% (w/v) bactoagar supplemented with ampicillin (50μg/ml) where appropriate.

2-2.11 *Drosophila* strains

Unless otherwise indicated, stocks were obtained from either the Indiana Stock centre, Bloomington, IA, or Umea Stock Centre, Sweden and are as described in Lindsay and Zimm (1992) or Flybase (www://flybase.bio.indiana.edu/). *P* element stocks used were characterised as part of the Berkeley Drosophila Genome Project (BDGP), and are described at www://fruitfly.berkeley.edu/ (The FlyBase consortium, 1999)

2-2.11.1 *Drosophila* strains used for generating germline clones

P{hs neo; ry^{+} ; FRT}$^{2L\text{-}40A}$; *ry*

P{w^{+}*mC*=ovoD1-18}2L1 *P{w*$^{+}$*mC*=*ovo*$^{D1\text{-}18}$}2L2 *P{ry*$^{+}$*t7.2*=neoFRT}40A/Dp(?;2)*bw*^{D},

S^{1} *wg*$^{Sp\text{-}1}$ *Ms(2)M1 bw*^{D}/*CyO*

*y w P{*mini *w*$^{+}$ *; hs-FLP}* ; *Gla Bc/CyO*

2-2.11.2 *Drosophila* cell cycle mutations used for analysis of genetic interactions

Fly stocks containing the specific cell cycle mutations that were tested are listed below. *cyclin A* (Lehner and O'Farrell 1989): *cyclin A*^{5}*/TM2* (*P*-element insertion), *Df(3L)vin3* (68C5-6; 68E3-4)/*TM3, Sb* (deletion); *string* (Edgar and O'Farrell, 1989; Edgar *et al.,* 1994): *stg*^{5}*/TM3, Sb* (strong EMS-generated allele); *stg*^{1}*/TM6B* (weak EMS-generated allele); *stg*^{AR2}*/TM3, Sb* (transcriptional null generated by *P*-element excision); *Df(3R)3450* (98A1-7; 99A6-8)/*TM6B* (deletion); *dE2F* (Duronio *et al.,* 1995): *dE2F*^{91}*/TM3, Sb, Ubx-lacZ* (EMS-generated allele); *dE2F*^{RM729}*/TM3, Sb, Ubx-lacZ* (*P*-element insertion); *dDP* (Duronio *et al.,* 1998): *dDP*^{vr10}*/CyO* (EMS-generated allele, semi-lethal); *dacapo* (de Nooij *et al.*, 1996; Lane *et al.,* 1996): *dap*^{4} (intragenic deletion generated by imprecise excision of the *dap*^{1} *P*-element); *cyclin B* (Knoblich and Lehner 1993): *Df(2R)59AB/CyO* (deletion); *cyclin B3* (Sigrist *et al.,* 1995): *Df(3R)XTAI* (96B; 96D) *th*^{1}, *st*^{1}, *ri*^{1}, *roe*^{1}, *p*^{1}*/Dp(3;3)* (94D; 96E*) SuM(3)w*^{13}, *st*^{1}, *e*^{1} (deletion); *cdc2* (Lehner and O'Farrell 1990b): *cdc2*$^{E1\text{-}9}$*/CyO* (EMS-generated allele); *cdc2c* (Stern *et al.,* 1993)*: Df(3R)H81 (*92E9; 92E15*)/TM2* (deletion); *Df(3R)H-B79* (92B2-3; 92F13-93A1)/TM2 (deletion); *Dmcks* (H. Richardson, P. Kylsten, B. J. Jennings, P. H. O'Farrell, S. I. Reed, and R. Saint, in preparation): *Df(3R)P14* (90C2-D1; 91A1-2) *sr*^{1}*/T(2:3)ap*^{Xa} (deletion); *cyclin C* (Leopold and O'Farrell 1991): *Df(3R)ry506-85C* (87D1-2; 88E5-6)/Dp(3:Y), ry (deletion); *RBF* (Du *et al.,* 1996): *Df(1)Su(s)83* (1B10; 1D6-E1*), y*^{1}, *cho*^{1}, *ras*^{1}, *v*^{1}*/Dp(1:Y)y*^{2}, *sc/C(1)DX, y*^{1}, *f*^{1} (deletion); *roughex* (Thomas *et al.,* 1994): *y*^{1}, *cho*^{1}, *rux*^{8}*/FM7c* (X-ray-generated allele, small deletion); *y*^{1}, *rux*^{7}*/FM7C* (X-ray

allele, rearrangement); and *rca1* (Dong *et al.*, 1997): *rca1*^{3300}/*CyO* (*P*-element allele). Previously described stocks of *DmcycE* mutations used in this study were the null allele *DmcycE*^{AR95}/*CyO* (Knoblich *et al.*, 1994), the deletions *Df(2L)TE35D-1/CyO* and *Df(2L)TE35D-3/CyO,* the hypomorphic EMS-generated alleles *DmcycE*^{P28}/*CyO* and *DmcycE*^{P41}/*CyO* (H. Richardson, unpublished data) and the partial female sterile allele *DmcycE*^{PZ01672}/*CyO* (Lilly and Spradling 1996).

Stocks for increasing the dosage of genes were, for *RBF* (Du *et al.*, 1996): *P*[w^+ *GMR-RBF*^{4} (homozygous for the *GMR-RBF*^{2} transgene on the third chromosome)]; for *roughex* (Thomas *et al.*, 1997): *P* [ry^+ *6.0C1* (third chromosome)]/*TM6B,* for *dacapo* (de Nooij *et al.*, 1996): *GMR-dacapo*^{56} (third chromosome) and human p21^{CIP1} (de Nooij and Hariharan 1995): *GMR*-p21^{CIP1} (third chromosome). Fly stocks were obtained from the Bloomington stock center or relevant laboratories.

2-2.11.3 X chromosome deficiency kit

Defiencies covering the X, 2nd and 3rd chromosomes (deficiency collections) were obtained from Bloomington Stock centre, Bloomington, Indiana. All fly stocks are as described in either Lindsay and Zimm (1992) or Flybase (www://flybase.bio.indiana.edu/).

Deficiency name	**Cytological region removed by deficiency**
Df(1)tBA1 w/FM7 ; Dp(1;2)E1 y$^+$/+	1A1 to 2A
Df(1)sc^{J4} *; Dp (1;f)z*^{a}*//C(1)DX y f*	1B to 3A3
Df(1)64c18 g^1 *sd*^{1}*/Dp(1;Y)w*$^+$*/C(1)DX y*^{1} *s*^{1} *f*^{1}	2E1-2 to 3E7-F1
Df(1)JC19/FM7c	2F6 to 3C5
Df(1)N-8/FM7c	3C2-3 to 3E3-4
Df(1)dm75e19/FM7c	3C11 to 3E4
Df(1)A113/C(1)DX, y w f ; Dp(1;2)w^{+64b}/+	3D6-E1 to 4F5
Df(1)JC10/FM7c sn$^+$	4C15-16 to 5A1-2
Df(1)C149/FM6	5A8-9 to 5C5-6
Df(1)N73/FM6	5C2 to 5D5-6
Df(1)sqh y/FM6b	5D1-2 to 5E
Df(1)JF5 f car/FM7	5E3-5 to 5E8
Df(1)G4e^L *H24i*^{R} *M f.FM7*	5E3-8 to 6B
Df(1)HA32/FM7c P(ry$^+$ *ftz:lacZ=lacC)*	6E4-5 to 7A6
Df(1)ct-J4, In(1)dl-49, f/C(1)DX y w f ; Dp(1;3)sn^{13a1}*/Ki*	7A2-3 to 7C1
Df(1)ct4b1, y sn/Binsn	7B2-4 to 7C3-4

X chromosome deficiencies cont….

Deficiency name	Cytological region removed by deficiency
Df(1)C128/FM6	7C1 to 7D5-6
Df(1)RA2/FM7c	7D10 to 8A4-5
Df(1)KA14/FM7c	7F1-2 to 8C6
Df(1)lz90b24, y w^a/FM7	8B5-8 to 8B8-9
Df(1)C52/FM6	8E to 9C-D
Df(1)v-L15/FM6	9B1-2 to 10A1-2
Df(1)v-N48/Dp(1;Y)B^{S-} v^+ y^+ Y/C(1)DX y f	9F to 10C3-5
Df(1)N71/FM6 ; Dp(1;2) v^{65b}/C(1)DX y f	10B2-8 to 10D3-8
Df(1)HA85/FM7c	10C1-2 to 11A1-2
Df(1)N105/FM6	10F7 to 11D1
Df(1)JA26/FM7c	11A1 to 11D-E
Df(1)C246/FM6	11D-E to 12A1-2
Df(1)N12, ras v/FM6	11D1-2 to 11F1-2
Df(1)g, f B/In(1)AM	12A to 12E
Df(1)RK2/FM7a y^{31d} sc^8 w^a v^{0f} B	12D2-E1 to 13A2-5
Df(1)RK4/FM7c y^{31d} sc^8 w^a B sn^{X2} v^{0f} g^4 B/Dp(1;Y) y sc^8 Y	12F5-6 to 13A9-B1
Df(1)sc72b/FM7	13F1 to 14B1
Df(1)4b18, y cv v f car/In(1) sc^{S1L} sc^{8R}+S, sc^8 sc^{S1} w^a B	14B8 to 14C1
Df(1)r-D1, v f/C(1)DX y w f ; Dp(1;4)r^+/+	14C2-4 to 15B2-C1
Df(1)r-D17/FM6, l(1)? ; Dp(1;4)r^+/+	14F6 to 15A6
Df(1)B/In(1) sc^7+AM, ptg^4 car = B263-20	16A2 to 16A6
Df(1)N19/FM6	17A1 to 18A2
Df(1)JA27/FM7c	18A5 to 20A
Df(1)ma13, y^2 ct^6 f/Dp(1;Y) y^+ ymal^{106}/C(1)RM, y v f mal^2	18F to 20h;Y
Df(1)DCB1-35b/FM6/Dp(1;Y) y^+ Ymal^{106}	19F1-2 to 20E-F
Df(1)JC4/FM7c	20A1 to 20E-F

2-2.11.4 2nd chromosome deficiency kit

Name of deficiency	Cytological region removed in deficiency
Df(2L)net-PMF/SM6a	21A1 to 21B7-8
Df(2L)a1/In(2L)Cy, Cy^1	21B8-C1 to 21C8-D1
Df(2L)ast2/SM1	21D1-2 to 22B2-3
Df(2L) dp-79DA cn^1/In(2LR) bw^1	22A2-3 to 22D5-E1
Df(2L)C144, dpp^{d-ho} ed^1/Gla^1 Bc^1 $Egfr^{E1}$	23A1-2 to 23C3-5
Df(2L)JS32, dpp^{d-ho}/SM6a	23C3-5 to 23D1-2
Df(2L)ed^1 al^1 b^1/SM5	24A3-4 to 24D3-4
Df(2L)sc19-8/SM6b	24C2-8 to 25C8-9
Df(2L)sc19-5/SM6b	25A4-5 to 25D5-7
Df(2L)cl-h3/SM6b	25D2-4 to 26B2-5
In(1)w^{m4}; Df(2L)E110/CyO	25F3-26A1 to 26D3-11

2nd chromosome deficiencies cont…

Deficiency name	Cytological region removed by deficiency
Df(2L)J-H/SM5	27C2-9 to 28B3-4
Df(2L)spd al^{1} dp^{ov1}/CyO	27D-E to 28C
Df1)w67c23 y^{1} ; Df(2L)Trf-C6R31/CyO	28DE (within)
In(1)w^{m4h} y^{1} ; Df(2L)/TE29Aa-11/CyO	28E4-7 to 29B2-C1
w* ; Df(2L)N22-3/CyO	29D1-2 to 30C4-D1
W^{1118} ; Df(2L)30A-C/CyO	29F7-30A1 to 30C2-5
w* ; Df(2L)N22-5/CyO	30A1-2 to 30C4-D1
Df(2L)s1402/CyO	30C to 30F
Df(2L)Mdh cn^{1}/Dp(2;)Mdh3 cn^{1}	30D-30F to 31F
Df(2L)J39/In(2L)Cy; Dp(2;Y)cb50, Dp (1;Y)B^{S} Yy^{+}/C(1)RM	35E1-2 to 36A6-7
Df(2L)Prl/CyO	32F1-3 to 33F1-2
Df(2L)prd1.7 b^{1} Adh^{n2} pr^{1} cn^{1} sca^{1}/CyO	33B2-3 to 34A1-2
Df(2L)b87e25/In(2L)NS	34B12-C1 to 35B10-C1
Df(2L)osp29 Adh^{UF} pr^{1}cn^{1}/CyO	35B1-3 to 35E6
Df(2L)r10 cn^{1}/In(2LR)O Cy^{1} dp^{1v1} pr^{1} cn^{2}	35E1-2 to 36A6-7
Df(2L)H20 b^{1} pr^{1} cn^{1} sca^{1}/CyO	36A8-9 to 36E1-2
Df(2L)TW137 cn^{1} bw^{1}/CyO Dp(2;2)M(2)m^{+}	36C2-4 to 37B9-C1
Df(2L)TW50 cn^{1} /CyO Dp(2;2)M(2)m^{+}	34E4-F1 to 38A6-7
Df(2L)TW84/CyO	37F5-38A1 to 39D3-E1
Df(2L)TW161 cn^{1} bw^{1}/CyO	38A6-B1 to 40A4-B1
Df(2R)M41A4/SM1	41A
In(2R)bw^{VDe2L} Cy^{R}/In(2LR)Gla	41A-B to 42A2-3
Df(2R)nap1/In(2LR)Gla Dp(2;)BG Gla	41D2-E1 to 42B1-3
Df(2R)nap9/In(2LR)Gla Dp (2;2)BD Gla	42A1-2 to 42E6-F1
Df(2R)ST1 Adh^{n5} pr^{1} cn*/CyO	42B3-5 to 43E15-18
In(2R)pk78s/CyO	42C1-7 to 43F5-8
Df(2R)cn9/SM6b Cy^{1} Roi^{1}	42E to 44C
w118 ; Df(2R)H3C1/CyO	43F to 44D
w^{118} ; Df(2R)H3E1/CyO	44D to 44F12
w^{1118} ; Df(2R)B5 px^{1} sp^{1}/CyO Adh^{nB}	46A to 46C
Df(2R)X1/CyO Adh^{nB}	46C to 47A1
Df(2R)Stan2 P{ry[+t7.2]neoFRT} 42D cn^{1} sp^{1}/CyO	46F1-2 to 47D1-2
Df(2R)E333/CyO-CR2	47A to 47F
Df(2R)en-A/CyO Cy^{1} dp^{1v1} pr^{1} cn^{2}	47D3 to 48B2-5
Df(2R)en30/Sm5;Dp(1;Ybb^{-})B^{S}	48A3-4 to 48C6-8
Df(2R)v135/CyO S* dp^{ov1} cn^{2} bw^{1}	49A-B to 49D-E
In(2R)vg-C/SM5	49A4-13 to 49E7-F1
Df(2R)CX1 b^{1} pr^{1}/SM1	49C1-4 to 50C23-D2
Df(2R)Trix/CyO	51A1-2 to 51B6
w^{a} N^{fa-g} ; Df(2R)Jp1/CyO	51C to 52F5-9

2nd chromosome deficiencies cont....

Deficiency name	Cytological region removed by deficiency
w^a N^{a-g} ; Df(2R)Jp8 w^+/CyO	52F5-9 to 52F10-53A1
Df(2R)Pcl7B/CyO	54E8-F1 to 55B9-C1
Df(2R)Pcl11B al^1 dp^{ov1} b^1 pr^1/CyO	54F5-55A1 to 55C1-3
Df(2R)PC4/CyO	55A to 55F
y^* w^*/Dp(1;Y)y+; Df(2R)P34/CyO	55E2-4 to 56B2-C1
Df(2R)017/SM1	56F5 to 56F15
Df(2R)AA21 c^1 px^1 sp^1/SM1	56F9-17 to 57D11-12
Df(2R)Pu-D17cn^1 bw^1 sp^1/SM1	57B4 to 58B
Dp(1;Y y^+/y^1 ; Df(2R)X58-7 pr^1 cn^1/CyO bw^1	58A1-2 to 58E4-10
Dp(1;Y y^+/y^1 ; Df(2R)X58-12 pr^1 cn^1/SM5	58D1-2 to 59A
w ; Df(2R)59AB/SM1*	59A1-3 to 59B1-2
Df(2R)or-BR6 cn^1 bw^1 sp^1/In(2LR)lt^{G16L} bw^{V32gR}	59D5-10 to 60B3-8
Df(2R)bw^{VDe2L} Px^{KR}/SM1	59D6-E1 to 60C-D
In(2LR)Px^4 dp^{ov1} b^1/CyO	60B to 60B1-2
Df(2R)Px2/SM5	60C5-6 to 60D9-10
Df(2R)M60E/In(2LR)bw^{V32g}	60E2-3 to 60E11-12
Dr(2R)ES1 b^1 pr^1 cn^1 wx^{wxt} If^1/SM1	60E6-8 to 60F1-2

2-2.11.5 3rd Chromosome deficiency kit

Name of deficiency	Cytological region removed in deficiency
Df(3L)emc5 red^1/TM2 emc^2 p^p Ubx^{130} e^s	61C3-4 to 62A8
Df(3L)R-G5 ve^1/TM6	62A10-B1 to 62C4-D1
Df(3L)R-G7 ve^1/TM6B Tb^+	62B8-9 to 62F2-5
Df(3L)M21 ri^1 p^p/Dp(3;3)$T33^Lf19^R$	62F to 63D
Df(3L)HR370/Dp(3;3)$T33^Lf19^R$	63A1 to 63D10
Df(3L)HR232/TM6B	63C6 to 63D3
Df(3L)HR119/TM6B	63C6 to 63E
w^{1118} ; Df(3L)GN50/TM8 1(3)DTS4^1 th^1 st^1 Sb^1 e^1	63E1-2 to 64B17
Df(3L)GN24/TM8 l(3)4^{DTS} th^1 st^1 Sb^1 e^1	63F4-7 to 64C13-15
Df(3L)ZN47 ry^{506}/TM3	64C to 65C
Df(3L)pbl-X1/TM6B	65F3 to 66B10
y^1 w^1 N^{spl-1} ; Df(3L)66C-G28/TM3	66B8-9 to 66C9-10
Df(3L)h-i22 Ki^1 roe^1 p^p/TM3	66D10-11 to 66E1-2
Df(3L)29A6 ri^1 p^p/TM3	66F5 to 67B1
Df(3L)AC1 roe^1 p^p/TM3	67A2 to 67D7-13 or 67A5 to 67D9-13
y^{17} ; Df(3L)lxd6/TM3 y^+ Sb^1 e^1 Ser^1	67E1-2 to 68C1-2
Df(3L)vin2/TM3	67F2-3 to 68D6
Df(3L)vin5 $e^{1?}$/TM3 Sb^1 Ser^1	68A2-3 to 69A1-3
Df(3L)vin7 $e^{1?}$/TM3	68C8-11 to 69B4-5
Df(3L)Ly mwh^1/TM1 jv	70A2-3 to 70A5-6

3rd chromosome deficiencies cont....

Deficiency name	Cytological region removed by deficiency
Df(3L)fz-GR3b/TM6b	70C1-2 to 70D4-5
Df(3L)fzM21/TM6	70D2-3 to 71E4-5
Df(3L)BK10 ri^1 e^1/TM3	71C to 71F
Df(3L)brm11/TM6C cu^1 Sb^1 e^1 ca^1	71F1-4 to 72D1-10
Df(3L)st-f13 Ki^1 roe^1 p^p/TM6B	72C1-D1 to 73A3-4
Df(3L)81K19/TM6B	73A3 to 74F
Df(3L)W10 ru^1 h^1 sbd^2/TM6B	75B3-6 to 75C
Df(3L)Cat/TM6 Hn^p ss^{p88} bx^{34e} Ubx^{P15} e^1	75B8 to 75F1
Df(3L)W4 ru^1 h^1 e^1 ca^1/TM6B	75B10 to 75 C1-2
Df(3L)VW3/TM3	76A3 to 76B2
Df(3L)rdgC th^1 st^1 in^1 ri^1 p^p/TM6C Sb^1 cu^1 e^s Tb^1	77A1 to 77D1
Df(3L)ri79C/TM3	77B-C to 77F-78A
Df(3L)Pc-MK/TM3 Sb^1 Ser^1	78A3 to 79E1-2
Dp(3;1)2-2 w^{1118} ; Df(3R)2-2/TM3	81F to 82F10-11
Df(3R)Tp110 Dp(3;3)Dfd^{rv1} ri^1 p^p/TM3	83C1-2 to 84B1-2
Df(3R)Scr p^p e^s/TM3	84A1-2 to 84B1-2
Df(3R)Antp17/TM3	84B1-2 to 84D11-12
Df(3R)p712 T(2;3)p712 red^1 e^1/TM3	84D4-6 to 85B6
Df(3R)p-XT103 ru^1 st^1 e^1 ca^1/TM3	85A2 to 85C1-2
Df(3R)by10 red^1 e^1/TM3	85D8-12 to 85E7-F1
Df(3R)by62 T(2;3)by^{62} red^1 e^1/TM1	85D11-14 to 85F6
Df(3R)Kx1/TM3 Sb^1 Ser^1	86C1 to 87B1-5
Df(3R)T-32 ri^1 cu^1 sr^1 e^s/MRS	86E2-4 to 87C6-7
Df(3R)ry615/TM3 Sb^1 Ser^1	87B11-13 to 87E8-11
Df(3R)$ry^{506-85C}$/MKRS; Dp(3;Y)$ry^{506-85C}$	87D1-2 to 88E5-6
Df(3R)red1/TM1	88B1 to 88D3-4
Df(3R)P115 e^{11}/TM1 ; Dp (3;1)P115 Tp(3;1)P115	89B7-8 to 89E7-8
Df(3R)C4 p^*/DP(3;3)P5 Sb^1	89E to 90A
Df(3R)P14 sr^1/T(2;3)ap^{Xa}	90C2-D1 to 91A1-2
Df(3R)ChaM7/TM6B	91A to 91F5
Df(3R)D1-BX12 ss^1 e^4 ro^1/TM6B	91F1-2 to 92D3-6
Df(3R)e-R1/TM3	93B3-5 to 93D2-4
Df(3R)e-N19/TM2	93B to 94
Df(3R)crbS87-4 st^1 e^1/TM3 Ser^1	95E8-F1 to 95F15
Df(3R)crbS87-5 st^1 e^1/TM3 Ser^1	95F7 to 96A17-18
Df(3R)XS Dp(3R) asp^1 ats^1 p^p/TM6B; y/Dp(1;Y)y^+In(3R)Ubx^{7LL} ats^R	96A1-7 to 96A21-25
Df(3R)XtAI th^1 st^1 ri^1 roe^1 p^1/Dp(3;3) SuM(3)w13 st^1 e^1	96B to 96D
Df(3R)T1-P e^1 ca^1/TM3 Ser^1	97A to 98A1-2
w^{1118} ; Df(3R)3450/TM6B	98E3 to 99A6-8
Df(3R)L127/TM6 ; Dp(3;1)B152	99B to 99E
Df(3R)B81 P{ry^+}F2-80 e^1/TM3 ; Dp(3;1)67A	99C8 to 100F5
Df(3R)awd-KRB ca^1/TM3 y^+ Sb^1 e^1 Ser^1	100C-D

2-2.11.6 Other *Drosophila* strains

Strain	Obtained from
w ; *DmcycE$^{14.11G}$*	J. Pispa, Columbia University, New York
w ; *DmcycE^{JP}*	J. Pispa, Columbia University, New York
w ; *DmcycE^{JP}* isogenic for 2nd and 3rd chromosomes	Generated by H. Richardson
dp b DmcycE^{JP} cn bw	Generated by H. Richardson
w^{1118} ; *Adh/SM6a* ; *Δ2-3/TM6B*	Laboratory stock
GMR-*GAL4, UAS*-type I *cyclin E*	H. Richardson
GMR-*GAL4, UAS*-type II *cyclin E*	D. Crack
phyl^{2245}	*phyllopod* null allele. G. Rubin
Df(2R) 3072r (deletes 51A5 to 51C1)	Deficiency used for *Su(DmcycE^{JP})2-2* analysis. Obtained from G. Rubin
Df(2L)E71, Df(2L)TW3, Df(2L)PR-A16 and Df(2L)OD15	Deficiencies used for defining *Su(DmcycE^{JP})2-3* critical region
l(2)36Fb^{1} rdo^{1} hk^{1}pr^{1}/CyO	EMS mutant used for analysis of *Su(DmcycE^{JP})2-3*
l(2)36Fd^{1} rdo^{1} hk^{1}pr^{1}/CyO	EMS mutant used for analysis of *Su(DmcycE^{JP})2-3*
l(2)36Fe^{1} rdo^{1} hk^{1}pr^{1}/CyO	EMS mutant used for analysis of *Su(DmcycE^{JP})2-3*
l(2)36Ff^{1} rdo^{1} hk^{1}pr^{1}/CyO	EMS mutant used for analysis of *Su(DmcycE^{JP})2-3*
l(2)36Fg^{1} rdo^{1} hk^{1}pr^{1}/CyO	EMS mutant used for analysis of *Su(DmcycE^{JP})2-3*
l(2)36Fh^{1} rdo^{1} hk^{1}pr^{1}/CyO	EMS mutant used for analysis of *Su(DmcycE^{JP})2-3*
l(2)36Fi^{1} rdo^{1} hk^{1}pr^{1}/CyO	EMS mutant used for analysis of *Su(DmcycE^{JP})2-3*
l(2)37Aa^{1} rdo^{1} hk^{1}pr^{1}/CyO	EMS mutant used for analysis of *Su(DmcycE^{JP})2-3*
l(2)37Ab^{1} rdo^{1} hk^{1}pr^{1}/CyO	EMS mutant used for analysis of *Su(DmcycE^{JP})2-3*
l(2)37Ac^{1} rdo^{1} hk^{1}pr^{1}/CyO	EMS mutant used for analysis of *Su(DmcycE^{JP})2-3*
Df(2R)AA21, Df(2R)PI13, Df(2R)PK1	Deficiencies used to refine *Su(DmcycE^{JP})2-6* region.
Df(2L)esc-P2-0, Df(2L)esc-P3-0, Df(2L)J39, Df(2L)J2, Df(2L)J3, Df(2L)J77, Df(2L)J27 and Df(2L)J1	Deficiencies used to refine *Su(DmcycE^{JP})2-1* critical region.

2-2.12 Fly media

Fortified (F1) Drosophila medium : 1% (w/v) agar, 18.75% compressed yeast, 10% treacle, 10% cornmeal (polenta) 2.5% tegosept mix (10% *para*-hydroxybenzoate in ethanol) and 1.5% acid mix (47% propionic acid/4.7% orthophosphoric acid).

Grape juice agar plates : 0.3% Agar, 25% grape juice 0.3% sucrose 0.03% tegosept mix.

2-3 Methods

Miscellaneous, well established molecular biological techniques were carried out according to protocols published previously (Ausubel, *et al.*, 1994)

2-3.1 Isolation of DNA

(i) Small scale preparation of plasmid DNA

A single colony was innoculated into 4ml of L-broth (supplemented with antibiotic), and incubated at overnight at 37°C with shaking. DNA was then isolated as described by Murphy and Kavanagh, 1988.

(ii) Large scale preparation of plasmid DNA

A single colony was innoculated into 50 ml of L-broth (supplemented with appropriate antibiotic), and incubated at overnight at 37°C with shaking. DNA was isolated using the QIAGEN kit.

(iii) Isolation of genomic DNA from *Drosophila.*

20-25 flies were collected and frozen at -20°C. Frozen flies were kept on ice and homogenised in 100µl ice cold 0.1M Tris pH 9.0, 0.1M EDTA in a microfuge tube. 100µl of pre-warmed (65°C) 2% SDS was added to the tubes and incubated at 65°C for 30 minutes. After allowing the samples to cool to room temperature, 42µl of 5M potassium acetate, pH5.2 was added and incubated on ice for 15 minutes. Cell debris was pelleted by centrifugation at 13,000rpm for 10 minutes. The supernatant from this was incubated on ice for a further 15 minutes and re-centrifuged. The supernatant from this was phenol/chloroform extracted three times and incubated with 1µl of 10mg/ml RNase for 15 minutes at 37°C. Following one more phenol/chloroform extraction, DNA was precipitated by adding an equal volume of isopropanol and incubation at room temperature for at least 15 minutes. DNA was pelleted by centrifugation at 13,000 for 15 minutes and the pellet washed twice in 70% ethanol and resuspended in 20µl of water.

2-3.2 Generation of recombinant plasmids

After the vector plasmid DNA was linearised by restriction enzyme digestion, 2 units of CIP were added to the restriction digestion mix and incubated at 37°C for 30 minutes. Linear vector molecules were then purified by phenol/chloroform extraction. Ligations of 100ng total DNA were performed with insert:vector ratio of 3:1 in 10-20µl 30mM tris-HCl

pH7.8, 10mM MgCl2, 10mM DTT, 0.5mM ATP and 1U T4 DNA ligase at room temperature for 2-15h.

Transformation procedure for plasmid recombinants.

500ml of L-broth was inoculated with 1/100 volume of a fresh overnight culture (usually DH5α) and cells were grown to A_{600} of 0.3-0.4 at 37°C. Bacterial cells were then harvested by centrifugation. The cells were then resuspended in 1 volume of ice-cold water, pelleted, resupended in ½ volume of ice-cold water, pelleted and resuspended in $^1/_{25}$ volume of ice-cold 10% sterile glycerol. The competent cells were then snap frozen and stored as 45ml aliquots at -80°C. For transformation, cells were thawed at room temperature, added to 1-5µl of salt free ligation reaction mixture and incubated on ice for 30 seconds. Cells were then transferred to an ice-cold electroporation cuvette and electroporated in a Bio-Rad "Gene Pulser" at 2000V. The cuvette was then washed out with 1 ml of SOC, and the suspension incubated at 37°C for 30 minutes. The cells were then plated on appropriate plates. If selection for β-galactosidase activity was required, 10 µl of 10% IPTG (in water) and 10µl of 20% BCIG (in DMSO) were added prior to plating.

2-3.3 DNA sequencing

Automated sequencing was done at the Department of Molecular Pathology at the Institute of Medical and Veterinary Science (IMVS).

2-3.4 Southern Analysis

Hybridisation of radiolabelled probes to membrane immobilised nucleic acids

DNA was digested, run on an agarose gel and transferred to nitrocellulose or nylon and crosslinked to the filter using 12000 microjoules/cm^2 UV irradiation ('Stratalinker'). Membranes were incubated in a hybridisation solution of 50% formamide, 100µg/ml denatured salmon sperm DNA, 0.5% skim milk powder at 42°C for at least 1 hour. DNA fragments to be used as probes were then radiolabelled using a 'Megaprime' DNA labelling kit (Amersham) and unincorporated nucleotides were removed by spinning the reaction through a sephadex G-25 column. Labelled probes were boiled for 5 minutes before being added to hybridisation mix and incubated at 42°C for 2-24 hours. After removal of the probe, membranes were washed twice at room temperature with a solution of 2 x SSC, 0.1% SDS then twice for 15 minutes at 65°C with a solution of 0.1 x SSC, 0.1% SDS. Membranes were exposed for various lengths of time to X-Omat film (kodak) in an autoradiography cassette at room temperature or -80°C with a calcium tungstate intesifying screen. Alternatively,

membranes were exposed to a phosphorimager capture screen followed by laser scanning and image analysis (Fujix BAS1000 scanner and MacBas software).

2-3.5 Mutagenesis of *Drosophila*

(i) X-ray mutagenesis

3-5 day old *Drosophila* males were placed into empty vials (~100 in each) and treated with 4000 rads of X-rays in a CIS biointernational X-ray machine using a ^{137}Cs radiation source (activity 3400Ci). Mutagenised flies were then allowed to recover for four hours with food before being added to three day old virgin females. The flies were turned into new bottles after two days, and removed after four days.

(ii) EMS mutagenesis

The generation of mutations in *Drosophila* using EMS was carried out as described in *'Drosophila*: A practical approach' D.B. Roberts pp40-42.

(iii) Nomenclature of second chromosome suppressors of $DmcycE^{JP}$

The 10 second chromosome suppressor complementation groups that have more than one allele were assigned a gene name based on the isolation of these genes as suppressors of the $DmcycE^{JP}$ rough eye phenotype. These genes were therefore named $Su(DmcycE^{JP})2\text{-}X$, where X is 1 to 10. In this thesis, when referring to the gene the full name is used (e.g. $Su(DmcycE^{JP})2\text{-}1$), but when a specific allele is referred to, it is abbreviated to $2\text{-}X^{allele\ name}$. Single alleles are referred to by their allele name only (e.g. *22S10*).

2-3.6 *Drosophila* embryo collection and fixation

Flies were allowed to lay on grape juice agar plates smeared with yeast. Embryos were then collected into a sieve from the plate using PBT. To dechorionate the embryos, the sieve was placed in a solution 2% solution of sodium hypochlorite for 2-3 minutes. Embryos were then washed thoroughly before being fixed in 2-phase mixture (equal volumes) of heptane and 4% paraformaldehyde in PBS. Embryos were shaken in this mixture for 15-20 minutes, and the paraformaldehyde removed. To remove the vitelline membrane, methanol was added the heptane and shaken vigorously for 30 seconds. De-vitellinised embryos fall from the interface to the bottom and were collected and put in a microfuge tube. Embryos were rinsed several times in methanol, then several times in ethanol. At this point, embryos were either stored at -20°C for later use or used immediately for either *in situ* hybridisation or antibody staining.

2-3.7 Whole mount antibody staining of embryos

Unless otherwise indicated, all washes and incubations were done with gentle agitation. Fixed embryos were re-hydrated in PBT for at least 30 minutes before being 'blocked' using blotto for at least 30 minutes.

(i) Detection of single antigen

Primary antibody was added to the embryos at the dilution specified in section 2-2.2 and incubated overnight at 4°C. An appropriate secondary antibody conjugated to biotin or a direct secondary antibody conjugated to HRP or a fluorophore was added to the embryos and incubated at room temperature for 2 hours. After washes in PBT, if a tertiary complex was required, streptavidin conjugated to HRP was added (ABC kit, vectastain) and incubated for at least 30 minutes at room temperature. HRP was detected colourmetrically after further washes using a solution of 0.5mg/ml DAB, 0.0045% H_2O_2 and 0.64µg/ml $NiCl_2$. (Blue precipitate). The colour reaction was stopped by several quick washes in PBT and the embryos mounted in 80% glycerol.

(ii) Detection of two antigens

Detection of two antigens was required when the *wg-lacZ* reporter was used to detect homozygous mutant embryos. In this case, both primary antibodies were added together and incubated at 4°C overnight. β-galactosidase was then detected by using a direct secondary antibody conjugated to HRP, and the other antigen via biotin. The two secondary antibodies were added together and incubated for 2 hours at room temperature. After washes, β-galactosidase signal was detected using the same for a single antigen except that no $NiCl_2$ was added to the mix causing the precipitate to be brown instead of blue. Any remaining HRP activity was then removed with the addition of 0.02% sodium azide to the first wash after the colour detection. The tertiary reagent (streptavidin-HRP) was then added to the embryos to detect the other antigen and this colour detection was done with the addition of $NiCl_2$ to give a blue precipitate.

2-3.8 Whole mount *in situ* hybridisation of embyros

(i) Generation of DIG labelled RNA probes

Antisense RNA probes were generated from plasmids containing the gene of interest, linearised using an enzyme that cuts at the 5' end of the clone. RNA was transcribed in vitro, incorporating DIG-11-UTP using T7 or T3 RNA polymerase using the promoter at the 3' end of the clone. The labelling reaction was done using a Boehringer Mannheim DIG RNA labelling kit (SP6/T7) according to suppliers instructions.

(ii) Hybridisation and colour detection

Embryos were fixed as described in section 2-3.6, and rehydrated in PBT for at least 30 minutes. Embryos were then rinsed in a 50:50 mix of PBT and hybridisation mix (50% formamide, 5 x SSC, 50mg/ml heparin, 0.1% Tween-20, 100mg/ml sonicated boiled salmon sperm DNA), then rinsed once in hybridisation mix and then put at 55°C for at least 2 hours. The RNA DIG probe then added to the embryos (50µl total volume) and incubated at 55°C overnight. The next day, embryos were washed once with pre-hybe mix (55°C), once with a 50:50 mix of PBT and pre-hybe (55°C), and 5 times (20 minutes each) in PBT (55°C). The embryos were then incubated with 1/2000 dilution anti-DIG-AP for 1 hour at room temperature, followed by four 20 minute washes in PBT. Embryos were then rinsed in AP buffer, then the AP detected by incubating embryos in a solution of (per 1ml) 3.5µl BCIP (50mg/ml in DMF) and 4.8µl NBT solution (100mg/ml in 70% DMF) in darkness. Embryos were then rinsed several times in PBT and mounted in 80% glycerol if no antibody staining was required subsequently. For immunostaining after *in situ* hybridisation, the staining was allowed to go a little darker and, after washing, embryos were blocked in blotto for at least 30 minutes, and continued with as per normal for immunostaining (section 2-3.7).

2-3.9 BrdU incorporation

(i) BrdU incorporation into embryos.

BrdU incorporation into embryos was done as described in Richardson *et al* 1993. If β-galactosidase was also being detected, this was done first, followed by hydrolysing embryos and detection of incorporated BrdU.

(ii) BrdU incorporation into larval tissues

Carried out as described in Secombe *et al.*, 1998.

2-3.10 Antibody staining of larval tissues

Discs were dissected in 1 x PBS and fixed in a mixture of 3 parts 1.5 x Brower fix (1.5 Brower fix: 0.15M PIPES, 3mM $MgSO_4$, 1.5mM EGTA, 1.5% NP-40 pH6.9), 0.5 part 16% Formaldehyde and 0.5 parts water for 30 minutes on ice. Discs were washed in PBT-BSA (1 x PBS, 0.3% triton-X100, 1mg/ml BSA) and blocked with a solution of 1 x PBS, 0.3% triton-X100, 5mg/ml BSA for at least 1 hour on ice. The primary antibody was added directly to the blocking solution and incubated at 4°C overnight. The next day, discs were washed extensively in PBT-BSA before adding the secondary antibody conjugated to biotin diluted in PBT-BSA. After several washes at 4°C, the tertiary antibody (streptavidin-FITC)

was added and incubated with the discs at 4°C for at least 2 hours. After further washes, the discs were stored in a solution of 30% glycerol, 50mM Tris pH8.8, 150mM NaCl, 0.02% sodium azide until ready to be mounted. To prevent photobleaching, discs were mounted in a solution of 30% glycerol, 50mM Tris pH8.8, 150mM NaCl, 0.02% sodium azide, 0.5mg/ml p-Phenylenediamine p1519 and photographed.

2-3.11 Preparation of adult wings

Adult *Drosophila* wings were placed in zylene for at least two hours, then mounted in Canadian Balsam on a microscope slide and a coverslip placed over the top.

2-3.12 Adult eye sections

Sectioning of *Drosophila* adult eyes was carried out as described by Lockett *et al.*, 1993.

2-3.13 Western analysis

(i) Protein extraction from larval eye discs

Eye discs were dissected in PBS and placed directly into protein sample buffer and frozen and stored at -20°C. Before loading, samples were vortexed for one minute and boiled for 3 minutes then loaded onto a SDS-polyacrylamide gel (10%) and Western blot carried out.

(ii) SDS-polyacrylamide gel electrophoresis

Protein extracts were run on a 0.8mm 10% SDS-polyacrylamide gel with a 4% stacking gel using the Mini-PROTEAN II Duel Slab Cell (Bio-Rad) according to the manufacturers instructions. Protein samples were loaded and electrophoresed at 200V for 45 minutes

(iii) Western blot

Gels to be transferred to nitrocellulose for Western blotting were soaked in protein gel transfer buffer for 1 hour. Protein was then transferred from the gel to nitrocellulose using a Bio-Rad semidry electroblotter according to suppliers instructions. After rinsing with PBT, the filter was blocked using Blotto at room temperature for 1-2 hours, and incubated with the primary antibody at 4°C overnight. After extensive washing in PBT, the secondary antibody (conjugated to HRP) was diluted in Blotto and incubated at room temperature for 2 hours. Signal was detected using ECL kit (Amersham)was used according to suppliers instructions.

2-3.14 Scanning electron microscopy

Drosophila eyes were prepared for scanning electron microscopy by dehydration in acetone. Flies were then mounted on studs and viewed without coating using a field emission scanning electron microscope at 1 kilovolt accelerating voltage and spot size 3.

2-3.15 Generation of germ-line and somatic clones

Germline clones were generated as described in Chou and Perrimon, 1996. Somatic clones were generated as described in Xu and Rubin, 1993.

2-3.16 Genetic interaction analysis

For genetic interaction analysis, $DmcycE^{JP}$ was made isogenic for the second and third chromosomes to minimise any variation in phenotype due to genetic background. In all cases a consistent phenotype was observed when flies were maintained at 25°. In order to test mutations in genes for dominant genetic interactions with $DmcycE^{JP}$, stocks were generated that contained $DmcycE^{JP}$ (either heterozygous over *CyO* or homozygous) together with the test allele over a balancer chromosome. For test alleles on the second chromosome, recombinants with $DmcycE^{JP}$ were generated using marked $DmcycE^{JP}$ stocks and the recombinant stock maintained over a *CyO* or a *Cy-Tb* second chromosome balancers. For test alleles on the X chromosome, the stock was balanced over the *FM7C* w^a, *y, B* chromosome. Test alleles on the third chromosome were maintained over *TM6B, Tb, Hu* or *TM2, Ubx* balancer chromosomes.

For analysis of genetic interactions with $DmcycE^{JP}$, stocks were outcrossed to $DmcycE^{JP}$ at 25° and progeny that were homozygous for $DmcycE^{JP}$ and heterozygous for the test allele (at least 50 progeny) were scored for their eye phenotype compared with $DmcycE^{JP}$. For BrdU-labelling experiments, stocks containing the test allele on the third chromosome were balanced over the *TM6B, Tb* chromosome while those containing the test allele on the second chromosome were balanced over a *Cy- Tb* chromosome. Flies were outcrossed to $DmcycE^{JP}$ and non-tubby larvae (those that were homozygous for $DmcycE^{JP}$ and heterozygous for the test allele) were selected for analysis. To obtain $DmcycE^{JP}$ larvae containing one copy of the *RBF* deficiency *Df(1)Su(s)83* chromosome, for BrdU-labelling, *Df(1)Su(s)83,* $white^+$ */FM7C,* $white^a$; $DmcycE^{JP}$ females were crossed to $white^+$; $DmcycE^{JP}$ males and from this cross female larvae with yellow Malpighian tubules ($white^+$ homozygotes) were selected.

2-3.17 Light microscopy and photography

Photography was done with either Kodak Ektachrome 160T film after viewing on a Zeiss Axiophot light microscope using 10X eyepiece lenses and Plan-Neofluar 20X/0.5 or 40X/0.75 objectives with DIC optics. Slides were scanned in with a Kodak RFS 2035 film scanner at >500dpi. Adobe photoshop 4.0 was used for image preparation. Colour prints were obtained using a Kodak XLT7720 Digital Continuous Tone Printer.

2-3.18 Computer analysis

(i) Analysis of DNA sequence from the 32A-E genomic region.

To estimate the number of genes in the 32A-E region, DNA sequence available from the BDGP was analysed using blastx (Altschul *et al.*, 1997) using the NCBI (National Center for Biotechnology Information) database (http://www.ncbi.nlm.nih.gov/), which translates the sequence in all six possible reading frames and compares this to protein sequences in the database. In addition, the sequence was analysed using the BDGP blastn (http://fruitfly.org/blast/), to determine whether there were any ESTs in the region.

(ii) Analysis of genes and mutations in a given cytological region.

To identify genes within a given cytological region, the 'cytosearch' program avaliable as part of Flybase was used (www://flybase.bio.indiana.edu/). This program enables genes, deficiencies, other lesions and P1 genomic clones within a given region to be identified.

2-4 Glossary

Markers

aristaeless (*al*)Homozygous viable mutation that results in strongly reduced aristae. Maps genetically to 2-0.4 and cytologically to 21C1-2.

black (*b*). Homozygous viable mutation that results in adults with very dark body colours. Maps genetically to 2-48.5 and cytologically to 34D4-6.

cinnabar (*cn*). Homozygous viable mutation that results in adults with bright orange eyes. Maps genetically to 2-57.5 and cytologically to 34E14-15.

curved (*c*) Homozygous viable. Homozygotes have wings that curve downward. Maps genetically to 2-75.5 and cytologically to 52D2-9.

brown (*bw*). Homozygous viable mutation that results in adults with brown eyes. Maps genetically to 2-104.5 and cytologically to 59E1-3.

Ovo^{D1} Dominant female sterile mutation. Females do not lay eggs. Used in generation of germ-line clones.

speck (*sp*) Homozygous viable mutation that results in axil of wings having a black speck. Maps genetically to 2-107.0 and cytologically to 60B13-60C5.

Tubby Dominant larval phenotype that results in third instar larvae being shorter and fatter than wild-type. Homozygous lethal.

Balancer chromosomes

Curly Dominant wing marker that results in flies with curled wings. Homozygous lethal.

*Cy*O. Second chromosome balancer. Carries the dominant marker *Curly*, and the recessive marker *cn*. Homozygous lethal.

TM6B Third chromosome balancer. Carries the dominant marker *Tubby*. Homozygous lethal

Cy-Tb Second chromosome balancer that has part of *TM6B* translocated onto the second. Has both *Curly* and *Tubby* dominant markers. Homozygous lethal.

Chapter 3: Characterisation of the *DmcycE^{JP}* phenotype

3-1 Introduction

As described in Chapter 1, one *white*$^-$ revertant of the adult viable *P*[*w*$^+$*m-lac*] element allele *14.11G* insertion was found to be homozygous viable, but adults had rough eyes when transheterozygous for *DmcycE* null alleles (Secombe *et al.*, 1998). This allele, *DmcycE^{JP}*, is an internal deletion of the *DmcycE$^{14.11G}$* *P* element that removes the *P* element *white* gene but maintains the *amp^R* and *lacZ* genes and the *P* element ends. This chapter describes the characterisation of the *DmcycE^{JP}* phenotype, and the analysis of genetic interactions between *DmcycE^{JP}* and genes known to have a role in the regulation of the cell cycle. These interactions demonstrate that *DmcycE^{JP}* is a hypomorphic *cyclin E* allele, and the rough eye phenotype of *DmcycE^{JP}* homozygotes is sensitive to the dose of *DmcycE* interacting genes.

3-2 Anaysis of the *DmcycE^{JP}* phenotype

Homozygous *DmcycE$^{14.11G}$* flies were found to be viable, fertile and indistinguishable from wild-type in their rate of development, but a minor eye defect was observed (Figure 3.1B compared to 3.1A). Eyes from *DmcycE$^{14.11G}$* adults appeared slightly reduced in size on the ventral side and occasionally contained regions of mild disorganisation. *DmcycE^{JP}* flies are also homozygous viable and fertile but have eyes with disorganised ommatidia, blistering on the surface of the eye and defects in bristle numbers and arrangement (Figure 3.1C). The eye is the only consistently affected tissue in *DmcycE^{JP}* flies, although at low frequency, wing defects such as small notches at the posterior/distal side of the wing and a shortening of the 5th vein were also observed (Figure 3.2; see also Table 3.1). A slight delay in development time was also observed (11 days vs 10 days to eclosion at 25°C), indicating that there may be other effects on cell proliferation in *DmcycE^{JP}* mutants.

To confirm that the *DmcycE^{JP}* rough eye and wing phenotypes were due to the presence of the internally deleted *P* element, *Δ2-3* transposase was used to mobilise the *P* element from *DmcycE^{JP}*. As the deletion within the *DmcycE^{JP}* *P* element removed the mini-*white* gene, which would normally serve as a marker for *P* element reversion, revertants were scored for loss of the rough eye phenotype when transheterozygous with *DmcycE^{JP}* (Figure 3.3; Figure 3.4). Reversion of the *DmcycE^{JP}* rough eye phenotype to wild-type occurred at a high frequency (90%), indicating that the internally deleted *P* element was responsible for the rough eye phenotype of *DmcycE^{JP}*. In addition, amplification of genomic DNA spanning the site of insertion demonstrated that in three out of four cases examined, the *P* element had

excised precisely (Figure 3.4). Thus, the *DmcycE*^{JP} rough eye mutant phenotype is due to the internally deleted *P* element. Similarly, the wing notching and vein defects associated with *DmcycE*^{JP} flies are also due to the presence of the *P* element, as these phenotypes are not present in at least one of the *DmcycE*^{JP} revertants obtained (Table 3.1).

Table 3.1 Wing phenotypes of *DmcycE*^{JP} and *DmcycE*^{JP} revertants

Strain	Number of flies with wing notches		Number of flies showing reduced 5th wing vein		Total
	One wing	Both wings	One wing	Both wings	
DmcycE^{JP}	10 (7.8%)	0	7 (5.5%)	2 (1.6%)	128
revertant #2	0	0	0	0	145
revertant #1	0	0	3 (2%)	0	146

Wings from *DmcycE*^{JP} and revertant adults were scored for the number of wing defects. At a low frequency, *DmcycE*^{JP} flies show wing notching in the posterior/distal portion of the wing, and a reduction in the 5th wing vein (Figure 3.2). The reduction or absence of these wing notchings and 5th wing vein shortenings in *DmcycE*^{JP} revertants demonstrates that these phenotypes are due to the presence of the *P* element.

3-3 The rough eye phenotype of *DmcycE*^{JP} is due to a reduction in S phases during eye development

As seen in figure 3.1C, flies homozygous for the *DmcycE*^{JP} allele have rough eyes. The degree of disorganisation of the eyes from *DmcycE*^{JP} homozygotes, however, was not as severe as that seen in eyes of individuals transheterozygous for *DmcycE*^{JP} and the amorphic allele, *DmcycE*^{AR95}, or the *DmcycE* deficiencies, *Df(2L)TE35D-3* or *Df(2L)TE35D-1* (Figure 3.5B, C compared with 3.5A; and data not shown). To test whether the converse was true, the dosage of *DmcycE* was increased by generating flies transheterozygous for *DmcycE*^{JP} and weak alleles of *DmcycE*. The first of these, *DmcycE*^{PZ01672}, is a homozygous viable partial female sterile *P* element allele that shows very mild eye roughening when placed over *DmcycE* null alleles (Lilly and Spradling 1996; data not shown). Two other weak alleles, *DmcycE*^{P28} and *DmcycE*^{P41} were also tested. *DmcycE*^{P28} is an EMS-generated allele that is homozygous viable, female sterile and has a milder rough eye phenotype than *DmcycE*^{JP} (data not shown). Flies transheterozygous for *DmcycE*^{JP} and *DmcycE*^{PZ01672} had essentially wild-type eyes (data not shown), whereas *DmcycE*^{JP}/*DmcycE*^{P41} and *DmcycE*^{JP}/*DmcycE*^{P28} flies showed a milder rough eye phenotype than homozygous

Figure 3.1 *DmcycE*^{JP} flies have rough eyes and a disorganised array of ommatidia.

Scanning electron micrographs of adult eyes from (A) wild-type, (B) *DmcycE*$^{14.11G}$, (C) *DmcycE*^{JP}. *DmcycE*$^{14.11G}$ shows only slight roughening and a slight reduction in the size of the eye on the ventral side compared to wild-type, whereas *DmcycE*^{JP} eyes show disorganisation in the arrangement of ommatidia, blistering on the surface of the eye and bristle number defects. Eyes are orientated with anterior to the right and dorsal side up.

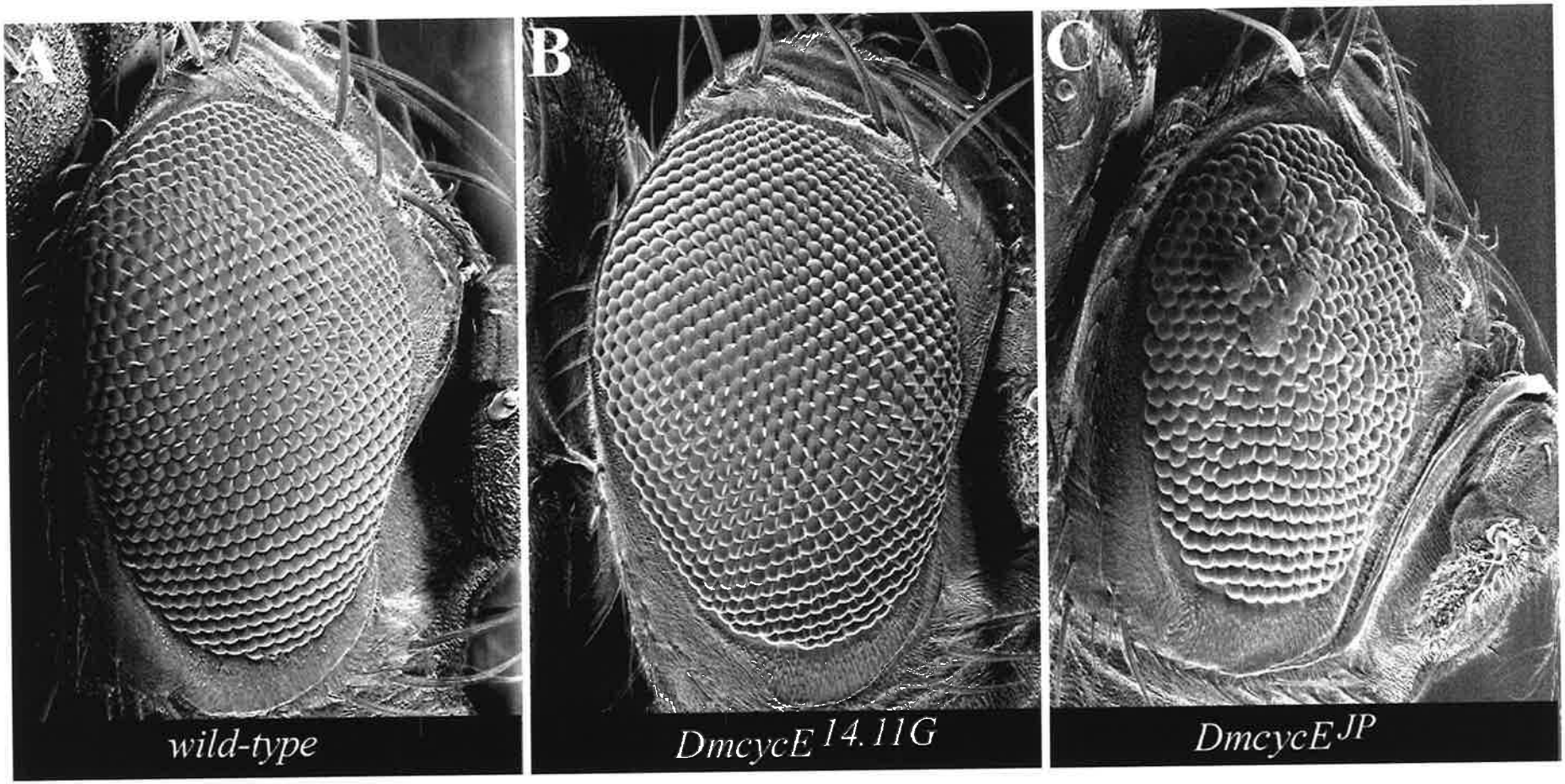
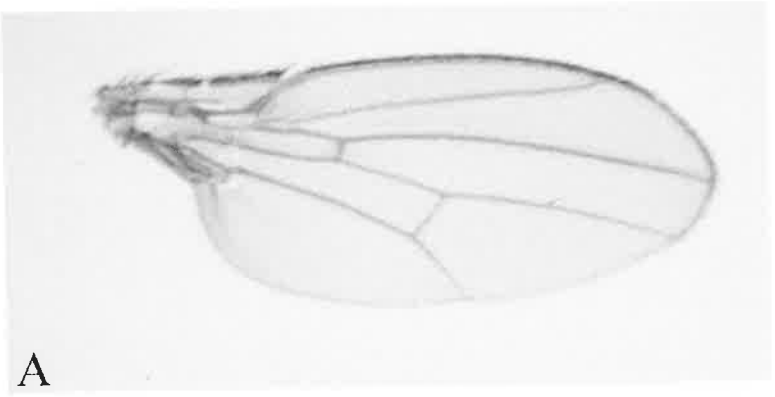
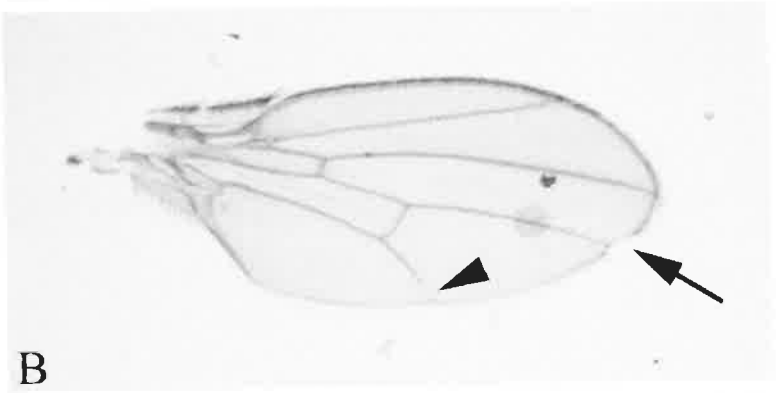

A
wild-type
B
DmcycE 14.11G
C
DmcycE JP

Figure 3.2 *DmcycE*^{JP} wings have notches and reduction in 5th wing vein.

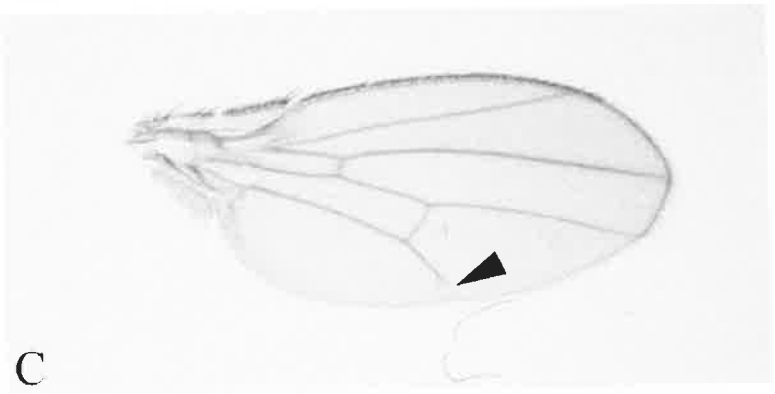
Wings from (A) wild-type and (B and C) homozgyous *DmcycE*^{JP} adults. At a low frequency, *DmcycE*^{JP} flies have wing notches at the posterior/distal part of the wing (indicated by arrow in B) and/or a reduction in the 5th wing vein (indicated by arrowhead in B and C). The frequency of these wing defects in *DmcycE*^{JP} revertants is markedly decreased or absent, indicating that they are due to the presence of the *DmcycE*^{JP} *P* element (see Table 3.1).



A



B



C

Figure 3.3 Reversion of the *DmcycE^{JP} P* element

To verify that the rough eye phenotype of *DmcycE^{JP}* flies was due to the internally deleted *P* element, *b DmcycE^{JP} bw* flies were crossed to a strain carrying Δ2-3 transposase. From the progeny of this cross, straight or curly winged males also carrying Δ2-3, which can be identified by the presence of the *Stubble* (*Sb*) dominant marker, were picked to cross to *b DmcycE^{JP} cn bw* flies. The progeny of this cross were scored for reversion of the rough eye phenotype to wild-type. Reversion to wild-type occurred at a frequency of approximately 70% if *DmcycE^{JP}/SM6a* males were used and at approximately 90% if *DmcycE^{JP}/Adh* males were used. The reason for the increase in reversion frequency with the *Adh* chromosome is presumably due to chromosome pairing and gene conversion after *P* element excision, which is inhibited by the *SM6a* balancer chromosome (Engels *et al.*, 1990). To generate stocks of revertants, *b DmcycE^{JP} bw/b DmcycE^{JP} cn bw* flies with wild-type eyes were crossed to *SM6a cn* and non-cn, curly progeny picked and crossed together.

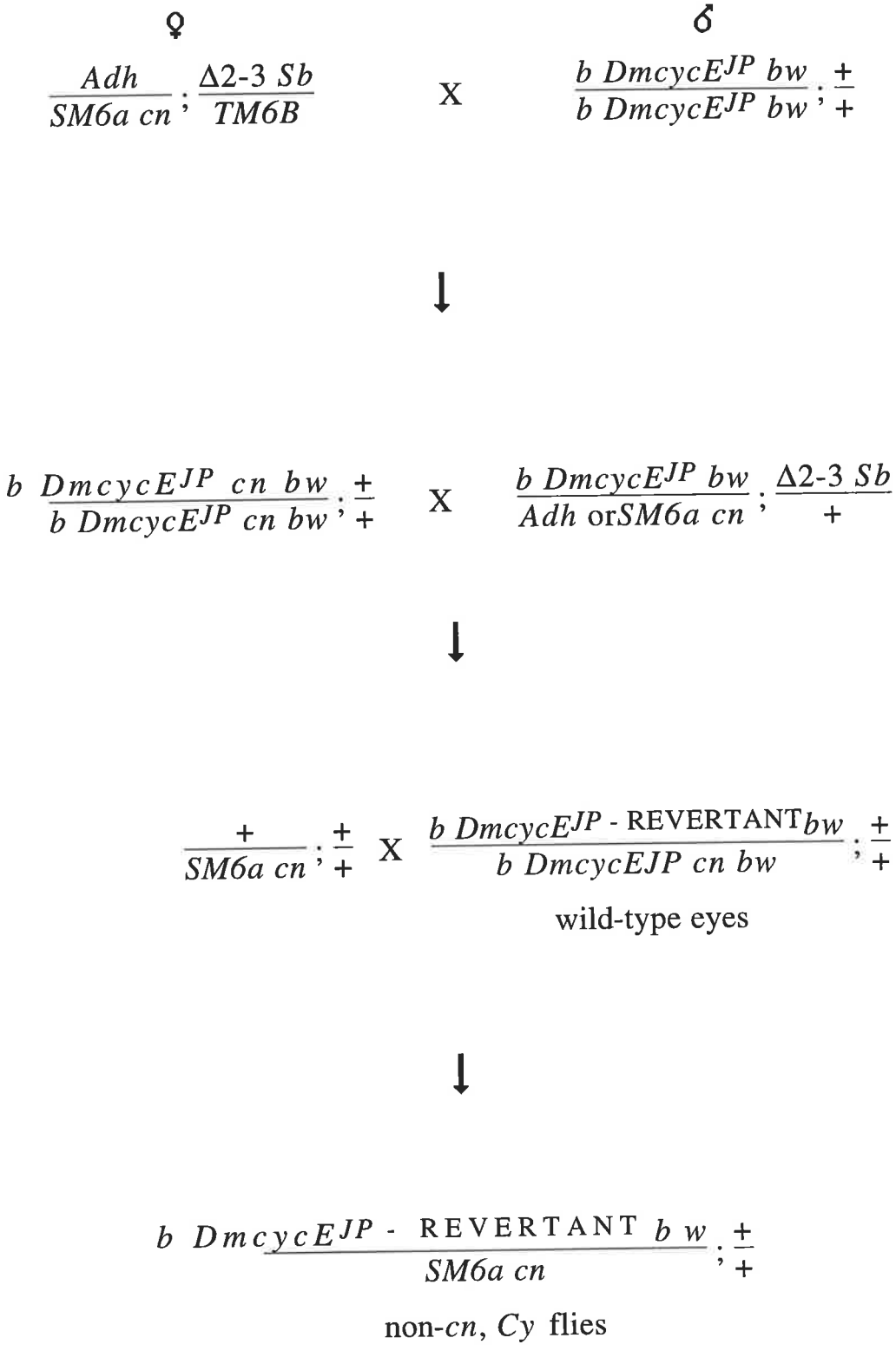
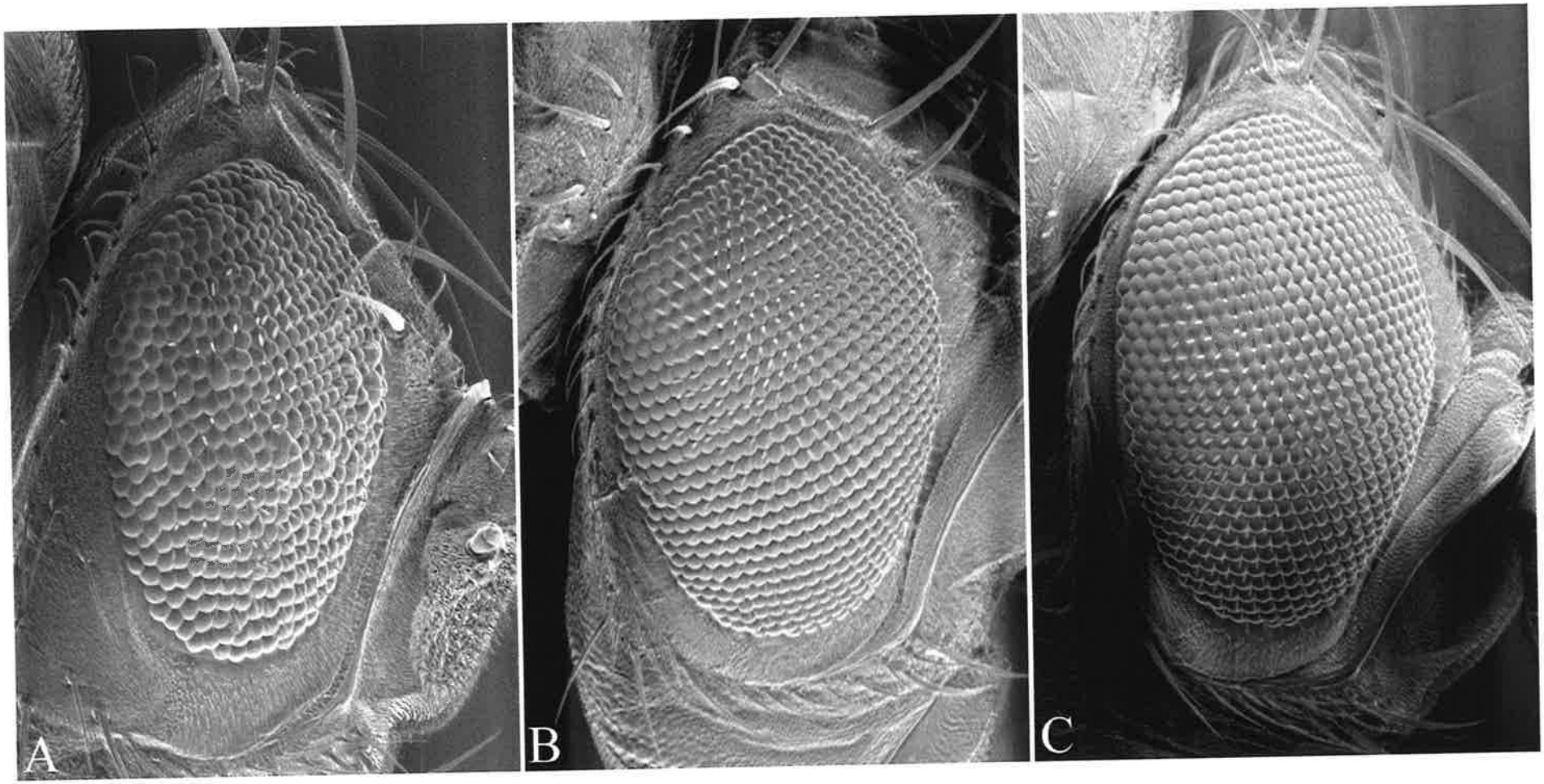

♀
♂
Adh / SM6a cn ; Δ2-3 Sb / TM6B
X
b DmcycE^JP bw / b DmcycE^JP bw ; + / +
b DmcycE^JP cn bw / b DmcycE^JP cn bw ; + / +
X
b DmcycE^JP bw / Adh or SM6a cn ; Δ2-3 Sb / +
+ / SM6a cn ; + / +
X
b DmcycE^JP - REVERTANT bw / b DmcycEJP cn bw ; + / +
wild-type eyes
b DmcycE^JP - REVERTANT b w / SM6a cn ; + / +
non-cn, Cy flies

Figure 3.4 *DmcycE*^{JP} revertants have wild-type eyes

Scanning electron micrographs of adult eyes from (A) *DmcycE*^{JP} (B) *DmcycE*^{JP} revertant #1 and (C) *DmcycE*^{JP} revertant #2. Stocks were generated from four revertants isolated as described in figure 3.4, all of which had wild-type eyes. (D) PCR analysis using genomic DNA from the four revertants. Primers flanking the site of insertion of the JP *P* element (described in materials and methods, section 2-2.6) were used to confirm that the *P* element had excised precisely to give wild-type eyes in these flies. Lane 1, amplification using genomic DNA from w^{1118} flies resulted in the expected 255 bp product (arrow). Lane 2, *DmcycE*^{JP}. No product is generated using *DmcycE*^{JP} genomic DNA, as the *P* insert is too large to allow amplification within the extension time used. Amplification from *DmcycE*^{JP} revertant #1, *DmcycE*^{JP} revertant #2, and *DmcycE*^{JP} revertant #4 genomic DNA (Lanes 3, 4 and 6, respectively) result in the same size product as w^{1118} indicating that precise excision has occurred in all of these cases. *DmcycE*^{JP} revertant #3 (Lane 5) gives a larger PCR product, ~400 bp (arrowhead), indicating that part of the original *P* element is probably still present in this strain, although *DmcycE*^{JP} revertant #3 flies have wild-type eyes (not shown).


A
B
C

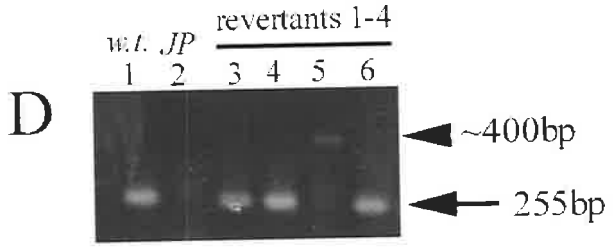
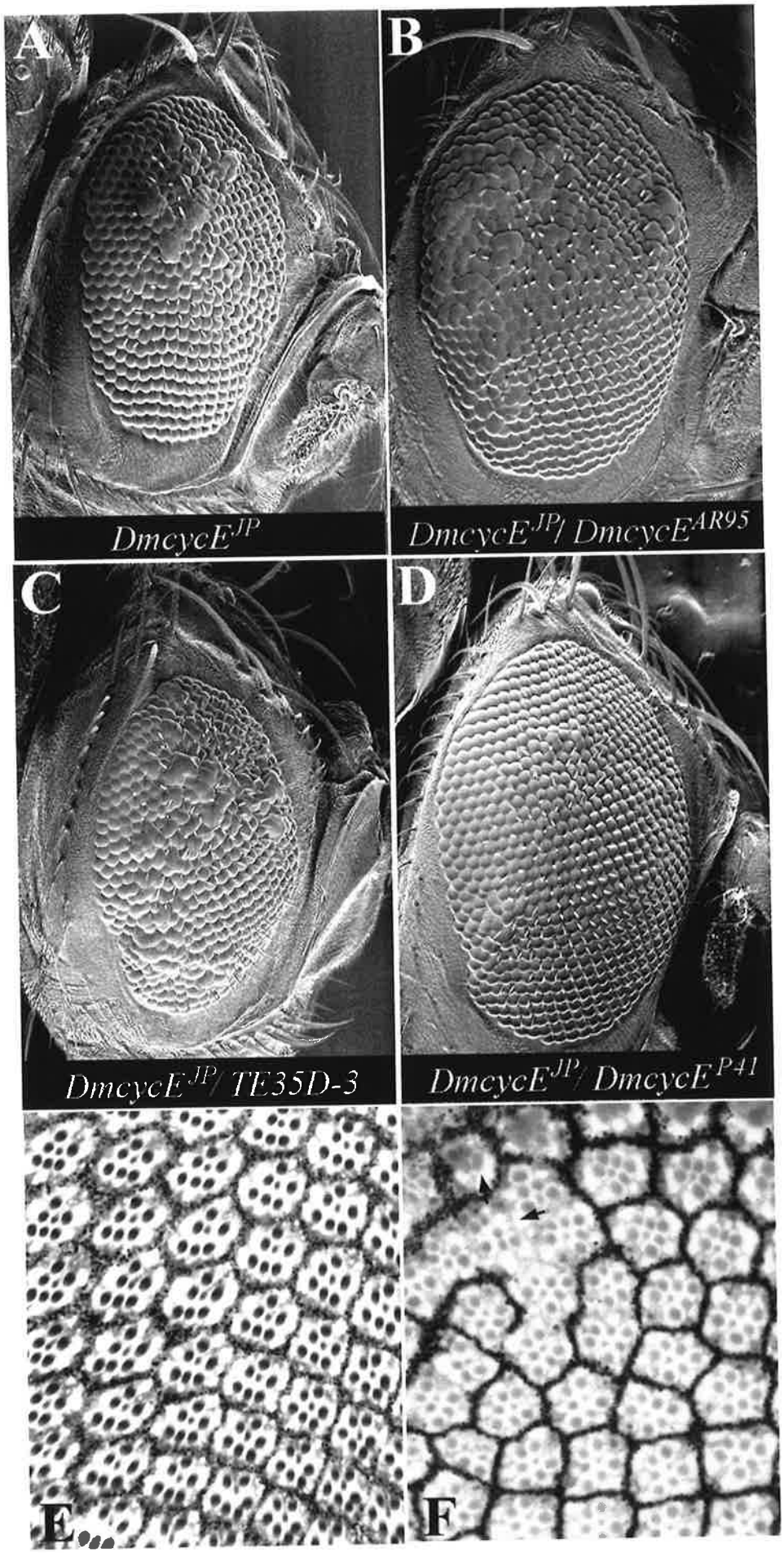

D
w.t.
JP
revertants 1-4
1 2 3 4 5 6
~400bp
255bp

Figure 3.5 The *DmcycE*^{JP} phenotype is sensitive to the dose of *cyclin E.*

Scanning electron micrographs of adult eyes from (A) *DmcycE*^{JP} (B) *DmcycE*^{JP}/*DmcycE*^{AR95}, (C) *DmcycE*^{JP}/*TE35D-3* (*DmcycE* deficiency) and (D) *DmcycE*^{JP}/*DmcycE*^{P41}, showing enhancement of the *DmcycE*^{JP} rough eye phenotype when the dosage of *DmcycE* is decreased further using the amorphic *DmcycE*^{AR95} allele or the *TE35D-3* deficiency or suppression when *DmcycE* dosage increased using the hypomorphic *DmcycE*^{P41} allele. Eyes are orientated with anterior to the right and dorsal side up. Transverse sections of adult eyes from (E) wild-type and (F) *DmcycE*^{JP}, showing the fusion of ommatidia due to the loss of pigment cells (stained black). The arrows (in F) point to examples of ommatidia containing reduced complements of R cells.


A
DmcycE^JP
B
DmcycE^JP/ DmcycE^AR95
C
DmcycE^JP/ TE35D-3
D
DmcycE^JP/ DmcycE^P41
E
F

DmcycE^{JP} flies (Figure 3.5D compared with 3.5A; and data not shown). The eyes of these transheterozygous flies were larger, the arrangement of ommatidia appeared more even and there was less blistering. Thus the *DmcycE*^{JP} phenotype is capable of being suppressed by increasing the dosage of *DmcycE* and enhanced by decreasing the dosage of *DmcycE.* These results suggested that the *DmcycE*^{JP} allele is affecting expression of *DmcycE* in the eye, but is not a null allele in the eye, and that the degree of disorganisation of *DmcycE*^{JP} adult eyes is sensitive to the dose of *DmcycE.*

To examine the cellular basis of the eye disorganisation, sections were taken of adult eyes from *DmcycE*^{JP} (Figure 3.5F). The most common defect observed in *DmcycE*^{JP} was the reduction or absence of pigment cells surrounding the photoreceptor cells, resulting in the fusion of ommatidia. The number and arrangement of photoreceptor (R) cells within each ommatidium was generally normal (Figure 3.5F compared with 3.5E). Occasionally, more severe defects were observed where there were less than seven R cells per ommatidium (Figure 3.5F; and data not shown). The reduction in pigment cells, one of the last types of cells to be recruited during eye development, suggests that *DmcycE*^{JP} eye imaginal discs are defective in the cell cycle that occurs posterior to the morphogenetic furrow (MF). This possibility was examined by monitoring S phases by Bromo-deoxyuridine (BrdU)-labelling (Figure 3.6C). While S phases in antennal discs from *DmcycE*^{JP} third instar larvae are similar to wild-type, there was a clear effect on eye imaginal disc S phases. In homozygous *DmcycE*^{JP} eye discs, the number of cells entering S phase both posterior to the morphogenetic furrow (MF), and in the undifferentiated asynchronously proliferating region anterior to the MF, were dramatically reduced (Figure 3.6C compared with 3.6A; Table 3.2). In addition, *DmcycE*^{JP} eye discs were smaller than wild-type, indicating that earlier in development cell proliferation of the undifferentiated eye disc cells is also reduced. The reduction in eye imaginal disc S phases accounts for the disorganisation and the reduction in size observed with *DmcycE*^{JP} adult eyes. Consistent with the adult eye phenotype of *DmcycE*$^{14.11G}$, the number of S phases from *DmcycE*$^{14.11G}$ eye discs was not noticeably different from wild-type (Figure 3.6B compared with 3.6A).

Table 3.2 Quantitation of the number of S phase cells posterior to the morphogenetic furrow in eye imaginal discs

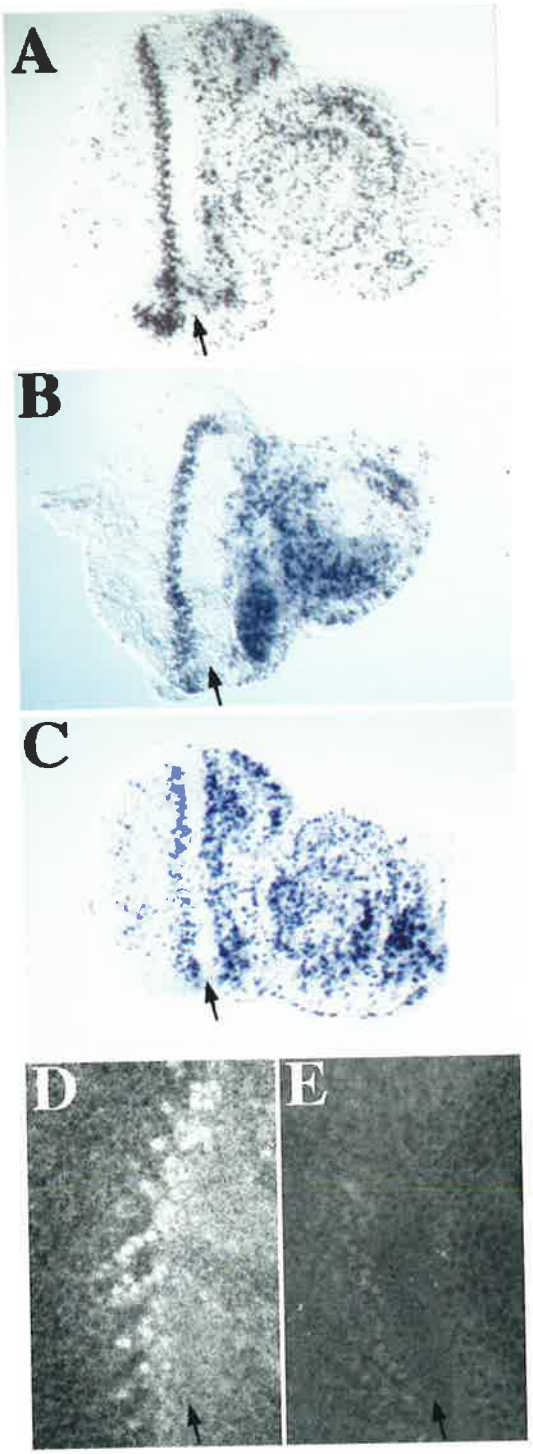
Strain	S phase cells (± standard deviation)
w^{1118} (wild-type)	54 ± 2.7
$DmcycE^{JP}$	31.2 ± 3.0
$DmcycE^{JP}$, $cdc2^{E1-9}/DmcycE^{JP}$, +	29.8 ± 2.1
Df RBF/+; $DmcycE^{JP}$	46.6 ± 3.4
GMR-RBF	40.8 ± 1.4
$DmcycE^{JP}$; *GMR-RBF*	24.7 ± 1.6
$DmcycE^{JP}$, $dap^4/DmcycE^{JP}$, dap^4	38.2 ± 3.5
GMR-p21^{CIP1}	5.6 ± 2.7
$DmcycE^{JP}$; *GMR*-p21^{CIP1}	0.2 ± 1.0
$DmcycE^{JP}$; $dE2F^{91}$/+	21.3 ± 1.3
$DmcycE^{JP}$, *dDP*/$DmcycE^{JP}$, +	24 ± 2.5
$DmcycE^{JP}$, *Df cyclin B*/$DmcycE^{JP}$, +	29 ± 1.0
$DmcycE^{JP}$; *cyclin* A^5/+	30 ± 3.3
$DmcycE^{JP}$; *string*^{AR2}/+	29 ± 2.3
$DmcycE^{JP}$, $rca1^{3300}/DmcycE^{JP}$, +	29.6 ± 3.7
$roughex^8$/+; $DmcycE^{JP}$	31 ± 2.6
$DmcycE^{JP}$; *P*[*ry*$^+$]*roughex*	30.5 ± 2.6

The average number of S phase cells immediately posterior to the MF was determined by counting S phase cells within a 200 micron region along the dorsal/ventral axis in the center of each eye imaginal disc. The sample number was at least five eye imaginal discs for each sample, except for *Df RBF/+* ; $DmcycE^{JP}$ where only three eye imaginal discs were counted.

The expression of *DmcycE* in $DmcycE^{JP}$ homozygotes was examined during embryogenesis and in various tissues during larval development by Cyclin E antibody staining (Figure 3.6D, E; data not shown). During embryogenesis, Cyclin E expression did not differ significantly from wild-type in mitotically proliferating or endoreplicating cells (data not shown). In $DmcycE^{JP}$ third instar larval brain lobes and wing, haltere, leg, and antennal imaginal discs, the level of Cyclin E protein appeared to be slightly lower than wild-type, although this did not appear to significantly affect S phases in these tissues (data not shown). In contrast, in

Figure 3.6 *DmcycE*^{JP} eye imaginal discs have a decreased number of S phase cells and reduced DmcycE protein levels.

S phase cells in eye imaginal discs, labelled with BrdU, from (A) wild-type, (B) *DmcycE*$^{14.11G}$ and (C) *DmcycE*^{JP} third instar larvae, showing that the number of S phase cells is significantly decreased in *DmcycE*^{JP} eye imaginal discs. Confocal micrographs of Cyclin E protein levels in eye imaginal discs from (D) wild-type and (E) *DmcycE*^{JP} third instar larvae, showing low levels of Cyclin E protein in *DmcycE*^{JP} eye imaginal discs relative to wild-type. Cyclin E protein was detected using an anti-Cyclin E antibody and visualised using a FITC-conjugate. D, E are shown at a higher magnification relative to A-C. The arrows indicate the morphogenetic furrow (MF). Eye imaginal discs are orientated with anterior to the right.


A
B
C
D
E

DmcycE^{JP} larval eye imaginal discs where the number of S phase cells was significantly reduced (Figure 3.6C), there was a marked reduction in the level of Cyclin E and in the number of cells expressing Cyclin E relative to wild-type (Figure 3.6E compared with 3.6D). This was confirmed by Western analysis, which showed that *DmcycE*^{JP} eye-antennal discs had significantly lower levels of Cyclin E protein compared with *DmcycE*$^{14.11G}$ and wild-type eye-antennal discs (Figure 3.7). Thus it appears that the *DmcycE*^{JP} phenotype results from a reduction of *DmcycE* expression that primarily affects the developing eye.

3-4 The *DmcycE*^{JP} rough eye phenotype represents a sensitive system for analysing *DmcycE* genetic interactors

As described above, the *DmcycE*^{JP} rough eye phenotype is sensitive to the dosage of *DmcycE*. To determine whether *DmcycE*^{JP} represents a genetically sensitive system capable of responding to the dosage of interacting genes, mutations in genes encoding products known to interact with Cyclin E were tested to determine whether they were able to modify the rough eye phenotype of *DmcycE*^{JP}.

First, the effect of halving the dosage of the *cdc2c* gene, encoding the Cdk partner of Cyclin E (Knoblich *et al.*, 1994) was tested. Surprisingly, when a deficiency known to remove *cdc2c*, *Df(3R)H*^{81}, was used to decrease the dosage of *cdc2c*, the *DmcycE*^{JP} rough eye phenotype was suppressed rather than enhanced as expected (data not shown). This may be due to the co-deletion of a gene that acts as a dosage sensitive suppressor of *DmcycE*^{JP}, although there are no obvious candidates for such a gene in the region. This observation is explored in more detail in Chapter 6, which describes the genetic interaction of *DmcycE*^{JP} with a specific *cdc2c* allele, *cdc2c*^{JS}, identified in this study. Importantly, altering the gene dosage of the G2/M regulatory protein kinase Cdk1, which does not physically interact with Cyclin E (Lehner and O'Farrell 1990b; Knoblich *et al.*, 1994), did not alter the eye phenotype or alter the S phase pattern in the eye imaginal disc of *DmcycE*^{JP} (Figures 3.8C and 3.9C; Table 3.2).

Two other genes that encode the RBF and p21^{Cip1} proteins that physically interact with Cyclin E, were also tested. The tumour suppressor protein, retinoblastoma (RBF), physically interacts with *Drosophila* Cyclin E, as Cyclin E/Cdc2c is able to phosphorylate RBF *in vitro* (Du *et al.*, 1996). Furthermore, *Drosophila RBF* has been shown to genetically interact with *DmcycE*. Decreasing the dosage of *DmcycE* by half enhances the rough eye phenotype of *GMR* (Glass Multimer Reporter)-induced ectopic expression of *RBF* posterior to the MF in eye imaginal discs (Du *et al.*, 1996). Another cell cycle regulator known to

Cyclin/Cdk activity and prevent entry into S phase (Sherr and Roberts, 1995). Ectopic expression of mammalian p21^{CIP1} using the *GMR* enhancer in the *Drosophila* eye imaginal disc has been shown to inhibit entry of cells posterior to the MF into S phase, resulting in a rough eye phenotype (de Nooij and Hariharan, 1995). This effect is overcome by overexpression of *DmcycE* (I. Hariharan, pers. com.), suggesting that the inhibition of S phase entry is due to the inhibition of Cyclin E/Cdc2c activity. A *Drosophila* homolog of mammalian p21^{CIP1}, *dacapo*, has recently been isolated and shown to inhibit Cyclin E/Cdk2 (de Nooij *et al.*, 1996; Lane *et al.,* 1996). To determine whether *RBF*, p21^{CIP1} or *dacapo* also interacted with *DmcycE*^{JP}, the dosage of these genes was increased or decreased in a *DmcycE*^{JP} background and the effect on the eye phenotype examined by scanning electron microscopy

Decreasing the dosage of *RBF* resulted in suppression of the rough eye phenotype of *DmcycE*^{JP}, whereas ectopic over-expression of *RBF,* using the *GMR* enhancer, enhanced the eye roughening observed in *DmcycE*^{JP} or *GMR-RBF* flies, which exhibit a slight eye roughening (Figure 3.8D-F compared with 3.8A). Likewise, *DmcycE*^{JP} increased the severity of the rough eye phenotype of *GMR*-p21^{CIP1} (Figure 3.8G, H). Furthermore, the *DmcycE*^{JP} rough eye phenotype was substantially enhanced by two copies of *GMR-dacapo*^{56}, which alone exhibited only a very mild eye roughening (data not shown; I. Hariharan, personal communication). Conversely, *DmcycE*^{JP} flies homozygous for the *dacapo* null mutant *dap*^{4} showed suppressed eyes (Figure3.8B). However in this case, halving the dosage of *dacapo* was not sufficient to achieve suppression of the *DmcycE*^{JP} rough eye phenotype.

To examine whether these interactions were occurring at the level of S phase, BrdU-labelling experiments were carried out (Figure 3.9B, D-F compared with 3.9A; Table 3.2). Reducing the dosage of *RBF* in a *DmcycE*^{JP} background increased the number of S phases throughout the eye disc (Figure 3.9D; Table 3.2). Increasing the dosage of *RBF* in cells posterior to the MF decreased the number of S phases in *DmcycE*^{JP} eye imaginal discs relative to *DmcycE*^{JP} or *GMR-RBF* eye discs (Figure 3.9E compared with 3.9F and 3.9A; Table 3.2). Ectopic expression of human p21^{CIP1} in cells posterior to the MF, using *GMR*-p21^{CIP1} results in a significantly decrease in S phase cells posterior to the MF (de Nooij and Hariharan 1995; Table 3.2; and data not shown). In *DmcycE*^{JP}; *GMR*-p21^{CIP1} flies these S phase cells posterior to the MF were almost completely abolished (Table 3.2). Conversely, discs homozygous for both *DmcycE*^{JP} and *dap*^{4} show an increase in the number of S phases seen posterior to the MF (Figure 3.9B; Table 3.2). These alterations in post-MF S phases, probably account for the effects seen on *DmcycE*^{JP} eye roughening.

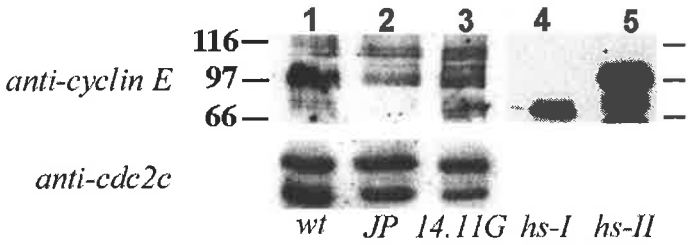


Figure 3.7 Western analysis of Cyclin E protein levels in *DmcycE*^{JP} eye-antennal imaginal discs.

Protein samples, prepared from dissected eye-antennal discs from third instar larvae, were analysed by immunoblotting using Cyclin E mouse monoclonal sera (upper panel) or with anti-Cdc2c sera (detects a doublet at ~34 kD; Knoblich *et al.*, 1994) as a loading control (lower panel), and detected using enhanced chemiluminescence. Tracks are as follows: track 1, wild-type; track 2, *DmcycE*^{JP}; track 3, *DmcycE*$^{14.11G}$; track 4, heat-shocked *hsp70-DmcycE* type I; track 5, heat-shocked *hsp70-DmcycE* type II. Tracks 4 and 5 are shown at a much shorter exposure time than tracks 1-3. Heat-shocked *hsp70-DmcycE* type I and type II serve as controls for the unmodified forms of DmcycE and give rise to bands at ~70 kD and ~ 95 kD, respectively, as well as lower molecular weight breakdown products. The DmcycE antisera detected protein bands at ~70 kD that co-migrate with the heat-shocked *hsp70-DmcycE* type I, as well as number of higher molecular weight bands that migrate between 97 kD and 116 kD in wild-type and *DmcycE*$^{14.11G}$ eye-antennal disc extracts (tracks 1 and 3; see Richardson *et al.*, 1995). Protein bands at these sizes were also detected in *DmcycE*^{JP} eye-antennal disc extracts (track 2), but the levels were significantly reduced compared with wild-type and *DmcycE*$^{14.11G}$ eye-antennal disc extracts. Molecular weight markers are indicated.

Figure 3.8 Altering the dose of *dacapo* or *RBF,* but not *cdc2,* and ectopic expression of human p21^{CIP1}, modifies the rough eye phenotype of *DmcycE*^{JP}.

Scanning electron micrographs of adult eyes from (A) *DmcycE*^{JP}, (B) *DmcycE*^{JP} *dap*^{4}/*DmcycE*^{JP}, *dap*^{4}, (C) *DmcycE*^{JP}, *cdc2*$^{E1-9}$/*DmcycE*^{JP}, +, (D) *Df(1)Su(s)83* (*RBF* deficiency)/+; *DmcycE*^{JP}, (E) *GMR-RBF,* (F) *DmcycE*^{JP}; *GMR-RBF,* (G) *GMR*-p21^{CIP1}, (H) *DmcycE*^{JP}; *GMR*-p21^{CIP1} showing that increasing the dosage of *RBF* or p21^{CIP1} enhances the rough eye phenotype of *DmcycE*^{JP} while reducing the dosage of *dacapo* or *RBF* suppresses the *DmcycE*^{JP} rough eye phenotype. Reducing the dosage of *cdc2* has no effect on the rough eye phenotype of *DmcycE*^{JP}. *GMR-RBF* by itself results in a mild rough eye phenotype and *GMR*-p21^{CIP1} alone results in a severe eye roughening, but both of these phenotypes are enhanced by *DmcycE*^{JP}. Eyes are orientated with anterior to the right and dorsal side up.

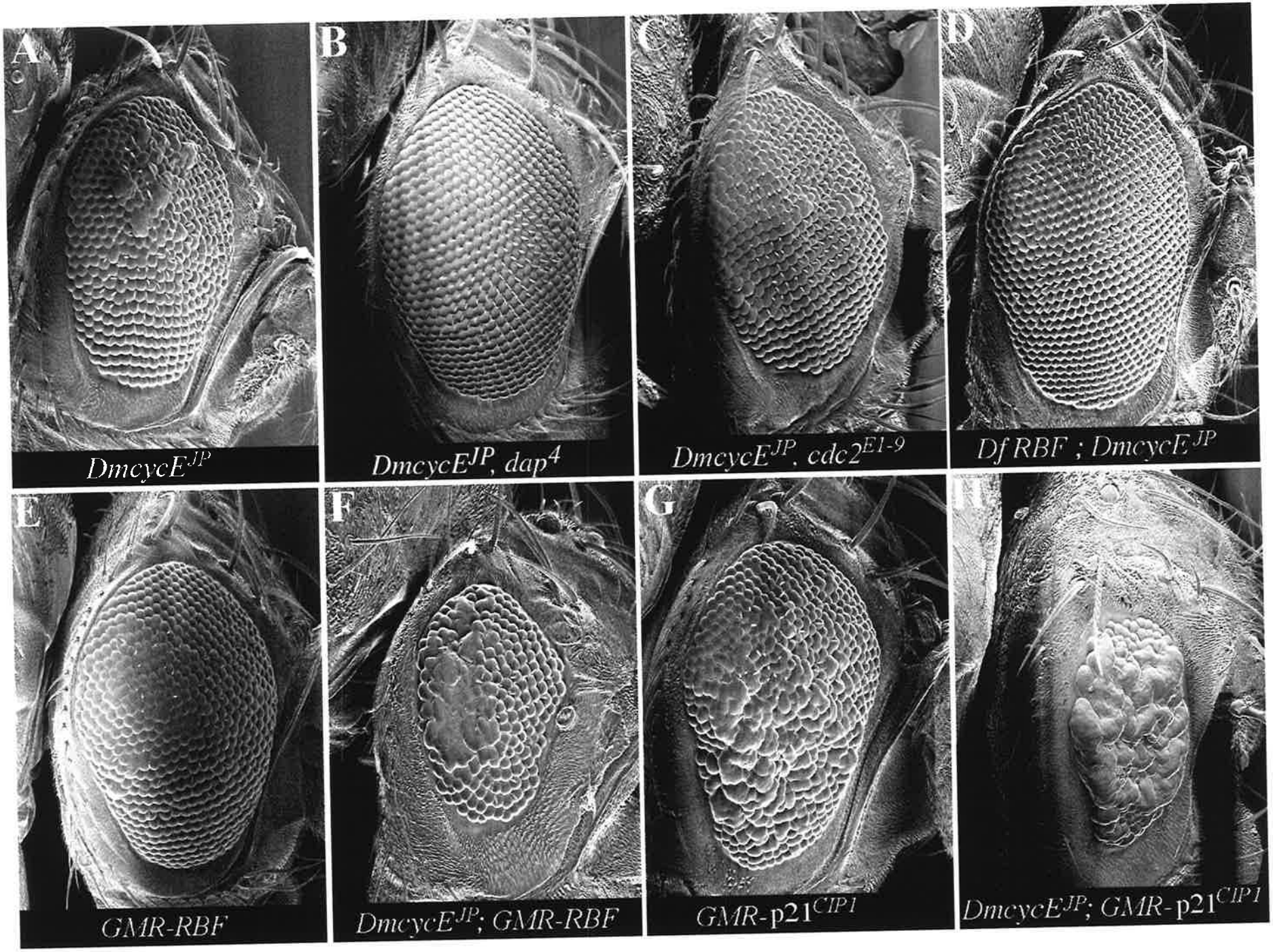
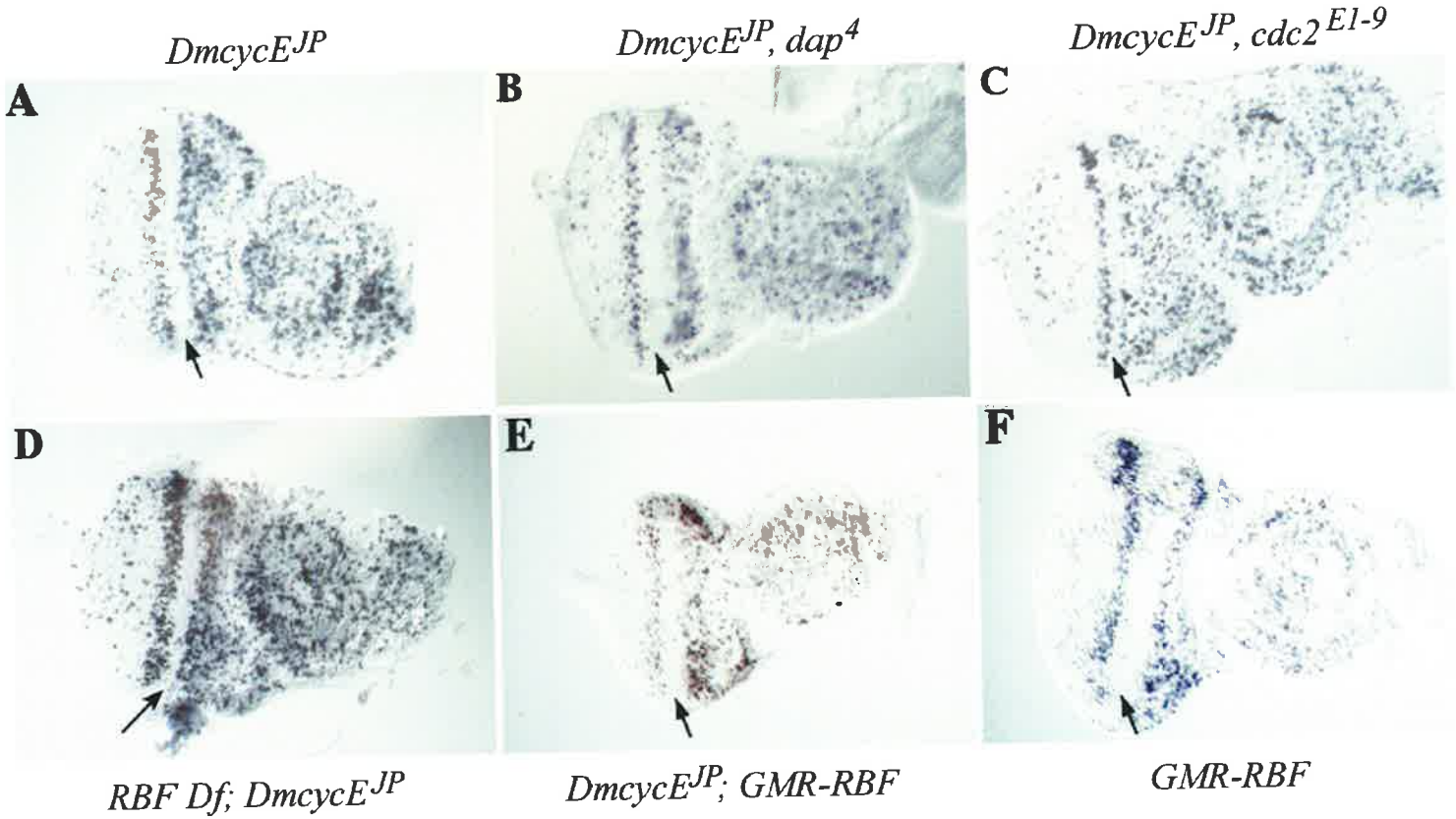

A
DmcycE^JP
B
DmcycE^JP, dap^4
C
DmcycE^JP, cdc2^E1-9
D
Df RBF ; DmcycE^JP
E
GMR-RBF
F
DmcycE^JP; GMR-RBF
G
GMR-p21^CIP1
H
DmcycE^JP; GMR-p21^CIP1

Figure 3.9 Altering the dose of *daacpo* and *RBF*, but not *cdc2* affects S phases in *DmcycE^{JP}* eye imaginal discs.

BrdU-labelling to reveal S phase cells in eye imaginal discs from (A) *DmcycE^{JP}*, (B) *DmcycE^{JP} dap^{4}/DmcycE^{JP}*, *dap^{4}*, (C) *DmcycE^{JP}*, *cdc2^{E1-9}/DmcycE^{JP}*, +, (D) *Df(1)Su(s)83* (*RBF* deficiency)/+; *DmcycE^{JP}*, (E) *DmcycE^{JP}*; *GMR-RBF* and (F) *GMR-RBF* showing that increasing the dosage of *RBF* reduces the number of S phase cells posterior to the MF in *DmcycE^{JP}* eye imaginal discs, while decreasing the dosage of *dacapo* or *RBF* increases the number of S phases posterior to the MF in *DmcycE^{JP}* eye imaginal discs. Reducing the dosage of *cdc2* has no effect the number of S phases relative to *DmcycE^{JP}*. *GMR-RBF* by itself results in a slight reduction of S phase cells posterior to the MF (see Table 3.2). Eye imaginal discs are orientated with anterior to the right. Arrow indicates MF.


DmcycE^JP
DmcycE^JP, dap^4
DmcycE^JP, cdc2^E1-9
A
B
C
D
E
F
RBF Df; DmcycE^JP
DmcycE^JP; GMR-RBF
GMR-RBF

posterior to the MF (Figure 3.9B; Table 3.2). These alterations in post-MF S phases, probably account for the effects seen on *DmcycE^{JP}* eye roughening.

These results show that the *DmcycE^{JP}* rough eye phenotype is sensitive to the dosage of the known G1 regulators *RBF, dacapo* and human p21^{CIP1}. Therefore the *DmcycE^{JP}* allele provides a unique dosage sensitive phenotype whereby genetic interactions with *DmcycE* can be explored.

3-5 Genetic interaction of *DmcycE* with other cell cycle regulatory genes

To assess the ability of other previously identified cell cycle genes to interact with *DmcycE^{JP}*, flies homozygous for *DmcycE^{JP}* and heterozygous either for specific mutations (where possible two different alleles) or for deficiencies removing these genes were generated. Genes known to have a role in the G1 to S phase transition, or required for the G2 to M phase transition in *Drosophila*, as well as genes with currently unspecified function in the cell cycle in *Drosophila*, were tested. The G1/S phase regulators tested were the S phase transcription factor genes *dE2F1* and *dDP1*, which are required for *DmcycE* transcription in endoreplicating tissues during embryogenesis (Duronio *et al.*, 1995; Royzman *et al.*, 1997; Duronio *et al.*, 1998). *Drosophila* G2 to M phase genes tested were *cyclin A, cyclin B, cyclin B3*, and the *S. pombe* Cdc25 mitotic inducer homologue, *string*. Two other cell cycle genes that have poorly defined functions in the *Drosophila* cell cycle were also tested. The first of these is the *Drosophila* homologue of the *suc1/CKS1*/p13 (*cks*) gene (*Dmcks*) (H. Richardson, P. Kylsten, B. J. Jennings, P. H. O'Farrell, S. I. Reed, and R. Saint, in preparation). Cks binds to Cdk proteins and has been shown to be required at both the G1-S phase transition and the G2-M phase transition in budding yeast, but is also capable of inhibiting entry into mitosis when overexpressed (reviewed by Pines, 1996). The second of these was *cyclin C,* which appears to play a role in general transcription (Leclerc *et al.*, 1996). The effect of altering the dosage of these genes on the *DmcycE^{JP}* rough eye phenotype was analysed by scanning electron microscopy and the results are summarised in Table 3.4.

A strong interaction was observed between *dE2F* or *dDP* and *DmcycE^{JP}*. Decreasing the dosage of either *dE2F* or *dDP* resulted in a dramatic enhancement of the *DmcycE^{JP}* rough eye phenotype (Figure 3.10B, D). BrdU-labelling experiments confirmed that the enhancement of *DmcycE^{JP}* eye roughening by halving the dosage of *dE2F* or *dDP* resulted from effects on S phase, since fewer S phase cells were observed both anterior and posterior to the MF in eye imaginal discs (Figure 3.11B, C; Table 3.2). These results show that *dE2F* and *dDP* genetically interact with *DmcycE* to promote the G1 to S phase transition.

or *Dmcks* (Figure 3.10C; data not shown). In contrast, mutations in *cyclin A* and the Cdc25 mitotic inducer, *string,* were found to be dominant enhancers of the $DmcycE^{JP}$ rough eye phenotype (Figure 3.12B, C). This result was initially surprising, given that *cyclin A* and *string* mutants arrest prior to mitosis in *Drosophila* embryogenesis (Lehner and O'Farrell, 1989; Edgar and O'Farrell, 1989). However, there is evidence that Cyclin A and String can play a role in S phase as mutations in these genes interact with *rux*, a gene required to establish the G1 arrest within the MF during eye development. Their identification as suppressors of *rux* is consistent with their ability to enhance the $DmcycE^{JP}$ rough eye phenotype.

Given the genetic interaction between $DmcycE^{JP}$ and *cyclin A* or *string*, the $DmcycE^{JP}$ rough eye phenotype was tested for sensitivity to the dosage of the Cyclin A regulators, *rux* and *rca1*. Fly stocks homozygous for $DmcycE^{JP}$ and heterozygous either for *rca1* or *rux* mutations, or homozygous for a *rux* genomic rescue construct, $P[ry^{+}]$ *rux*, were generated and adult eyes examined. Decreasing the dosage of *rca1* or increasing the dosage of *rux* resulted in an enhancement of the $DmcycE^{JP}$ rough eye phenotype while decreasing the dosage of *rux* resulted in suppression of this phenotype (Figure 3.12D-F). These genetic interactions are consistent with those observed with *cyclin A* or *string* mutations with $DmcycE^{JP}$.

Mutations in *rux* result in an adult rough eye phenotype due to ectopic S phases within the MF during eye development (Thomas *et al.,* 1994). Reducing the dose of *string* or *cyclin A* suppressed the *rux* eye phenotype by reducing the number of ectopic S phases (Dong *et al.,* 1997). Considering this evidence that Cyclin A and String are capable of functioning in entry into or progression through S phase, it was important to determine whether the interactions observed between *string* or *cyclin A* alleles and $DmcycE^{JP}$ were due to an effect on S phases. BrdU-labelling experiments were therefore carried out on larvae homozygous for $DmcycE^{JP}$ and heterozygous for *cyclin A*, *string, rux* or *rca1* mutations, or containing $P[ry^{+}]$ *rux* (Figure 3.13B-F; Table 3.2). Contrary to expectations, BrdU-incorporation revealed that decreasing the dosage of *cyclin A* or *string* in $DmcycE^{JP}$ homozygotes had no significant effect on the number of S phase cells posterior to the MF (Figure 3.13B,C, compared with 3.13A; Table 3.2). Likewise, manipulating the dosage of *rux* or *rca1* also had no significant effect on the number of S phases posterior to the MF in $DmcycE^{JP}$ eye discs (Figure 3.13D-F; Table 3.2). In addition, although more difficult to accurately quantitate, no significant effect on $DmcycE^{JP}$ S phases in the anterior asynchronously proliferating region was observed when the dosage of *cyclin A, string, rca1* or *rux* were altered (Figure 3.13B-F). Thus, unlike the genetic interaction observed between *rux* and *cyclin A, string* or *rca1* (Dong *et al.,* 1997), the

Figure 3.10 Altering the dose of *dE2F, dDP,* but not *cyclin B* modifies the *DmcycE*^{JP} rough eye phenotype.

Scanning electron micrographs of adult eyes from (A) *DmcycE*^{JP}, (B) *DmcycE*^{JP}; *dE2F*^{91}/+, (C) *DmcycE*^{JP}, *Df(2R)59AB* (*cyclin B* deficiency)/*DmcycE*^{JP}, +, (D) *DmcycE*^{JP},*dDP*^{5D}/*DmcycE*^{JP}, +, showing that reducing the dosage of *dE2F* or *dDP* enhances the rough eye phenotype of *DmcycE*^{JP}, while decreasing the dosage of *cyclin B* has no effect on the *DmcycE*^{JP} rough eye phenotype. Eyes are orientated with anterior to the right and dorsal side up.

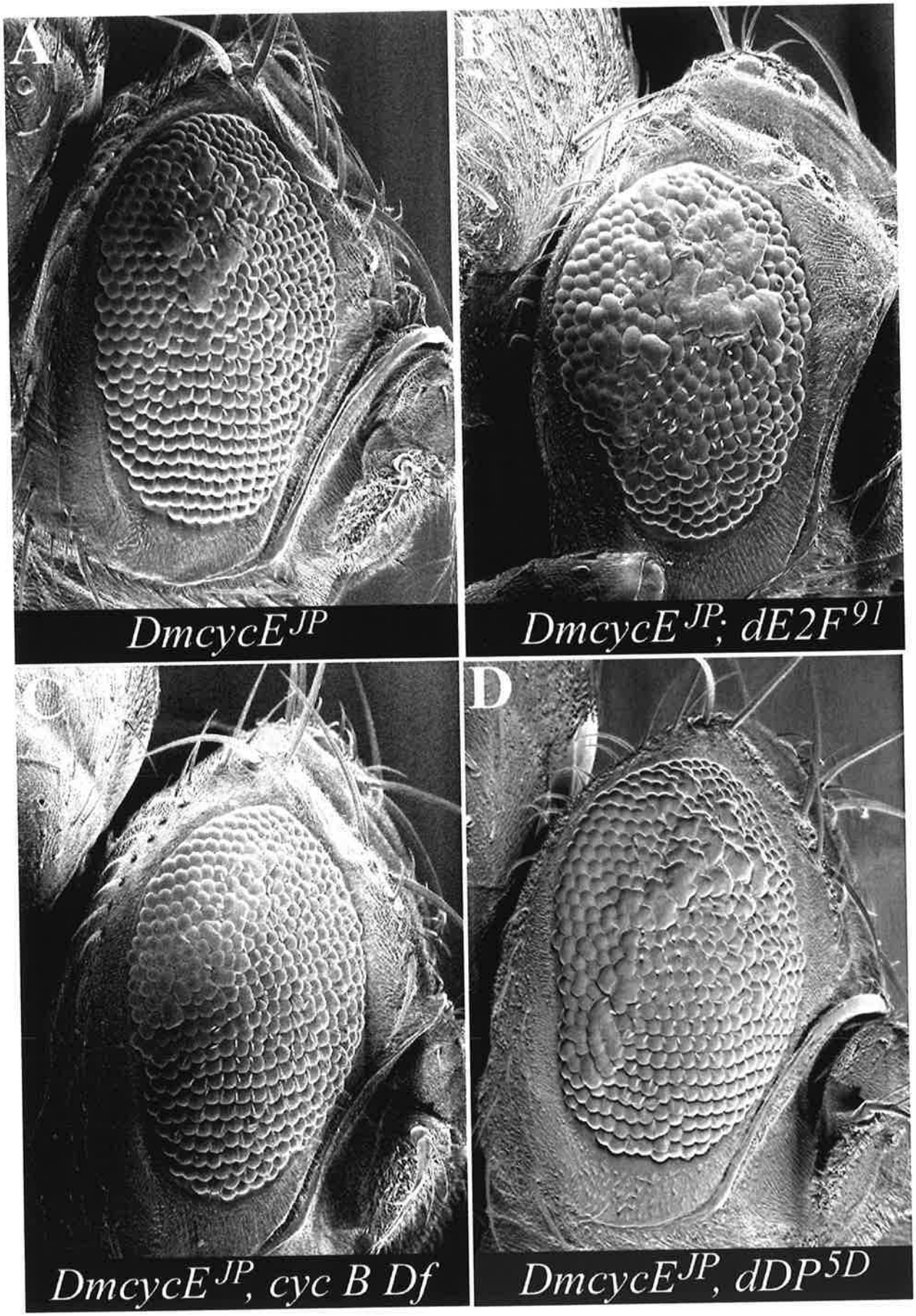
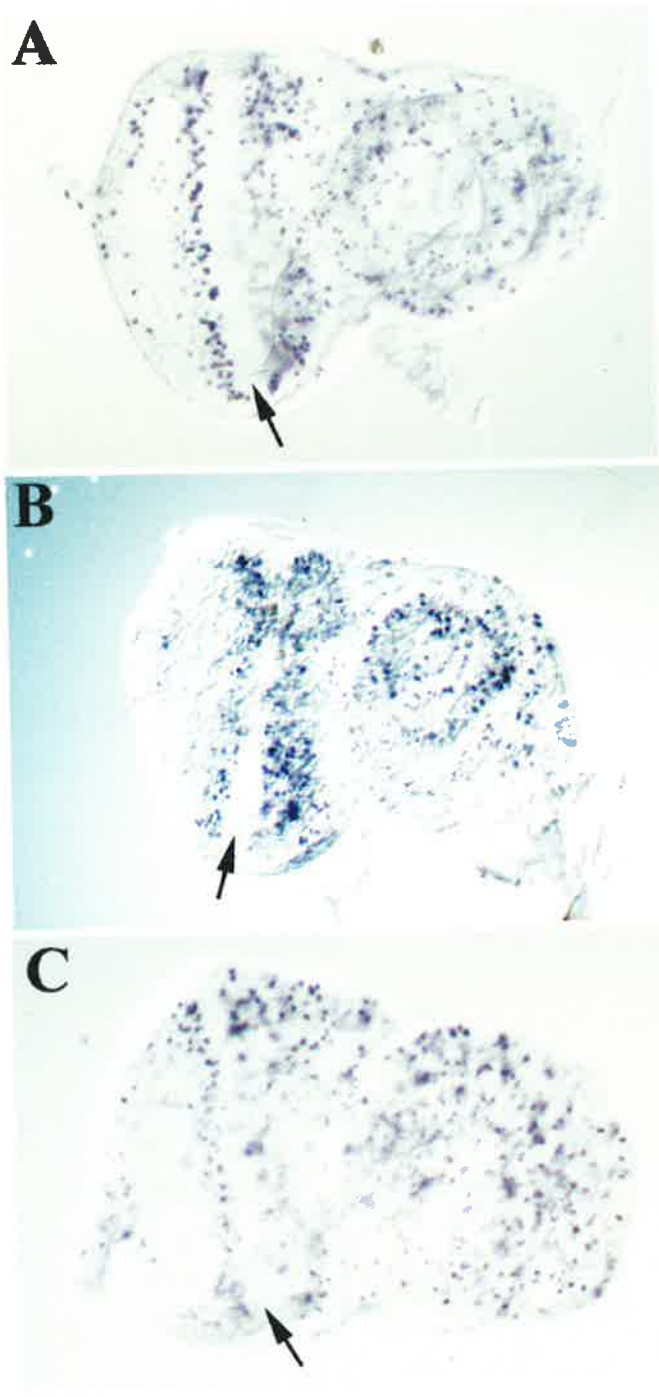

A
DmcycE^JP
DmcycE^JP; dE2F^91
DmcycE^JP, cyc B Df
D
DmcycE^JP, dDP^5D

Figure 3.11 Reducing the dose of *dE2F* or *dDP* enhances the *DmcycE*^{JP} phenotype by reducing the number of S phases

BrdU-labelling to reveal S phase cells in eye imaginal discs from (A) *DmcycE*^{JP}, (B) *DmcycE*^{JP}; *dE2F*^{91}/+, (C) *DmcycE*^{JP}; *dDP*^{5D}/+, showing that decreasing the dosage of *dE2F* or *dDP* reduces the number of S phase cells posterior to the MF in *DmcycE*^{JP} eye imaginal discs. Eye imaginal discs are orientated with anterior to the right. Arrow indicates MF.



DmcycE^{JP}

DmcycE^{JP}; dE2F^{91}

DmcycE^{JP}, dDP^{5D}

Figure 3.12 Altering the dose of *cyclin A*, *string* (*stg*) *roughex* (*rux*) or *rca1* modifies the *DmcycE*^{JP} rough eye phenotype.

Scanning electron micrographs of adult eyes from (A) *DmcycE*^{JP}, (B) *DmcycE*^{JP}; *cyclin A*^{5}/+ , (C) *DmcycE*^{JP}; *string*^{AR2}/+, (D) *DmcycE*^{JP}, *rca1*^{3300}/*DmcycE*^{JP}, +, and (E) *DmcycE*^{JP}, *P*[*ry*$^{+}$]*roughex* and (F) *roughex*^{7}/+; *DmcycE*^{JP} showing that reducing the dosage of *cyclin A*, *string* or *rca1* enhances the rough eye phenotype of *DmcycE*^{JP}, while decreasing the dosage of *roughex* suppresses the *DmcycE*^{JP} rough eye phenotype. Eyes are orientated with anterior to the right and dorsal side up.

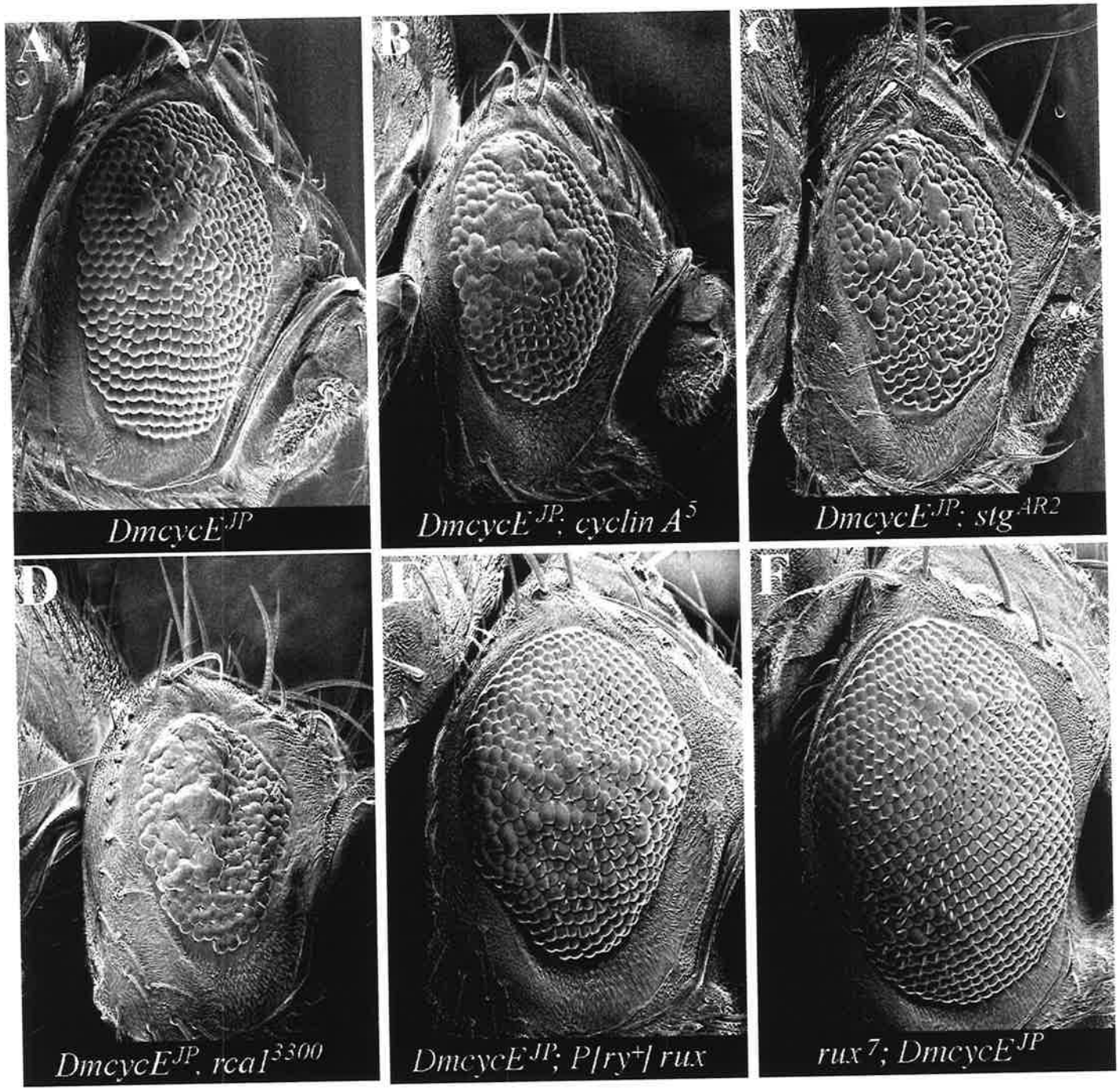
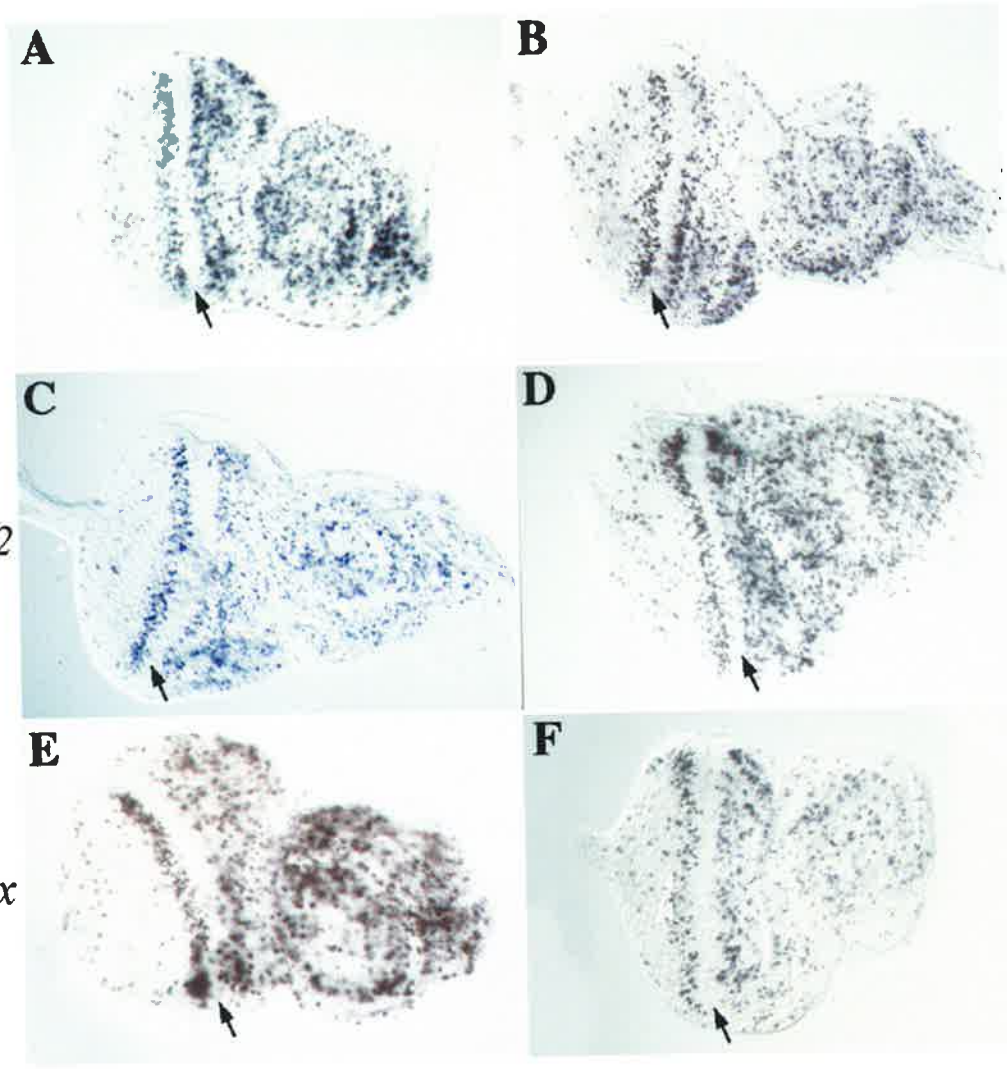

A
DmcycE^JP
B
DmcycE^JP; cyclin A^5
C
DmcycE^JP; stg^AR2
D
DmcycE^JP, rca1^3300
E
DmcycE^JP; P[ry+] rux
F
rux^7; DmcycE^JP

Figure 3.13 Altering the dose of *cyclin A, string* (*stg*), *roughex* (*rux*) or *rca1* does not affect S phases in *DmcycE*^{JP} eye imaginal discs.

BrdU-labelling to reveal S phase cells in eye imaginal discs from (A) *DmcycE*^{JP}, (B) *DmcycE*^{JP}; *cyclin A*^{5}/+ , (C) *DmcycE*^{JP}; *string*^{AR2}/+, (D) *DmcycE*^{JP}, *rca1*^{3300}/*DmcycE*^{JP}, +, (E) *DmcycE*^{JP}, *P*[*ry*$^{+}$]*roughex* and (F) *roughex*^{7}/+; *DmcycE*^{JP} showing that decreasing the dosage of *cyclin A*, *string rca1* or *rux*, or increasing the dosage of *rux*, has no significant effect on S phases. Eye imaginal discs are orientated with anterior to the right. Arrow indicates MF.



DmcycE^{JP}

DmcycE^{JP}; cyc A^{5}

DmcycE^{JP}; stg^{AR2}

DmcycE^{JP}, rca1^{3300}

DmcycE^{JP}, p[ry^{+}] rux

rux^{7}; DmcycE^{JP}

the dosage of *cyclin A, string, rca1* or *rux* were altered (Figure 3.13B-F). Thus, unlike the genetic interaction observed between *rux* and *cyclin A, string* or *rca1* (Dong *et al.,* 1997), the genetic interaction observed between $DmcycE^{JP}$ and *cyclin A, string, rux* or *rca1* does not appear to be occurring by affecting the number of S phase cells.

Considering their established role in entry into mitosis, the effect of decreasing the dosage of *cyclin A* or *string* on mitoses in $DmcycE^{JP}$ eye imaginal discs was examined. The monoclonal antibody MPM-2 which recognises an M phase phosphorylated epitope (Davis *et al.,* 1983; Westendorf *et al.,* 1994) has been widely used to examine mammalian mitotic cells. To confirm that it was also able to recognise mitotic cells in *Drosophila* tissues, eye imaginal discs were co-stained with the MPM-2 antisera and the DNA stain Hoechst 33258 (Figure 3.14A-C). A subset of cells anterior and posterior to the MF stained with the MPM-2 antibody (Figure 3.14A and 3.14D). These MPM-2 stained cells contained condensed DNA and mitotic figures as revealed by Hoechst 33258 staining (Figure 3.14B, C; and data not shown) consistent with cells in mitosis. Thus MPM-2 also stains mitotic cells in *Drosophila* tissues. To examine the effect on mitoses when the dosage of *cyclin A* or *string* was halved in $DmcycE^{JP}$ eye imaginal discs, MPM-2 staining was carried out on $DmcycE^{JP}$, $DmcycE^{JP}$; $cyclin\ A^{5}/+$ and $DmcycE^{JP}$; $stg^{AR2}/+$ and wild-type eye imaginal discs. Interesting, MPM-2 staining revealed that cells from eye imaginal discs homozygous for $DmcycE^{JP}$ were larger than from wild-type imaginal discs (Figure 3.14E-F compared with 3.14D). This is consistent with a delay in G1 phase expected for $DmcycE^{JP}$ where cells can continue to grow. Despite the lower number of S phase cells, $DmcycE^{JP}$ eye imaginal discs showed only a slightly lower number of M phase cells compared with wild-type (Figure 3.14E compared with 3.14D; Table 3.3). Significantly, halving the dosage of *cyclin A* or *string* reduced the number of mitotic cells posterior to the furrow in $DmcycE^{JP}$ eye imaginal discs (Figure 3.14F, G compared with 3.14E; Table 3.3). These results suggest that *cyclin A* and *string* enhance the $DmcycE^{JP}$ eye imaginal disc phenotype by decreasing the number of mitoses.

Table 3.3 Quantitation of the number of MPM-2 stained (M phase) cells posterior to the MF in eye imaginal discs.

Strain	M phase (± standard deviation)	S phase (± standard deviation)
w1118 (wild-type)	25.2 ± 2.6	54 ± 2.7
DmcycE^{JP}	21.5 ± 1.1	31 ± 3 0
DmcycE^{JP}; cyclin A^{5}/+	15.7 ± 1.9	30 ± 3.3
DmcycE^{JP}; stg^{AR2}/+	15 ± 2 0	29 ± 2.3

The average number of M phase cells immediately posterior to the MF was determined by counting MPM-2 stained (M phase) cells within a 200 micron region along the dorsal/ventral axis in the center of each eye imaginal disc. The sample number was at least five eye imaginal discs for each sample. The number of S phase cells in an equivalent region is from Table 2.

Table 3.3 Summary of the interaction of *DmcycE^{JP}* with other cell cycle genes

Gene	Increase dosage	Decrease dosage	Interaction
DmcycE	Suppression	Enhancement	-
cdc2	-	No effect	None
dE2F	-	Enhancement	Positive
dDP	-	Enhancement	Positive
Hs-p21^{CIP1}	Enhancement	-	Negative
dacapo	Enhancement	Suppression[a]	Negative
RBF	Enhancement	*Df*[b] - Suppression	Negative
cyclin B	-	*Df* - No effect	None
cyclin B3	-	*Df* - No effect	None
cyclin A	-	Enhancement	Positive
string	-	Enhancement	Positive
roughex	Enhancement	Suppression	Negative
rca1	-	Enhancement	Positive
Dmcks	-	*Df* - No effect	None
cyclin C	-	*Df* - No effect	None

The effect of increasing or decreasing the dosage of cell cycle genes on the severity of the rough eye phenotype was scored relative to *DmcycE^{JP}*. Alleles used are described in the materials and methods (section 2-2.12.2).

[a] The suppression observed in this case only occurred when the *dacapo* mutation was homozygous. [b] *Df* indicates that a deficiency removing the gene was used.

Figure 3.14 The effect of halving the dosage of *cyclin A* and *string* on mitoses in *DmcycE*^{JP} eye imaginal discs.

To demonstrate the specificity of the mammalian mitosis-specific MPM-2 antibody, eye imaginal discs from wild-type larvae were stained with (A) MPM-2 antisera using FITC (green) and with the (B) DNA stain Hoechst 33258 (false coloured red). The merge of the two stainings (C) shows that cells in mitosis with condensed DNA are stained with the MPM-2 sera. An example of a cell in mitosis, stained with the MPM-2 antibody, is indicated by the arrrowhead. Staining with MPM-2 antiserum was used to compare the number of mitotic cells in (D) wild-type, (E) *DmcycE*^{JP}, (F) *DmcycE*^{JP} ; *cyclinA*^{5}/+ and (G) *DmcycE*^{JP}; *string*^{AR2}/+, eye imaginal discs, showing that decreasing the dosage of *cyclin A* and *string* reduces the number of mitotic cells posterior to the MF (arrow). Anterior is to the right.

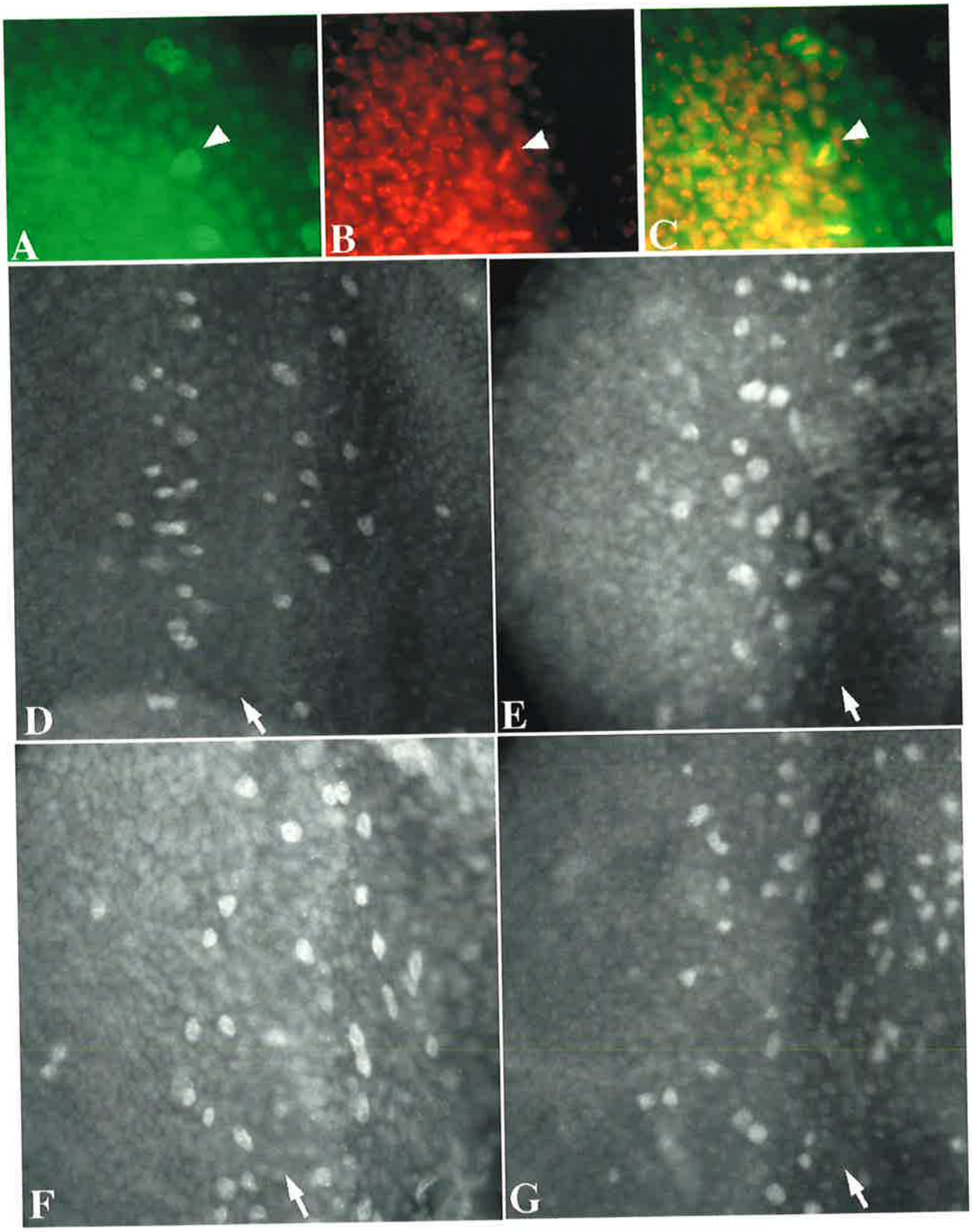
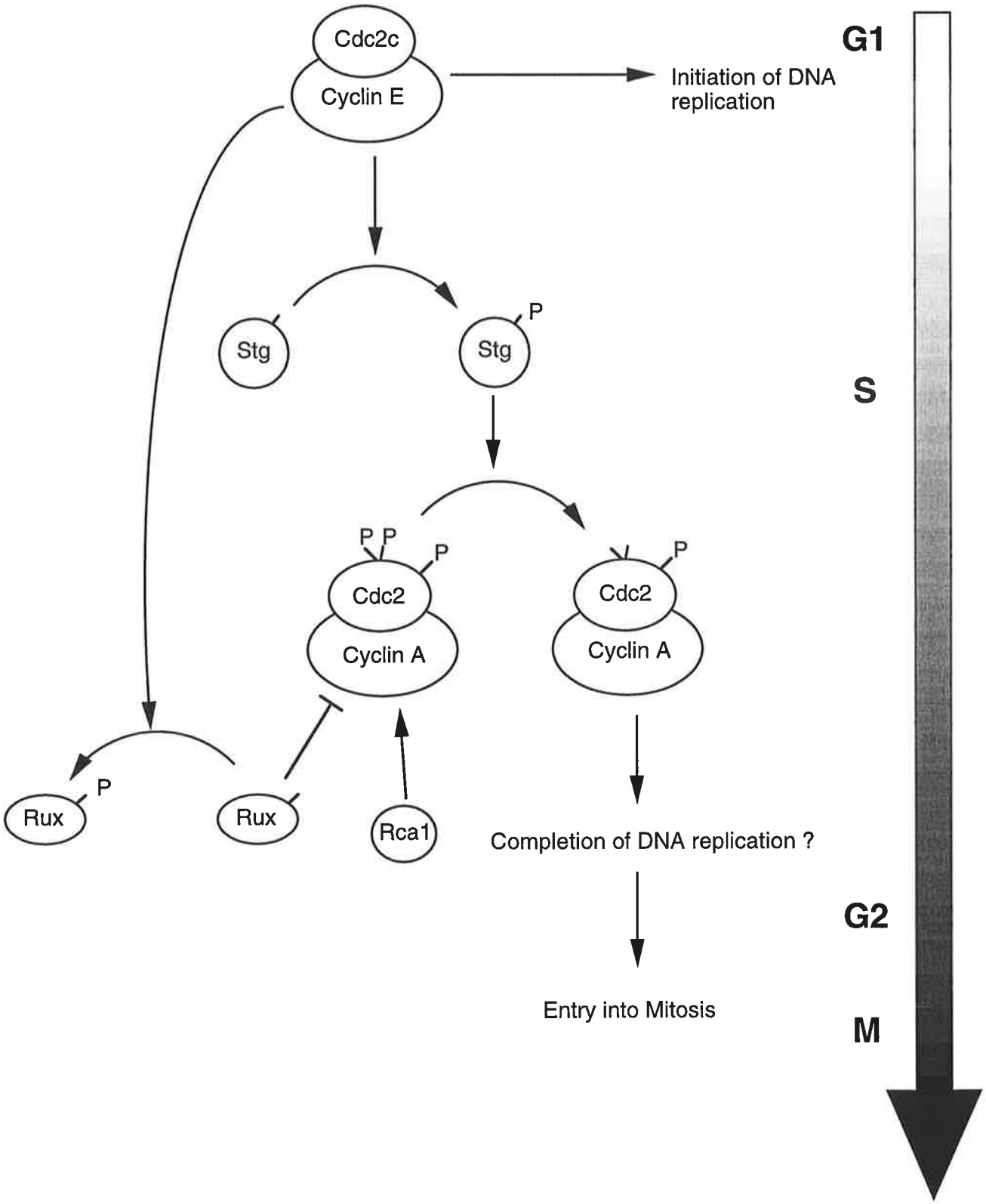

A
B
C
D
E
F
G

Figure 3.15 Model for the control of entry into and through S phase by Cyclin E.

In addition to its established function at the initiation of S phase, possibly involving phosphorylation of replication initiation proteins, Cyclin E/Cdc2c is proposed to have a role in the activation of Cyclin A/Cdc2 protein kinase in two ways. Firstly, as already shown, by the phosphorylation of the Cyclin A inhibitor, Rux, which leads to the inactivation of Rux and activation of Cyclin A (Thomas *et al.*, 1997). Rca1 appears to play a role in stabilising Cyclin A (Dong *et al.*, 1997). Second, Cyclin E/Cdc2c may have a role in the activation of Cyclin A/Cdc2 by the phosphorylation and activation of String. The phosphorylation state of Cdc2 is important for its activity. The active Cdc2 protein kinase is phosphorylated on Thr 161 and dephosphorylated on Thr 14 and Tyr 15. Phosphorylation of String (Cdc25) by Cyclin E/Cdc2c is proposed to activate the String phosphatase, which then acts to dephosphorylate the critical Thr 14 and Tyr 15 residues of Cdc2, thereby activating Cyclin A/Cdc2 activity. Active Cyclin A/Cdc2 may play a role in the completion of S phase as well as a role in the G2 to M phase transition.


Cdc2c
Cyclin E
Initiation of DNA replication
G1
Stg
Stg
P
S
P P
P
Cdc2
Cyclin A
Cdc2
Cyclin A
P
Rux
P
Rux
Rca1
Completion of DNA replication ?
G2
Entry into Mitosis
M

3-6 Discussion and conclusions

This chapter has described the characterisation of a hypomorphic allele of *Drosophila cyclin E*, $DmcycE^{JP}$, that results in a rough eye phenotype due to the presence of an internally deleted *P* [w^+ *m-lac*]-element inserted in the first intron of the *DmcycE* type II transcription unit. It is curious that an internal deletion within a *P* element can create a new phenotype. It is possible that the internal deletion permits the *P* element to more efficiently sequester particular *DmcycE* enhancers or enhancers that are inaccessible to the w^+ *P* element allele, $DmcycE^{14.11G}$, resulting in greater disruption of *DmcycE* transcription in particular tissues. These possibilities are consistent with a difference observed in the LacZ enhancer trap expression patterns in $DmcycE^{14.11G}$ and $DmcycE^{JP}$. While $DmcycE^{JP}$ and $DmcycE^{14.11G}$ showed a Cyclin E-like pattern of LacZ expression in embryos, the patterns third instar larval tissues differed. $DmcycE^{14.11G}$ showed *lacZ* expression in all imaginal discs, while $DmcycE^{JP}$ showed none (data not shown). A change in the *lacZ* expression pattern of $DmcycE^{JP}$ with respect to $DmcycE^{14.11G}$ is consistent with different enhancers being affected.

The $DmcycE^{JP}$ rough eye phenotype results largely from the failure to form the full quota of pigment and bristle cells surrounding each ommatidium, resulting in fusion of ommatidia and general disorganisation. This phenotype is similar to that observed when the human Cdk inhibitor, $p21^{CIP1}$, is expressed from the *glass* response element in the posterior region of the eye disc (de Nooij and Hariharan, 1995) and suggests that there are too few cells for proper eye development in homozygous $DmcycE^{JP}$ eye imaginal discs. During eye development, the precluster photoreceptor cells (R8, R2-R5) arise in the morphogenetic furrow (MF) and sequentially recruit the R1, R6 and R7 cells, cone cells, pigment cells and bristle cells from cells that have undergone the post-MF cell division (Wolff and Ready, 1993). $DmcycE^{JP}$ eye imaginal discs have much lower levels of Cyclin E and, as a consequence, fewer S phase cells in the band of proliferating cells posterior to the MF. This would be expected to reduce the number of cells that can be recruited into the developing ommatidia. In addition, $DmcycE^{JP}$ third instar larval eye imaginal discs are smaller than wild-type, indicating a general proliferation defect in the eye imaginal disc during larval development. Consistent with this, $DmcycE^{JP}$ eye imaginal discs have fewer S phases in the anterior undifferentiated proliferating region. Thus the small and rough eye exhibited by $DmcycE^{JP}$ can be accounted for by the reduction of S phases in the undifferentiated region and in the region posterior to the MF, respectively.

3-6.1 Interactions of *DmcycE^{JP}* with other cell cycle genes

The *DmcycE^{JP}* rough eye phenotype is responsive to the level of *DmcycE*, therefore establishing the *DmcycE^{JP}* allele as a sensitive system for examining interacting genes. This system has been tested using the *Drosophila, retinoblastoma (RBF), dacapo (dap)* and human p21^{CIP1} genes for which there is evidence for interaction with *DmcycE* in *Drosophila* (de Nooij and Hariharan, 1995; de Nooij *et al.,* 1996; Du *et al.,* 1996). Modifying the dosage of these genes altered the *DmcycE^{JP}* rough eye phenotype consistent with their previously defined roles in the cell cycle. In addition, halving the dosage of the S phase transcription factor genes *dE2F* or *dDP* enhanced the rough eye phenotype of *DmcycE^{JP}* by decreasing the number of S phases in *DmcycE^{JP}* eye imaginal discs, indicating that in the eye, as in some embryonic tissues, *dE2F* and *dDP* interact with *DmcycE* to promote the G1 to S phase transition. It is not clear whether dE2F/dDP acts predominantly upstream or downstream of *DmcycE* in the eye imaginal disc, as during embryogenesis, dE2F can activate *DmcycE* transcription as well as be activated by Cyclin E/Cdc2c activity.

3-6.2 Implications of the genetic interaction of *DmcycE* with *cyclin A* and *string*

cyclin A and *string (cdc25)* have been shown to be involved in entry into mitosis during *Drosophila* embryogenesis (Lehner and O'Farrell, 1989; Edgar and O'Farrell, 1989), but can also function in entry into or through S phase during eye development (Thomas *et al.,* 1994; Dong *et al.,* 1997). We found that halving the dosage of *cyclin A, string* or *rca1*, but not other genes required for entry into mitosis (*cdc2, cyclin B* or *cyclin B3*), enhanced the *DmcycE^{JP}* rough eye phenotype. In contrast, halving the dosage of *rux*, an inhibitor of Cyclin A required to establish G1 arrest in the eye imaginal disc, suppressed the *DmcycE^{JP}* rough eye phenotype. However, unlike the suppression of *rux* by *cyclin A, string* or *rca1* (Thomas *et al.,* 1994; Dong *et al.,* 1997), these genes did not modify the *DmcycE^{JP}* phenotype by altering the number of S phases. Rather, they led to a decrease in M phase cells posterior to the MF. These interactions are, however, unlikely to be due to a G2/M defect imposed upon a G1/S defect, since reducing the dosage of other G2/M genes such as *cyclin B*, does not enhance the *DmcycE^{JP}* rough eye phenotype and, conversely, halving the dosage of *string* in a *cyclin E^{+}* background has no effect on mitoses. The genetic interactions between *DmcycE^{JP}* and *cyclin A*, *rca1* or *rux* are consistent with the mechanism for the activation of Cyclin A described previously (Thomas *et al.,* 1997; Sprenger *et al.,* 1997; Dong *et al.,* 1997). Cyclin A is normally destabilised by Rux in the MF, which causes the relocalisation of Cyclin A to the

nucleus, where it is degraded (Thomas *et al.*, 1997; Sprenger *et al.*, 1997). Phosphorylation and inactivation of Rux by Cyclin E/Cdc2c then allows the accumulation of Cyclin A, aided by Rca1.

Existing mechanisms do not explain the genetic interaction between *DmcycE* and *string* that we observed or between *roughex* and *string* (Thomas *et al.*, 1994). In *Drosophila* embryos at least, phosphorylation of the tyr 15 and thr 14 residues of Cdk1 in Cyclin A/Cdk1 complexes inhibits Cdk activity (Sprenger *et al.*, 1997). String (Cdc25) acts to dephosphorylate these residues and activate Cdk activity. In mammalian cells Cyclin E/Cdk2 phosphorylates and activates the Cdc25A phosphatase in the G1 to S phase transition (Hoffman *et al.*, 1994). One possible explanation, therefore is that Cyclin E/Cdc2c acts to phosphorylate and activate String phosphatase activity, leading to the activation of Cyclin A/Cdk1 activity (see Figure 3.15).

Why is there a specific genetic interaction between *DmcycE* and *cyclin A,* but not *cyclin B,* if they both are involved in entry into mitosis? The precise role of Cyclin A in the cell cycle is still not well defined and the specific interaction with *DmcycE* hints at unique roles for Cyclin A/Cdk in the cell cycle. Based on the fact that Cyclin E, but not Cyclin A, is required for endoreplication cycles (Knoblich *et al.*, 1994; Lehner and O'Farrell, 1990a), a role for Cyclin A in S phase would be expected to be distinct from that of Cyclin E in the initiation of S phase. Endoreplication cycles are unique in that the late-replication of heterochromatic DNA does not occur (Lilly and Spradling, 1996; reviewed by Spradling and Orr-Weaver, 1987). Since Cyclin A is normally absent in these cycles, Cyclin A/Cdk1 may play a role in the completion of S phase as well as in entry into mitosis in *Drosophila.* Further analysis is needed to provide evidence for a role for Cyclin A and String in the completion of S phase.

In summary, the $DmcycE^{JP}$ allele provides a dosage sensitive system in which to examine the role of cell cycle regulatory genes in G1/S phase. Analysis of genetic interactions between $DmcycE^{JP}$ and other cell cycle mutations has raised the possibility that Cyclin E controls cell cycle progression by a novel mechanism involving the activation of Cyclin A and String. However, this *DmcycE* hypomorphic allele is of far greater significance than just analysing the role of known cell cycle regulatory components. Dosage-sensitive alleles have been used extensively in mutagenic screens to identify novel interacting genes (for example see Simon *et al.*, 1991; Raftery *et al.*, 1995; Dong *et al.*, 1997). On the basis of the results, a genetic screen using the dosage-sensitive allele, $DmcycE^{JP}$, has been carried out (described in Chapter 5) with the aim of identifying novel modifiers of *cyclin E* transcription and activity operating during the development of the *Drosophila* eye imaginal disc.

Chapter 4: Chromosomal regions that genetically interact with *DmcycE^{JP}*

4-1 Introduction

Correct spatial and temporal regulation of *cyclin E* transcription and activity is necessary for proper development (Knoblich *et al.*, 1994; Richardson *et al.*, 1995). Evidence for this stems from mutant and overexpression studies using *cyclin E*, demonstrating that *cyclin E* expression is necessary and sufficient for entry into S phase. There is, however, relatively little known about the upstream signals that lead to *cyclin E* transcription, and the downstream targets that lead to the initiation of DNA replication. The *DmcycE^{JP}* rough eye phenotype has provided an opportunity to identify genes involved in the regulation of *cyclin E* transcription and function by screening for mutations that dominantly enhance or suppress this phenotype. This chapter describes a pilot study to identify chromosomal regions that genetically interact with *DmcycE^{JP}* using deficiencies covering parts of the X and third chromosomes.

4-2 X and third chromosome deficiencies that modify *DmcycE^{JP}*

The analysis described in Chapter 3 demonstrated that the *DmcycE^{JP}* rough eye phenotype is sensitive to the gene dose of interacting genes. As a pilot study to obtain an estimate of how many interactors were expected from a random mutagenesis, available X and third chromosome deficiencies were tested to determine how many of these were able to modify the *DmcycE^{JP}* phenotype. A complete list of deficiencies included in this analysis can be found in materials and methods (sections 2-2.11.3 and 2-2.11.5). Deficiencies spanning the second chromosome were not tested for their effect on the *DmcycE^{JP}* phenotype in this study, as this required recombining each deficiency onto the *DmcycE^{JP}* chromosome. Once a chromosomal region was defined as modifying the *DmcycE^{JP}* phenotype, genes in the region were identified using the Flybase Cytosearch program as described in materials and methods (section 2-3.18).

A total of 20 suppressor regions and 16 enhancer regions on the X and third chromosomes were identified by generating homozygous *DmcycE^{JP}* flies that were also heterozygous for the deficiency chromosome (Table 4.1 and 4.2). Consistent with results described in Chapter 3, deficiencies removing genes already known to interact with *DmcycE^{JP}* such as *RBF*, *rux*, *E2F* and *string* behaved as expected (Tables 4.1 and 4.2) with the exception of *Df(3R)vin2*, which removes *cyclin A*. Unlike specific *cyclin A* mutations,

Df(3L)vin5, suppressed the *DmcycE*^{JP} rough eye phenotype. The most likely explanation for this is that these deficiencies also delete a dose-sensitive suppressor of *DmcycE*^{JP}, although there are no candidates for such a gene in the region (Table 4.2). In addition to identifying regions that remove genes previously known to genetically interact with *DmcycE*^{JP}, regions removing genes predicted to be involved in restricting or promoting entry into S phase were identified. These included homologues of tumour suppressors or oncogenes, genes involved in the initiation of DNA replication, in degradation pathways or in chromatin remodelling. Possible candidates within deficiencies that enhanced or suppressed the *DmcycE*^{JP} rough eye phenotype will now be discussed.

Table 4.1 X chromosome regions that modify the *DmcycE*^{JP} phenotype

Deficiency	Region removed by deficiency	Effect on *DmcycE*^{JP}	Candidate genes in the region
Df(1)tBA1	**1A1 to 2A**	**Suppression**	***RBF*** #
Df(1)JC19	2F6 to 3C5	Suppression	none
Df(1)N73 and Df(1)sqh	**5D1-2 to 5D5-6**	**Suppression**	***roughex***
Df(1)KA14	7F1-2 to 8C6	Mild suppression	none
***Df(1)v*^{N48}**	**9F to 10C3-5**	**Suppression**	***hopscotch***
Df(1)C246	11D-E to 12A1-2	Suppression	*BAP60* *
Df(1)RK4	12F5-6 to 13A9-B10	Suppression	none
Df(1)sc72b	13F1 to 14B1	Suppression	none
Df(1)4b18	14B8 to 14C1	Enhancement	none
Df(1)N19	17A1 to 18A2	Enhancement	none
Df(1)ma13	**19A1-2 to 20E-F**	**Suppression**	***RpS6***

Summary of X chromosome deficiencies that modify the *DmcycE*^{JP} phenotype and the most obvious candidate gene(s) in these regions. In the cases where deficiencies that overlap have the same effect on *DmcycE*^{JP}, the region common to both deficiencies is given as the cytological region. Those highlighted in bold have a candidate gene within the region that has been tested and found to have the same effect on the *DmcycE*^{JP} phenotype as the corresponding deficiency. * indicates that no mutation is available to test the interaction. Gene abbreviations used are *Retinoblastoma protein* (*RBF*), *Brahma associated protein 60* (*BAP60*) and *Ribosomal protein S6* (*RpS6*). # indicates that while *RBF* has only been shown to suppress the *DmcycE*^{JP} phenotype by using *RBF* deficiencies, overexpression of *RBF* enhances the *DmcycE*^{JP} rough eye phenotype.

Table 4.2 Regions of the third chromosome that modify *DmcycE^{JP}*

Deficiency	Region removed by deficiency	Effect on *DmcycE^{JP}*	Candidate genes in the region
Df(3L)emc5	61C3-4 to 62A8	Enhancement	*Rac1* *
Df(3L)R^{G7}	62B8-9 to 62F2-5	Enhancement	none
Df(3L)HR370	63A1 to 63D10	Enhancement	none
Df(3L)HR232 and *Df(3L)HR119*	63C6 to 63D3	Suppression	none
Df(3L)GN50	63E1-2 to 64B17	Enhancement	*RfC40, cdc4* *
Df(3L)GN24	63F4-7 to 64C13-15	Suppression	*Ubiquitin-63E**, *Rpd3*
Df(3L)66C^{G28}	66B8-9 to 66C10	Enhancement	*Cbl* *
Df(3L)h^{i22}	66D10-11 to 66E1-2	Enhancement	*mcm7* *
Df(3L)AC1	67A2 to 67D7-13	Enhancement	none
Df(3L)vin2 and *Df(3L)vin5*	68A2-3 to 68D6	Suppression	none
Df(3L)brm11	**71F1-4 to 72D1-10**	**Suppression**	***brahma***
Df(3L)81K19	73A3 to 74F	Suppression	*Pros 26*
Df(3L)Cat	75B8 to 75F1	Enhancement	Replication deficient region *
Df(3L)rdgC	77A1 to 77D1	Enhancement	none
Df(3L)Pc^{MK}	78A3 to 79E1	Enhancement	none
Df(3R)Tp110	83C1-2 to 84B1-2	Enhancement	none
Df(3R)p712	84D4-6 to 85B6	Suppression	none
Df(3R)by10	85D8-12 to 85F1	Suppression	*hyd*
Df(3R)ChaM7	91A to 91F5	Suppression	none
Df(3R)e^{N19}	**93B to 94**	**Enhancement**	***E2F***
Df(3R)crbS87^{4}	95E8-F1 to 95F15	Enhancement	*yes95CD* *
Df(3R)XS	96A1-7 to 96A21-25	Suppression (mild)	none
Df(3R)T1^{P}	97A to 98A1-2	Suppression (mild)	*l(3)mbt*
Df(3R)3450	**98E3 to 99A6-8**	**Enhancement**	***string***
Df(3R)awd^{KRB}	100C6-7 to 100D3-4	Suppression	none

Summary of third chromosome regions that have an effect on the *DmcycE^{JP}* rough eye phenotype. In the cases where deficiencies that overlap have the same effect on *DmcycE^{JP}*, the region common to both deficiencies is given as the cytological region. Bold indicates that the candidate gene has been tested and has the same effect on the *DmcycE^{JP}* phenotype as the corresponding deficiency. * indicates that no mutant is available to test the genetic interaction between the candidate gene and *DmcycE^{JP}*. Gene abbreviations used are *Replication factor-C40* (*RfC40*), *Histone deacetylase* (*Rpd3*), *minichromosome maintenance 7* (*mcm7*), *Proteosome subunit 26* (*Pros 26*), *hyperplastic discs* (*hyd*), and *lethal(3)malignant brain tumour* (*l(3)mbt*). Candidate genes that are underlined have been tested, and did not show any effect on the *DmcycE^{JP}* phenotype.

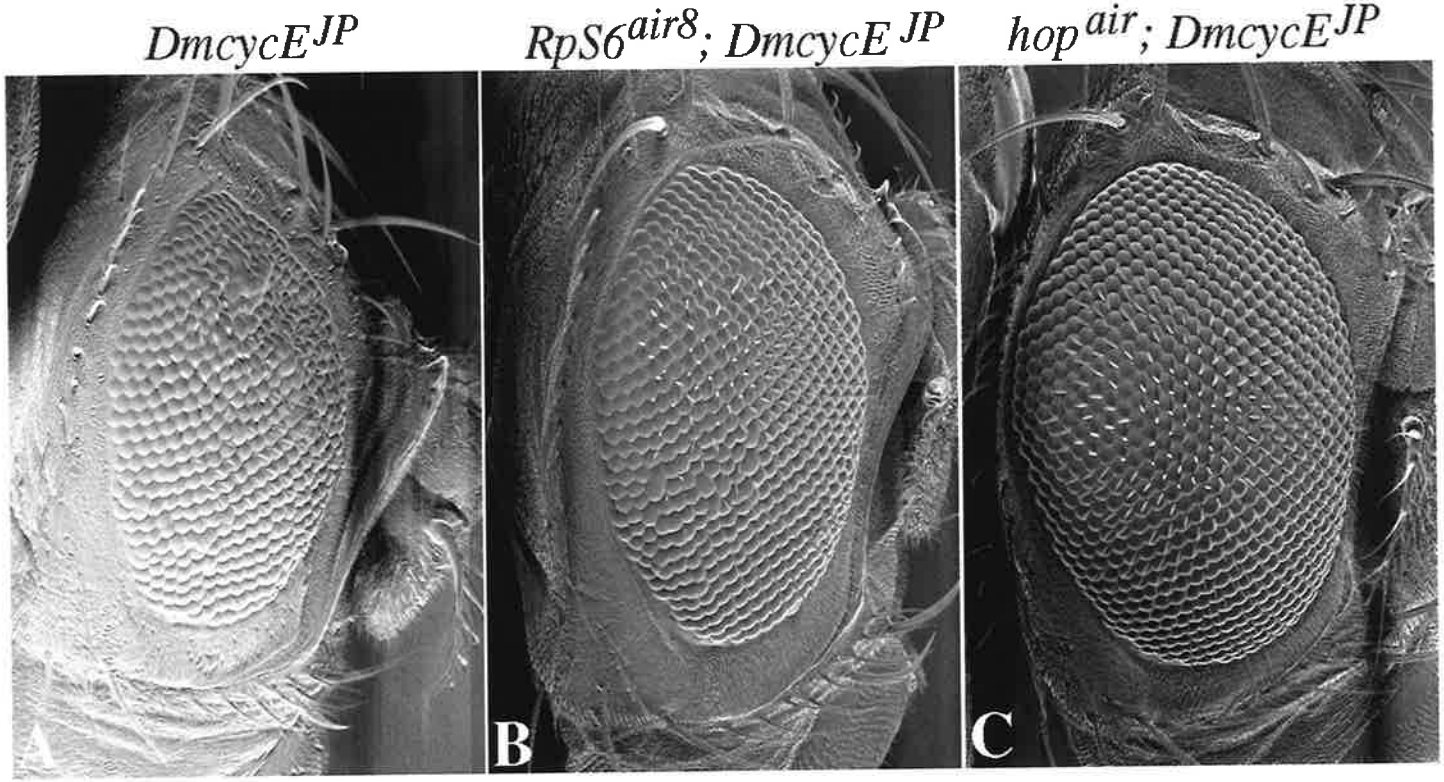
4-2.1 Tumour suppressors

Consistent with the expectation that reducing the dose of negative regulators of proliferation would result in suppression of the *DmcycE*^{JP} rough eye phenotype, two suppressor regions on the X chromosome and two on the third chromosome remove tumour suppressor genes. Two of these genes, *hopscotch* (*hop*) and *Ribosomal protein S6* (*RpS6*) (Watson *et al.*, 1991; Stewart and Denell, 1993) have been tested specifically, and suppress the *DmcycE*^{JP} rough eye phenotype (Figure 4.1). *hop* and *RpS6* were identified as mutations that result in overproliferation of larval hematopoietic tissues, small imaginal discs, and give rise to variable melanotic tumour phenotypes (Watson *et al.*, 1991). *hop* encodes a non-receptor protein tyrosine kinase of the Janus kinase (JAK) family, which, in mammalian cells, is activated in response to a variety of cytokines and growth factors (reviewed by Schindler and Darnell, 1995). Activation of any of the JAK family in mammalian cells by association with ligand bound receptors leads to the phosphorylation and activation of a signal transducer and activator of transcription (STAT) protein. Phosphorylated STAT proteins then translocate to the nucleus and directly activate gene transcription. While in mammalian cells activation of the JAK/STAT pathway correlates with a mitogenic response, there is little evidence that this pathway is required for proliferation (Ihle and Kerr, 1995). Similarly, in *Drosophila*, the role of the JAK/STAT pathway in proliferation is not well characterised (Harrison *et al.*, 1995), although the homozygous mutant phenotype suggests it has a positive role in proliferation of imaginal discs but a negative role in the proliferation of blood cells. Hop however, is known to be necessary for establishing a sub-set of the *even skipped* transcription pattern during embryogenesis (Binari and Perrimon, 1994). While the embryonic function of Hop requires the STAT, Marelle (Hou *et al.*, 1996), mutations in this gene have no effect on the *DmcycE*^{JP} phenotype (data not shown). This indicates that Marelle is not a limiting component of Hop signalling or a different pathway may be required for Hop function during eye development. As Hop appears to act as a positive cell cycle regulator in eye imaginal discs, the basis of the suppression of *DmcycE*^{JP} by halving the dosage of *hop* is not understood.

The ribosomal-associated protein RpS6 is phosphorylated in response to mitogen stimulation (Pelech and Sanghera, 1992; Posada and Cooper, 1992), and phosphorylated RpS6 is preferentially incorporated into polysomes resulting in an increased rate of translation (Traugh and Pendergast, 1986). Phophorylated RpS6 is then presumed to specifically increase the translation of positive cell cycle regulators, although there is no direct evidence for this. Based on the positive role in cell proliferation expected for RpS6, the

Figure 4.1 Halving the dosage of *hop* or *RpS6* suppresses the $DmcycE^{JP}$ rough eye phenotype

(A, B, C) Scanning electron micrographs of adult eyes from (A) $DmcycE^{JP}$, (B) $RpS6^{air8}$/+ ; $DmcycE^{JP}$ and (C) hop^{air}/+ ; $DmcycE^{JP}$ showing that reducing the dosage of *hop* or *RpS6* suppresses the $DmcycE^{JP}$ rough eye phenotype. Anterior is to the right, dorsal side up in all panels.


DmcycE^JP
RpS6^air8; DmcycE^JP
hop^air; DmcycE^JP
A
B
C

reasons for the hematopoietic overproliferation phenotype of *RpS6* mutants is not understood (Watson *et al.*, 1994). *RpS6* mutants have small imaginal discs, consistent with the suspected function of RpS6 as a positive cell cycle regulator. However, based on this phenotype, it is difficult to explain the suppression of the $DmcycE^{JP}$ rough eye phenotype resulting from halving the dosage of *RpS6*. Determining the precise role for the Hop and RpS6 proteins during eye development may explain the observed suppression of $DmcycE^{JP}$.

Two other tumour suppressor genes, *lethal (3) malignant brain tumour* (*l(3)mbt*) (Gateff *et al.*, 1993) and *hyperplastic discs* (*hyd*) (Mansfield *et al.*, 1994) were identified as candidates in the regions 97A to 98A1-2 and 85D8-12 to 85E10-13, respectively. Mutations in *hyd* result in overproliferation of imaginal discs, indicating that Hyd is normally a negative regulator of proliferation (Mansfield *et al.*, 1994). *hyd* is likely to encode a E3 ubiquitin-protein ligase involved in targeting proteins for ubiquitin-mediated proteolysis (Callaghan *et al.*, 1998), so may be involved in the proteolysis of Cyclin E. Larvae homozygous for mutations in *l(3)mbt* show overproliferation of the larval brain hemispheres and large imaginal discs (Gateff *et al.*, 1993), suggesting that *l(3)mbt* encodes a negative regulator of the G1 to S phase transition. Although likely candidates for suppressors of $DmcycE^{JP}$, specific mutations in either *l(3)mbt* or *hyd* have no effect on the $DmcycE^{JP}$ phenotype (A. Brumby, pers. com.), indicating that *l(3)mbt* and *hyd* are not dose-dependent regulators of the Cyclin E pathway. Halving the dosage of *l(3)mbt* or *hyd* is therefore unlikely to be solely responsible for the suppression of $DmcycE^{JP}$ observed using $Df(3R)T1^{P}$ or *Df(3R)by10*.

4-2.2 Degradation pathways

Genes encoding products potentially involved in the degradation of Cyclin E or inhibitors of Cyclin E/Cdk2 activity were identified in regions that suppressed the $DmcycE^{JP}$ phenotype. Genes that may be directly involved in the degradation of Cyclin E include the *Ubiquitin-63E* (*Ubi-p63E*) (Arribus *et al.*, 1986) and *Proteosome 26kD subunit* (*Pros26*) (Saville and Belote, 1993), that are within the regions 63F4-7 to 64C13-15 and 73A3 to 74F, respectively. In mammals, Cyclin E is degraded by the ubiquitin pathway (Won and Reed, 1996; Clurman *et al.*, 1996). Thus, reducing the dose of gene products involved in this process may be expected to suppress the $DmcycE^{JP}$ phenotype by stabilising the low levels of Cyclin E present in $DmcycE^{JP}$ eye discs. There are, however, multiple *Ubi* genes in *Drosophila* (Lee *et al.*, 1988), so it is unlikely that reducing the gene-dose of one of these genes would significantly reduce ubiquitin levels. Unfortunately, no mutations exist in *Ubi-63E*, so this genetic interaction cannot be tested. Like ubiquitin, the proteosome, of which

Pros26 encodes a subunit, is required for the degradation of many proteins. *Pros26* mutations are available, but have not been tested for an interaction with $DmcycE^{JP}$.

A gene within the $DmcycE^{JP}$ enhancer region 63E1-2 to 64B17, *cdc4*, may be involved in the degradation of a Cyclin E/Cdk2 inhibitor. *Drosophila cdc4* has recently been identified by homology to *Sacharomyces cerevisiae* and human *cdc4* (H. Richardson, pers. com.), although no function has yet been attributed to this gene. In yeast, CDC4 is an F box protein in the SCF ubiquitin ligase complex that triggers entry into S phase by ubiquitinating the Cdk inhibitor SIC1 (reviewed by Krek, 1998). Ubiquitinated SIC1 is then targeted for destruction by the proteosome, triggering entry into S phase. The components of this complex are evolutionarily conserved (Lyapina *et al.*, 1998), although the targets of this ubiquitin-protein ligase complex are not known for multicellular organisms. By analogy to the function of CDC4 in yeast, *Drosophila* Cdc4 may be involved in the targeting of a Cyclin E/Cdc2c inhibitor for destruction. Halving the dosage of this gene would be expected to increase the levels of a Cdk inhibitor, resulting in decreased levels of active Cyclin E/Cdc2c and enhancement of the $DmcycE^{JP}$ rough eye phenotype. Unfortunately, no mutations are presently available in *Drosophila cdc4* to test this interaction.

4-2.3 Initiation of DNA replication

Two genes known to be involved in the initiation of DNA replication, *Replication-factor-C 40kD* subunit (*RfC40*) and *mini-chromosome maintenance 7* (*mcm7*), are removed by *Df(3L)GN50* and $Df(3L)h^{i22}$ respectively, which were identified as enhancers of $DmcycE^{JP}$ (Table 4.2). *RfC40* encodes an ATP-dependent helicase protein, and null mutations in this gene result in larvae with very small imaginal discs, consistent with the role for the product of this gene in the initiation of DNA replication (Harrison *et al.*, 1995). Although mutations in the *RfC40* gene have been isolated, they have not been tested for enhancement of the $DmcycE^{JP}$ phenotype. *mcm7* encodes a DNA replication licensing factor (Su *et al.*, 1997; Ohno *et al.*, 1998). Reducing the dose of this gene would therefore be predicted to enhance the $DmcycE^{JP}$ rough eye phenotype. Unfortunately, no mutations have yet been isolated in this gene, so the genetic interaction between $DmcycE^{JP}$ and *mcm7* cannot be specifically tested.

Another gene essential for DNA replication is predicted to be within the region 75B8-75F1 (Smith *et al.*, 1993). Analysis of deficiencies that result in defects in DNA replication during embryogenesis has revealed that embryos homozygous for *Df(3L)Cat* are replication defective (Smith *et al.*, 1993). Embryos homozygous for *Df(3L)Cat* cease DNA replication

after the 16 mitosis, indicating that a factor essential for DNA replication is removed by this deficiency. The enhancement of *DmcycE^{JP}* seen using *Df(3L)Cat* is consistent with the deletion of a gene essential for the initiation of DNA replication. Specific alleles of the gene within 75B8 to 75F1 required for DNA replication have not been identified, so it was not possible to test this interaction directly.

4-2.4 Homologues of human oncogenes

Homologues of human oncogenes were also found in regions that enhanced *DmcycE^{JP}*. These were *Rac1* (Harden *et al.*, 1995; Sasamura *et al.*, 1997), *Cbl* (Hime *et al.*, 1997; Meisner *et al.*, 1997) and *yes95CD* (Bhargava *et al.*, 1991) for regions 61C3-4 to 62A8, 66B8-9 to 66C10 and 95D1-2 to 96A2 respectively. In mammalian systems, Cbl has been demonstrated to be a negative regulator of protein tyrosine kinase-mediated signalling, although the mechanism by which Cbl leads to cell transformation remains unclear (Thein and Langdon, 1998). Similarly, in *Drosophila*, Cbl acts as a negative regulator of epidermal growth factor (EGF)-mediated signalling during the development of the R7 photoreceptor (Meisner *et al.*, 1997), although a direct role for *Drosophila* Cbl in regulating cell proliferation has not been determined. As Cbl has been implicated as a proto-oncogene in mammalian cells, halving the dosage of *Cbl* may be expected to enhance the *DmcycE^{JP}* rough eye phenotype.

Very little is known about *Drosophila yes95CD*, which was isolated based on homology to the *v-yes* human oncogene (Bhargava et al., 1991). Human *c-yes* encodes a membrane-associated protein tyrosine kinase, the enzymatic activity of which is elevated in many human carcinomas (Pena *et al.*, 1995). The precise function of the Yes95CD protein in the regulation of differentiation or proliferation has not been determined, therefore the mechanism by which it may act to enhance the *DmcycE^{JP}* rough eye phenotype is not clear. *Cbl* and *yes95CD* were identified based on homology to human oncogenes and there are no corresponding mutations of these genes that could be tested for their interaction with *DmcycE^{JP}*.

Like *Cbl* and *yes95CD*, there are no mutations available for the *Rac1* gene, a candidate for the enhancer of the *DmcycE^{JP}* rough eye phenotype within the region removed by *Df(3L)emc5* (61C3-4 to 62A8). Rac1 is member of the Rho sub-class of Ras-like GTPases, and there is evidence that members of this sub-class of GTPases have a role in the regulation of proliferation in mammalian cells (reviewed by Hall, 1998). Tissue culture cells overexpressing *Rac1* exhibit reduced serum requirements, anchorage independent growth,

and lead to tumour formation when injected into nude mice (Khosravi *et al.*, 1995). In addition, Rac1 is required for Ras-induced transformation, and overexpression of constitutively active Rac1 is sufficient to cause malignant transformation of rodent fibroblasts (van Leeuwen *et al.*, 1995). While no *Rac1* mutations have been isolated, specific mutations are available for *Drosophila* RhoA, another member of this class of small GTPases (Strutt *et al.*, 1997). RhoA, like Rac1, has also been shown to have a role in proliferation, and is required for Ras-induced transformation (Khosravi *et al.*, 1995). In addition, overexpression of wild-type and dominant negative forms of RhoA have been shown to upregulate mammalian Cyclin E/Cdk2 activity and induce progression from G1 into S phase (Hu *et al.*, 1999). Consistent with a role for this class of GTPases in proliferation, reducing the dose of *RhoA* enhances the $DmcycE^{JP}$ phenotype (H. Richardson, pers. com.).

4-2.5 Chromatin remodelling

In addition to identifying genes previously known to have a role in proliferation, genes involved in chromatin remodelling were also identified. Deficiencies removing *brahma* (*brm*) (Kennison, 1995), and *brahma associated protein 60* (*BAP60*) (Papoulas *et al.*, 1998), suppressed the $DmcycE^{JP}$ phenotype (Tables 4.1 and 4.2). *brm* is a trithorax group gene that encodes a putative helicase with homology to the SWI2 member of the SWI/SNF family of proteins from yeast and humans (Tamkun *et al.*, 1992). Brm is thought to act as part of a multiprotein complex that includes BAP60, and affects chromatin structure leading to changes in DNA accessibility to transcription factors (Schnitzler *et al.*, 1998). Several lines of evidence support the involvement of Brm in cell cycle regulation. In *Drosophila*, *brm* alleles have been identified as dominant enhancers of overexpression of *dE2F* and *dDP* in the eye imaginal disc (Staehling-Hampton *et al.*, 1999), indicating that *brm* is a negative regulator of proliferation. This interaction is consistent with data from mammalian systems showing that the two human Brm homologues, hBRM and BRG1, act as co-repressors with pRb to downregulate E2F-dependent transcription (Trouche *et al.*, 1997). It has also been shown recently that BRG1 is phosphorylated by Cyclin E/Cdk2 and that overexpression of BRG1 in tissue culture results in cells arresting in G1 (Shanahan *et al.*, 1999). The G1 arrest induced by overexpression of BRG1 can be abrogated by the overexpression of *cyclin E*, thus Cyclin E/Cdk2 may result in the activation of E2F-dependent transcription by the phosphorylation and inactivation of both RBF and Brahma. While specific alleles of *brm* have been shown to suppress the $DmcycE^{JP}$ phenotype (A. Brumby, pers. com.), there are no specific alleles of BAP60 available, therefore this interaction could not be tested.

A deficiency that removes the *Drosophila* histone deacetylase gene (*rpd3*), *Df(3L)GN24*, was also identified as a suppressor of the $DmcycE^{JP}$ rough eye phenotype. In mammalian cells, pRb mediated repression of E2F-dependent transcription occurs by the recruitment of the histone deacetylase, HDAC1 (Brehm, *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998). HDAC1 removes the negatively charged acetyl group from histones, altering chromatin structure and preventing access by transcription factors. Halving the gene dosage of *Drosophila rpd3* may therefore be expected to suppress the $DmcycE^{JP}$ rough eye phenotype by relieving repression of E2F target genes. A mutation in *rpd3* has been tested, but does not affect the $DmcycE^{JP}$ phenotype (A. Brumby, pers. com.), indicating that this histone deacetylase is not a limiting component of Cyclin E-regulated entry into S phase, and is not responsible for the enhancement of $DmcycE^{JP}$ by *Df(3L)GN24*. Since there are at least two other HDACs in *Drosophila* (Bornemann *et al.*, 1999), halving the dosage of *rpd3* may not be sufficient to affect E2F-dependent gene transcription and modify the $DmcycE^{JP}$ rough eye phenotype.

Discussion and Conclusions

The rough eye phenotype caused by the $DmcycE^{JP}$ *P* element allele has provided an opportunity to identify genes that interact with *cyclin E* in a dose-dependent manner. These are expected to include genes involved in the regulation of *cyclin E* transcription, Cyclin E/Cdc2c function and downstream effectors. To test this system, a series of deficiencies with defined breakpoints were used to identify regions of the X and 3rd chromosomes that modified the $DmcycE^{JP}$ phenotype. In addition to identifying regions known to encode cell cycle regulatory proteins, many other regions were identified that removed genes expected to be involved in initiation of DNA replication, degradation of Cyclin E or Cyclin E/Cdc2c inhibitors, in promoting or inhibiting entry into S phase or in chromatin remodelling.

Although identifying deficiencies that modify the $DmcycE^{JP}$ rough eye phenotype has identified numerous chromosomal regions that are likely to include regulators of *cyclin E* transcription and function, there are several drawbacks to this kind of screen. The most significant of these is that the use of deficiencies relies on previously known genes or mutations being responsible for the enhanced or suppressed phenotype observed. Of the 36 regions that modify $DmcycE^{JP}$, 18 do not have a candidate gene or mutation mapping within the deficiency breakpoints, and may therefore be the result of reducing the dose of a novel gene. In some cases, a candidate has been identified but no mutations were available to test

the effect of reducing the dose of this gene on the *DmcycE*^{JP} phenotype. In other cases, a candidate gene was identified, but testing specific mutations demonstrated that halving the dosage of this gene did not account for the modification observed with the deficiency. In addition, as the chromosomal regions removed by many of the deficiencies are quite large, there is the potential for the deletion of more than one modifier. In some cases, this would result in the co-deletion of enhancer and suppressor mutations, resulting in no modification of the *DmcycE*^{JP} phenotype and preventing both genes from being identified. For these reasons, a random mutagenesis was carried out to generate specific alleles of modifiers that could be further characterised (described in Chapter 5).

Chapter 5: The *DmcycE^{JP}* genetic interaction screen

5-1 Introduction

Chapter 4 described the identification of numerous chromosomal regions that enhanced or suppressed the *DmcycE^{JP}* rough eye phenotype. A candidate gene responsible for the observed interaction with *DmcycE^{JP}* could not be assigned for many of these chromosomal regions, highlighting the need for a random mutagenesis to identify novel components of Cyclin E regulated entry into S phase. To isolate alleles of *DmcycE^{JP}* modifiers that could then be characterised further, a genetic interaction screen was carried out using the mutagens ethyl methanesulphonate (EMS) and X-irradiation. As many more modifiers were isolated than could be characterised within the scope of this study, only suppressor mutations mapping to the second chromosome were characterised and are described in this chapter.

5-2 Choice of mutagen for the genetic interaction screen

Commonly, three approaches are used to generate mutations in *Drosophila*, *P* element insertion, X-irradiation and chemical mutagenesis such as EMS. To randomly generate mutations that could then be examined for their effect on the *DmcycE^{JP}* phenotype, two of these mutagens, EMS and X-rays, were utilised. X-rays, in general, tend to cause deletions and chromosomal re-arrangements (Sankaranarayanan and Sobels, 1976), which may be useful in the subsequent cloning of novel modifiers of *DmcycE^{JP}*. To complement the type of alleles generated by X-rays, an EMS mutagenesis was also carried out. EMS usually results in nucleotide substitutions (Lifschytz and Falk, 1968), resulting in missense or nonsense mutations. Unlike an X-ray generated mutation, which can affect multiple genes, an EMS-induced mutation usually only affects one gene. While there are many advantages to using *P* element insertional mutagenesis for the cloning of novel genes identified as enhancers of suppressors of *DmcycE^{JP}*, this type of mutagenesis was not undertaken for several reasons. The most significant of these is that the *DmcycE^{JP}* mutation is itself a *P* element insertion, so a *P* insertional mutagenesis would have led to *DmcycE^{JP}* reversion. Previously generated *P* mutations on the X and third chromosomes could have be used. However, this would be very labour intensive, requiring three crosses to generate flies homozygous for *DmcycE^{JP}* and heterozygous for the *P* insert. In addition, the use of *P* elements in dominant genetic interaction screens has been found to be problematic due to variations in the *P* element genetic background and to the generally hypomorphic nature of *P* alleles (Karim *et al.*, 1996).

For these reasons, *P* element alleles were not used in the genetic interaction screen for $DmcycE^{JP}$ modifiers. However, it was expected that once X-ray or EMS-induced modifiers of $DmcycE^{JP}$ were mapped, cytologically characterised *P* alleles could be identified from the Berkeley *Drosophila* genome project (BDGP) collection.

5-3 The fly screen

Both the EMS and X-ray mutageneses required isogenic $DmcycE^{JP}$ males to be mutagenised (see materials and methods for details, section 2-3.5), and crossed *en masse* to *b* $DmcycE^{JP}$ females. The progeny from this cross were scored for dominant modification of the $DmcycE^{JP}$ rough eye phenotype (Figure 5.1). Flies selected as having enhanced or suppressed eyes were crossed to a $DmcycE^{JP}$ strain to ensure that the modification of the $DmcycE^{JP}$ rough eye phenotype observed initially was consistent and to generate a stock. In addition, F1 progeny were scored for black bodied flies to estimate the mutation frequency. All flies in this generation should be heterozygous for *black* unless a mutation in this gene was induced during mutagenesis of the $DmcycE^{JP}$ males.

As a large number of X and third chromosome deficiencies modified the $DmcycE^{JP}$ phenotype (Section 4-2), it was expected that a large number of X-ray and EMS-induced enhancers and suppressors would be generated. For this reason, it was decided to restrict the analysis of modifiers to those mapping to the second and third chromosomes. As described in Figure 5.2, modifiers of $DmcycE^{JP}$ that gave a consistent phenotype when crossed back to $DmcycE^{JP}$ were crossed to second and third chromosome balancer stocks to generate stocks of modifiers on these chromosomes. Once a stock was generated, flies were crossed to a $DmcycE^{JP}$ strain to ensure that the enhancer or suppressor mutation segregated away from the balancer chromosome. Crossing the modifiers obtained, particularly enhancers, to second chromosome balancer stocks also enabled any mutations that result in a dominant eye roughening in the absence of $DmcycE^{JP}$ to be detected. The effect of dominant eye roughening mutations on the $DmcycE^{JP}$ rough eye phenotype may not be due to a specific effect on *cyclin E*, but merely the result of combining two eye roughening phenotypes. One such mutant was obtained, and was discarded.

So that each modifier could easily be traced back to the bottle from which it was derived, each suppressor or enhancer was given an allele name derived from the bottle from which it was selected. This was important, as mutations arising from one bottle have the potential to be a result of a pre-meiotic mutation, and therefore represent the same allele. To distinguish X-ray and EMS generated alleles, EMS generated allele names have an E prefix.

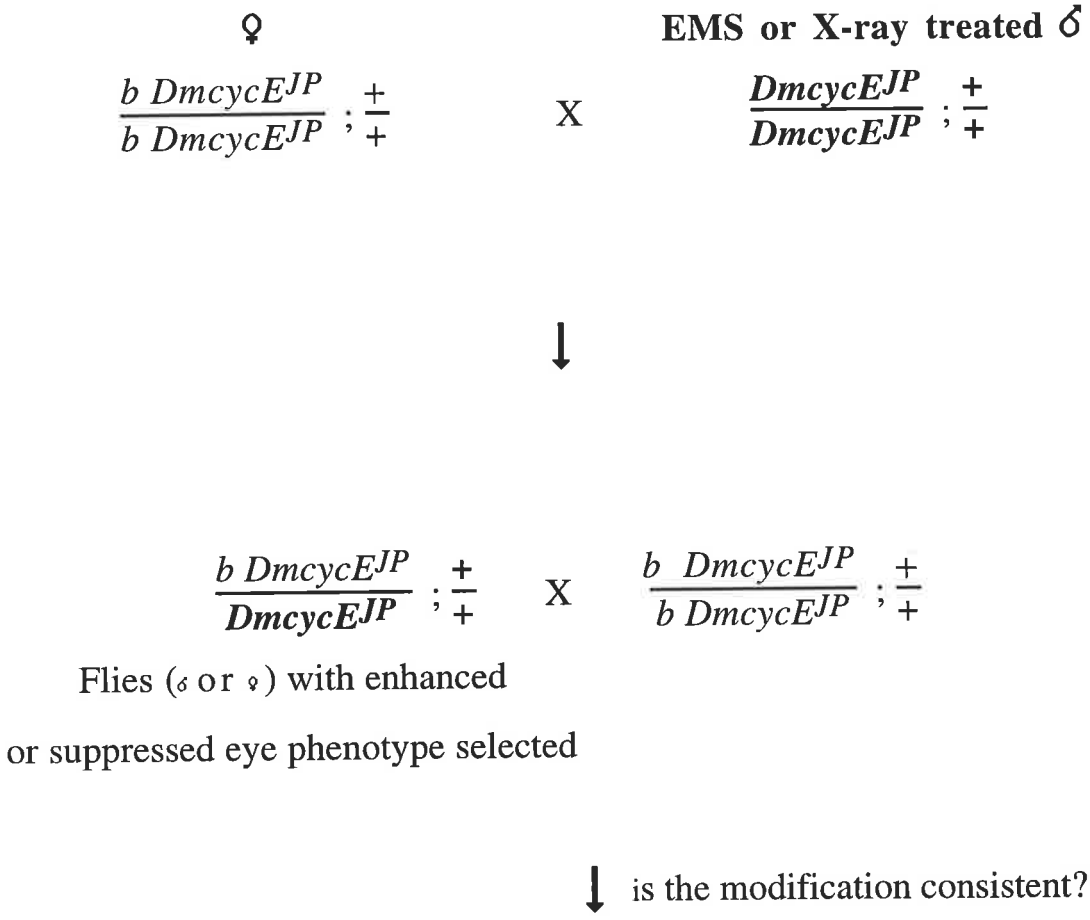


Figure 5.1 Isolation of dominant modifiers of *DmcycE^JP*.

To generate random mutations in the genome of *Drosophila*, homozygous *DmcycE^JP* males were treated with either X-rays or EMS (see material and methods, section 2-3.5 for details) and crossed to *b DmcycE^JP* virgins. From the progeny of this cross, flies with more severe (enhanced) or less severe (suppressed) rough eyes than *DmcycE^JP* were selected. F1 progeny were scored for the recessive black phenotype, which results in adults with a dark body colour, to monitor the frequency of mutation induced by X-rays or EMS in the *black* (*b*) gene. Modified flies were individually crossed to *b DmcycE^JP* to verify that the modification of the *DmcycE^JP* rough eye phenotype was consistent and to generate stocks for further analysis (see Figure 5.2). Bold indicates chromosomes exposed to mutagen.

Figure 5.2 Generation of stocks on the second or third chromosome

For each modifier isolated in the mutagenesis, two sets of crosses (A and B) were carried out to determine whether the modifier was on the second or third chromosome, and to generate a stock for further examination. A mutation that modifies the *DmcycE^{JP}* phenotype on the second or third chromosome is indicated by *

(A) Generating a stock for a modifier on the second chromosome. Modified male flies from the final cross described in Figure 5.1 were crossed to a *Cy*O balancer chromosome stock carrying a mutation in the *black* gene. This allowed the mutagenised second chromosome to be differentiated from the *b DmcycE^{JP}* chromosome. From the progeny of this cross, non-black males and females balanced over *b CyO* were crossed together to obtain a stock. In addition, non-black flies were crossed back to *DmcycE^{JP}* flies to determine whether the modifier was located on the second chromosome. If an enhancer or suppressor was on the second chromosome, all straight winged progeny from the cross to *DmcycE^{JP}* flies would be expected to be modified.

(B) Generating a stock of a third chromosome modifier. Modified males from the final cross described in Figure 5.1 were crossed to a homozygous *DmcycE^{JP}* strain carrying the third chromosome balancer chromosomes *TM2* and *TM6B*. From this cross, modified males and females carrying either the *TM2* or *TM6B* balancer chromosome were crossed together to generate a stock. Modified flies from this cross were also crossed back to *DmcycE^{JP}* flies. A modifier on the third chromosome would be expected to segregate away from the balancer chromosome in this cross.

(A) 2nd chromosome modifier?

♀ ♂

$$\frac{b}{b\ CyO} \quad X \quad \frac{b\ DmcycE^{JP}}{\boldsymbol{DmcycE^{JP}\ *}}$$

↓

$$\frac{\boldsymbol{DmcycE^{JP}\ *}}{b\ CyO}$$

Non-*black* males and females selected to generate a stock

↓ test cross X $\frac{DmcycE^{JP}}{DmcycE^{JP}}$

Are all straight winged flies modified?

(B) 3rd chromosome modifier?

♀ ♂

$$\frac{DmcycE^{JP}}{DmcycE^{JP}} ; \frac{TM2}{TM6B} \quad X \quad \frac{b\ DmcycE^{JP}}{DmcycE^{JP}} ; \frac{+*}{+}$$

↓

$$\frac{b\ DmcycE^{JP}bw \text{ or } DmcycE^{JP}}{DmcycE^{JP}} ; \frac{+*}{TM6B \text{ or } TM2}$$

Select modified flies to generate a stock

↓ test cross X $\frac{DmcycE^{JP}}{DmcycE^{JP}}$

Does modifier segregate away from balancer?

In addition, the name given to each modifier indicates whether it acted as an enhancer or suppressor of the *DmcycE^{JP}* phenotype, (E or S respectively) and a number to identify each modifier individually. For example, *65S12* would identify a modifier as an X-ray generated allele selected from bottle 65, suppressor number 12.

With assistance from Dr. Helena Richardson, an X-ray mutagenesis was carried out and a total of 39,234 F1 flies were screened for suppressed or enhanced eye phenotypes compared with *DmcycE^{JP}*. From these, 651 flies were selected as suppressors and were crossed to *b DmcycE^{JP}* to determine whether the modified phenotype was consistent. Of these 651 suppressors, 295 died before they were able to mate or were sterile, and 252 did not consistently suppress the *DmcycE^{JP}* rough eye phenotype or did not have a suppressor mutation that mapped to the second or third chromosome. Thus, stocks of 104 suppressors that consistently modified the *DmcycE^{JP}* phenotype were generated (Table 5.1). Of the 74 suppressors that mapped to the second chromosome, 60 were homozygous lethal or gave escaper homozygous flies at a frequency of less than 5%. In addition, two second chromosome suppressors, *61S5* and *59S4*, behaved as reciprocal 2::Y translocations. As only males ever carry the suppressor mutation in these cases, it is unknown whether they represent homozygous viable or lethal mutations. Of the 30 suppressors that mapped to the third chromosome, 26 were homozygous lethal, three were homozygous viable and one was a dominant female-lethal suppressor, *1S1*. As no modified *1S1* females are ever obtained to cross to the modified males, this allele cannot be classified as either homozygous viable or lethal.

499 flies were originally selected as X-ray-induced enhancers of *DmcycE^{JP}*. Of these, 156 died before mating or were sterile and 284 did not give a consistent phenotype or did not have an enhancer mutation that mapped to the second or third chromosome. 59 enhancer mutants were therefore isolated in the X-ray mutagenesis (Table 5.1). A total of 21 enhancers mapped to the second chromosome, 13 of which were homozygous lethal. 38 enhancers mapped to the third chromosome and 29 of these were homozygous lethal.

To complement the types of alleles expected to be isolated in the X-ray mutagenesis, an EMS mutagenesis was carried out with the help of Dr. H. Richardson, Dr. A. Brumby and Dr. J. Horsfield. A total of 15,049 F1 flies were screened and 364 flies with suppressed eyes were selected. Of these, 147 died or were sterile and 188 did not map to the second or third chromosome or did not give a consistent phenotype. Stocks were generated of the 29 suppressors, 24 of which mapped to the second chromosome and five to the third chromosome. While five of the 24 alleles mapping to the second chromosome were

homozygous viable, no homozygous viable EMS alleles were isolated on the third chromosome. Of the 613 enhancers selected, 340 died or were sterile and 219 did not give a consistent phenotype or did not map to the second or third chromosome. Thus, 54 EMS induced enhancer mutations were isolated. Of the 14 mapping to the second chromosome, 13 were homozygous lethal and of the 40 on the third chromosome 38 were homozygous lethal (Table 5.1).

Table 5.1 Summary of the number of modifiers identified in the screen

		2nd chromosome		3rd chromosome	
		Homozygous lethal	Homozygous viable	Homozygous lethal	Homozygous viable
Suppressors	EMS	19	5	5	0
	X-ray	60	12	26	3
Enhancers	EMS	13	1	38	2
	X-ray	13	8	29	9

Summary of the number of homozygous viable and homozygous lethal second and third chromosome modifiers obtained from the EMS and X-ray mutageneses. Not included in the table are three X-ray-generated suppressor mutations for which it is not possible to know whether they are homozygous viable or lethal. Two of these are likely to be 2::Y translocations, and the other is dominant female lethal. A total of 246 modifiers were obtained.

From the X-ray mutagenesis, nine alleles of *black* were generated, giving an approximate mutation frequency of 2.3×10^{-3}. As all nine black-bodied flies arose from different bottles, these represent independent events. The EMS mutagenesis produced a total of six black-bodied flies, although since some of these flies came from the same bottle, this may represent 4 to 6 independent *black* alleles. As it cannot be easily determined whether these are independent mutations in the *black* gene, the mutation frequency can only be estimated as a range of 2.8×10^{-4} to 4.2×10^{-4}. The mutation frequencies obtained for the X-ray and EMS mutageneses are within the ranges described by previous studies (Grigliatti, 1986), and indicate that both of the mutagenic regimes used were efficiently inducing mutations.

As such a large number of modifiers were isolated in the X-ray and EMS mutageneses, this study was restricted to the further characterisation of homozygous lethal suppressor mutations mapping to the second chromosome. These suppressors mutants are expected to be negative regulators of proliferation and may include homologues of tumour

suppressor genes. Mutations mapping to the second chromosome were chosen as the Berkeley *Drosophila* Genome Project (BDGP) has begun a concerted effort to sequence this chromosome and characterise *P* element insertions. This therefore maximised the chances of isolating a novel gene for which either a *P* allele or genomic sequence was already available.

5-4 Second chromosome suppressor complementation groups

Of the 98 suppressors of $DmcycE^{JP}$ mapping to the second chromosome, 75 were found to be homozygous lethal (Table 5.1). In addition, four stocks, *E9S22, 23S8*, *51S5* and *55S8*, give rise to homozygous flies at a frequency of less than 5%, and were also classed as homozygous lethal. The number of genes represented by these 79 second chromosome suppressors was estimated by complementation analysis. This involved *inter se* crosses to determine which combinations of alleles resulted in transheterozygous lethality. This analysis revealed that there were 46 complementation groups, 10 with more than one allele and 36 single alleles.

Basic characterisation was then carried out on those complementation groups for which more than one allele was isolated. This basic characterisation included determining whether the suppression of $DmcycE^{JP}$ was occurring at the level of S phases during eye development, and whether the modifier was likely to be acting upstream or downstream of *cyclin E* transcription. The cytological location of the lethal mutation was also determined for some complementation groups by crossing suppressors to deficiencies available from the Bloomington stock centre. In addition, the stage of lethality was determined for some complementation groups. More detailed mutant phenotype characterisation was limited to $Su(DmcycE^{JP})2\text{-}2$ and $Su(DmcycE^{JP})2\text{-}1$, which is described in Chapters 7 and 8.

To determine whether a suppressor was acting at the level of S phase regulation, modifiers were crossed to the *Curly-Tubby* (*Cy-Tb*) second chromosome balancer, which carries the *Tubby* dominant larval marker. By crossing suppressors balanced using *Cy-Tb* to a homozygous $DmcycE^{JP}$ strain, $DmcycE^{JP}$, *suppressor*/$DmcycE^{JP}$, + third instar larvae could be distinguished from other genotypes for examination of S phases by BrdU labelling. In addition, balancing a suppressor stock using *Cy-Tb* enabled any homozygous modifier larvae that survive to or beyond the third larval instar to be identified and the stage of lethality determined.

To determine whether any suppressors identified in the screen were acting downstream of *cyclin E* transcription, the effect of reducing the dose of these genes on the adult eye phenotype caused by overexpression of *cyclin E* was examined. In this assay,

cyclin E was overexpressed in the developing eye disc using the UAS/GAL4 system (Brand and Perrimon, 1993). Gal4 expression driven by the *GMR* enhancer was used to indirectly overexpress *cyclin E* in all cells posterior to the MF during eye development (Hay *et al.*, 1994), resulting in ectopic S phases and adults with a mild rough eye phenotype. Suppressors of $DmcycE^{JP}$ acting downstream of *cyclin E* transcription, such as inhibitors, would be expected to enhance the rough eye phenotype generated by overexpression of *cyclin E*. Conversely, a suppressor acting upstream of *cyclin E* transcription or acting in a parallel pathway would not be able to have an effect on this phenotype. In addition to testing the effect of second chromosome suppressors on the overexpression phenotype of type I *cyclin E*, the effect on the type II *cyclin E* overexpression phenotype was also determined. The effect was tested on both of these phenotypes as type I and II Cyclin E differ in their amino terminus, and have different abilities to induce S phases within the MF when ectopically expressed (Crack *et al.*, 1999).

The effect of second chromosome suppressors was also tested on the eye phenotype of a hypomorphic *roughex* (*rux*) mutant. The *rux* rough eye phenotype results from the inappropriate persistence of Cyclin A in the MF leading to ectopic S phases. As discussed in Chapter 3, reducing the dose of either *cyclin A* or *string* suppresses the *rux* phenotype by reducing the number of ectopic S phases (Dong *et al.*, 1997). *string* and *cyclin A* also genetically interact with $DmcycE^{JP}$. Reducing the dose of *cyclin A* or *string* enhance the $DmcycE^{JP}$ phenotype and this occurs not at the level of S phase, but by reducing the number of mitoses posterior to the MF (Chapter 3; Secombe *et al.*, 1998). Consistent with other genes interacting with both *rux* and $DmcycE^{JP}$, several third chromosome deficiencies that modify $DmcycE^{JP}$ also affect the *rux* phenotype (B. Thomas, pers. com.). The identification of a suppressor of $DmcycE^{JP}$ as an enhancer of the *rux* rough eye phenotype may indicate that this gene is also a negative regulator of Cyclin A. It was therefore of interest to test whether reducing the dose of $DmcycE^{JP}$ suppressors identified in the screen were able to affect the *rux* phenotype. The characterisation of each of the homozygous lethal second chromosome suppressor complementation groups will now be discussed. For a description of nomenclature used in this thesis for second chromosome suppressors isolated, see materials and methods, section 2-3.5.

5-4.1 *Su(DmcycE^{JP})2-1* and *Su(DmcycE^{JP})2-2*

The complementation group *Su(DmcycE^{JP})2-1* has two X-ray induced, *2-1^{23S9}* and *2-1^{27S3}*, and two EMS induced, *2-1^{E2S31}* and *2-1^{E6S2}*, alleles (Table 5.2). This complementation group was characterised in more detail than other groups, and is discussed in more detail in Chapter 8. The *Su(DmcycE^{JP})2-2* complementation group has three X-ray-induced alleles, *2-2^{28S2}*, *2-2^{38S4}* and *2-2^{39S2}* (Table 5.3), the characterisation of which is described in Chapter 7.

Table 5.2 Complementation analysis of *Su(DmcycE^{JP})2-1*

Strain	*23S9*	*27S3*	*E2S31*	*E6S2*
23S9	-	0/200	0/102	0/165
27S3	-	-	0/92	0/147
E2S31	-	-	-	0/187
E6S2	-	-	-	-

Complementation analysis for *Su(DmcycE^{JP})2-1*. All combinations of *Su(DmcycE^{JP})2-1* alleles are lethal when transheterozygous. Data are presented as number of transheterozygous flies/total number scored.

Table 5.3 Complementation analysis of *Su(DmcycE^{JP})2-2*

Strain	*28S2*	*38S4*	*39S2*
28S2	-	0/198	0/285
38S4	-	-	0/147
39S2	-	-	-

Complementation analysis for *Su(DmcycE^{JP})2-2*. Crossing any of the three X-ray-induced *Su(DmcycE^{JP})2-2* alleles together results in lethality. Data are presented as number of transheterozygous flies/total number scored.

5-4.2 *Su(DmcycE^{JP})2-3*

Two X-ray induced non-complementing (0 transheterozygotes out of 170 scored) alleles of *Su(DmcycE^{JP})2-3* were isolated and named *2-3^{59S16}* and *2-3^{65S12}*. Both alleles are strong suppressors of the *DmcycE^{JP}* rough eye phenotype resulting in near wild-type eyes (Figure 5.3). To examine whether *Su(DmcycE^{JP})2-3* suppressed the *DmcycE^{JP}* rough eye phenotype by increasing the number of S phases seen during eye development, BrdU incorporation was carried out on *DmcycE^{JP}*, *2-3/DmcycE^{JP}*, + eye discs. As can be seen from Figure 5.3, decreasing the dose of *Su(DmcycE^{JP})2-3* in a *DmcycE^{JP}* background results

in an increase in the number of S phases, accounting for the suppressed eye phenotype observed. *Su(DmcycE^{JP})2-3* also appears to act upstream of *cyclin E* transcription, or in a parallel pathway and independently of Rux, as reducing the dose of this modifier had no effect on the rough eye phenotype generated by overexpression of either type I or type II *cyclin E* or on the *rux* mutant phenotype (data not shown).

Both *Su(DmcycE^{JP})2-3* alleles were generated by X-irradiation, which often results in chromosomal rearrangements that affect meiotic recombination. To determine whether either of the two alleles of *Su(DmcycE^{JP})2-3* were likely to be chromosomal re-arrangements, both alleles were tested for their effect on the recombination frequencies between the recessive *black* (*b*) *cinnabar* (*cn*) and *brown* (*bw*) second chromosome markers. While *2-3^{59S16}* did not affect recombination between these markers, *2-3^{65S12}* resulted in a reduced frequency of recombination between the *b* and *cn* markers (data not shown). Both alleles were therefore crossed to deficiencies covering the region between these two markers, which map cytologically to 34D4-6 and 43E3-14, respectively. *2-3^{65S12}* and *2-3^{59S16}* failed to complement *Df(2L)TW137* (36C3-4 to 37B9-C1), *Df(2L)TW50* (36E4-F1 to 38A6-7), *Df(2L)E71* (36F2-6 to 37C6-D1), *Df(2L)TW3* (36F7-37A1 to 37B2-8) and *Df(2L)OD15* (36F7-9 to 37B9-C1), while both alleles complemented *Df(2L)H20* (36A8-9 to 36E3-4), and *Df(2L)PR-A16* (37B2-12 to 38D2-5). Thus the region defining *Su(DmcycE^{JP})2-3* is 36F7-37A1 to 37B2-8 (Figure 5.4).

A summary of genes and their known function or phenotypes within the 36F7-37A1 to 37B2-8 region is provided in Table 5.4. As none of the genes with known function in the region are strong candidates for *Su(DmcycE^{JP})2-3*, alleles were crossed to the numerous EMS and *P* element alleles in the region for which little information was available. Crossing *2-3^{59S16}* and *2-3^{65S12}* to EMS mutants in the region revealed that *2-3^{59S16}* is likely to be a deficiency removing at least six complementation groups, including *l(2)36Fd* and *l(2)37Ac* (Table 5.5). However, *2-3^{65S12}* was found to be lethal over *l(2)36Fd*, and give ~5% escapers over *l(2)37Ac*. *2-3^{65S12}* is therefore likely to be a smaller lesion affecting both of these genes. To determine which of these EMS alleles was likely to represent an allele of *Su(DmcycE^{JP})2-3*, both *l(2)36Fd* and *l(2)37Ac* were recombined onto the *DmcycE^{JP}* chromosome and their eye phenotypes examined. While *l(2)37Ac* had no effect on the *DmcycE^{JP}* phenotype, *l(2)36Fd* suppressed the phenotype (data not shown). Since *l(2)36Fd* completely fails to complement both *2-3^{59S16}* and *2-3^{65S12}*, and suppresses the *DmcycE^{JP}* rough eye phenotype, it is likely that *l(2)36Fd* is an allele of *Su(DmcycE^{JP})2-3*.

Figure 5.3 *Su(DmcycE^{JP})2-3* suppresses *DmcycE^{JP}* by increasing S phases

(A, B, C) Scanning electron micrographs of adult eyes from (A) *DmcycE^{JP}*, (B) *DmcycE^{JP}, 2-3^{59S26}/DmcycE^{JP}*, + and (C) *DmcycE^{JP}, 2-3^{65S12}/DmcycE^{JP}*, + showing suppression of the *DmcycE^{JP}* rough eye phenotype. (D, E, F) BrdU labelling of eye discs from (D) *DmcycE^{JP}* (E) *DmcycE^{JP}, 2-3^{59S26}/DmcycE^{JP}*, + and (F) *DmcycE^{JP}, 2-3^{65S12}/DmcycE^{JP}*, + third instar larvae, demonstrating that suppression of the *DmcycE^{JP}* rough eye phenotype results from an increase in the number of S phases posterior to the MF. Anterior is to the right. Bars indicate the MF in D, E and F.

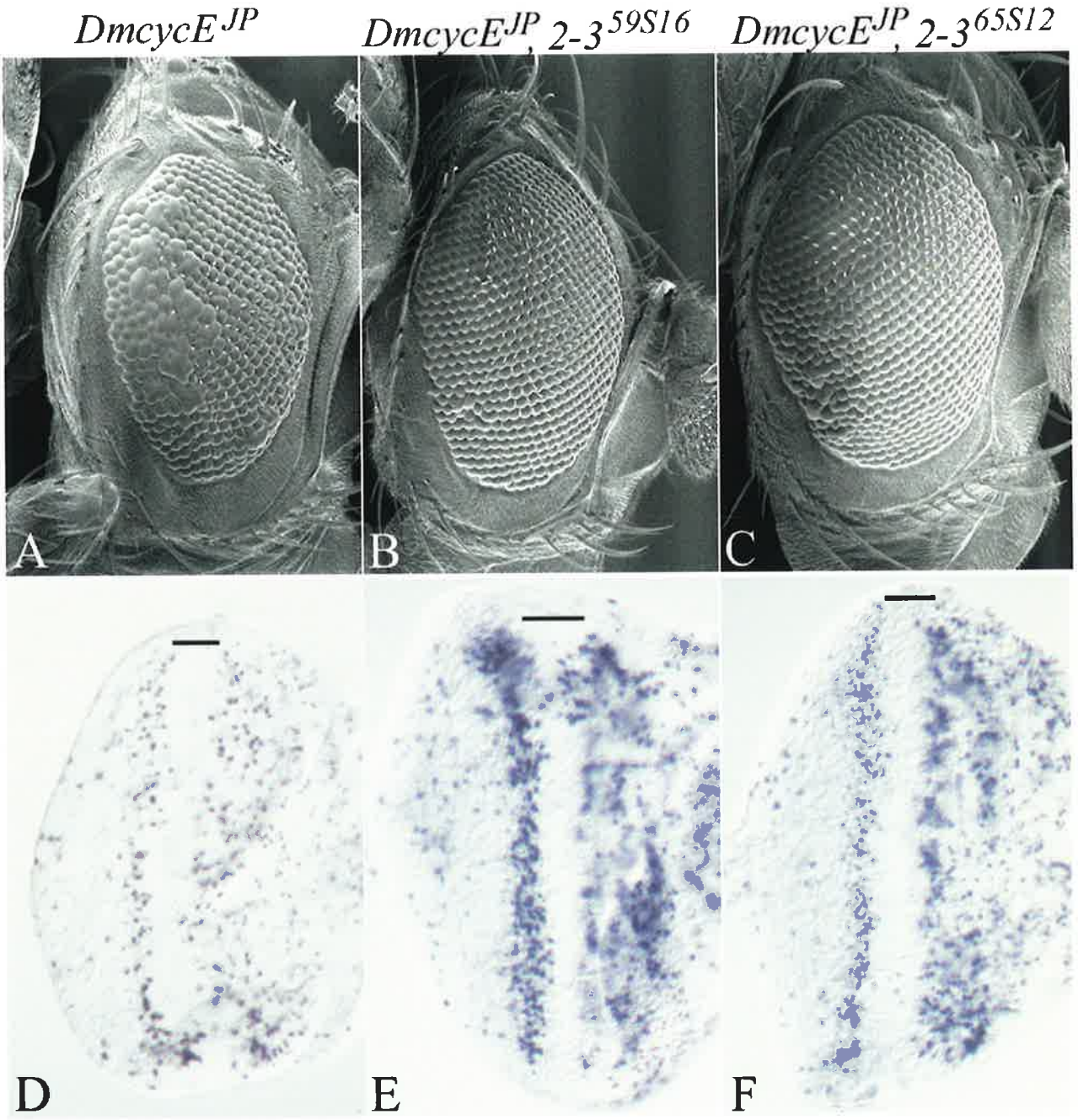

DmcycE^JP
DmcycE^JP, 2-3^59S16
DmcycE^JP, 2-3^65S12
A
B
C
D
E
F

Figure 5.4 Cytological location of *Su(DmcycE^{JP})2-3*

Schematic representation of the cytological location of *Su(DmcycE^{JP})2-3*. The region to which this complementation group maps was determined by crossing *2-3^{59S16}* and *2-3^{65S2}* to deficiencies with known breakpoints. Both alleles were lethal over *Df(2L)TW137*, *Df(2L)TW50*, *Df(2L)E71*, *Df(2L)TW3* and *Df(2L)OD15*, and viable over *Df(2L)H20* and *Df(2L)PR-A16*, defining the region for *Su(DmcycE^{JP})2-3* as 36F7-37A1 to 37B2-8. Complementation data presented on left hand side of figure are given as the number of flies transheterozygous for the *Su(DmcycE^{JP})2-3* allele and the deficiency over the total number of flies scored. Black bars represent the region deleted in deficiencies, with dashes at the end of each bar indicating breakpoint uncertainty regions. * The one escaper *2-3^{65S12}/Df(2L)TW137* fly observed was wild-type in appearance and fertile.

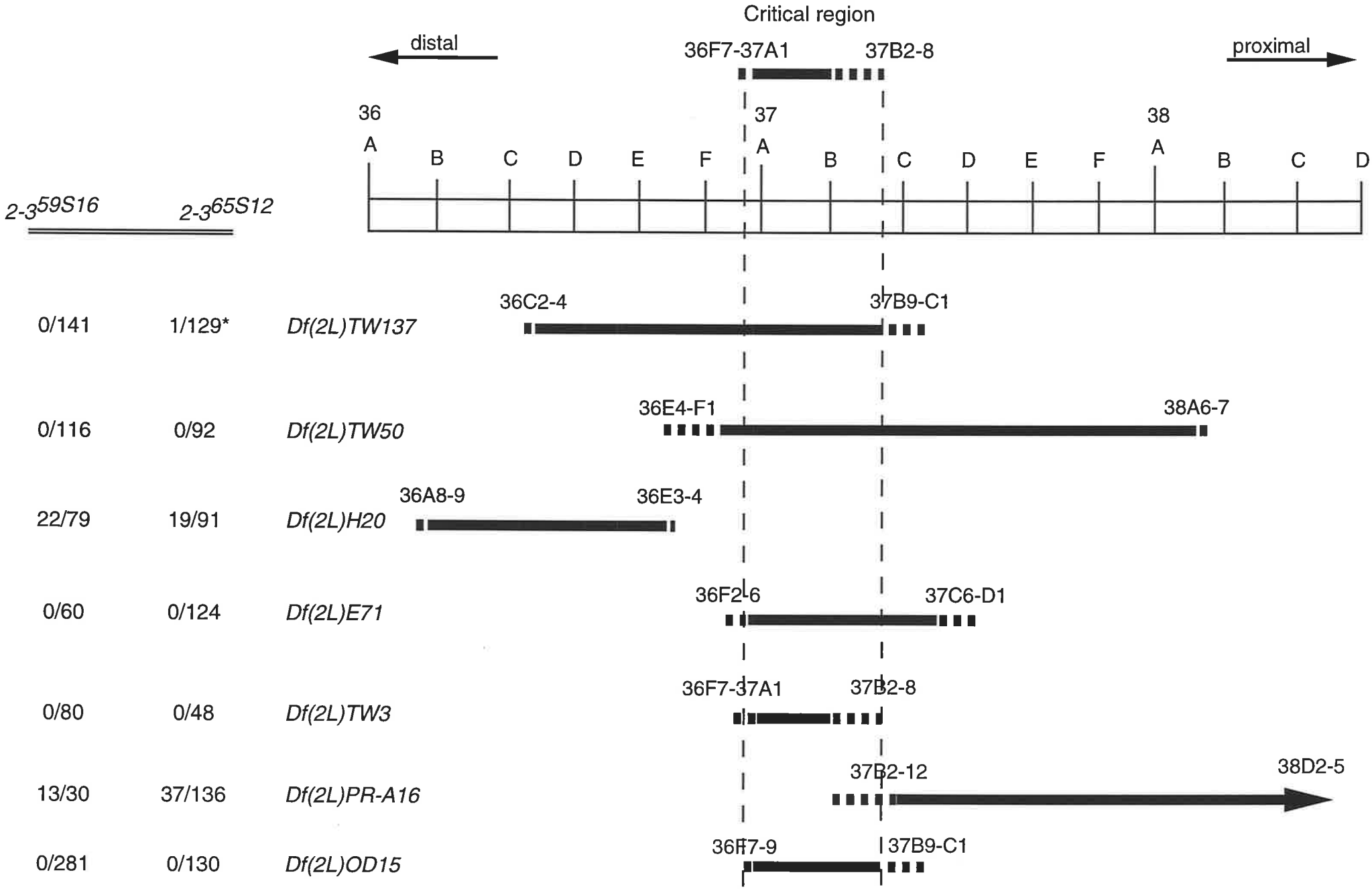

Critical region
distal
proximal
36F7-37A1
37B2-8
36
37
38
A
B
C
D
E
F
2-3 59S16
2-3 65S12
0/141
1/129*
Df(2L)TW137
36C2-4
37B9-C1
0/116
0/92
Df(2L)TW50
36E4-F1
38A6-7
22/79
19/91
Df(2L)H20
36A8-9
36E3-4
0/60
0/124
Df(2L)E71
36F2-6
37C6-D1
0/80
0/48
Df(2L)TW3
13/30
37/136
Df(2L)PR-A16
37B2-12
38D2-5
0/281
0/130
Df(2L)OD15
36F7-9
37B9-C1

Table 5.4 Summary of genes in the region 36F7 to 37B8

Gene	Cytology	Known function/phenotype
presto	36E4-37C1	Maternal effect only
fs(2)eo3	36E4-38A7	Female sterile
fs(2)lo-F	36E4-38A7	Female sterile
fs(2)lo-G	36E4-38A7	Female sterile
fs(2)1to2	36E4-38A7	Female sterile
fs(2)1to3	36E4-38A7	Female sterile
l(2)00455	**36F1-2**	***P* element allele**
M(2)36F	36F2-6	Strong dominant minute phenotype, with small bristles and chunky body
l(2)36Fc	**36F4-37A1**	**EMS allele. See Table 5.5**
l(2)36Fb	**36F4-6**	**EMS allele. See Table 5.5**
Mst36Fa	36F6-10	Male specific transcript
Mst36Fb	36F6-10	Male specific transcript
Tosca	36F6-10	Expressed only in female germline
l(2)36Ff	**36F7-37A1**	**EMS allele. See Table 5.5**
l(2)36Fg	**36F7-37A1**	**EMS allele. See Table 5.5**
l(2)36Fh	**36F7-37A1**	**EMS allele. See Table 5.5**
l(2)36Fi	**36F7-37A1**	**EMS allele. See Table 5.5**
male specific lethal 1	36F7-37A1	Male specific effect
Rough eye	36F7-37B8	Mutants show dominant eye roughening
l(2)36Fd	**36F7-9**	**EMS allele. See Table 5.5**
l(2)36Fe	**36F7-9**	**EMS allele. See Table 5.5**
l(2)37Aa	**36F8-37B7**	**EMS allele. See Table 5.5**
Tufted	36F8-37B7	Dominant bristle phenotype
NaCP37B	36F8-37B7	Sodium channel protein
l(2)k13805	**37A1-2**	***P* element allele**
l(2)k05402	**37B7-10**	***P* element allele**
l(2)01265	**37B8-9**	***P* element allele**
l(2)k16106	**37B8-9**	***P* element allele**
l(2)02660	**37B8-9**	***P* element allele**
l(2)k05424	**37B8-12**	***P* element allele**
α-*methyl dopa resistant* (*amd*)	37B9-C1	Required for neurotransmitter production

Summary of the genes and mutations in the critical region for *Su(DmcycE^{JP})2-3*. Genes indicated in bold are considered to be candidates for *Su(DmcycE^{JP})2-3*.

Table 5.5 Complementation tests of EMS alleles with *Su(DmcycE^{JP})2-3*

EMS mutation	*Su(DmcycE^{JP})2-3^{59S16}*	*Su(DmcycE^{JP})2-3^{65S12}*
l(2)36Fb	28/63	71/160
l(2)36Fd	**0/77**	**0/75**
l(2)36Fe	8/32	47/122
l(2)36Ff	**0/76**	27/81
l(2)36Fg	**0/86**	75/161
l(2)36Fh	**0/122**	22/66
l(2)36Fi	21/41	32/82
l(2)37Aa	**0/91**	60/142
l(2)37Ab	67/156	13/51
l(2)37Ac	**0/242**	**31/381**

To test whether *Su(DmcycE^{JP})2-3* corresponds to any previously identified mutation, *Su(DmcycE^{JP})2-3^{59S16}* and *Su(DmcycE^{JP})2-3^{65S12}* were crossed to unordered EMS mutants in the region. While *2-3^{59S16}* removes at least six complementation groups, *2-3^{65S12}* only affects *l(2)36Fd* and *l(2)37Ac*. Those crosses that resulted in complete or partial lethality of transheterozygous flies are indicated in bold.

Although two X-ray alleles were generated in the mutagenesis, and subsequently one EMS allele has been defined, the advantages of obtaining a *P* element allele are enormous. *P* alleles greatly assist in the expeditious cloning of a gene due to genomic DNA flanking the *P* insert being easily rescued and used to screen cDNA and genomic DNA libraries. With this in mind, *Su(DmcycE^{JP})2-3* alleles were crossed to *P* elements in the region to which *Su(DmcycE^{JP})2-3* mapped. All of the *P* alleles in the region presented in Table 5.4 of *Su(DmcycE^{JP})2-3* complemented *2-3^{59S16}* and *2-3^{65S12}* (data not shown), indicating that no *P* alleles of this modifier exist.

To determine the stage at which *Su(DmcycE^{JP})2-3* alleles are lethal, *2-3^{59S16}*, *2-3^{65S12}* and *l(2)36Fd* were crossed to the *Cy-Tb* balancer. Homozygous, non-tubby larvae and pupae were identified for *2-3^{65S12}* and *l(2)36Fd*. Homozygous *2-3^{65S12}* and *l(2)36Fd* pupae do not, however, eclose and they die as pharate adults with no obvious defects (data not shown). Consistent with *2-3^{59S16}* deleting multiple genes, no non-tubby larvae were detected when this allele was balanced using *Cy-Tb*, indicating that homozygotes die before the third larval instar. It remains to be determined whether *Su(DmcycE^{JP})2-3* homozygotes have any proliferative defects and why these mutants fail to eclose.

5-4.3 *Su(DmcycE^{JP})2-4*

Su(DmcycE^{JP})2-4 has three X-ray generated alleles, *26S8*, *57S6* and *59S3* (Table 5.6), that suppress the *DmcycE^{JP}* phenotype by increasing the number of S phases posterior to the MF during eye disc development (Figure 5.5). Like *Su(DmcycE^{JP})2-3*, reducing the dose of *Su(DmcycE^{JP})2-4* has no effect on the eye phenotype caused by overexpression of type I or type II *cyclin E*, or on the *rux* phenotype. In addition, crossing alleles of *Su(DmcycE^{JP})2-4* to *Cy-Tb* demonstrated that all three alleles are lethal before the third larval instar when homozygous. The *Su(DmcycE^{JP})2-4* gene product is therefore likely to be required early in development.

Table 5.6 Complementation analysis of *Su(DmcycE^{JP})2-4*

Strain	*26S8*	*57S6*	*59S3*
26S8	-	0/123	0/157
57S6	-	-	0/205
59S3	-	-	-

All three alleles of *Su(DmcycE^{JP})2-4* are lethal when transheterozygous. Data are presented as number of transheterozygous flies/total number scored.

Preliminary genetic mapping indicated that *Su(DmcycE^{JP})2-4* was located between the *b* and *cn* markers, as two out of three alleles modified recombination between these markers. No recombinants were detected between *b* and *cn* for *2-4^{59S3}*, while recombination between *cn* and *bw* was normal (data not shown). Similarly, the recombination frequency observed between *b* and *cn* in the *2-4^{57S6}* strain was decreased, while recombination between *cn* and *bw* was normal (data not shown). In contrast, *2-4^{26S8}* showed a normal level of recombination between *b* and *cn*, but showed recombination suppression between *cn* and *bw*, although this allele complemented all deficiencies in this region (data not shown). Consistent with *Su(DmcycE^{JP})2-4* mapping between the *b* and *cn* markers, all three alleles failed to complement *In(2R)bw^{VDe2L}* (41A-B to 42A3-4) and *Df(2R)nap1* (41D2-E1 to 42B1-3) and complemented *Df(2R)nap9* (42A1-2 to 42E6-F1), defining the region for *Su(DmcycE^{JP})2-4* as 41D2-E to 42A1-2 (Figure 5.6). The *2-4^{26S8}* allele therefore has two lesions, one between *cn* and *bw* that results in suppression of recombination between these two markers, which may or may not be associated with a lethal mutation, and the other at 41D2-E to 42A1-2 causing the mutation in the *Su(DmcycE^{JP})2-4* gene.

All of the *P* alleles available in the 41D-42A region complement *Su(DmcycE^{JP})2-4* alleles (data not shown), indicating that there are no previously characterised *P* insertions in this gene. One of the *P* insertions that complemented the *Su(DmcycE^{JP})2-4* alleles, *l(2)06210*, is an allele of the Ecdysone receptor, which has been shown to be required for MF progression during eye development (Brennan *et al.*, 1998). The viability of this *P* insert over *Su(DmcycE^{JP})2-4* alleles eliminates this gene as a candidate. Examination of the remaining genes in the region of *Su(DmcycE^{JP})2-4* revealed one candidate for this suppressor, *Act42A*, which encodes the actin-related protein Act1 protein (Table 5.7). Peptide sequencing of proteins associated with *Drosophila* Brahma identified Act1 and/or Act2 (encoded by the *Act5c* gene on the X chromosome) as being as being part of this multi-subunit complex (Papoulas *et al.*, 1998). Although immunoprecipitation experiments have been unable to demonstrate a clear association between Act1 or Act2 and Brahma *in vivo*, Actin and an actin-related protein have also been identified as subunits of the human hBRM and BRG complexes (Papoulas *et al.*, 1998). The association between actin and the Brahma complex is somewhat surprising given that Act1 has only ever been reported to be cytoplasmic (Fyrberg *et al.*, 1983), and Brahma acts on chromatin structure in the nucleus. This interaction therefore suggests that there may be low levels of Act1 or Act2 protein in the nucleus. As described previously (section 4-2.5), there is substantial evidence for the involvement of Brahma in the negative regulation of proliferation. Unfortunately, there are no mutations available in *Act42A*, so allelism between *Su(DmcycE^{JP})2-4* and *Act42A* could not be tested directly. As both *2-4^{57S6}* and *2-4^{59S3}* suppress recombination and are therefore likely to be chromosomal re-arrangements, genomic Southern analysis could be used to determine whether the *Act42c* region is re-arranged in these *Su(DmcycE^{JP})2-4* alleles. As there are no other clear candidates for *Su(DmcycE^{JP})2-4*, this suppressor may be *Act42A*, or a novel gene.

Figure 5.5 *Su (DmcycE^{JP})2-4* suppresses *DmcycE^{JP}* by increasing the number of S phase cells during eye development

(A, B, C, D) Scanning electron micrographs of adult eyes from (A) *DmcycE^{JP}*, (B) *DmcycE^{JP}, 2-4^{26S8}/DmcycE^{JP}*, +, (C) *DmcycE^{JP}, 2-4^{57S6}/DmcycE^{JP}*, + and (D) *DmcycE^{JP}, 2-4^{59S3}/DmcycE^{JP}*, + showing suppression of the *DmcycE^{JP}* rough eye phenotype. (D, E, F, G) BrdU labelling of eye discs from (D) *DmcycE^{JP}* (E) *DmcycE^{JP}, 2-4^{26S8}/DmcycE^{JP}*, + (F) *DmcycE^{JP}, 2-4^{57S6}/DmcycE^{JP}*, + and (G) *DmcycE^{JP}, 2-4^{59S3}/DmcycE^{JP}*, + third instar larvae, demonstrating that suppression of the *DmcycE^{JP}* rough eye phenotype results from an increase in the number of S phases posterior to the MF. Anterior is to the right. Bars in E, F, G, and H indicate the MF.

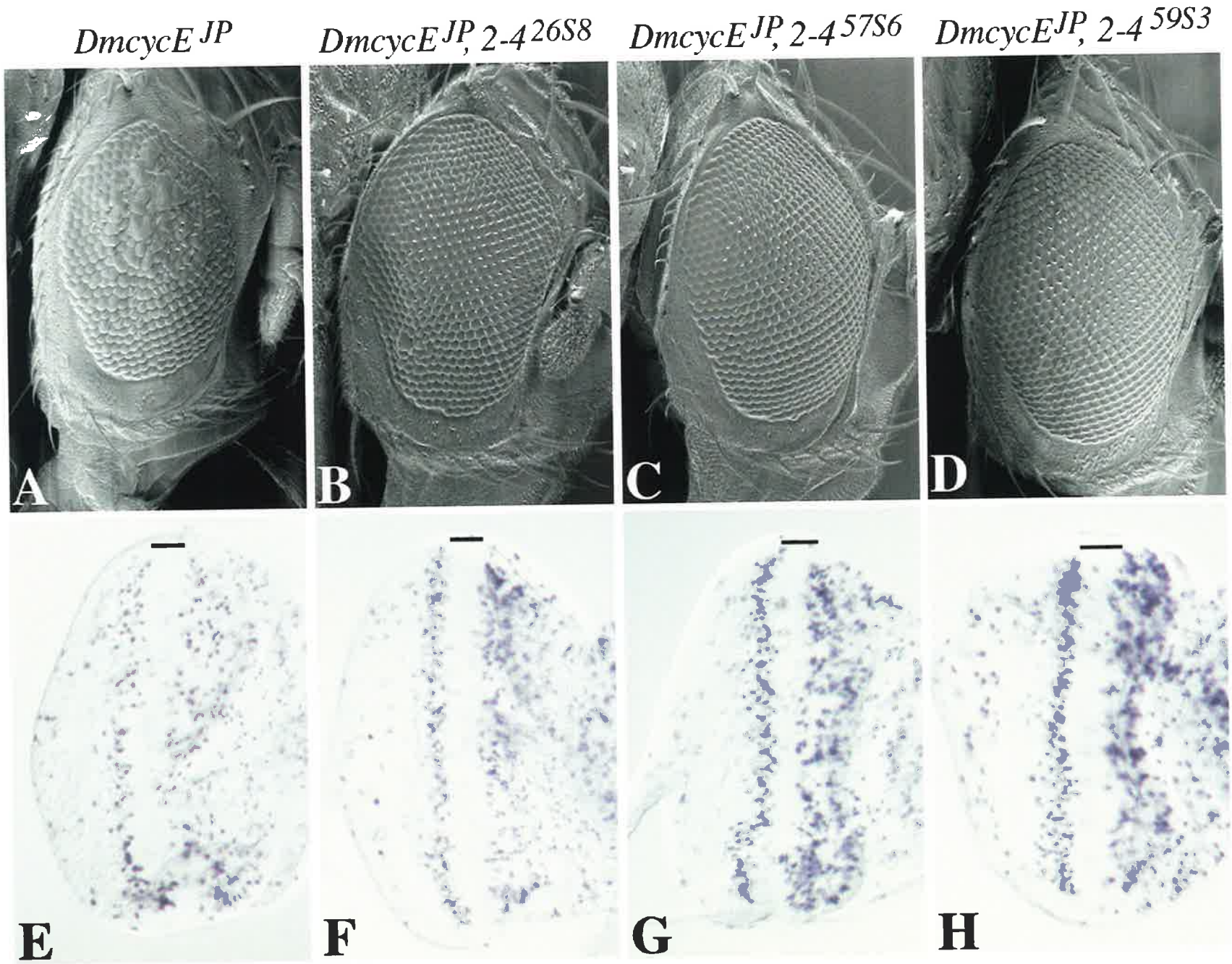

DmcycE JP
DmcycE JP, 2-4 26S8
DmcycE JP, 2-4 57S6
DmcycE JP, 2-4 59S3
A
B
C
D
E
F
G
H

Figure 5.6 Cytological location of *Su(DmcycE^{JP})2-4*

Schematic representation of the cytological region to which *Su(DmcycE^{JP})2-4* maps. The three alleles of *Su(DmcycE^{JP})2-4*, *2-4^{26S8}*, *2-4^{57S6}* and *2-4^{59S3}*, were lethal over *In(2R)bw^{Vde2L}* and *Df(2R)nap1* and viable over *Df(2R)nap2*, indicating that *Su(DmcycE^{JP})2-4* maps between 41D2-E1 and 42A1-2. Complementation data are shown for only *2-4^{59S3}* allele, but similar results were obtained with *2-4^{26S8}* and *2-4^{57S6}*. Black bars represent the region deleted in deficiencies, with the dashed regions at the end of each bar indicating the breakpoint uncertainty regions.

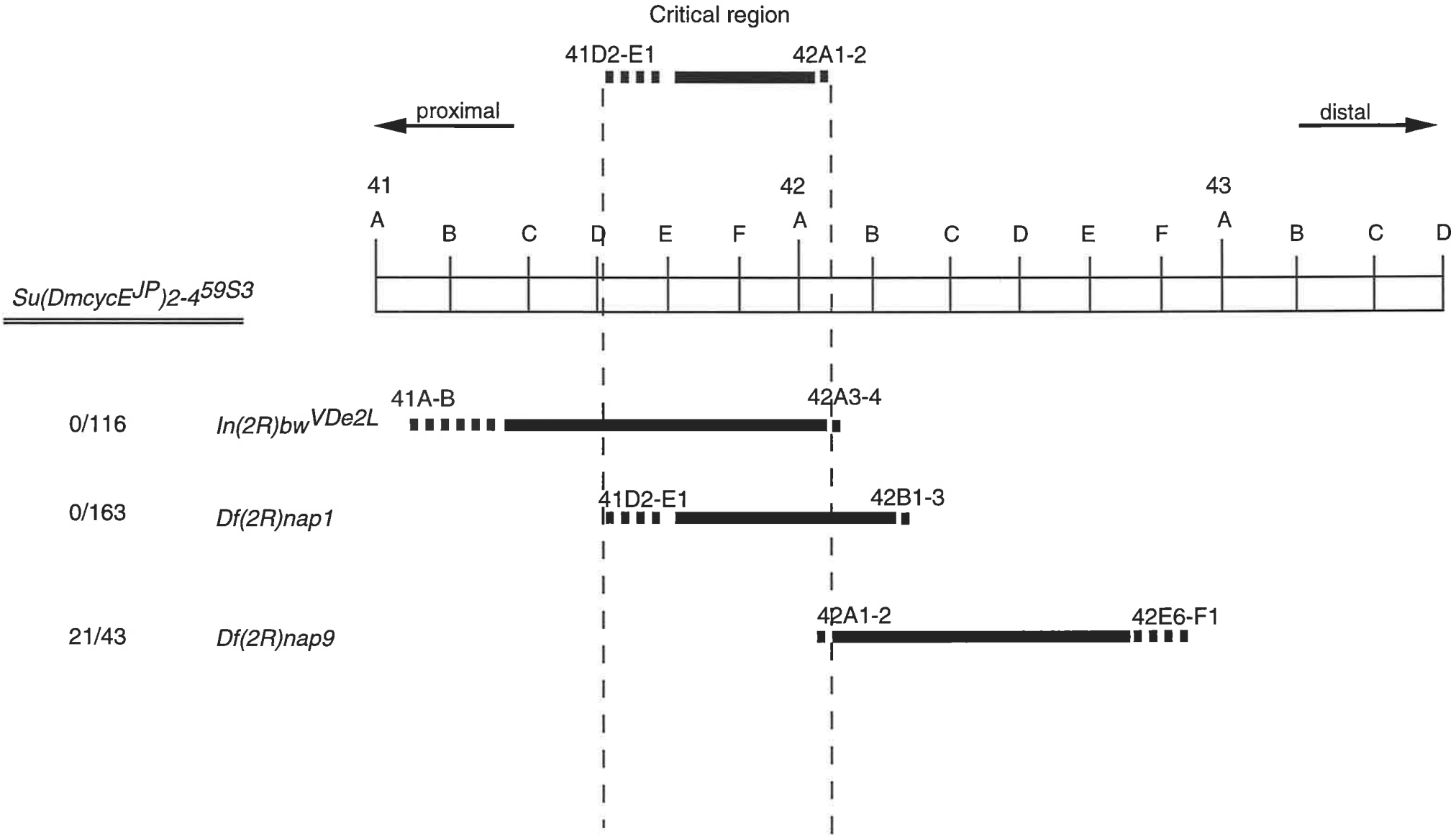

Critical region
41D2-E1
42A1-2
proximal
distal
41
42
43
A
B
C
D
E
F
A
B
C
D
E
F
A
B
C
D
Su(DmcycE^JP)2-4^59S3
0/116
In(2R)bw^VDe2L
41A-B
42A3-4
0/163
Df(2R)nap1
41D2-E1
42B1-3
21/43
Df(2R)nap9
42A1-2
42E6-F1

Table 5.7 Genes in *Su(DmcycE^{JP})2-4* region

Gene	Cytology	Known function/phenotype
cripple	38B5-43E14	Visible recessive leg phenotype
arista	40-47	Dominant effect on aristae
smoq	h35 to 49D3	Acts in production of cuticular hydrocarbons
misformed	h43 to 42A3	Visible, recessive eye, wing and leg defect
Dip-A	h46 to 42A2	Dipeptidase protein required for digestion
E(da)	41	Affects sex ratios
neuromuscular	41F	Required for terminal differentiation of a sub-set of neurons and muscles
l(2)07022	**41F8-9**	***P* element allele**
l(2)k08138	**41F8-9**	***P* element allele**
roughish	41F9-	Homozygous viable, visible phenotype
apterous	41F9-11	Required for correct wing formation and courtship behaviour. Strong alleles are homozygous lethal
Act42A	**42A**	**Recently identified as a Brahma associated protein 47 (BAP47)**
tRNAs	42A	tRNAs would not be expected to modify *DmcycE^{JP}*
Vm42A	42A	Specifically expressed in pole cells
indora	42A	Expressed only in pole cells
22W	42A	Homozygotes have mitotic defects. Highly condensed chromosomes
lie	42A1-19	Recessive male sterile
l(2)05315	**42A1-2**	***P* element allele**
l(2)k10108	**42A1-2**	***P* element allele**
l(20k03113	**42A1-2**	***P* element allele**
l(2)09851	**42A1-2**	***P* element allele**
maleless	42A1-3	Male specific lethal
Src42A	42A1-4	Homologue of *c-src* proto-oncogene. Loss of function alleles would be predicted to enhance *DmcycE^{JP}* phenotype
thick	42A1-B1	Recessive visible bristle phenotype
l(2)k09848	**42A8-12**	***P* element allele**
Ecdysone receptor	**42A10**	**Ecdysone receptor gene. Allelic to *l(2)06210***
Stambha A	42A8-19	Conditional recessive paralysis phenotype

Summary of the genes and mutants known to be within the region 41D2-E to 42A1-2. tRNAs refers to 19 tRNA genes within this region, which are all equally unlikely candidates for a suppressor of *DmcycE^{JP}*. Gene abbreviations used are *Dipeptidase A* (*Dip-A*), *Vitelline membrane mRNA* (*Vm42A*), *Actin 42A* (*Act42A*), *long island expressway* (*lie*) and *Enhancer of daughterless* (*E(da)*). Genes that are considered candidates for *Su(DmcycE^{JP})2-4* are highlighted in bold.

5-4.4 *Su(DmcycE^{JP})2-5*

Two X-ray generated suppressors, *42S11* and *58S12*, gave no transheterozygous flies when crossed together (Table 5.8). These alleles therefore make up the fifth complementation group identified, *Su(DmcycE^{JP})2-5*. Both of these alleles appear to suppress the *DmcycE^{JP}* rough eye phenotype to the same extent, and this occurs by increasing the number of S phases during eye development (Figure 5.7). Thus, *Su(DmcycE^{JP})2-5* is likely to be a regulator of proliferation. *2-5^{42S11}* or *2-5^{58S12}* did not have any effect on the eye phenotype caused by overexpression of *cyclin E* type I or II (data not shown), suggesting that this suppressor acts upstream of *cyclin E* transcription or in a parallel pathway. In addition, no effect was observed when reducing the dose of *Su(DmcycE^{JP})2-5* in a *rux* mutant background. *Su(DmcycE^{JP})2-5* is therefore likely to act in a different pathway to Rux.

Crossing *Su(DmcycE^{JP})2-5* alleles to the *Cy-Tb* balancer revealed that *2-5^{42S11}* homozygotes die during pupal development and that *2-5^{58S12}* is lethal before the third larval instar. This is consistent with *2-5^{58S12}* being a more severe allele than *2-5^{42S11}*, or a deficiency or re-arrangement affecting more than one gene. The stage at which *2-5^{42S11}/ 2-5^{58S12}* transheterozygotes die has not been determined.

Although no genetic mapping has been carried out with this complementation group, *Su(DmcycE^{JP})2-5* alleles were crossed to a sub-set of the second chromosome deficiencies available to determine a cytological location for this gene. *2-5^{42S11}* failed to complement the overlapping deficiencies *Df(2R)nap9* (42A1-2 to 42E6-F1) and *Df(2R)ST1* (42B3-5 to 43E15-18), and complemented *Df(2R)nap1* (41D2-E1 to 42B1-3) and *In(2R)pk78s* (42C1-7 to 43F5-8) (Figure 5.8). In addition to failing to complement *Df(2R)nap9* and *Df(2R)ST1*, *2-5^{58S12}* was also lethal over *Df(2R)nap1*, indicating that this allele is a deficiency or re-arrangement that affects a larger region than *2-5^{42S11}*. Thus the minimal region for *Su(DmcycE^{JP})2-5* is 42B3-5 to 42C1-7.

Analysis of the genes in the 42B3-42C7 region did not reveal any obvious candidates for *Su(DmcycE^{JP})2-5* (Table 5.9). *Su(DmcycE^{JP})2-5* alleles were therefore crossed to *P* element alleles available in the region in the hope of finding a *Su(DmcycE^{JP})2-5 P* allele. Of the 11 *P* element alleles tested, one was found to be semi-lethal in combination with *2-5^{42S11}* (Table 5.8). The few escaper flies, transheterozygous for *2-5^{42S11}* and *l(2)04524*, do not have any gross abnormalities but appear less healthy than their heterozygous siblings

Figure 5.7 *Su(DmcycE^{JP})2-5* suppresses the *DmcycE^{JP}* phenotype by increasing the number of S phases during eye development

(A, B, C) Scanning electron micrographs of adult eyes from (A) *DmcycE^{JP}*, (B) *DmcycE^{JP}, 2-5^{42S11}/DmcycE^{JP}*, +, and (C) *DmcycE^{JP}, 2-5^{58S12}/DmcycE^{JP}*, + showing suppression of the *DmcycE^{JP}* rough eye phenotype. (D, E, F) BrdU labelling of eye discs from (D) *DmcycE^{JP}* (E) *DmcycE^{JP}, 2-5^{42S11}/DmcycE^{JP}*, + and (F) *DmcycE^{JP}, 2-5^{58S12}/DmcycE^{JP}*, + third instar larvae, showing that suppression of the *DmcycE^{JP}* rough eye phenotype results from an increase in the number of S phases posterior to the MF. Anterior is to the right. Bars indicate the MF in D, E, and F.

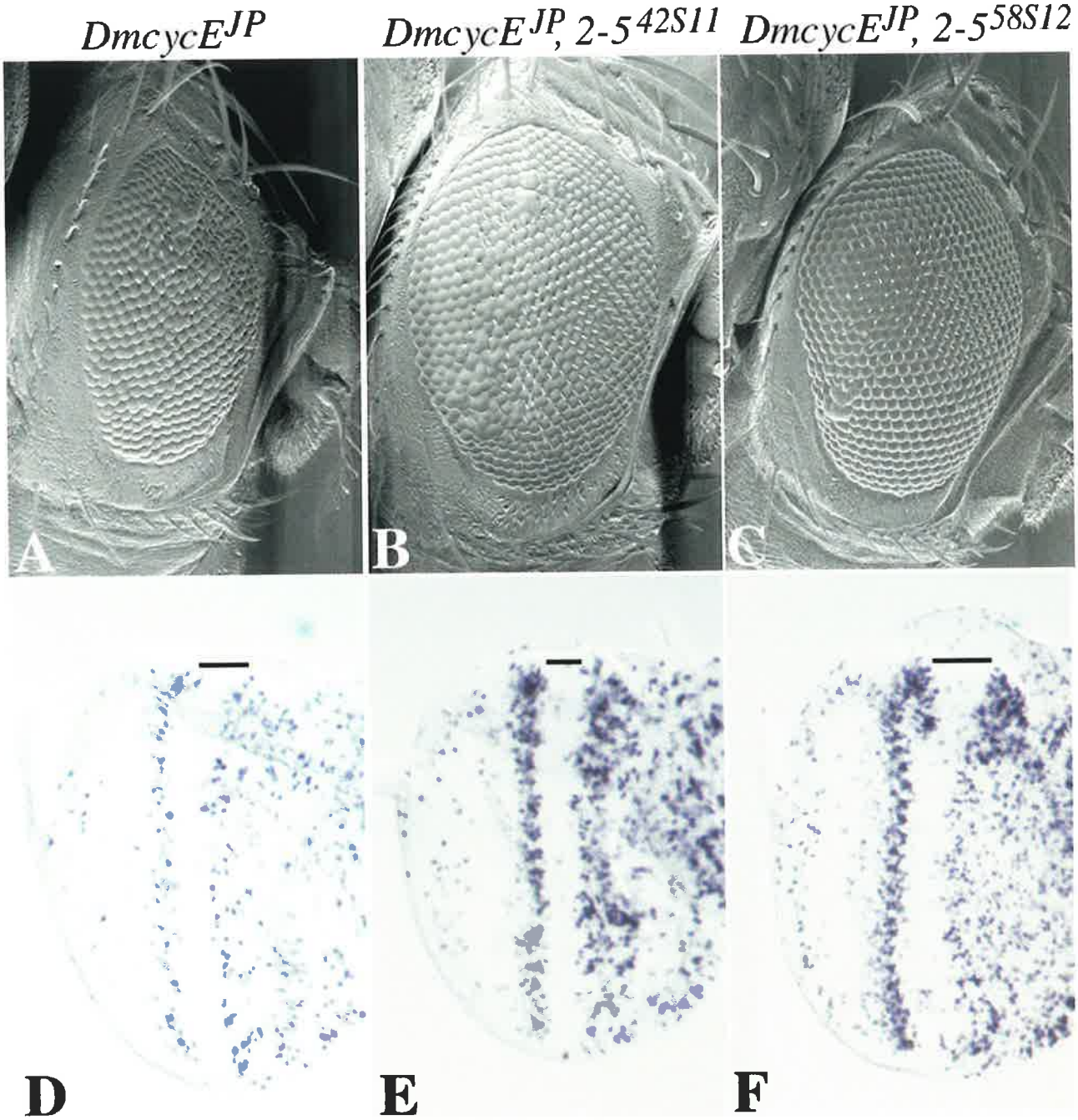
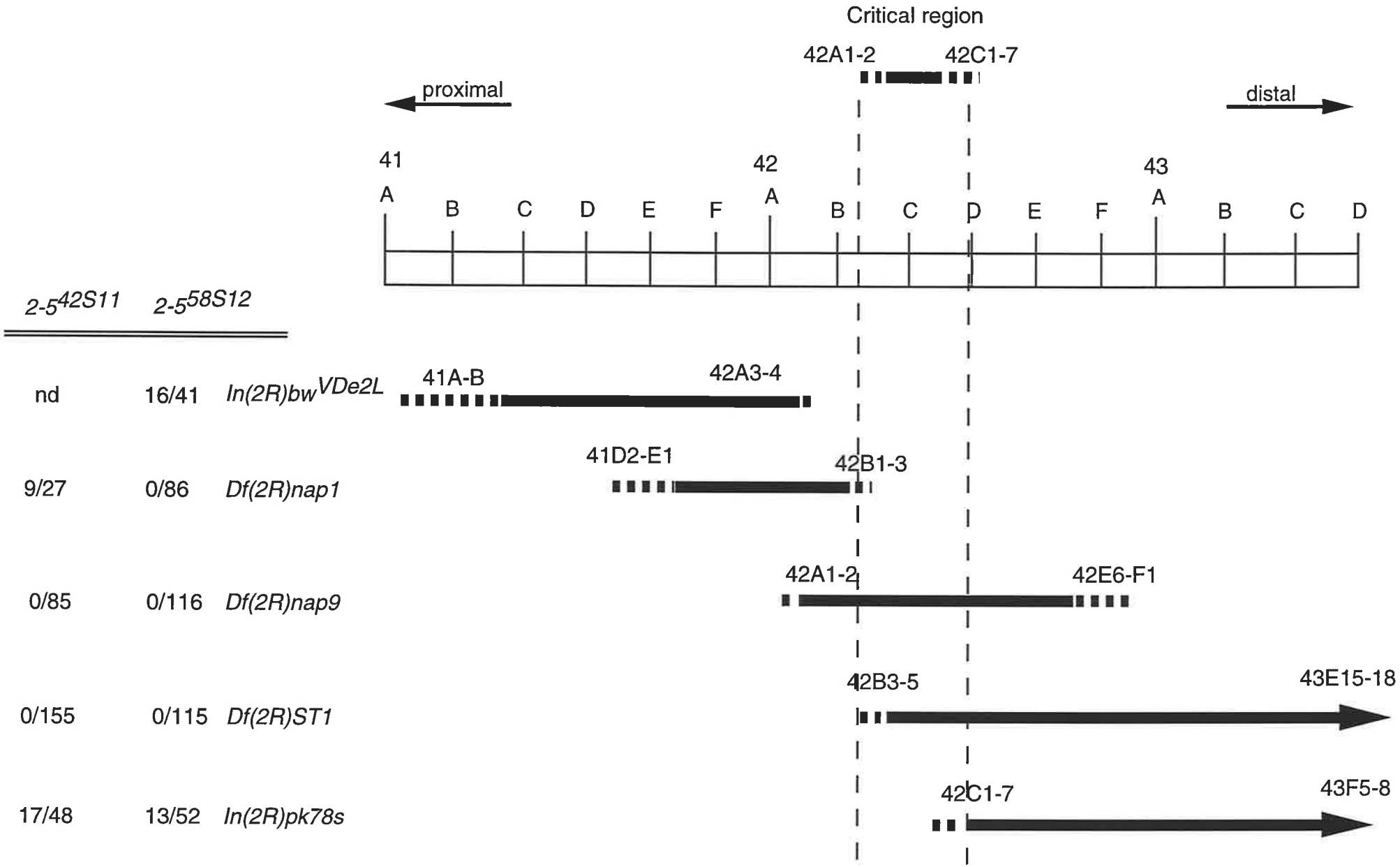

DmcycE^JP
DmcycE^JP, 2-5^42S11
DmcycE^JP, 2-5^58S12
A
B
C
D
E
F

Figure 5.8 Cytological location of *Su(DmcycE^{JP})2-5*

Schematic representation of the cytological region to which *Su(DmcycE^{JP})2-5* maps. *2-5^{42S11}* and *2-5^{58S12}* are lethal over *Df(2R)nap9* and *Df(2R)ST1* and viable over *In(2R)pk78s*. *2-5^{58S12}* is also lethal over *Df(2R)nap1*, which complements *2-5^{42S11}*, indicating that *2-5^{58S12}* is a deficiency or re-arrangement that affects a larger region than *2-5^{42S11}*. Thus the cytological region defining *Su(DmcycE^{JP})2-5* is 42B3-5 to 42C1-7. Black bars represent the regions deleted in deficiencies, with the dashed regions at the end of each bar indicating the breakpoint uncertainty regions. Arrows indicate that the deficiency extends further distal than indicated on map.


Critical region
42A1-2
42C1-7
proximal
distal
41
A
B
C
D
E
F
42
A
B
C
D
E
F
43
A
B
C
D
2-5^42S11
2-5^58S12
nd
16/41
In(2R)bw^VDe2L
41A-B
42A3-4
9/27
0/86
Df(2R)nap1
41D2-E1
42B1-3
0/85
0/116
Df(2R)nap9
42A1-2
42E6-F1
0/155
0/115
Df(2R)ST1
42B3-5
43E15-18
17/48
13/52
In(2R)pk78s
42C1-7
43F5-8

and die within a day of eclosing. Unfortunately, *l(2)04524* could not be crossed to $2\text{-}5^{58S12}$, as this stock was very unhealthy and eventually died.

Table 5.8 Complementation analysis of *Su(DmcycE^{JP})2-5*

Strain	*42S11*	*58S12*	*l(2)04524*
42S11	-	0/148	4/92
58S12	-	-	nd
l(2)04524	-	-	-

Two X-ray generated alleles, *42S11* and *58S12* fail to complement each other, indicating that they are alleles of the same gene. In addition, a *P* element allele in the region, *l(2)04524* is semi-lethal over *42S11*, indicating that this may also be an allele of *Su(DmcycE^{JP})2-5*. Data are presented as the number of transheterozygous flies/total number scored. nd = not determined.

Table 5.9 Genes in the 42B3 to 43C7 region

Gene	Cytology	Known function/phenotype
cripple	38B5-43E14	Visible recessive mutation resulting in leg defects
Arista	40A1-67A9	Mutations have dominant effect on aristae
Fs(2)Ket	h37-49D3	Dominant female sterile mutation
Smoq	h37-49D3	Required for production of cuticular hydrocarbons
l(2)k03204	**42B1-3**	***P* element allele**
l(2)01094	**42B2-3**	***P* element allele**
1.28	42B1-5	Transcriptional target of Dfd specifically in the maxillary head segment
l(2)04524	**42B**	***P* element allele**
rambling	42B3-43E18	Homozygous viable. Mutants have disrupted bolwigs nerve path and morphology
ek6	42B4-E7	Dominant enhancer of overexpression of Notch
Rab2	42C1-10	GTP-binding protein. Mammalian homologue involved in vesicular transport
l(2)04535	**42C1-2**	***P* element allele**
l(2)k14019	**42C1-2**	***P* element allele**
l(2)k09107	**42C1-2**	***P* element allele**
Adf1	42C2-7	Transcription factor. Allelic to *l(2)04065 P* element allele
l(2)01349	**42C3-4**	***P* element allele**
l(2)10521	**42C3-7**	***P* element allele**
l(2)01289	**42C7**	***P* element allele**

Summary of genes in the *Su(DmcycE^{JP})2-5* region. Genes indicated in bold are considered to be candidates. Abbreviations used are *Adh transcription factor 1* (*Adf1*) and *Rab-protein-2* (*Rab2*).

Both the BDGP and Harvie *et al.* (1998) have rescued genomic DNA flanking the *l(2)04524* insert, but the sequence obtained by these two groups differs. Harvie *et al.*, (1998), identified *l(2)04524* as an enhancer trap line that resulted in β-galactosidase expression in the ring gland during all stages of larval development. The sequence obtained from the *P* element rescue described by Harvie *et al.* (1998), has homology to a 66.5kD hypothetical protein identified during the sequencing of *Caenorhabditis elegans* chromosome III (Wilson *et al.*, 1994). Although the gene identified in *l(2)04524* and the 66.5kD protein have limited homology to propionyl-CoA carboxylases, the similarity is not enough to predict function. In contrast, the sequence flanking *l(2)04524* identified by the BDGP has homology to the human EB1 protein (BDGP; Figure 5.9), and is likely to be the *Drosophila* homologue of this gene.

Human EB1 was identified as a protein that binds to the carboxy terminus of the adenomatous polyposis coli (APC) tumour suppressor protein *in vitro* and *in vivo* (Su *et al.*, 1995). Both EB1 and APC have been shown to associate with microtubules in mammalian cells (Schwartz et al., 1997; Berrueta *et al.*, 1998; Morrison *et al.*, 1998) and in yeast an EB1-related protein, BIM1, has been shown to be important for microtubule function (Beinhauer *et al.*, 1997). There is also evidence for the involvement of APC at the G1 to S phase transition, although no evidence to date has directly implicated EB1 function in the regulation of entry into S phase. Circumstantial evidence for the involvement of EB1 has come from analysis of APC mutations found in colorectal cancers. Almost all APC mutations found in these cancers result in the production of a truncated protein, removing the domain required for binding to EB1 and implicating this interaction in cell cycle control. One group has investigated the potential role for EB1 in colorectal tumourigenesis (Jais *et al.*, 1998), however, none of the 21 tumour samples examined carried mutations in the EB1 coding region. This, however, does not rule out a role for EB1 in the control of proliferation.

Although the functions of EB1 are unknown, the binding of this protein to APC suggests that it is required for APC function. There are two possible functions of APC that, if EB1 was involved in or required for, would explain the observed suppression of the $DmcycE^{JP}$ rough eye phenotype observed by halving the dosage of EB1. The first involves the regulation of β-catenin (Armadillo in *Drosophila*) degradation by APC, which has been characterised in both mammalian cells and *Drosophila* (reviewed in Polakis, 1997; Ahmed *et al.*, 1998). In response to the Wnt/Wingless signalling pathway, cytosolic β-catenin becomes nuclear localised and binds to T-cell factor 4 (Tcf-4) and acts as a co-activator, resulting in transcriptional upregulation of target genes (reviewed by Polakis, 1997). APC is involved in targeting free cytosolic β-catenin to the proteosome for degradation, thus downregulating

Figure 5.9 Alignment of *l(2)04524* EST and EB1 related proteins.

Using the sequence available from the BDGP of genomic DNA flanking the *l(2)04524* insert, an EST (clot 431) was found that overlapped this sequence. The EST, clot 431, encodes an incomplete protein of 238 amino acids that shows homology to mammalian EB1 proteins. The alignment shown compares all 238 amino acids of the putative *Drosophila* EB1 homologue with human EB1 (Su *et al.*, 1995), and human T-cell APC binding protein (Renner *et al.*, 1997). In addition, the alignment of 119 conserved amino acids with BIM1 from *Saccharomyces cerevisiae* (Schwartz *et al.*, 1997) and 114 amino acids of MAL3 from *Schizosaccharomyces pombe* (Beinhauer *et al.*, 1996) is shown. Human EB1 shows 51% identity and 62% similarity over the region shown, and conserved amino acids between human and *Drosophila* EB1 are shown above the alignment. + indicates similar amino acid, grouped as follows: hydrophobic; M, L, I, V; aromatic; Y, F, W; basic; K, R, H; acidic and acid amide; D, E, N, Q; small aliphatic and hydroxyl; S, T, A, G, P. The level of conservation between the putative *Drosophila* EB1 and human EB1 is approximately the same as between human EB1 and human T-cell APC binding protein (59% identity and 69% similarity).

```
          MAVNVYST VTS+NLSRHDMLAW+N+ LQ + +KIE+LC+GAAYCQFMDMLFP S+ +K+

Dros EB1  MAVNVYSTNVTSENLSRHDMLAWVNDCLQSQFSKIEELCTGAAYCQFMDMLFPNSVPVKR
HS EB1    MAVNVYSTSVTSDNLSRHDMLAWINESLQLNLTKIEQLCSGAAYCQFMDMLFPGSIALKK
HS TCBP   MAVNVYSTSITQETMSRHDIIAWVNDIVSLNYTKVEQLCSGAAYCQFMDMLFPGCISLKK
BIM1                    SRTELLTWLNGLLNLNYKKIEECGTGAAYCQIMDSIY-GDLPMNR
MAL3                    SRQELLAWINQVTSLGLTRIEDCGKGYAMIQIFDSIYQD-IPLKK

          VKF + LEHEYIQNFKILQAGFK+M+VDKIIP+DKLVKG+FQDNFEF+QWFKKFFDANYD

Dros EB1  VKFRTNLEHEYIQNFKILQAGFKKMSVDKIIPIDKLVKGRFQDNFEFLQWFKKFFDANYD
HS EB1    VKFQAKLEHEYIQNFKILQAGFKRMGVDKIIPVDKLVKGKFQDNFEFVQWFKKFFDANYD
HS TCBP   VKFQAKLEHEYIHNFKLLQASFKRMNVDKVIPVEKLVKGRFQDNLDFIQWFKKFYDANYD
BIM1      VKFNATAEYEFQTNYKILQSCFSRHGIEKTVYVDKLIRCKFQDNLEFLQWLKKHWIRHKD
MAL3      VNFECNNEYQYINNWKVLQQVFLKKGIDKVVDPERLSRCKMQDNLEFVQWAKRFWDQYYP

          G+DYD  A R+G   + + + V          ++SS      P +T R A +     P+V

Dros EB1  GRDYDASAVREGAPMGFGSGAVKSLPGTAASGVSSSYR--RGPSATTRPAMTS-AVKPTVS
HS EB1    GKDYDPVAARQGQETAVAPSLVAPALNKPKKPLTSSSAAPQRPISTQRTAAAP-KAGPGVV
HS TCBP   GKEYDPVEARQGQ-----DAIPPPDPGEQIFNLPKKSH--HANSPTAGAAKSSPAAKP--G
BIM1      ESVYDPDARRKYRPI----------------------------------------------
MAL3      GGDYDALARR----------------------------------------------------

          +  P   N+                              +++  E+ +QV  +++ +E LEKER

Dros EB1  KVLPRTNNAAPASRINACANSTGTVKKNDVSNSVNNQQIEEMSNQVMDMRINLEGLEKER
HS EB1    RKNPGVGNG-------------------------DDEAAELMQQVNVLKLTVEDLEKER
HS TCBP   STPSRPSSAKRASSSGSASKS----------DKDLETQVIQLNEQVHSLKLALEGVEKER
BIM1      ----------------------------------------------------------
MAL3      ----------------------------------------------------------
```

targeting free cytosolic β-catenin to the proteosome for degradation, thus downregulating transcription of Tcf-4 target genes. *c-MYC* has recently found to be transcriptionally regulated by Tcf-4/β-catenin, with mutations in APC resulting in elevated levels of *c-MYC*, leading to increased proliferation (He *et al.*, 1998). As c-Myc has been implicated in the transcriptional activation of *cyclin E* in human cells (Dang, 1999), any up-regulation of *c-MYC* expression would be expected to increase *cyclin E* transcription. If EB1 was involved in a similar pathway in *Drosophila*, this would be expected to suppress the $DmcycE^{JP}$ phenotype.

The second characterised role of APC that could explain the suppression of the $DmcycE^{JP}$ rough eye phenotype by halving the dosage of EB1, involves the role of APC in the regulation of Cyclin E/Cdk2 activity in mammalian cells (Baeg *et al.*, 1995). Overexpression of APC in tissue culture cells results in a G1 arrest by leading to inhibition of Cyclin E/Cdk2, but not Cyclin D/Cdk4 kinase activity (Baeg *et al.*, 1995). The mechanism by which APC affects Cyclin E/Cdk2 activity is not clear, although as EB1 and APC are localised to the cytosol, this regulation is presumably indirect. Inconsistent with this second model, $Su(DmcycE^{JP})2\text{-}5$ does not enhance the *cyclin E* overexpression rough eye phenotype. This may, however, merely reflect the fact that the *cyclin E* overexpression phenotype is not sensitive enough to detect the action of a modifier acting several steps upstream of inhibition of Cyclin E/Cdc2c activity.

If EB1 acts by modulating APC activity, APC alleles should also suppress the $DmcycE^{JP}$ rough eye phenotype. Mutations in the recently identified *Drosophila APC* gene, *dAPC* (Ahmed *et al.*, 1998), should therefore be tested to determine whether reducing the dose of *dAPC* suppresses the $DmcycE^{JP}$ rough eye phenotype. A deletion removing *dAPC*, *Df(3R)3450*, has been tested as part of screening third chromosome deficiencies, and shown to enhance, rather than suppress the $DmcycE^{JP}$ phenotype. However, this enhancement is likely to be due to the co-deletion of the *string* gene, which is known to enhance the $DmcycE^{JP}$ phenotype. Recently, there has also been another APC-like gene identified in both *Drosophila* and humans (van Es *et al.*, 1999). It has not yet been determined whether this second human APC gene is involved in tumourigenesis, and no mutations have yet been described for the *Drosophila* homologue.

While $2\text{-}5^{42S11}$ is semi-lethal over *l(2)04524*, it is possible that *l(2)04524* is not an allele of $Su(DmcycE^{JP})2\text{-}5$ simply because $2\text{-}5^{42S11}$ is an X-ray generated deletion removing more than one gene. To clarify this, the *l(2)04524* allele should now be tested to determine whether it suppresses $DmcycE^{JP}$. It is possible, however, that this *P* allele will have no or little effect on the $DmcycE^{JP}$ phenotype, as it is likely to be a weak allele given

of more severe alleles of *l(2)04524* by imprecise *P* element excision and subsequent analysis of the effect of these alleles on *DmcycE^{JP}* will enable this to be tested. Molecular characterisation of the lesion associated with the *2-5^{42S11}* allele should also clarify whether this allele affects more than one gene. In addition, the *P* element plasmid rescue should be repeated to resolve the discrepancy in genomic sequence obtained by the BDGP and Harvie *et al*, (1998).

5-4.5 *Su(DmcycE^{JP})2-6*

Su(DmcycE^{JP})2-6 has ten alleles; *41S1, 42S7, 42S13, 42S14, 65S4, 66S4, 67S7, E3S17, E3S18,* and *E3S31* (Table 5.10), although it should be noted that alleles originating from the same bottle may be due to a mutation that occurred pre-meiotically. All of these alleles are strong suppressors of the *DmcycE^{JP}* phenotype and this suppression is the result of an increase in the number of S phases during eye development (Figure 5.10; and data not shown). No *Su(DmcycE^{JP})2-6* alleles affect the overexpression of *cyclin E* rough eye phenotypes, or the *rux* phenotype, suggesting that *Su(DmcycE^{JP})2-6* acts upstream of *cyclin E* transcription or in a parallel pathway and independently of the Rux pathway.

Crossing the *Su(DmcycE^{JP})2-6* alleles *2-6^{42S14}*, *2-6^{65S4}*, *2-6^{E3S17}* and *2-6^{E3S18}* to the *Cy-Tb* balancer revealed that while *2-6^{65S4}* homozygotes die before the third larval instar, homozygotes of the other three alleles result in larval/pupal lethality. It is likely that *2-6^{65S4}* has more than one lethal mutation resulting in lethality before the third larval instar, as transheterozygous combinations of these alleles result in larvae that are larger than wild-type and delayed in pupariation. These pupae do not develop, indicating that *Su(DmcycE^{JP})2-6* mutants die soon after pupariation.

To determine a cytological location for *Su(DmcycE^{JP})2-6*, alleles were crossed to deficiencies spanning the second chromosome. All ten alleles were lethal over *Df(2R)Pu-17*, that removes the cytological region 57B4 to 58B (Figure 5.11). To further refine this region, at least one allele of *Su(DmcycE^{JP})2-6* was crossed to deficiencies flanking *Df(2R)Pu-17*, *Df(2R)X58-7* (58A1-2 to 58E4-10), *Df(2R)AA21* (56F9-17 to 57D11-12), *Df(2R)PI13* (57B13-14 to 57D8-9), and *Df(2R)PK1* (57C5 to 57F6). *Su(DmcycE^{JP})2-6* alleles complemented all of these deficiencies, refining the region to which *Su(DmcycE^{JP})2-6* maps as 57F5-6 to 58A1-2 (Figure 5.11).

Table 5.10 Complementation analysis of *Su(DmcycE^{JP})2-6*

	41S1	*42S7*	*42S13*	*42S14*	*65S4*	*66S4*	*67S7*	*E3S17*	*E3S18*	*E3S31*
41S1	-	0/113	0/120	0/206	0/146	0/129	0/99	0/136	0/120	0/99
42S7	-	-	0/105	0/144	0/121	0/179	0/57	0/70	0/163	0/88
42S13	-	-	-	0/156	0/158	0/105	0/132	0/248	0/95	0/75
42S14	-	-	-	-	0/146	0/105	0/194	0/157	0/120	0/105
65S4	-	-	-	-	-	0/127	0/212	0/156	0/103	0/143
66S4	-	-	-	-	-	-	0/107	0/127	0/90	0/110
67S7	-	-	-	-	-	-	-	0/93	0/100	0/75
E3S17	-	-	-	-	-	-	-	-	0/90	0/239
E3S18	-	-	-	-	-	-	-	-	-	0/133
E3S31	-	-	-	-	-	-	-	-	-	-

Summary of complementation data obtained for the ten alleles of *Su(DmcycE^{JP})2-6*. All alleles fail to complement each other indicating that they are allelic. Those alleles originating from the same bottle have the potential to be due to a pre-meiotic mutation, although *42S13* and *42S14* represent independent events since these alleles have different second site lethal mutations.

A summary of genes known to be within the region 57F5-6 to 58A1-2 is shown in Table 5.11. In addition to *P* elements in the region, all of which complement *Su(DmcycE^{JP})2-6* alleles, five candidates for *Su(DmcycE^{JP})2-6* were identified, *lethal(2) tumorous antennal, labial, clypeo-labral imaginal discs* (*l(2)talc*), *HMG-D*, *HMG-Z*, *Su(var)57D58D,* and *Su(tor)2-180*. The only described allele of *l(2)talc*, *l(2)k106/22*, behaves as a tumour suppressor, resulting in overgrown imaginal discs and larval lethality (Török *et al.*, 1993). Unlike all other described tumour suppressors, which affect all larval imaginal discs, *l(2)talc* results in the overproliferation of only a subset of imaginal discs. It has recently been found that the *P* element insertion in the *l(2)k106/22* strain is not associated with the phenotype, and the overgrowth phenotypes have recently been found to be due to a mutation in *Additional sex combs* (*Asx*) gene, which maps to 51A (Török and Gorjanacz, 1998). *l(2)talc* is therefore not a candidate for *Su(DmcycE^{JP})2-6*.

Given the evidence already described implicating chromatin structure in cell cycle regulation, three genes in the 57F-58A region, *HMG-D*, *HMG-Z* and *Su(var)57D58D*, are considered candidates for *Su(DmcycE^{JP})2-6*. *HMG-Z* encodes a site-specific DNA binding protein, that may confer a superhelical DNA conformation (Churchill *et al.*, 1995), although the physiological relevance of this has not been established. *HMG-D* encodes a protein of unknown function that is 65% identical to *HMG-Z* (Ner *et al.*, 1993). Mutations are not available for either of these genes. Testing these candidates will therefore require genomic Southern analysis using *Su(DmcycE^{JP})2-6* X-ray alleles. Similarly, there are no specific mutations in *Su(var)57D58D*, although the involvement of HMG-Z in the alteration of chromatin structure suggests that this may correspond to *Su(var)57D58D*. *Su(var)57D58D* is defined as a region that when triploid in a cell, suppresses the mottled phenotype associated with the *white* allele, *In(1)w^{m4h}* (Wustman *et al.*, 1989). Whether *Su(DmcycE^{JP})2-6* corresponds to this suppressor of position effect variagation should therefore be tested by crossing this modifier of *DmcycE^{JP}* into a *w^{m4}* background. If *Su(DmcycE^{JP})2-6* corresponds to *Su(var)5758D*, *Su(DmcycE^{JP})2-6* alleles would be expected to enhance the *w^{m4}* phenotype when halved in dosage.

The previously identified mutation *Su(tor)2-180* is implicated in signal transduction, and is also a candidate for *Su(DmcycE^{JP})2-6*. *Su(tor)2-180* was isolated as a mutation that dominantly suppressed an embryonic *torso* (*tor*) gain-of-function phenotype (Doyle and Bishop, 1993). *Su(tor)2-180* also suppresses the rough eye phenotype associated with the epidermal growth factor (EGF) receptor gain-of-function allele *Ellipse*, suggesting that *Su(tor)2-180* functions downstream of several receptor tyrosine kinase pathways. The *Ellipse*

Figure 5.10 *Su(DmcycE^{JP})2-6* acts at the level of S phase

(A, B, C, D) Scanning electron micrographs of adult eyes from (A) *DmcycE^{JP}*, (B) *DmcycE^{JP}, 2-6^{42S14}/DmcycE^{JP}*, +, (C) *DmcycE^{JP}, 2-6^{E3S17}/DmcycE^{JP}*, + and (D) *DmcycE^{JP}, 2-6^{E3S18}/DmcycE^{JP}*, +, showing suppression of the *DmcycE^{JP}* rough eye phenotype. (E, F, G, H) BrdU labelling of eye imaginal discs from (E) *DmcycE^{JP}* (F) *DmcycE^{JP}, 2-6^{42S14}/DmcycE^{JP}*, +, (G) *DmcycE^{JP}, 2-6^{E3S17}/DmcycE^{JP}*, + and (H) *DmcycE^{JP}, 2-6^{E3S18}/DmcycE^{JP}*, + third instar larvae, showing that suppression of the *DmcycE^{JP}* rough eye phenotype results from an increase in the number of S phases posterior to the MF. Anterior is to the right. Bars indicate the MF.

While eye phenotypes and BrdU incorporation is shown for only four alleles of this complementation group, the other six alleles are also good suppressors of the *DmcycE^{JP}* rough eye phenotype, but have not been tested to determine whether they rescue *DmcycE^{JP}* S phases.

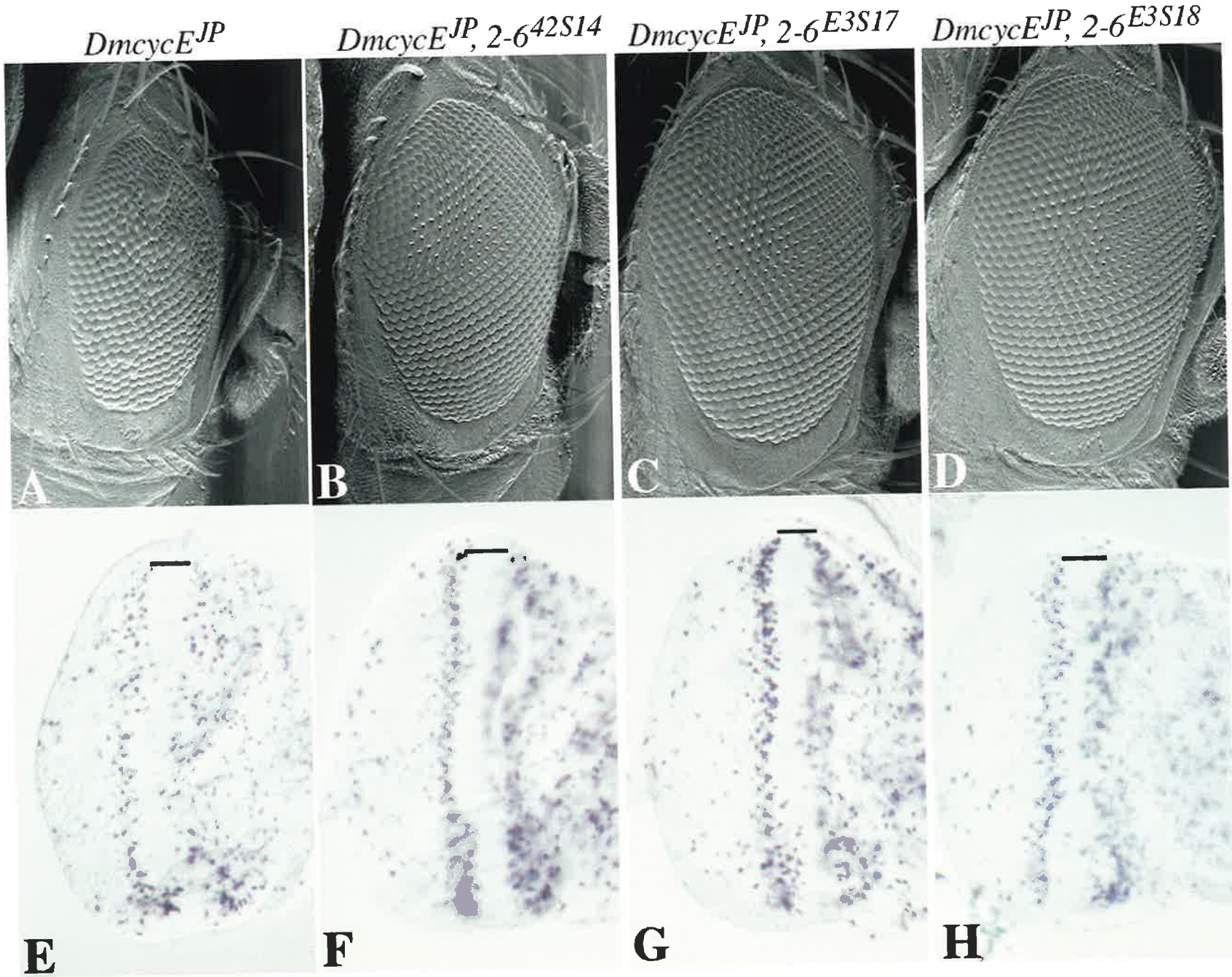
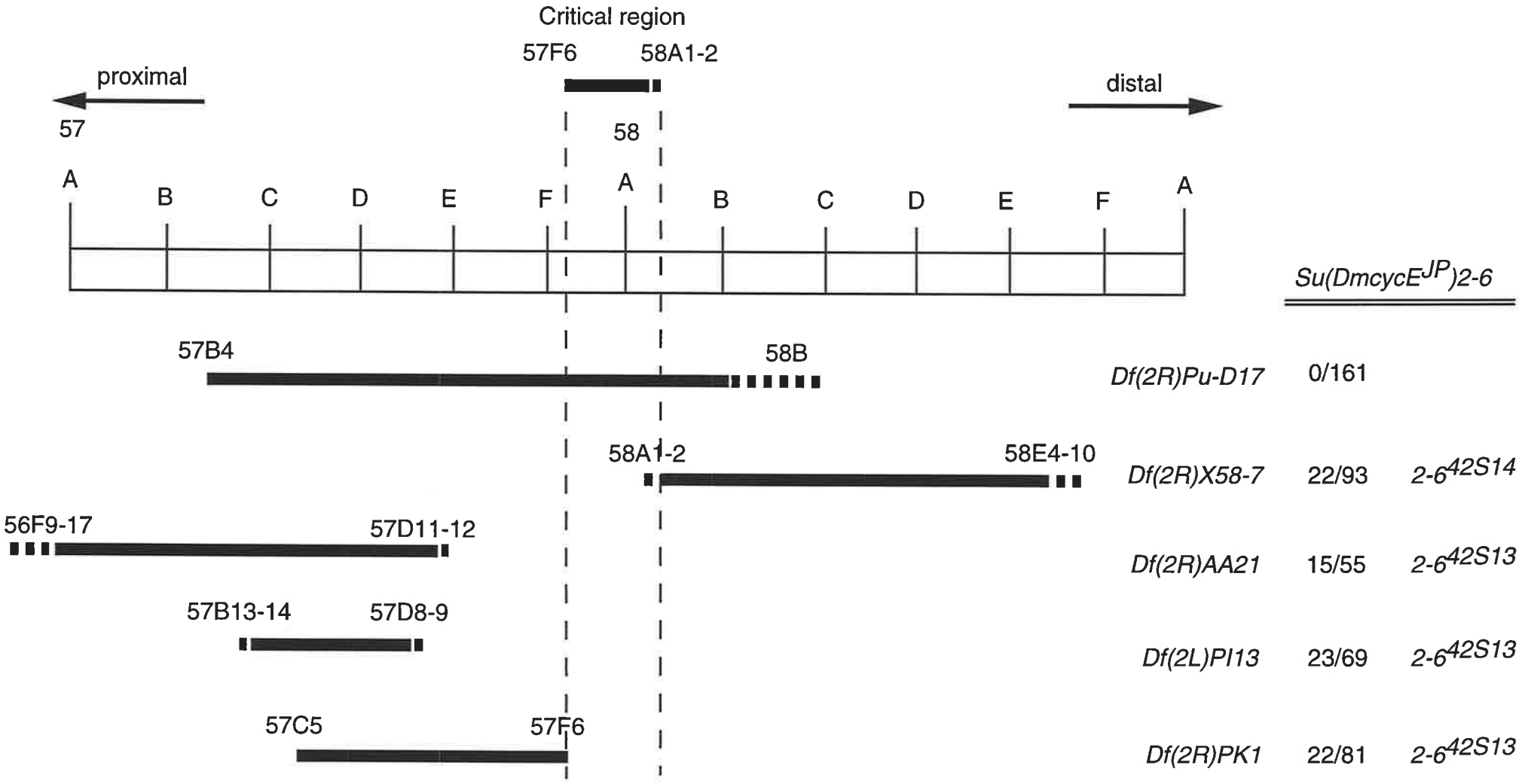

DmcycE^JP
DmcycE^JP, 2-6^42S14
DmcycE^JP, 2-6^E3S17
DmcycE^JP, 2-6^E3S18
A
B
C
D
E
F
G
H

Figure 5.11 Cytological location of *Su(DmcycE^{JP})2-6*

Schematic representation of the cytological region to which *Su(DmcycE^{JP})2-6* maps. All ten alleles of *Su(DmcycE^{JP})2-6* fail to complement *Df(2R)Pu-D17* (data are presented for *2-6^{65S4}* allele, although similar results were obtained with other *Su(DmcycE^{JP})2-6* alleles), and at least one allele from this group complements the deficiencies *Df(2R)X58-7*, *Df(2R)AA21*, *Df(2R)PI13*, and *Df(2R)PK1*. Thus the *2-6* region is defined as 57F6 to 58A1-2. Complementation data presented on the right hand side of the figure are given as the number of transheterozygous flies over the total number scored. Bars indicate region deleted in each of the deficiencies, with the dashed regions at the extremes of each bar indicating breakpoint uncertainty regions.


Critical region
57F6
58A1-2
proximal
distal
57
58
A
B
C
D
E
F
A
B
C
D
E
F
A
Su(DmcycE^JP)2-6
57B4
58B
Df(2R)Pu-D17
0/161
58A1-2
58E4-10
Df(2R)X58-7
22/93
2-6^42S14
56F9-17
57D11-12
Df(2R)AA21
15/55
2-6^42S13
57B13-14
57D8-9
Df(2L)PI13
23/69
2-6^42S13
57C5
57F6
Df(2R)PK1
22/81
2-6^42S13

rough eye phenotype results from ectopic S phases in the MF and apoptosis, leading to adults with rough eyes (Baker and Rubin, 1992). Halving the dosage of *Su(tor)2-180* suppresses the *Ellipse* phenotype, indicating that the *Su(tor)2-180* gene product is a positive regulator of EGF signalling. Halving the dosage of *Su(tor)2-180* would therefore not be expected to suppress the $DmcycE^{JP}$ rough eye phenotype, although still should be tested for allelism with $Su(DmcycE^{JP})2\text{-}6$.

Table 5.11 Genes in the region of 57F5-6 to 58A1-2

Gene	Cytology	Known function/phenotype
Additional veins	48B-58E	Dominant addition of wing veins phenotype
fs(2)TLM	51A2-60D1	Male and female recessive sterile
E(f)	52D3-58E4	Recessive visible bristle mutation
l(2)S3	52D3-58E4	Homozygotes die as small and dumpy pupae
Su(tor)2-180	**52D3-59E1**	**Involved in EGF pathway during eye development**
Strapped on 2	52D3-60C2	Homozygous males have incising of wing margin
Humpy	56F9-58A1	Homozygous viable, male and female sterile mutation
l(2)talc	**57**	**Tumour suppressor**
Pratfall	57B5-58B2	Homozygous embryonic lethal with segmentation defects
mat(2)N	57D8-58B2	Female sterile. Notch-like
l(2)k08108	**57D11-12**	***P* element allele**
Su(var)57D58D	**57E4-59A4**	**Suppressor of PEV**
ER2-5	57F-58A	MAPK-specific tyrosine phosphatase. Homozygous viable (F. Karim, pers. com.).
l(2)k05204	**57F5-6**	***P* element allele**
Tbp	57F8-10	Required for transcription from TATA promoters
l(2)03605	**57F8-10**	***P* element allele**
HMG-D	**57F8-11**	**Binds DNA and bends it**
HMG-Z	**57F8-11**	**Encodes a protein similar to HMG-D**
l(2)07837	**58A3-4**	***P* element allele**
PpN58A	58A	Testes specific phosphatase
l(2)Su(H)	58A-F	Mutants die as pupae between head involution and beginning of eye pigmentation

Summary of the genes and mutations within the critical region for $Su(DmcycE^{JP})2\text{-}6$. Gene abbreviations used are *Enhancer of forked (E(f))*, *lethal (2) tumorous antennal, labial, clypeo-labral imaginal discs* (*l(2)talc*), *High mobility group protein D* (*HMG-D*), *High mobility group protein Z* (*HMG-Z*), *TATA binding protein* (*Tbp*), *Protein phosphatase N at 58A* (*PpN58A*), and *maternal lethal (2) Notchlike* (*mat(2)N*). Genes considered to be candidates for $Su(DmcycE^{JP})2\text{-}6$ are indicated in bold. PEV is an abbreviation for position effect variegation, EGF is an abbreviation for epidermal growth factor.

In addition to a lesion at 57F5-6 to 58A1-2, several $Su(DmcycE^{JP})2\text{-}6$ alleles have at least one other lethal mutation. Evidence for this has come from the lethality of some of the alleles of this group with other suppressors. $2\text{-}6^{42S13}$ fails to complement a single allele

suppressor, *22S9* resulting in no transheterozygous flies from 186 scored, while all other members of *Su(DmcycE^{JP})2-6* complement this allele. The second lesion on the *2-6^{42S13}* chromosome is likely to be at 36C2-4 to 37B9-C1, as both *22S9* and *2-6^{42S13}* are lethal over a deficiency that removes this region, *Df(2L)TW137*. Whether this region co-incides with the *22S9* suppressor mutation is unknown. In addition, another allele, *2-6^{42S14}*, is lethal when transheterozygous with *55S2*, an allele of *Su(DmcycE^{JP})2-7*. *2-7^{55S2}* is lethal in combination with *Df(2R)M60E*, which removes 60E2-3 to 60E11-12 (see section 5-5.6), but is viable over *Df(2R)Pu-D17* that removes *Su(DmcycE^{JP})2-6*, demonstrating that *55S2* is not simply a large deletion or re-arrangement that affects both regions. Similarly, 2-6^{42S14} is viable in combination with *Df(2R)M60E*, indicating that this region is not affected. Given that other *Su(DmcycE^{JP})2-6* alleles are not lethal over 2-7^{55S2}, the simplest explanation is that a second aberration has been induced in both these chromosomes, resulting in lethality when transheterozygous. The additional mutations associated with *2-6^{42S13}* and *2-6^{42S14}* however, demonstrate that these two alleles selected from the same bottle are independent mutations and are not due to the same pre-meiotic event.

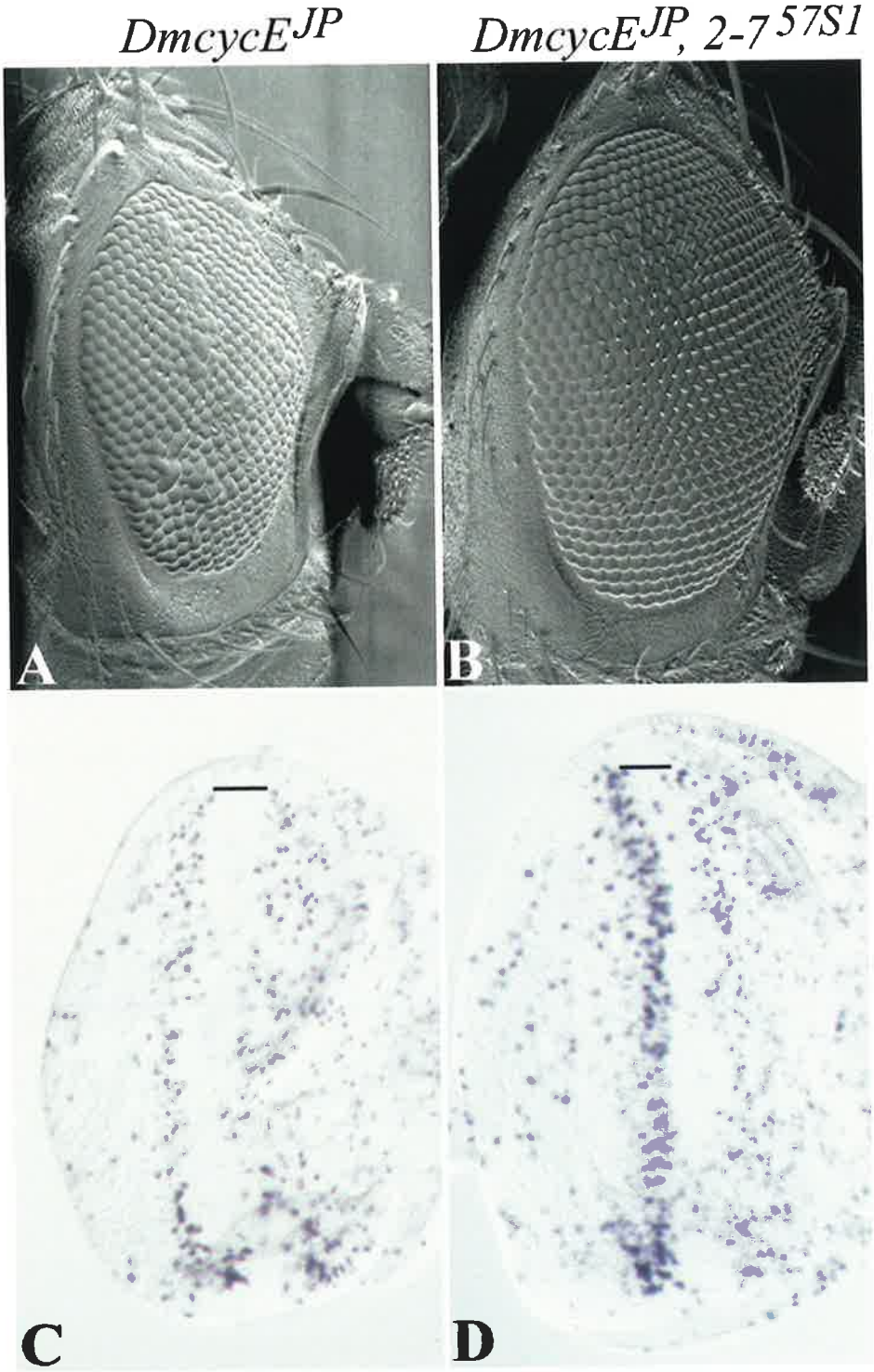
5-4.6 *Su(DmcycE^{JP})2-7*

The *Su(DmcycE^{JP})2-7* complementation group has 12 alleles, *14S3*, *19S3*, *40S5*, *42S3*, *55S2*, *57S1*, *62S9*, *64S10*, *64S19*, *65S19*, *65S23* and *E10S15*, although not all combinations of these alleles result in complete failure to complement (Table 5.12). In cases where *Su(DmcycE^{JP})2-7* transheterozygous flies were obtained, these flies had very rough eyes, held out wings, and died within a few days of eclosing. One allele of *Su(DmcycE^{JP})2-7*, *2-7^{57S1}*, was tested to determine whether suppression of the *DmcycE^{JP}* phenotype occurred by increasing the number of S phases during eye development. As seen in Figure 5.12, this allele of *Su(DmcycE^{JP})2-7* increases *DmcycE^{JP}* S phases, and is therefore likely to be a negative regulator of the G1 to S phase transition. In addition, 2-7^{55S2}, 2-7^{57S1} and 2-7^{64S19} had no effect on the *GMR-cyclin E* type I, *GMR-cyclin E* type II, or *rux* rough eye phenotypes.

The stage at which *Su(DmcycE^{JP})2-7* results in lethality has not been determined. However, no non-tubby larvae were seen in the *2-7^{57S1}* stock balanced using *Cy-Tb*, indicating that *2-7^{57S1}* homozygotes die before the third larval instar. To determine the stage at which *Su(DmcycE^{JP})2-7* transheterozygotes die, other *Su(DmcycE^{JP})2-7* alleles balanced using *Cy-Tb* need to be crossed together.

Figure 5.12 *Su(DmcycE^{JP})2-7* suppresses the *DmcycE^{JP}* eye phenotype by increasing the number of S phases during eye development

(A, B) Scanning electron micrographs of adult eyes from (A) *DmcycE^{JP}*, and (B) *DmcycE^{JP}, 2-6^{57S1}/DmcycE^{JP}*, + showing suppression of the *DmcycE^{JP}* rough eye phenotype. (C, D) BrdU labelling of eye imaginal discs from (C) *DmcycE^{JP}* (D) *DmcycE^{JP}, 2-7^{57S1}/DmcycE^{JP}*, + third instar larvae, showing that suppression of the *DmcycE^{JP}* rough eye phenotype results from an increase in the number of S phases posterior to the MF. Anterior is to the right. Bars indicate the MF in C and D. While SEMs and BrdU incorporation is shown for only one *Su(DmcycE^{JP})2-7* allele, other alleles of this complementation group suppress of the *DmcycE^{JP}* rough eye phenotype to a similar extent. The other six alleles, *2-7^{55S2}*, *2-7^{62S9}*, *2-7^{64S10}*, *2-7^{64S19}*, *2-7^{65S19}*, *2-7^{65S23}* have not been tested to determine whether they rescue *DmcycE^{JP}* S phases.


DmcycE^JP
DmcycE^JP, 2-7 57S1
A
B
C
D

The cytological position to which *Su(DmcycE^{JP})2-7* maps was determined by crossing alleles of this complementation group to the second chromosome deficiencies. Seven out of the 12 *Su(DmcycE^{JP})2-7* alleles were completely lethal in combination with *Df(2R)M60E*, which removes the cytological region 60E6-9 to 60E11 (Table 5.13). The remaining five alleles, *2-7^{19S3}*, *2-7^{40S5}*, *2-7^{42S3}*, *2-7^{62S9}* and *2-7^{64S10}*, were semi-lethal over this deficiency. *2-7^{19S3}*, *2-7^{40S5}*, *2-7^{42S3}*, *2-7^{62S9}* and *2-7^{64S10}* are therefore likely to be hypomorphic alleles. Transheterozygous *2-7^{19S3}*, *2-7^{40S5}*, *2-7^{42S3}*, *2-7^{62S9}* or *2-7^{64S10}/Df(2R)M60E* flies also had a similar phenotype to transheterozygous *Su(DmcycE^{JP})2-7* alleles, having a short life span, rough eyes and wing defects. The cytological region for *Su(DmcycE^{JP})2-7* was refined by crossing *2-7^{55S2}*, *2-7^{64S19}* and *2-7^{65S23}* to the adjacent deficiency *Df(2R)ES1*, which removes 60E6 to 60F1-2. These *Su(DmcycE^{JP})2-7* alleles complemented *Df(2R)ES1*, limiting the cytological region for this suppressor to 60E6. A summary of genes in the region of 60E6 is shown in Table 5.14. There are only a few genes known to be within this cytological region, none of which are candidates for *Su(DmcycE^{JP})2-7*. *Su(DmcycE^{JP})2-7* is therefore likely to be a novel gene.

Su(DmcycE^{JP})2-7 alleles also show a genetic interaction with the single allele *61S10*. *Su(DmcycE^{JP})2-7* alleles are semi-lethal over *61S10* and transheterozygous flies have rough eyes and held out wings. *61S10* is viable over *Df(2R)M60E* (Table 5.13), but is lethal over the deficiency adjacent to *Df(2R)M60E*, *Df(2R)ES1* that removes 60E6 to 60F1-2, indicating that *61S10* is perhaps a deficiency that affects both of these regions. Consistent with *61S10* being a deficiency, this modifier deletes or disrupts two genes in the 60E region, *zipper* (*zip*) and *Dll* (*Distal-less*). However, since *Su(DmcycE^{JP})2-7* alleles are viable over *Df(2R)ES1*, and *61S10* is viable over *Df(2R)M60E* it is unlikely that *61S10* is a large deficiency that overlaps the *Su(DmcycE^{JP})2-7* region. As *61S10* shows genetic interactions with *Su(DmcycE^{JP})2-7* alleles, but not with *Df(2R)M60E*, the interaction may depend on the *DmcycE^{JP}* mutation being homozygous, and may indicate that *61S10* acts in the same pathway as *Su(DmcycE^{JP})2-7*.

Table 5.12 Complementation analysis of *Su(DmcycE^{JP})2-7*

	14S3	*19S3*	*40S5*	*42S3*	*55S2*	*57S1*	*62S9*	*64S10*	*64S19*	*65S19*	*65S23*	*E10S15*
14S3	-	17/116	8/94	22/80	33/239*	15/208	49/174	39/153	5/161	0/135	5/174	33/228
19S3	-	-	13/113	9/60	13/107	32/177	30/160	21/122	10/130	26/155	16/146	33/176
40S5	-	-	-	14/67	7/142	4/62	32/164	16/132	5/110	40/160	25/168	8/142
42S3	-	-	-	-	4/70	6/44	27/141	9/73	2/74	22/187	13/120	10/151
55S2	-	-	-	-	-	31/239	10/139	5/204	0/87	0/123	1/152	8/163
57S1	-	-	-	-	-	-	3/90	2/198	0/174	2/108	7/204	5/140
62S9	-	-	-	-	-	-	-	14/203	0/146	9/117	24/189	11/117
64S10	-	-	-	-	-	-	-	-	35/176	10/125	8/148	17/149
64S19	-	-	-	-	-	-	-	-	-	0/52	0/190	2/152
65S19	-	-	-	-	-	-	-	-	-	-	7/251	3/122
65S23	-	-	-	-	-	-	-	-	-	-	-	15/126
E10S15	-	-	-	-	-	-	-	-	-	-	-	-

Summary of alleles that form *Su(DmcycE^{JP})2-7* complementation group. Unlike all other complementation groups identified, alleles of *Su(DmcycE^{JP})2-7* are not completely lethal when transheterozygous. The number of transheterozygous flies scored from these crosses varied greatly, but always less than the expected 33%, indicating that these combinations are semi-lethal. Transheterozygous flies obtained had very rough eyes, held out wings, and died within a few days of eclosing. * Transheterozygous *2-7^{14S3}/2-7^{55S2}* females are sterile and do not lay eggs, but males are fertile.

Table 5.13 *Su(DmcycE^{JP})2-7* alleles are lethal over *Df(2R)M60E*

Allele	x *Df(2R)M60E*
2-7^{14S3}	**0/91**
2-7^{19S3}	**8/100 (8%)**
2-7^{40S5}	**37/194 (19%)**
2-7^{42S3}	**12/93 (13%)**
2-7^{55S2}	**0/104**
2-7^{57S1}	**0/77**
2-7^{62S9}	**3/132 (2.3%)**
2-7^{64S10}	**20/317 (6.3%)**
2-7^{64S19}	**0/82**
2-7^{65S19}	**0/114**
2-7^{65S23}	**0/70**
2-7^{E10S15}	**0/109**
61S10	21/91

Summary of the interactions between *Df(2R)M60E*, *Su(DmcycE^{JP})2-7* alleles and the single allele *61S10*. Flies transheterozygous for *Df(2R)M60E* and *19S3*, *40S5*, *42S3*, *62S9* or *64S10* were seen at a reduced frequency than expected (33%), and have very rough eyes. Similar results were obtained when these alleles were crossed to *Su(DmcycE^{JP})2-7* alleles. Those highlighted in bold are classified as failure (or near failure) to complement. The 'escaper' transheterozygous flies seen from the crosses of *2-7^{19S3}*, *2-7^{40S5}*, *2-7^{42S3}*, *2-7^{62S9}* or *2-7^{64S10}* and *Df(2R)M60E* also had very rough eyes, misformed wings and died within a day of eclosing.

Table 5.14 Summary of genes within *Su(DmcycE^{JP})2-7* region

Gene	Cytology	Known function/phenotype
smell impaired 60E	60E	Viable, sensory mutant
ESTS:17F25	60E	Potential metalloproteinase
NaCP60E	60E5-11	Sodium channel protein expressed in central and peripheral nervous system

Summary of genes in the 60E6 cytological region. There are no candidate genes for *Su(DmcycE^{JP})2-7*.

5-4.7 *Su(DmcycE^{JP})2-8*

The two EMS generated alleles of *Su(DmcycE^{JP})2-8*, *2-8^{E6S4}* and *2-8^{E6S19}* fail to complement each other, resulting in no transheterozygous flies from 107 scored. Both of these alleles are good suppressors of the *DmcycE^{JP}* mutation, although *2-8^{E6S4}* is a stronger suppressor than *2-8^{E6S19}*, indicating that although these alleles were derived from the same bottle, they are likely to be independent mutations in the same gene (Figure 5.13). As with all other suppressor groups described in this chapter, the suppression of *DmcycE^{JP}* by *Su(DmcycE^{JP})2-8* occurs by increasing the number of S phases seen during eye development (Figure 5.13), indicating a role for *Su(DmcycE^{JP})2-8* in proliferation. In addition, neither of the *Su(DmcycE^{JP})2-8* alleles modify the eye phenotypes of overexpression of type I or II *cyclin E* or *rux*. The cytological location of this suppressor has not yet been determined. In addition, the stage at which *2-8^{E6S4}/2-8^{E6S19}* transheterozygotes die has not been examined, although no non-tubby larvae were observed when *2-8^{E6S4}* or *2-8^{E6S19}* were balanced using *Cy-Tb* indicating that homozygotes are lethal before the third larval instar.

5-4.8 *Su(DmcycE^{JP})2-9* and *Su(DmcycE^{JP})2-10*

No characterisation has been done on *Su(DmcycE^{JP})2-9* and *Su(DmcycE^{JP})2-10*, the last two complementation groups identified. *Su(DmcycE^{JP})2-9* has one X-ray-induced and one EMS-induced allele, *25S11* and *E1S4*, respectively (Figure 5.14), that gave no transheterozygous flies from 160 scored. While *2-9^{25S11}* is an excellent suppressor of the *DmcycE^{JP}* rough eye phenotype, *2-9^{E1S4}* is a mild suppressor, indicating that this EMS-induced allele is weaker than the X-ray-induced *2-9^{25S11}*. *Su(DmcycE^{JP})2-10* has two X-ray-induced alleles, *65S15* and *65S13*, and one EMS-induced allele, *E10S34*, that fail to complement each other (Table 5.15), and suppress the *DmcycE^{JP}* rough eye phenotype (Figure 5.15). The cytological position of these two suppressor groups remains to be determined. Likewise, whether these suppressors act by increasing the number of *DmcycE^{JP}* S phases during eye development has not been examined.

Figure 5.13 *Su(DmcycE^{JP})2-8* suppresses the *DmcycE^{JP}* phenotype by increasing the number of eye disc S phases

(A, B, C) Scanning electron micrographs of adult eyes from (A) *DmcycE^{JP}*, (B) *DmcycE^{JP}, 2-8^{E6S4}/DmcycE^{JP}*, +, and (C) *DmcycE^{JP}, 2-8^{E6S19}/DmcycE^{JP}*, +, showing suppression of the *DmcycE^{JP}* rough eye phenotype. (D, E, F) BrdU labelling of eye discs from (D) *DmcycE^{JP}* (E) *DmcycE^{JP}, 2-5^{E6S4}/DmcycE^{JP}*, + and (F) *DmcycE^{JP}, 2-8^{E6S19}/DmcycE^{JP}*, + third instar larvae, showing that suppression of the *DmcycE^{JP}* rough eye phenotype results from an increase in the number of S phases posterior to the MF. Anterior is to the right. Bars indicate the MF in D, E and F.

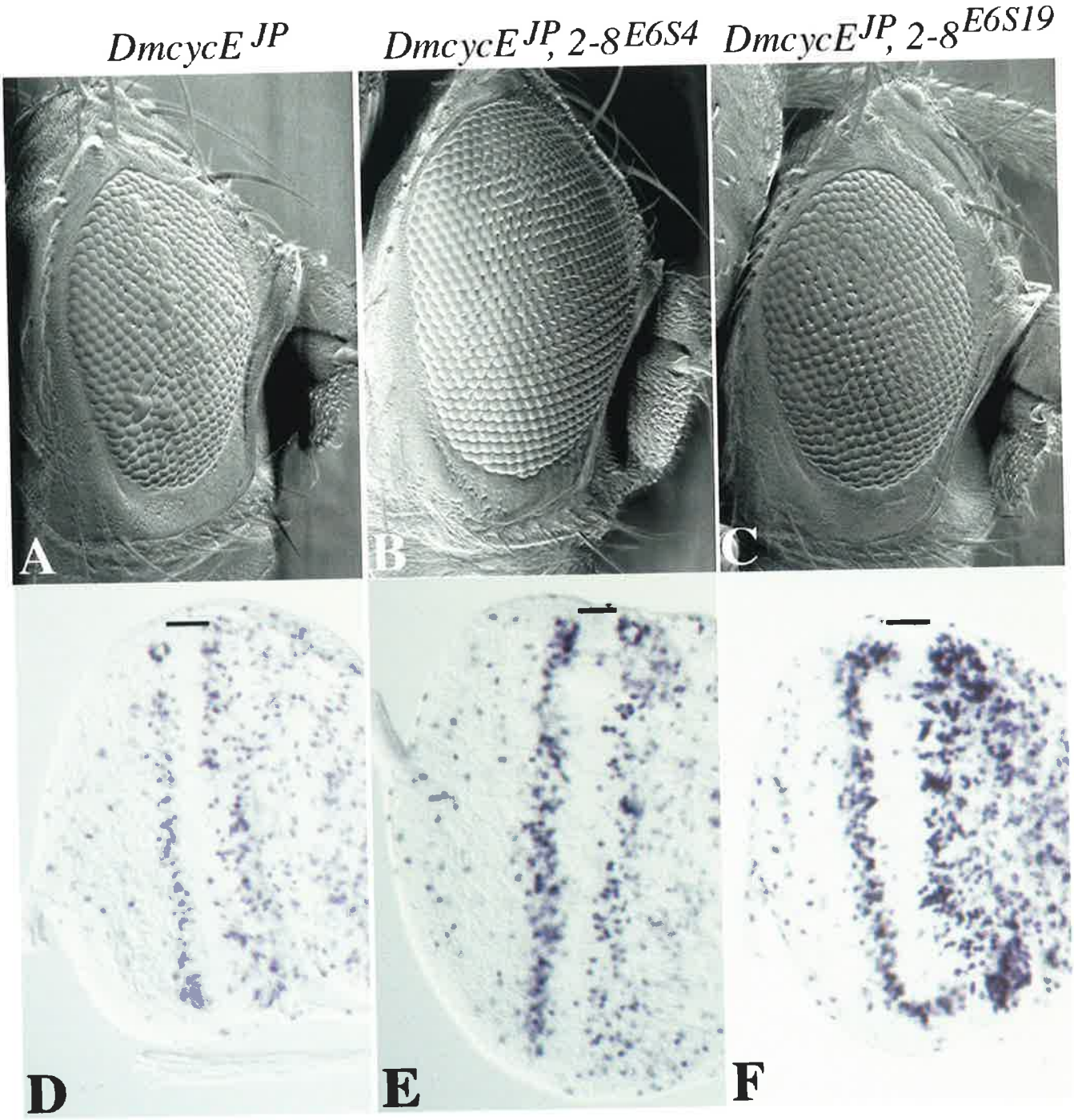
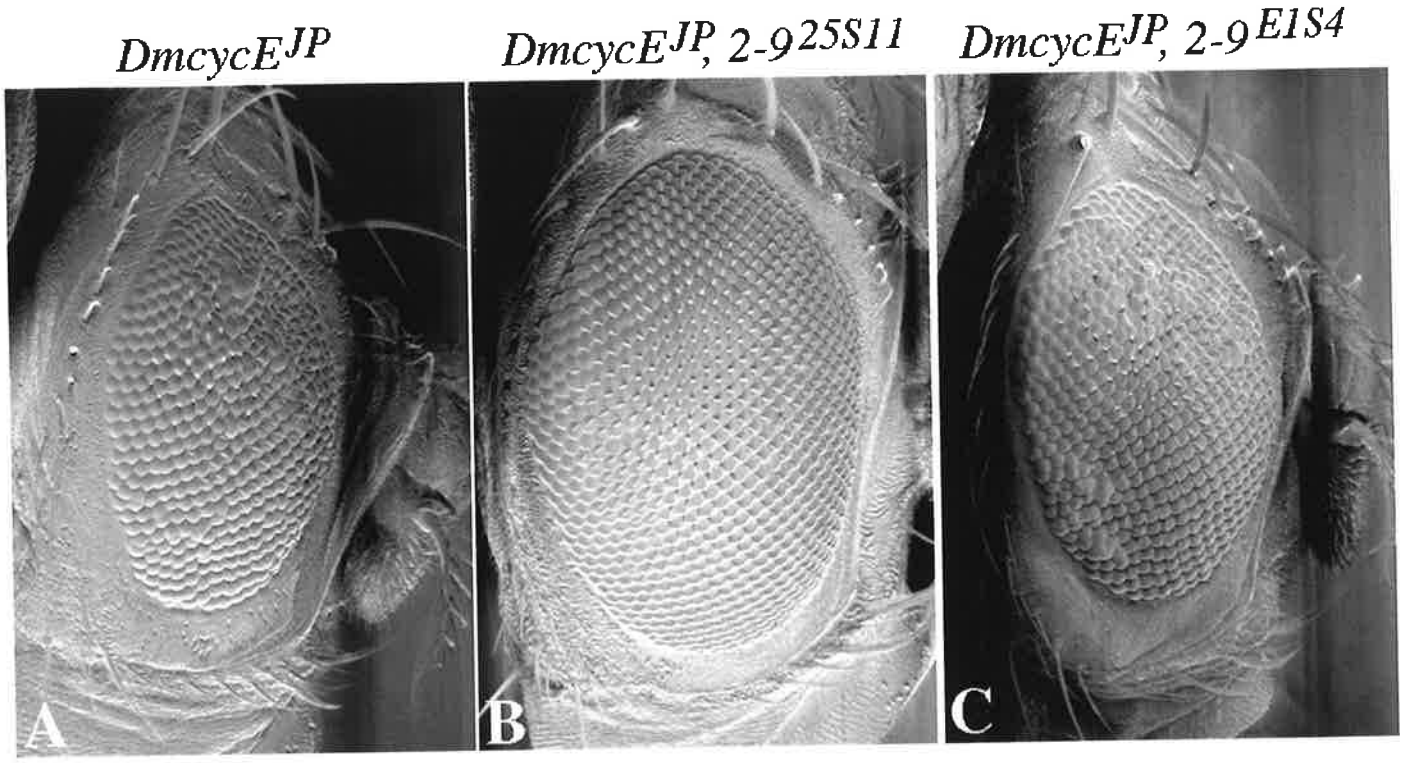

DmcycE JP
DmcycE JP, 2-8 E6S4
DmcycE JP, 2-8 E6S19
A
B
C
D
E
F

Figure 5.14 *Su(DmcycE^{JP})2-9* suppresses the *DmcycE^{JP}* rough eye phenotype

(A, B, C) Scanning electron micrographs of adult eyes from (A) *DmcycE^{JP}*, (B) *DmcycE^{JP}, 2-9^{25S11}/DmcycE^{JP}*, + and (C) *DmcycE^{JP}, 2-9^{E1S4}/DmcycE^{JP}*, + showing that both the alleles of this complementation group suppress the *DmcycE^{JP}* rough eye phenotype. Anterior is to the right, dorsal side up.

Figure 5.15 *Su(DmcycE^{JP})2-10* suppresses the *DmcycE^{JP}* rough eye phenotype

(A, B, C) Scanning electron micrographs of adult eyes from (A) *DmcycE^{JP}*, (B) *DmcycE^{JP}, 2-10^{65S13}/DmcycE^{JP}*, + and (C) *DmcycE^{JP}, 2-10^{E10S34}/DmcycE^{JP}*, + showing that these alleles suppress the *DmcycE^{JP}* rough eye phenotype. The suppression of *DmcycE^{JP}* by *2-10^{65S5}* is not shown, but is similar to *2-10^{65S13}*, thus these suppressors may represent the same pre-meiotic mutation. Anterior is to the right, dorsal side up.


DmcycE^JP
DmcycE^JP, 2-9^25S11
DmcycE^JP, 2-9^E1S4
A
B
C

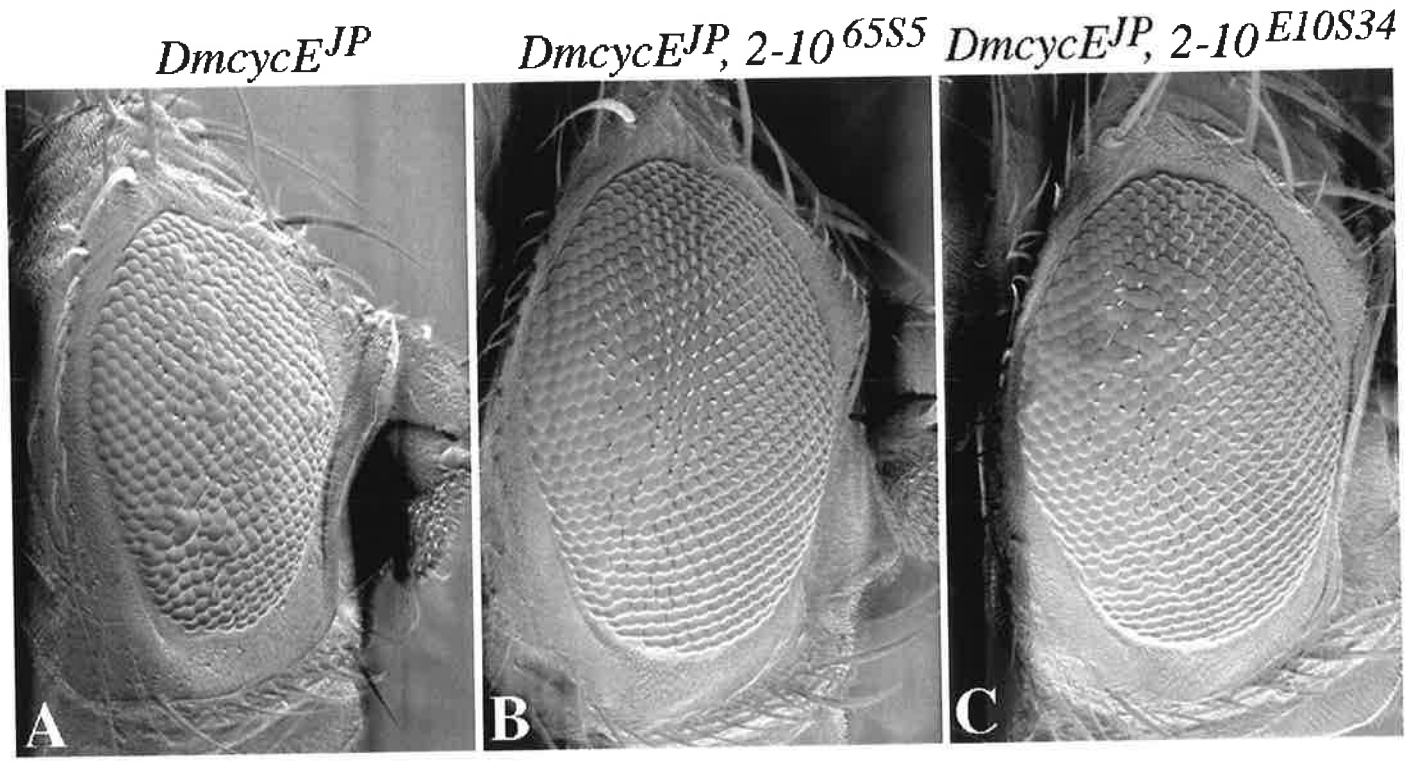

DmcycE^JP
DmcycE^JP, 2-10^65S5
DmcycE^JP, 2-10^E10S34
A
B
C

Table 5.15 Complementation analysis of *Su(DmcycE^{JP})2-10*

	65S5	*65S13*	*E10S34*
65S5	-	0/121	0/109
65S13	-	-	0/156
E10S34	-	-	-

The three alleles of *Su(DmcycE^{JP})2-10* fail to complement each other. Data are presented as the number of transheterozygous flies/total number scored.

5-5 Discussion and conclusions

Genetic interaction screens have been used extensively in *Drosophila* to dissect numerous regulatory pathways. Such screens have been successful in elucidating pathways involving the Sevenless receptor tyrosine kinase (Simon *et al.*, 1991), Rux (Dong *et al.*, 1997), Dpp (Raftery *et al.*, 1995), and most recently E2F (Staehling-Hampton *et al.*, 1999). This chapter has described the generation of 246 X-ray and EMS-induced mutations that modify the *DmcycE^{JP}* phenotype, and the subsequent characterisation of homozygous lethal suppressor mutations mapping to the second chromosome. While stocks were generated of 246 modifiers of *DmcycE^{JP}*, originally 2127 flies were selected as having an eye phenotype that was enhanced or suppressed compared with *DmcycE^{JP}*. There are several reasons for this, the most obvious of which is that many modifiers either died or were sterile. The reasons for such high mortality and sterility rates are not clear. In addition, many modifiers were classed as not giving a consistent phenotype or not mapping to the second or third chromosomes. Many of these can be accounted for simply because flies originally selected had only slightly enhanced or suppressed eyes. In general, these did not give a consistent modified phenotype when crossed back to *DmcycE^{JP}* and may, therefore, not have been modified at all, but rather exhibited phenotypes at the extremes of the range normally seen in a *DmcycE^{JP}* strain. The remainder can be accounted for by modifiers mapping to the X or fourth chromosomes, and those modified phenotypes resulting from multiple gene effects. Also, specifically with EMS mutagenesis, a significant number of flies originally selected as enhancers or suppressors were mosaic for the modified phenotype. More often than not, these mosaics did not carry the modifier in the germ-line, and were therefore discarded. This problem has been reported previously for F1 screens (Grigliatti, 1986), and results from EMS affecting only one strand of the DNA double helix, leading to only one out of two daughter

cells carrying the mutation. The combination of all of these reasons accounts for the large number of fertile modifiers which were discarded or for which stocks were not generated.

5-5.1 Saturation of the X-ray and EMS mutageneses

The number of mutations generated in the *black* gene was used as an estimate of the mutation frequency for both the EMS and X-ray mutageneses. This may not have been an ideal choice of marker to monitor the rate of mutagenesis, as the *black* locus has not been cloned, thus the size of this gene is unknown. Assuming that all genes are equally likely to be hit, each modifier of $DmcycE^{JP}$ would be expected to have nine X-ray alleles and four EMS alleles. This is obviously not the case, as many modifiers isolated are single alleles. It should, however, be noted that this estimate assumes that all modifiers survived and reproduced. Approximately one third of the X-ray and approximately half of the EMS modifiers originally selected died or were sterile, thus any expectations about the number of alleles for each modifier should take this into account. Six X-ray alleles and two to four EMS alleles should therefore have been isolated for each modifier. This, however, is also affected by the strength of the alleles generated, with milder alleles not necessarily having a significant effect on the $DmcycE^{JP}$ phenotype. In addition, a proportion of X-ray and EMS mutants are expected to be rare gain-of-function or overexpression alleles, which are generally unlikely to fall into a lethal complementation group. It is also possible that the high number of single alleles is, in part, due to the suppressor mutation in these stocks being homozygous viable, with additional mutations induced accounting for the homozygous lethal phenotype. The additional lethal mutations associated with these suppressors would therefore not be expected to fall into complementation groups. Genetic mapping of the suppressor and the lethal mutation in these cases will enable this to be clarified.

Given the number of single alleles identified in the screen, and that the number of alleles predicted to be isolated for each modifier was only obtained for two complementation groups, *Su(DmcycE^{JP})2-6* and *Su(DmcycE^{JP})2-7*, it is unlikely that the mutageneses were saturating. Confirmation that the mutageneses were not saturating came from testing specific genes to determine whether alleles were isolated in the screen. Amorphic alleles of *E2F* and *string* have been shown to enhance the $DmcycE^{JP}$ phenotype (see Chapter 3; Secombe *et al.*, 1998). Alleles of *E2F* or *string* were not isolated among the enhancers that mapped to the third chromosome (A. Gardiakos and H. Richardson, unpublished). Furthermore, not all of the deficiencies identified as suppressors of $DmcycE^{JP}$ using the third chromosome deficiency collection have a corresponding X-ray or EMS generated suppressor (A. Brumby,

J. Horsfield, and H. Richardson, unpublished). The reason for this may be that the suppression originally observed using the deficiencies was due to multiple gene effects, or that the screen was not saturating.

Given the evidence that the mutageneses carried out appear not to be saturating for the second and third chromosomes, it is unlikely that alleles were generated of all modifiers of *DmcycE^{JP}*. However, many *DmcycE^{JP}* modifiers were isolated, some of which may be novel genes. Thus, the screen has been successful, and has provided many interesting mutations for future analysis.

5-5.2 The second chromosome suppressors

46 homozygous lethal second chromosome suppressor complementation groups were identified in this study, 10 of which have multiple alleles. Of the 10 groups with multiple alleles, six (described in this chapter) have been shown to suppress the *DmcycE^{JP}* phenotype by increasing the number of S phases during eye development. This indicates that these six genes encode proteins involved in negatively regulating entry into S phase. These suppressors also appear to act upstream of *cyclin E* transcription or in a parallel pathway, as reducing the dose of these genes had no effect on the rough eye phenotypes due to overexpression of type I or type II *cyclin E*. It is not surprising that these suppressors do not enhance the overexpression of *cyclin E* phenotype, as most downstream targets of the Cyclin E/Cdc2c complex would be expected to be proteins required for the initiation of DNA replication, mutations in which would not be expected to suppress *DmcycE^{JP}*. Enhancers of *DmcycE^{JP}*, which are expected to include downstream effectors of Cyclin E-mediated entry into S phase, may be more likely to modify the *GMR-cyclin E* rough eye phenotype.

Halving the dosage of second chromosome suppressors tested also had no effect on the eye phenotype of a *rux* hypomorphic allele. *rux* mutants have inappropriate Cyclin A in the MF, leading to ectopic S phases and rough eyes (Thomas *et al.*, 1994). It might therefore be predicted that a suppressor of *DmcycE^{JP}* may act by increasing the levels of Cyclin A in and posterior to the MF, thereby bypassing the requirement for Cyclin E for entry into S phase. As none of the suppressors of *DmcycE^{JP}* enhanced the *rux* phenotype, these genes do not act through the Rux pathway, and are therefore unlikely to be involved in the regulation Cyclin A.

Cytological locations have been determined for five of the second chromosome complementation groups described in this chapter, enabling candidate genes for these suppressors to be identified. *Su(DmcycE^{JP})2-3* and *Su(DmcycE^{JP})2-5* are likely to be allelic

to the previously identified, but uncharacterised, mutations *l(2)36Fd* and *l(2)04524*, respectively. In addition, candidate genes or mutations have been assigned for *Su(DmcycE^{JP})2-4*, and *Su(DmcycE^{JP})2-6*. These now need to be tested by characterisation of the lesions associated with these suppressors. No candidates were proposed for *Su(DmcycE^{JP})2-7*, which may be novel and *Su(DmcycE^{JP})2-8*, *Su(DmcycE^{JP})2-9* and *Su(DmcycE^{JP})2-10*, which need to be mapped.

5-5.3 Suppressors involved in chromatin remodelling

As described in Chapter 4, analysis of chromosomal regions that modify the *DmcycE^{JP}* rough eye phenotype revealed that a deficiency removing the chromatin remodelling gene *brahma* suppressed the *DmcycE^{JP}* phenotype. Consistent with this, alleles of *brahma* have been identified among the third chromosome suppressors generated in the *DmcycE^{JP}* genetic interaction screen (A. Brumby, pers. com.). In addition, an allele of *moira*, which encodes a protein known to physically associate with Brahma, was also identified as a suppressor of the *DmcycE^{JP}* phenotype (A. Brumby, pers. com.). Both of these genes have also been identified as enhancers of *GMR-dE2F/dDP* phenotype (Staehling-Hampton *et al.*, 1999), confirming their cell cycle regulatory role and implicating chromatin remodelling in the regulation of cell proliferation. *Su(DmcycE^{JP})2-4* and *Su(DmcycE^{JP})2-6* may also encode proteins involved in chromatin remodelling. Act42A, a component of the Brahma complex, is within the critical region for *Su(DmcycE^{JP})2-4* (Papoulas *et al.*, 1998). Similarly, two HMG proteins involved in altering chromatin structure are within the critical region for *Su(DmcycE^{JP})2-6* (Ner *et al.*, 1993; Churchill *et al.*, 1995), although these are not the only candidates in this region. There are no previously characterised mutations in these genes. Determining whether *Su(DmcycE^{JP})2-4* and *Su(DmcycE^{JP})2-6* are alleles of *Act42A* and *HMG-D* or *HMG-Z* will therefore require molecular characterisation of the lesions associated with these alleles.

5-5.4 Future work

Further analysis of the second chromosome suppressors described in this chapter should include determining whether *Su(DmcycE^{JP})2-9* and *Su(DmcycE^{JP})2-10* and the single alleles act by increasing *DmcycE^{JP}* S phases. In addition, cytological locations need to be determined for *Su(DmcycE^{JP})2-8*, *Su(DmcycE^{JP})2-9* and *Su(DmcycE^{JP})2-10* so that candidate genes can be identified. For those suppressors that are novel genes, or genes not previously implicated in the control of proliferation, analysis of homozygous mutant phenotypes needs to be carried out. Initially this would include examining S phases and

cyclin E expression in homozygous mutant embryos and larvae to determine whether any result in over-proliferation defects. Those suppressors that are novel need to be positionally cloned.

There are many factors now that will aid in the cloning of the second chromosome suppressors of $DmcycE^{JP}$. A major contributor to this will be the availability of the *Drosophila* genome sequence, which is expected to be finished in the year 2000. Genomic DNA sequence analysis will enable genes within the critical region for each suppressor to be predicted based on similarity to other known genes or proteins using database searches and gene prediction programs. It is therefore important to assign cytological locations for all of the modifiers obtained in the screen. Candidate genes for complementation groups with X-ray alleles can be tested relatively easily, as these alleles are likely to be deletions or chromosomal re-arrangements that should be revealed by genomic Southern analysis. Consistent with the prediction that X-ray generated alleles are likely to be deletions or re-arrangements, alleles of $Su(DmcycE^{JP})2\text{-}3$, and $Su(DmcycE^{JP})2\text{-}4$ show recombination suppression indicative of chromosomal re-arrangements, and $2\text{-}3^{59S16}$ is likely to be a deletion. Determining the breakpoints of these deletions and re-arrangements will aid in the isolation of these genes. The cloning and molecular characterisation of second chromosome suppressors will provide insights into the mechanisms by which these modifiers act to regulate of the G1 to S phase transition during development.

Chapter 6: Characterisation of an allele of *cdc2c*

6-1 Introduction

As described in Chapter 3, reducing the gene dose of negative regulators of the G1 to S phase transition, such as *RBF* or *dacapo*, suppressed the $DmcycE^{JP}$ rough eye phenotype. Conversely, reducing the dose of the positive S phase regulators, *dE2F* or *dDP*, enhanced the $DmcycE^{JP}$ phenotype, consistent with the previously established roles for these genes in the G1 to S phase transition. Surprisingly, reduction of the dose of the Cdk partner for Cyclin E, *cdc2c*, using the deficiency, $Df(3R)H^{81}$, resulted in suppression of the $DmcycE^{JP}$ rough eye phenotype. This suppression was not altered by the addition of one copy of a *cdc2c* genomic DNA transgene, indicating that the suppressed phenotype was not due to reduced levels of Cdc2c (H. Richardson, pers. com.). It is therefore likely that in addition to deleting *cdc2c*, $Df(3R)H^{81}$ also deletes a dose sensitive suppressor of $DmcycE^{JP}$. As there are no described specific alleles of *cdc2c*, the genetic interaction between *cdc2c* and $DmcycE^{JP}$ was unable to be tested specifically. The genetic interaction screen described in Chapter 5 is expected to result in the identification of positive and negative regulators of the G1 to S phase transition. Thus, alleles of *cdc2c* may be expected among the enhancers of $DmcycE^{JP}$. This chapter describes the initial characterisation of one of these $DmcycE^{JP}$ enhancers that corresponds to a *cdc2c* mutation.

6-2 *E1E6* is an allele of *cdc2c*

The *Drosophila* cdk2 homologue, *cdc2c*, has been mapped cytologically to 92E9-15 on the right arm of the third chromosome (Lehner and O'Farrell, 1990b; Stern *et al.*, 1993), and is defined by the breakpoints of $Df(3R)H^{81}$. As an initial assay to determine whether any alleles of *cdc2c* were isolated in the genetic interaction screen as $DmcycE^{JP}$ enhancer mutations, all 67 homozygous lethal third chromosome enhancers were crossed to $Df(3R)H^{81}$. From these crosses, two EMS-induced mutations, *E9E1* and *E1E6*, failed to complement $Df(3R)H^{81}$ (A. Gardiakos and H. Richardson, unpublished results; Table 6.1), and were therefore candidate *cdc2c* mutations. Since both *E9E1* and *E*1E6 were lethal in combination with $Df(3R)H^{81}$, these two mutants were crossed together to determine whether they represented the same complementation group. Flies transheterozygous for *E9E1* and *E1E6* were viable and fertile, demonstrating that they represent different genes. To test if either of these mutations corresponded to *cdc2c*, a *P* element insert containing genomic DNA spanning *cdc2c* (C. Lehner, pers. com.) was used to attempt to rescue the lethality of *E9E1*

and *E1E6*. The lethality of *E1E6*, but not *E9E1*, in association with $Df(3R)H^{81}$ was able to be rescued by one copy of a genomic *cdc2c* construct, demonstrating that *E1E6* is an allele of *cdc2c* (Table 6.1). *E1E6* was therefore renamed $cdc2c^{JS}$ (Secombe *et al.*, 1998). The *cdc2c* mutation is not the only lethal mutation carried on the $cdc2c^{JS}$ chromosome, as the genomic *cdc2c* transgene was not able to rescue the lethality of $cdc2c^{JS}/cdc2c^{JS}$ homozygous flies.

Table 6.1 *E1E6* is an allele of *cdc2c*.

	x $Df(3R)H^{81}$	x $Df(3R)H^{81}$, *P*{genomic *cdc2c*}
E1E6 ($cdc2c^{JS}$)	0/150	21/102
E9E1	0/50	0/140

Number of flies transheterozygous for either $Df(3R)H^{81}$, which removes *cdc2c*, or $Df(3R)H^{81}$ with a *cdc2c* genomic *P* element insert. The lethality of *E1E6* over $Df(3R)H^{81}$ is rescued by the presence of one copy of the genomic *cdc2c* rescue transgene, whereas *E9E1* is not. Data are presented as the number of transheterozygous flies/ total number of flies scored.

6-3 Reducing the dose of *cdc2c* enhances $DmcycE^{JP}$

The identification of the *cdc2c* allele, $cdc2c^{JS}$, as an enhancer of the $DmcycE^{JP}$ rough eye phenotype (Figure 6.1A, B), is consistent with the characterised role for Cdc2c as the Cyclin E associated kinase (Knoblich *et al.*, 1994; Sauer *et al.*, 1995). To determine whether the enhancement of $DmcycE^{JP}$ was occurring by reducing the number of S phases during eye development, BrdU incorporation was carried out on $DmcycE^{JP}$ larvae heterozygous for $cdc2c^{JS}$. Compared with $DmcycE^{JP}$ discs, halving the dose of *cdc2c* resulted in a reduction in the number of S phases (Figure 6.1C, D; Table 6.2), accounting for the enhanced rough eye phenotype. As at least one other lethal mutation is linked to $cdc2c^{JS}$, it was possible that the enhanced eye phenotype and reduction in S phases was due to other mutations on the $cdc2c^{JS}$ chromosome. To confirm that it was reduced Cdc2c levels that resulted in the enhancement of $DmcycE^{JP}$, the genomic *cdc2c* transgene was used to restore wild-type levels of Cdc2c in a $DmcycE^{JP}$ strain heterozygous for $cdc2c^{JS}$. The eye phenotype of $DmcycE^{JP}$ flies heterozygous for both $cdc2c^{JS}$ and the *cdc2c* genomic transgene was indistinguishable from that of $DmcycE^{JP}$ alone (data not shown), indicating that reducing the dose of *cdc2c* was causing the enhanced rough eye phenotype.

Figure 6.1 Reducing the dose of *cdc2c* enhances the *DmcycE*^{JP} phenotype.

(A, B) Scanning electron micrographs of adult eyes from (A) *DmcycE*^{JP} and (B) *DmcycE*^{JP}; *cdc2c*^{JS}/+ showing that reducing the dose of *cdc2c* enhances the *DmcycE*^{JP} rough eye phenotype. (C, D) BrdU labelling of eye discs from (C) *DmcycE*^{JP} and (D) *DmcycE*^{JP}; *cdc2c*^{JS}/+ 3rd instar larvae, showing that the number of S phases anterior and posterior to the MF is decreased when the dosage of *cdc2c* is halved. Anterior is to the right in all panels. Bar indicates MF.

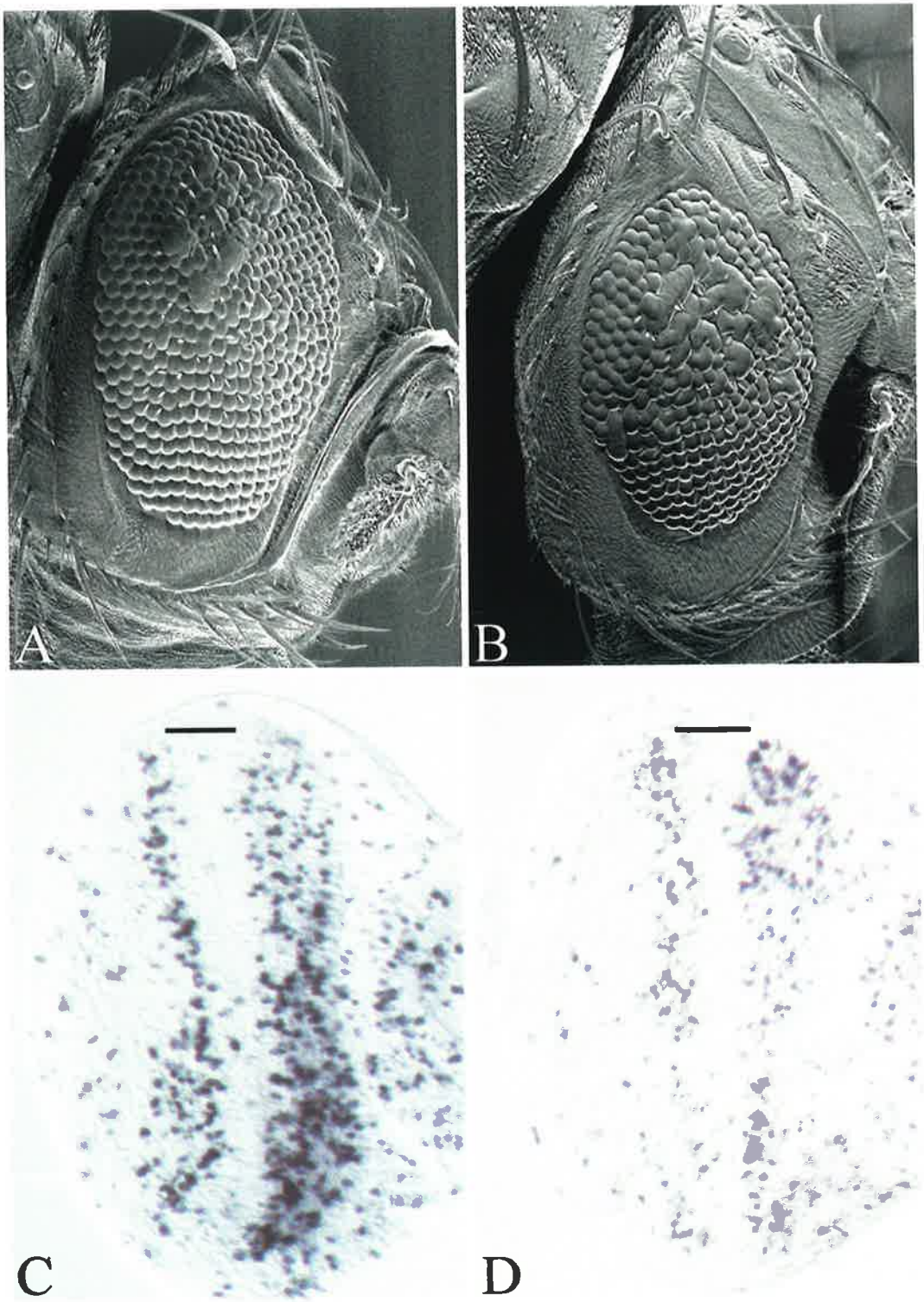

A
B
C
D

Table 6.2 Genetic interaction between *DmcycE^{JP}* and *cdc2c*.

Strain	S phases (± SE)
w^{1118}	54 ± 2.7
DmcycE^{JP}	31.2 ± 3.0
DmcycE^{JP} ; *cdc2c^{JS}*/+	23.2 ± 2.3

The number of S phase cells within a 200 μm region immediately posterior to the MF were counted from at least ten eye discs and presented as an average ± standard error (SE). Reducing the dose of *cdc2c* using the *cdc2c^{JS}* allele resulted in a decrease in the number of S phases posterior to the MF.

6-4 Preliminary phenotypic characterisation of *cdc2c^{JS}*

As the *cdc2c^{JS}* chromosome had at least one other lethal mutation, analysis of mutant phenotypes could not be carried out on *cdc2c^{JS}* homozygotes. Preliminary analysis was therefore carried out after crossing *cdc2c^{JS}* away from *DmcycE^{JP}*, and as transheterozygotes with *Df(3R)H^{81}*. To test whether *cdc2c^{JS}/Df(3R)H^{81}* resulted in embryonic lethality, *cdc2c^{JS}* and *Df(3R)H^{81}* flies were crossed together, allowed to lay on grape agar plates, and the number of unhatched and hatched embryos scored (Table 6.3). Approximately one quarter of embryos from this cross were not expected to hatch, as this proportion are predicted to be homozygous for the balancer chromosome. If *cdc2c^{JS}/Df(3R)H^{81}* results in embryonic lethality, half the embryos from this cross would not be expected to hatch. Approximately one quarter (28%) of embryos remained unhatched from the cross of *cdc2c^{JS}* and *Df(3R)H^{81}*, demonstrating that *cdc2c^{JS}/Df(3R)H^{81}* embryos develop and hatch into larvae. To determine whether *cdc2c^{JS}/Df(3R)H^{81}* survive to, or beyond, the third larval instar stage, *cdc2c^{JS}* and *Df(3R)H^{81}* stocks balanced using the *TM6B* balancer were crossed together. *TM6B* carries the dominant *Tubby* larval marker, allowing *cdc2c^{JS}/Df(3R)H^{81}* larvae to be distinguished from other genotypes. From this cross, non-tubby third instar larvae were observed that were slightly developmentally delayed, smaller than wild-type, and were unable to pupate normally. Thus, *cdc2c^{JS}/Df(3R)H^{81}* larvae die at the third instar larval/pupal boundary, although it cannot be ruled out that some *cdc2c^{JS}/Df(3R)H^{81}* larvae die during the first and second larval instars.

Table 6.3 *cdc2c^{JS}/Df(3R)H^{81}* is embryonic viable

Strain	Number of embryos unhatched	Total number of embryos counted	% unhatched embryos
w^{1118}	5	154	3%
cdc2c^{JS}/TM3 x *Df(3R)H^{81}/TM3*	62	223	28%

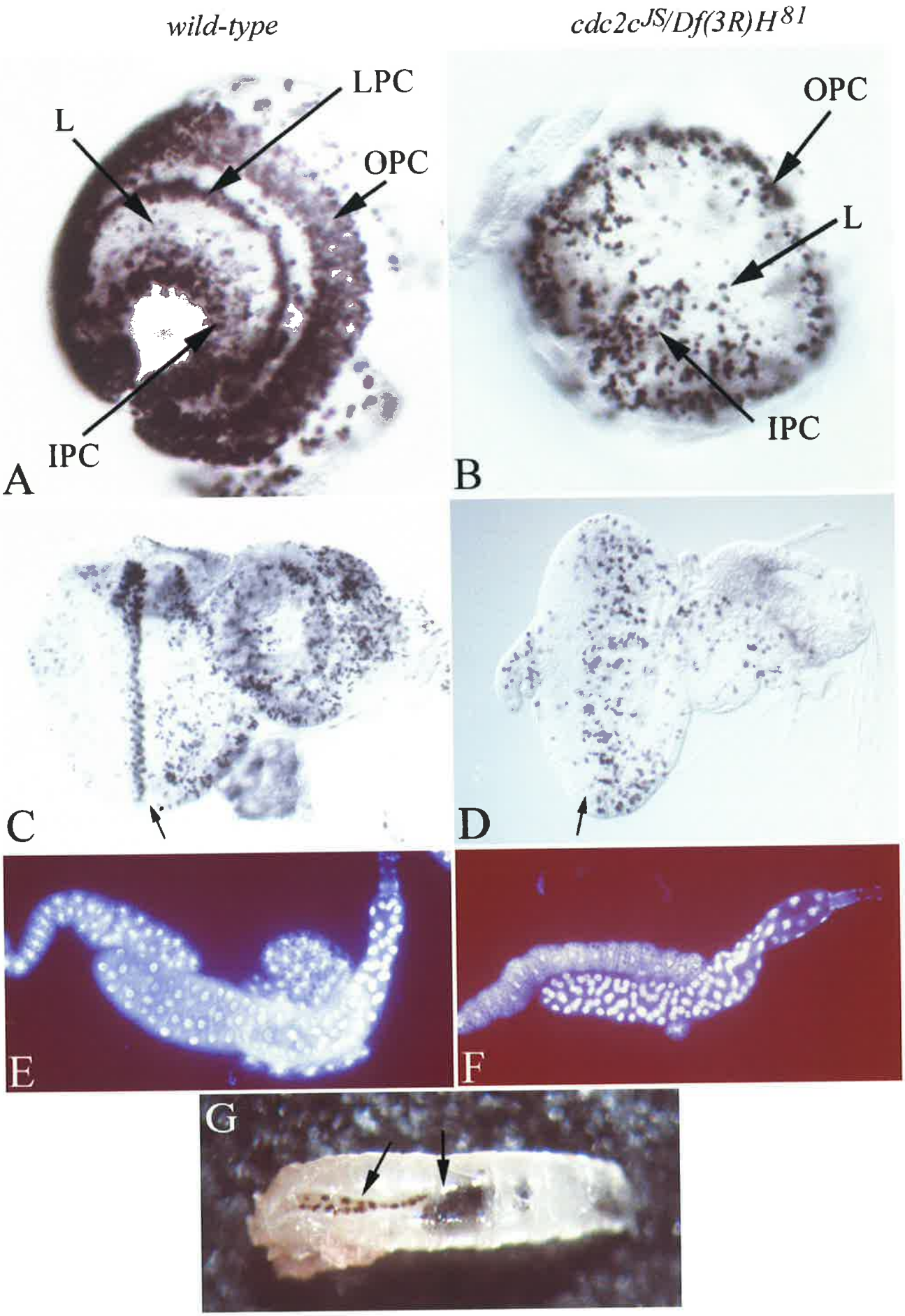
Flies of the appropriate genotype were allowed to lay on grape agar plates before counting the total number of embryos laid. These embryos were then aged for 24 hours to allow embryos to hatch and the number of unhatched and hatched embryos counted. The unhatched embryos seen from *cdc2c^{JS}/TM3* x *Df(3R)H^{81}/TM3* are due to the lethality of the *TM3* balancer chromosome when homozygous, accounting for approximately one quarter of embryos laid.

To examine *cdc2c^{JS}/Df(3R)H^{81}* larvae for proliferative defects, BrdU incorporation was carried out to reveal S phases. Larval brain lobes and all imaginal discs examined showed a reduced number of S phases (Figure 6.2; and data not shown), demonstrating a requirement for Cdc2c in mitotically dividing cells. In addition, polytene salivary glands of *cdc2c^{JS}/Df(3R)H^{81}* larvae were smaller than wild-type, indicating a possible effect on endoreplication (Figure 6.2E, F). Significantly, there were two tissues in which a sub-set of S phases were not detected. The first of these occurred in the epithelium of the larval brain lobe. While a reduced number of S phase cells were seen in the outer proliferative centre (OPC), inner proliferative centre (IPC), and lamina (L), no S phases were detected in lamina precursor cells (LPC) (Figure 6.2B compared with 6.2A). The second case in which there was an absence of S phase cells occurred during differentiation of the eye imaginal disc. Like other imaginal discs, there is a general reduction in the number of S phase cells throughout the disc, but no S phases were detected immediately posterior to the MF (Figure 6.2D compared with 6.2C). The reason for *cdc2c^{JS}* having such specific effects on a subset of S phases is not clear.

In addition to proliferative defects, *cdc2c^{JS}/Df(3R)H^{81}* third instar larvae also develop melanotic masses (Figure 6.2G). Although it is not clear why these melanotic masses appear, it is presumably due to the melanization of tissues that do not develop properly in *cdc2c* mutant larvae. Encapsulation and melanization normally occurs during larval development in response to infection, although the presence of melanotic masses has also been reported for a number of mutations that affect imaginal disc and hematopoietic cell divisions (Watson *et al.*, 1991; Rodriguez *et al.*, 1996). The melanotic masses seen in

Figure 6.2 *cdc2c* mutant larvae have fewer S phases, reduced salivary glands and melanotic masses

BrdU labelling to show S phases from wild-type (A, C, E) and $cdc2c^{JS}/Df(3R)H^{81}$ (B, D, F) 3rd instar larvae. (A, B) The number of S phases in the outer proliferative centre (OPC) inner proliferative centre (IPC) and lamina (L) of $cdc2c^{JS}/Df(3R)H^{81}$ larval brain lobes is reduced relative to wild-type and lamina precursor cell (LPC) S phases were not detected. (C, D) The number of S phases in $cdc2c^{JS}/Df(3R)H^{81}$ eye discs is also reduced relative to wild-type and post-MF S phases are absent. (E, F) Hoechst 33258 staining showing that $cdc2c^{JS}/Df(3R)H^{81}$ salivary glands are reduced in size compared with wild-type. (G) $cdc2c^{JS}/Df(3R)H^{81}$ third instar larva showing melanotic masses (indicated by arrows). Anterior is to the right in all panels and arrows in C and D indicate the MF.


wild-type
cdc2c^JS/Df(3R)H^81
LPC
L
OPC
IPC
A
OPC
L
IPC
B
C
D
E
F
G

cdc2c^{JS} larvae may result in lethality, as melanin formation is associated with reactions involving phenoloxidases, and the production of toxic intermediates (reviewed by Wright, 1997). The failure of *cdc2c*^{JS}/*Df(3R)H*^{81} larvae to pupate may be due to reduced proliferation of ring gland cells in *cdc2c*^{JS}/*Df(3R)H*^{81} larvae, compromising the release of ecdysteroids required for pupation to occur (reviewed by Riddiford, 1993).

6-5 Molecular basis of the *cdc2c*^{JS} mutation

As *cdc2c*^{JS} was induced using the mutagen EMS, it was predicted to be a mis-sense or nonsense mutation within the *cdc2c* coding region. To determine the molecular basis of this mutation, genomic DNA spanning the *cdc2c* locus was PCR amplified from *cdc2c*^{JS}/*Df(3R)H*^{81} larvae and from the *DmcycE*^{JP} parental strain. As the genomic structure of *cdc2c* was unknown, the *cdc2c* locus was PCR amplified in two overlapping fragments (Figure 6.3), to allow for the presence of introns. Both the 5' and 3' PCR products amplified from genomic DNA were larger than the corresponding PCR products using a *cdc2c* cDNA template, indicating the presence of at least one intron within *cdc2c* (data not shown). These PCR products were then cloned into pBluescript, and the nucleotide sequence of two independent clones of each fragment was determined using vector and *cdc2c* primers (described in materials and methods section 2-2.6; Figure 6.3). The sequence of *cdc2c* from the parental *DmcycE*^{JP} strain revealed six polymorphisms relative to the published sequence, only one of which resulted in an amino acid change (Figure 6.3). Since this change results in a conservative amino acid substitution of lysine to arginine, it is not expected to alter the function of the protein. In addition, six introns containing the conserved residues required for splicing were identified (Breathnach and Chambon, 1981; Hawkins, 1988; Figure 6.3). Surprisingly, sequencing the coding region and introns of *cdc2c*^{JS} failed to find any nucleotide differences between *cdc2c*^{JS} and *cdc2c* from the parental *DmcycE*^{JP} strain. The absence of any changes within coding and intron sequences may indicate that the mutation is within 5' or 3' untranslated regions, or in the promoter region.

6-6 Discussion and conclusions

The identification of an allele of the *Drosophila cdk2* gene, *cdc2c*, has allowed the role of Cdk2 in cell cycle progression to be examined in the context of a whole animal. Previously, evidence for the involvement Cdk2 in the G1 to S phase transition has come from tissue culture and *in vitro* studies. Overexpression of dominant negative forms of Cdk2 (van den Heuval and Harlow, 1993) or microinjection of anti-Cdk2 antibodies (Tsai *et al.*, 1993) into mammalian tissue culture cells results in G1 arrest. In addition, immunodepletion studies

have demonstrated that Cdk2 activity is essential for efficient DNA replication but not for mitosis in *Xenopus* cell-free extracts (Fang and Newport, 1991). Thus, Cdk2 is required specifically for progression from G1 into S phase in these assays. Preliminary evidence in *Drosophila* has also implicated Cdc2c in cell cycle control, as overexpression of peptide aptamers that block the function of Cdc2c during eye development results in a rough eye phenotype (Kolonin and Finley, 1998). This experiment did not, however, address whether Cdc2c was functioning at the G1 to S phase transition.

As described in Chapter 3, a deficiency known to remove *cdc2c*, $Df(3R)H^{81}$, did not enhance the $DmcycE^{JP}$ phenotype as expected, but suppressed it. Although this was assumed to be caused by the co-deletion of a gene that resulted in suppression of the $DmcycE^{JP}$ phenotype, the identification of a specific *cdc2c* allele has enabled the effect of reducing the dose of this gene to be tested directly. To identify alleles of *cdc2c* isolated in the mutagenesis, enhancers of $DmcycE^{JP}$ on the third chromosome were tested to determine whether any mapped within the $Df(3R)H^{81}$ deficiency. Consistent with the prediction that reducing the dose of the Cyclin E-associated kinase would enhance the $DmcycE^{JP}$ phenotype, one EMS mutant was identified that was lethal in combination with $Df(3R)H^{81}$, and this lethality was able to be rescued by a genomic *cdc2c* transgene. This *cdc2c* allele, $cdc2c^{JS}$, is the first allele of *cdc2c* described, and was therefore characterised further.

The crystal structure for human Cdk2 has been determined (De Bondt *et al.*, 1993), and the residues critical for Cdk activity and binding to Cyclin A characterised (Jeffrey *et al.*, 1995). It was therefore of interest to ascertain exactly which residue is mutated in $cdc2c^{JS}$. Surprisingly, sequencing the coding region and introns of $cdc2c^{JS}$ failed to identify any differences between $cdc2c^{JS}$ and *cdc2c* from the parental $DmcycE^{JP}$ strain. The $cdc2c^{JS}$ mutation must therefore lie outside the sequenced region. Although all of the *cdc2c* coding region, and all introns were sequenced, the primers used for amplification of $cdc2c^{JS}$ were located in the 5' and 3' untranslated regions (Figure 6.3, underlined nucleotides). Consequently, nucleotides 1-62 of the 5' untranslated region and 1122-1140 of 3' untranslated region were not sequenced. It is therefore possible that the mutation lies within these regions, and affects Cdc2c levels by reducing the stability of the mRNA or preventing translation. Completing the sequencing of the *cdc2c* genomic region, including immediately upstream of the transcription start site may identify a mutation that may act to abolish or reduce transcription. In addition, *in situ* hybridisation, Northern and Western analysis should be carried out to demonstrate that *cdc2c* transcript and proteins levels are reduced in $cdc2c^{JS}/Df(3R)H^{81}$ larvae compared with wild-type.

Figure 6.3 Exon/intron boundaries of *cdc2c*.

(A) Sequence of *cdc2c* showing the start (ATG) and stop (TGA) codons (bold and underlined), and sequence for 5', 3' and internal primers used for PCR amplification and sequencing (underlined). The position of six introns are indicated within the cDNA sequence. No sequence changes were found between the $DmcycE^{JP}$ strain and $cdc2c^{JS}$, demonstrating that the mutation in $cdc2c^{JS}$ is not within the coding region. Six polymorphisms were found between the published *cdc2c* (Lehner and O'Farrell, 1990b) sequence and the sequence of the parental $DmcycE^{JP}$ strain (shown as alternate residues, published nucleotide first). One of these polymorphisms (italic, underlined) would result in lysine at amino acid position 76 being changed to arginine. This is a highly conservative alteration, and is the amino acid normally found at this position in Xenopus Cdk2 (Fang and Newport, 1991).

(B) Table summarising the size of the six introns and the sequence flanking exon/intron boundaries in *cdc2c*. The consensus sequence for the 5' splice donor site and 3' spice acceptor site are $^{A}/_{C}$AG * $\underline{GT}^{A}/_{G}$AGT and $^{C}/_{T}{}^{C}/_{T}{}^{C}/_{T}{}^{C}/_{T}{}^{C}/_{T}{}^{C}/_{T}N^{C}/_{T}\underline{AG}$ * G$^{G}/_{T}$ respectively (Breathnach and Chambon, 1981). * indicates the exon/intron boundary. Underlined nucleotides are critically conserved and required for splicing to occur.

A

GACCAAAGAAGAAGAATTTCCCTCCAAAATATTAGTTTATTTTGCCAACATCGAT
CTCAAGGATTGTTTACGAGGTTATGACCACCATTCTAGATAACTTTCA$^{A}/_{G}$CGCGC
CGAAAAGATTGGCGAGGGCACCTACGGTATAGTTTACAAAGCGCGTAGCAACTCC
ACCGGCCAGGATGTGGCCCTCAAAAAGATTCGGCTAGAAGG |[1] CGAAACGGAG
GGTGTTCCTTCGACGGCCATTCGAGAGAT$^{C}/_{T}$TCCCTGCTGAAGAACCTTAAGCAC
CCAAATGTGGTCCAACTATTTGACGTAGTCATTTCCGGCAACAATCTGTACATGA
TATTCGAGTACCTGAACATGGATCTAAAGAAGCTGATGGA$^{T}/_{C}$A$^{A}/_{G}$GAAAAAAGA^{C}
$/_{T}$GTGTTCACCCCTCAGTTGATAAAG |[2] AGCTATATGCATCAGATATTAGATGC
CGTCGGCTTTTGCCACACGAATCGTATCCTGCATCGCGATCTCAAGCCCCAGAAC
CTTCTCGTAGACACGGCGGGCAAAATAAAG |[3] TTGGCTGACTTTGGCCTAGCA
AGGGCCTTCAACGTGCCTATGCGGGCGTACACACACGAAGTCGTCACCCTCTGGT
ACCGAGCTCCAGAGATTCTGTTGGGCACGAAATTCTACTCCACGGGCGTGGACAT
CTGGAGTCTAGGCTGCATTTTCTCTGA$^{A}/_{G}$ATG |[4] ATTATGCGCCGCTCCTTGT
TTCCTGGAGACAGCGAGATCGATCAACTTTATAGGATTTTCCGTACCTTAAGCAC
ACCTGATGAAACAAATTGGCCTGGTGTGACGCAGCTGCCAGACTTTAAGACCAAG
TTCCCTAGATGGGAGGGAACTAACATGCCACAACCCATAACCGAACACGAGGCGC
ACGAACTCATAATG |[5] TCAATGCTGTGCTATGATCCCAACCTGCGCATCTCAG
CCAAGGACGCACTGCAGCACGCTTACTTCCGCAATGTGCAGCATGTTGACCATGT
AGCCCTGCCTGTAGATCCCAATGCCGGCAGCGCTTCGCGTCTAACGCGGCTCGTC
TGATCGTGTCCAATAGCCCCAGATCTAGCAAATTACTTGTGTTTTCTAGTATA
|[6] CTGCCACCTCATTATCATGCATTCCCCATCCTCACTCGCACGTAACATACG
ATAAATACACGATCATCCATAAGT

B

Intron number	Intron length (bp)	5' donor site	3' acceptor site
1	265	AGG*GTGCGT	CGATTCACAG*CG
2	55	AAG*GTTGAT	CACTTTGCAG*AG
3	59	AAG*GTGAGA	TCAACTCCAG*TT
4	59	ATG*GTAGTG	AGAATTACAG*AT
5	55	ATG*GTGAGT	TACCTTACAG*TC
6	75	ATA*GTAAGT	TATTTTACAG*CT

The enhancement of the *DmcycE*^{JP} rough eye phenotype and reduction of S phases during eye development by halving the dose of *cdc2c* is consistent with the role of Cdc2c as the Cyclin E regulated kinase. To gain further evidence for the involvement of Cdc2c in regulating the G1 to S phase transition, preliminary phenotypic characterisation of *cdc2c*^{JS} mutants was undertaken. Unlike mutations in many other cell cycle genes such as *cyclin E*, *cyclin A* and *string*, which result in embryonic lethality (Lehner and O'Farrell, 1989; Knoblich *et al.*, 1994; Edgar and O'Farrell, 1990), *cdc2c*^{JS}/*Df(3R)H*^{81} is lethal at the larval/pupal boundary, at which point maternally deposited *cdc2c* transcript is presumably depleted. It is possible that *cdc2c*^{JS}/*Df(3R)H*^{81} is lethal at the larval/pupal boundary because *cdc2c*^{JS} is a hypomorphic allele. Alternatively, *cdc2c*^{JS} may be a null or severe allele, as mutations in the mitotic Cdk, *cdc2* also result in larval/pupal lethality (Stern *et al.*, 1993). This presumably reflects the stable nature of Cdks, which are not broken down with each cell cycle.

While mutations in *cdc2* affect mitotically dividing cells and result in a G2 arrest, *cdc2c*^{JS} affects both mitotically dividing and endoreplicating cells. In mitotically dividing cells, this is reflected in the reduction in the number of cells labelling with BrdU in imaginal discs and brain lobes in *cdc2c*^{JS}/*Df(3R)H*^{81} mutant larvae. Endoreplicative cycles may also be affected in *cdc2c*^{JS}/*Df(3R)H*^{81} larvae as salivary gland cells are smaller than wild-type in *cdc2c*^{JS}/*Df(3R)H*^{81} larvae, although nuclear size is not significantly different. While decreasing Cdc2c activity is not expected to affect cell growth or size directly (Neufeld and Edgar, 1998), mild effects on endoreplication in *cdc2c*^{JS}/*Df(3R)H*^{81} salivary glands may result in a small reduction in polyteny and in smaller cells. As Cyclin E is required for mitotic and endoreplicative cycles (Knoblich *et al.*, 1994; Sauer *et al.*, 1995), it is not surprising that a mutation in *cdc2c* also affects both types of cycles.

In addition to a general reduction in the number of S phases seen in *cdc2c*^{JS}/*Df(3R)H*^{81} larvae, there were no S phases detected immediately posterior to the MF during eye development or in the lamina precursor cells (LPC) of the larval brain epithelium. LPCs undergo a developmentally regulated S phase requiring direct innervation by developing photoreceptor neurons from the eye disc to generate adult neurons of the optic lobe (Selleck *et al.*, 1992). There are several possible scenarios to explain why these LPC S phases do not occur. It is possible that eye imaginal disc photoreceptors or photoreceptor neurons do not develop normally in *cdc2c*^{JS} larvae, thus, LPCs do not receive the signal to undergo S phase. The presence of photoreceptors can be checked by staining *cdc2c*^{JS} mutant eye discs for the Elav or 22C10 neuronal markers. In addition, both LPC and post-MF

S phases could be affected if these developmentally regulated proliferative events are the first to require zygotic transcription of *cdc2c*, with all other proliferation relying on maternally-derived Cdc2c. As no mutation was found within the $cdc2c^{JS}$ coding region, it is likely that the lesion associated with this allele lies in the promoter region. It is possible that a specific enhancer responsible for *cdc2c* expression in LPC and post-MF cells is modified in $cdc2c^{JS}$. This, however, is unlikely given that in addition to the specific effect observed on LPC and post-MF S phases, there is a reduction in the number of S phase cells in all tissues of $cdc2c^{JS}$ larvae. It is more likely that the mutation associated with $cdc2c^{JS}$ reduces or abolishes all zygotic transcription, and the LPC and post-MF cells are the first to be affected by this. The identification and characterisation of more *cdc2c* alleles will clarify whether the phenotypes are due to a general effect on proliferation or a specific effect on LPC and post-MF S phases.

Although only one allele of *cdc2c* was isolated and further characterised, the mutant phenotype of $cdc2c^{JS}$ and its genetic interaction with $DmcycE^{JP}$ indicates that Cdc2c is involved in G1 to S phase transition in *Drosophila*. Further characterisation of *cdc2c* mutant phenotypes will require the isolation of more alleles. Several alleles have been isolated as dominant suppressors of a *cyclin E* overexpression adult eye phenotype screen (C. Lehner, pers. com.). The analysis of these alleles will complement the work described in this chapter.

Chapter 7: Characterisation of *Su(DmcycE^{JP})2-2*

7-1 Introduction

The genetic interaction screen described in Chapter 5 was carried out with the aim of identifying novel genes involved in the regulation of the G1 to S phase transition during *Drosophila* development. Consistent with this expectation, several of the complementation groups identified in this screen may be mutations in novel genes. This chapter describes the initial mutant phenotype characterisation and cytological location of *Su(DmcycE^{JP})2-2*, which has three X-ray generated alleles, *2-2^{28S2}*, *2-2^{38S4}* and *2-2^{39S2}*.

7-2 *Su(DmcycE^{JP})2-2* suppresses *DmcycE^{JP}* by increasing S phases during eye development

The three alleles of *Su(DmcycE^{JP})2-2* suppress the *DmcycE^{JP}* rough eye phenotype, although *2-2^{38S4}* and *2-2^{39S2}* are stronger suppressors than *2-2^{28S2}* (Figure 7.1A-D). To determine whether reducing the dose of *Su(DmcycE^{JP})2-2* acts to suppress the *DmcycE^{JP}* phenotype by increasing the number of cells in S phase during eye development, eye imaginal disc S phases were examined by BrdU-labelling (Figure 7.1E-H). All three alleles of *Su(DmcycE^{JP})2-2* suppress the *DmcycE^{JP}* phenotype by increasing the number of S phases posterior to the MF (7.1F, G, H compared with 7.1E). Consistent with the weak suppression of *DmcycE^{JP}* by *2-2^{28S2}*, this allele only marginally increases S phases during eye development, while *2-2^{38S4}* and *2-2^{39S2}* increase the number of S phases to near wild-type levels. The suppression of the *DmcycE^{JP}* phenotype by increasing S phase cells indicates that *Su(DmcycE^{JP})2-2* encodes a negative regulator of the G1 to S phase transition.

7-3 Cytological position of *Su(DmcycE^{JP})2-2*

To allow *Su(DmcycE^{JP})2-2* to be recombined away from the *DmcycE^{JP}* mutation for mutant phenotype analysis, the cytological position for this modifier was determined. As all three *Su(DmcycE^{JP})2-2* alleles were generated using X-irradiation, chromosomal re-arrangements may have been induced that would alter chromosome pairing during meiosis, affecting recombination. To determine whether *Su(DmcycE^{JP})2-2* alleles result in recombination suppression, the recombination frequencies between the recessive second chromosome markers *black* (*b*), *curved* (*c*) and *speck* (*sp*) were examined. This revealed that *2-2^{38S4}* and *2-2^{39S2}* reduced recombination between the *b* and *c* markers relative to the *DmcycE^{JP}* parental strain (Table 7.1). In addition, *2-2^{39S2}* resulted in recombination suppression between *c* and *sp*, indicating that this region is also affected in this allele (data

not shown). The *b* and *c* markers map cytologically to 34D and 52D receptively, thus *Su(DmcycE^{JP})2-2* alleles were crossed to deficiencies available between these two markers (deficiencies are described in materials and methods, section 2-2.11.4). Consistent with *Su(DmcycE^{JP})2-2* mapping between *b* and *c*, all three alleles failed to complement *Df(2R)trix*, which removes the cytological region 51A1-2 to 51B6 (Table 7.2). This region was then refined using *Df(2R)3072r*, which deletes 51A5 to 51C1. All three alleles complemented this deficiency, defining the *Su(DmcycE^{JP})2-2* critical region as 51A1-5. The closest deficiency proximal to *Df(2R)trix* is *Df(2R)CX1*, which removes 49C1-4 to 50C23-D2. *Su(DmcycE^{JP})2-2* alleles complement this deficiency (data not shown), although this does not refine the cytological position of *Su(DmcycE^{JP})2-2*. Consistent with *Df(2R)trix* removing *Su(DmcycE^{JP})2-2*, recombining this deficiency onto the *DmcycE^{JP}* chromosome suppressed the *DmcycE^{JP}* rough eye phenotype (data not shown).

Table 7.1 *2-2^{39S4}* alters recombination between *black* and *curved*

Strain	Number of recombinants	Total number of flies scored	% recombination
DmcycE^{JP}	70	299	23.4%
39S2	42	256	16.4%
38S4	19	208	9.1%
28S2	nd	nd	nd

Examination of the recombination frequencies between *black* (*b*) and *curved* (*c*) in *2-2^{38S4}* and *2-2^{39S2}* revealed recombination suppression between *b* and *c*, indicating that these alleles may have chromosomal re-arrangements in this region. *b* maps genetically to 48.5 and *c* to 75.5, thus a recombination frequency of 27% would be expected. This frequency was not, however, obtained using parental *DmcycE^{JP}* flies, indicating that recombination between these markers is compromised in this strain. A maximum of 23.4% would therefore be expected with *2-2^{38S4}* or *2-2^{39S2}*. nd = not determined.

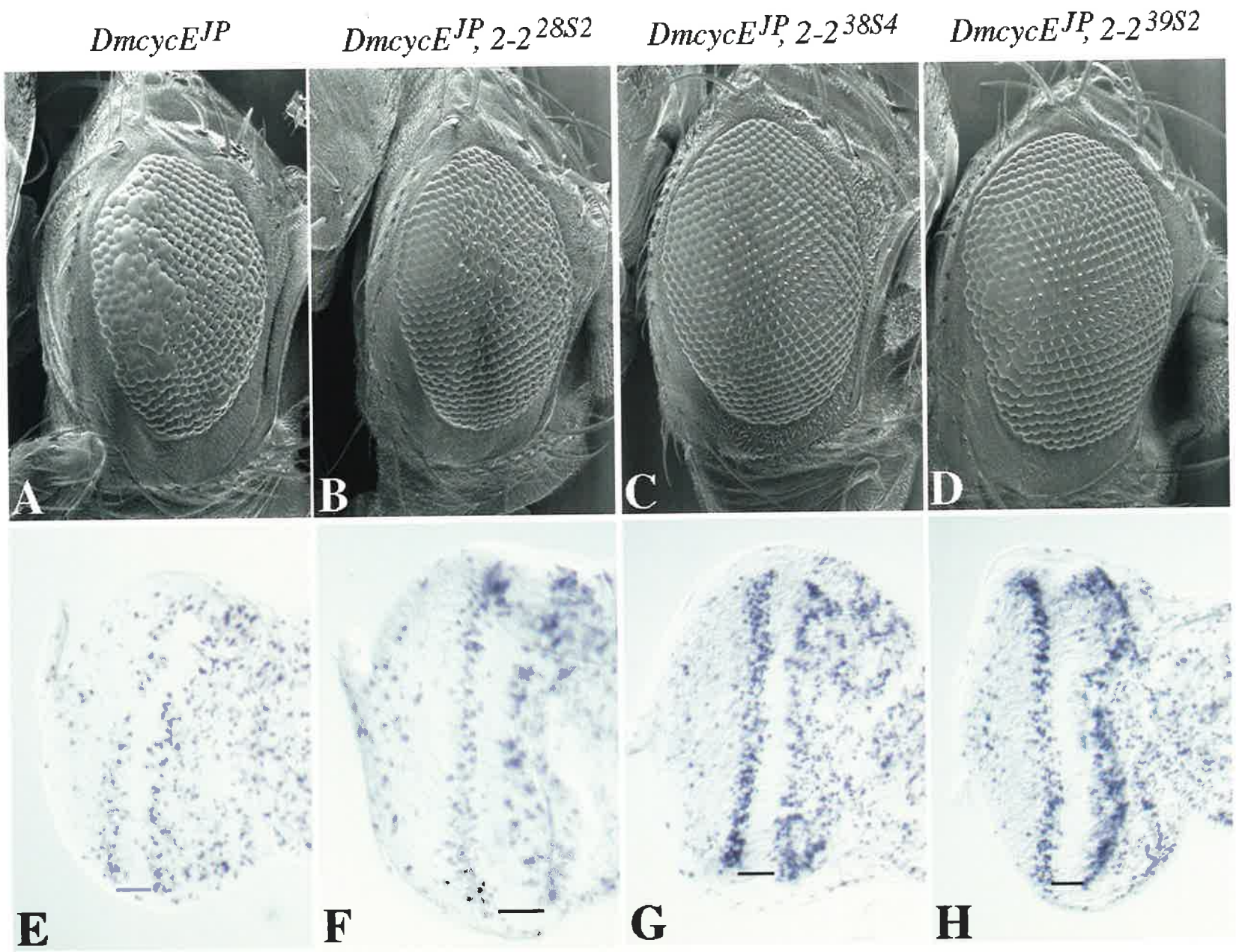
Table 7.2 *Su(DmcycE^{JP})2-2* maps to 51A1-5

Allele	*Df(2R)trix*	*Df(2R)3072r*
2-2^{28S2}	0/61	23/69
2-2^{38S4}	0/61	19/62
2-2^{39S2}	0/203	45/110

Crossing *Su(DmcycE^{JP})2-2* alleles to deficiencies available as part of the deficiency collection revealed that all three alleles failed to complement *Df(2R)trix*, which deletes the cytological region 51A1-2 to 51B6. In addition, *Su(DmcycE^{JP})2-2* alleles complemented *Df(2R)3072r*, which removes 51A5 to 51C1. *Su(DmcycE^{JP})2-2* therefore maps to 51A1-5. Data are presented as the number of transheterozygous flies/total number scored.

Figure 7.1 *Su(DmcycE^{JP})2-2* suppresses the *DmcycE^{JP}* phenotype by increasing the number of S phases during eye development.

(A, B, C, D) Scanning electron micrographs of adult eyes from (A) *DmcycE^{JP}*, (B) *DmcycE^{JP}*, *2-2^{28S2}/DmcycE^{JP}*, +, (C) *DmcycE^{JP}*, *2-2^{38S4}/DmcycE^{JP}*, + and (D) *DmcycE^{JP}*, *2-2^{39S2}/DmcycE^{JP}*, +, showing suppression of the *DmcycE^{JP}* rough eye phenotype. (E, F, G, H) BrdU labelling to show S phases of (E) *DmcycE^{JP}*, (F) *DmcycE^{JP}*, *2-2^{28S2}/DmcycE^{JP}*, +, (G), *DmcycE^{JP}*, *2-2^{38S4}/DmcycE^{JP}*, +, and (H) *DmcycE^{JP}*, *2-2^{39S2}/DmcycE^{JP}*, + third instar larvae eye imaginal discs. The number of S phases posterior to the MF is increased when the dose of *Su(DmcycE^{JP})2-2* is reduced. Anterior is to the right, and dorsal side is up for A, B, C and D. The bars in E, F, G and H indicate the MF.


DmcycE^JP
DmcycE^JP, 2-2^28S2
DmcycE^JP, 2-2^38S4
DmcycE^JP, 2-2^39S2
A
B
C
D
E
F
G
H

7-4 Phenotypic analysis of *Su(DmcycE^{JP})2-2* mutants

Since *2-2^{38S4}* and *2-2^{39S2}* showed stronger suppression of the *DmcycE^{JP}* phenotype than *2-2^{28S2}*, *DmcycE^{JP}* was recombined away from *2-2^{38S4}* and *2-2^{39S2}* and these flies used for all subsequent analysis. *2-2^{38S4}* and *2-2^{39S2}* were recombined away from *DmcycE^{JP}* using a second chromosome carrying the recessive *aristaless* (*al*), *b*, *c* and *sp* markers. The relative order of these genes is *al*, *b*, *cyclin E*, *Su(DmcycE^{JP})2-2*, *c*, *sp*. Recombinants carrying *Su(DmcycE^{JP})2-2*, but lacking *DmcycE^{JP}* were therefore selected as *al*, *b*, non-*c*, non-*sp* and stocks generated. Homozygous lethal *2-2^{38S4}* and *2-2^{39S2}* recombinants were crossed back to *DmcycE^{JP}* to determine whether recombinants were wild-type for *cyclin E*, and crossed to *Su(DmcycE^{JP})2-2* alleles and *Df(2R)trix* to ensure the presence of *Su(DmcycE^{JP})2-2*. Recombinant stocks generated for *2-2^{38S4}* and *2-2^{39S2}* did not exhibit any dominant phenotypes.

To determine whether *Su(DmcycE^{JP})2-2* acts downstream of *cyclin E* transcription, the effect of halving the dose of this suppressor on the rough eye phenotype generated by overexpression of type I or II *cyclin E* in all cells posterior to the MF during eye development was tested. Reducing the dose of *2-2^{38S4}* or *2-2^{39S2}* did not modify the rough eye phenotype associated with overexpression of *cyclin E* (data not shown), indicating that this modifier either acts upstream of *cyclin E* transcription or in a parallel pathway. Similarly, reducing the dose of *Su(DmcycE^{JP})2-2* alleles did not affect the rough eye phenotype associated with the hypomorphic *rux^{3}* mutation (data not shown).

7-4.1 *Su(DmcycE^{JP})2-2* is embryonic lethal

As *Su(DmcycE^{JP})2-2* was identified as a suppressor of the *DmcycE^{JP}* rough eye phenotype, it is predicted to encode a negative regulator of proliferation. To examine whether *Su(DmcycE^{JP})2-2* homozygotes have proliferative defects, the stage at which *Su(DmcycE^{JP})2-2* homozygotes die was determined. To test whether embryos homozygous for mutations in *Su(DmcycE^{JP})2-2* survive and hatch into larvae, the number of hatched and unhatched embryos from balanced *2-2^{38S4}* or *2-2^{39S2}* stocks was determined (Table 7.3). As *2-2^{38S4}* or *2-2^{39S2}* may have more than one lethal mutation, the embryonic lethality of *2-2^{38S4}* or *2-2^{39S2}* transheterozygous with *Df(2R)trix* was also examined (Table 7.3). Both of these crosses gave rise to ~50% of embryos failing to hatch, indicating that *2-2^{38S4}/Df(2R)trix* and *2-2^{39S2}/Df(2R)trix* are embryonic lethal. *2-2^{38S4}* homozygotes are also embryonic lethal, however homozygous *2-2^{39S2}* embryos survive and hatch into larvae,

as only the proportion of embryos expected to be homozygous for the balancer chromosome do not hatch. To determine whether *2-2^{39S2}* homozygotes survive to the third larval instar, this stock was balanced using *Cy-Tb*. No non-tubby third instar larvae were seen in the *2-2^{39S2}/Cy-Tb* stock, indicating that *2-2^{39S2}* homozygotes die during the first and/or second larval instars. Thus, while *2-2^{38S4}* is likely to be a null allele of *Su(DmcycE^{JP})2-2*, *2-2^{39S2}* is hypomorphic. Analysis of the *Su(DmcycE^{JP})2-2* mutant phenotype was therefore carried out using homozygous *2-2^{38S4}*, *2-2^{38S4}/Df(2R)trix* and *2-2^{39S2}/Df(2R)trix* embryos.

Table 7.3 Embryonic lethality of *Su(DmcycE^{JP})2-2* alleles

Strain	Number of unhatched embryos	Total number of embryos	% unhatched
w^{1118}	66	1213	5%
39S2/CyO x *Df(2R)trix/CyO*	246	619	40%
38S4/CyO x *Df(2R)trix/CyO*	548	934	58%
39S2/CyO	115	409	28%
38S4/CyO	440	771	57%

The *Su(DmcycE^{JP})2-2* alleles *2-2^{39S2}* and *2-2^{38S4}* were used to determine whether mutations in *Su(DmcycE^{JP})2-2* result in embryonic lethality. Flies of the appropriate genotype were allowed to lay on grape agar plates, and the number of unhatched and hatched embryos counted after 24 hours. *2-2^{38S4}* either homozygous, or transheterozygous with *Df(2R)trix* results in embryonic lethality, indicating that this is a severe allele. Homozygous *2-2^{39S2}* are embryonic viable, whereas this allele in combination with *Df(2R)trix* results in almost half of the embryos not hatching. *2-2^{39S2}* is therefore a hypomorphic allele.

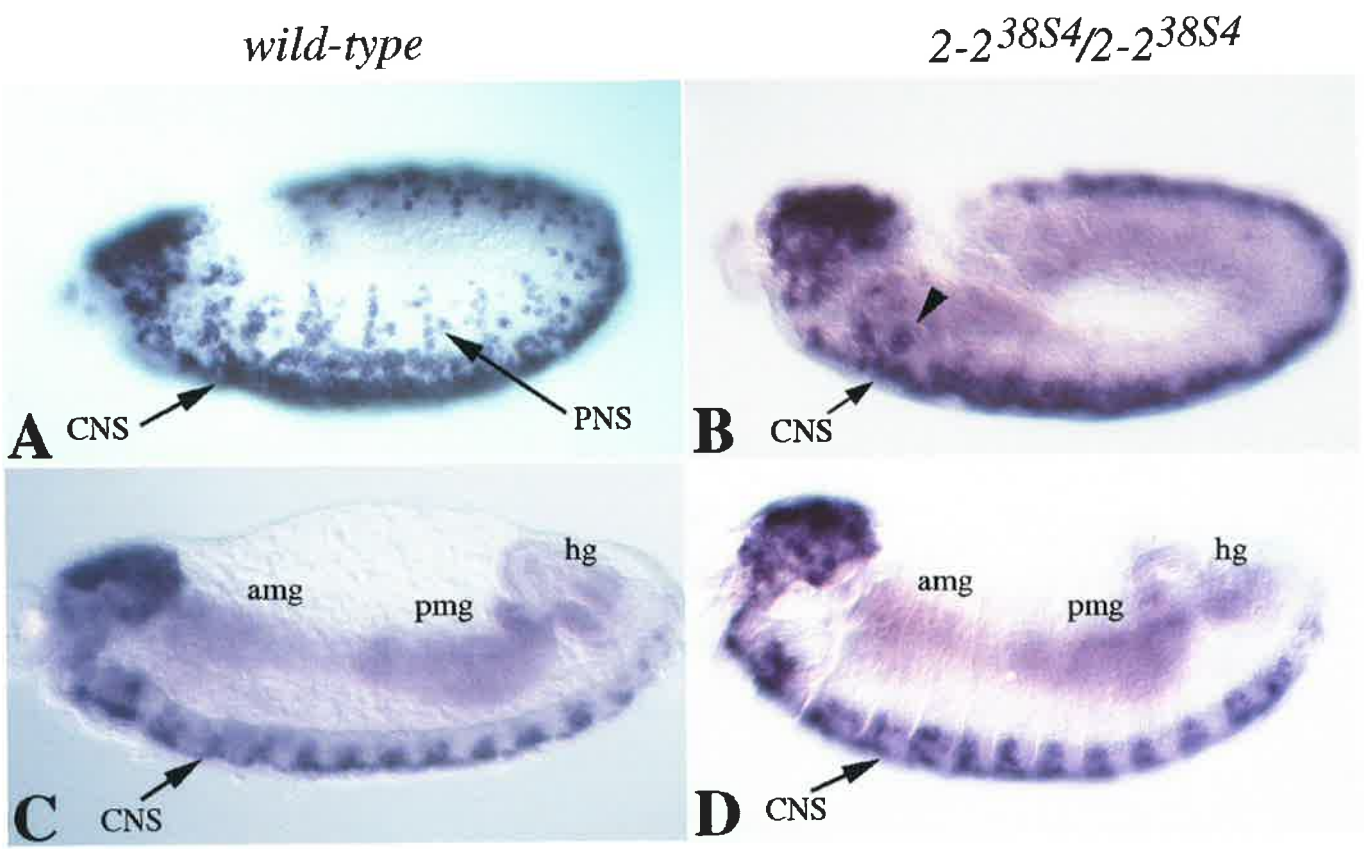
7-4.2 *Su(DmcycE^{JP})2-2* embryos have an altered pattern of *cyclin E* expression

cyclin E transcription is developmentally regulated during embryogenesis (Knoblich *et al.*, 1994; Jones, 1997), with expression correlating with mitotically dividing and endoreplicating tissues (Richardson *et al.*, 1993). As reducing the dose of *Su(DmcycE^{JP})2-2* did not affect the overexpression of type I or type II *cyclin* E rough eye phenotypes, the *Su(DmcycE^{JP})2-2* gene product may act on *cyclin E* transcription. To determine whether the pattern of *cyclin E* transcription is altered in *Su(DmcycE^{JP})2-2* zygotic mutants, whole mount *in situ* hybridisation to detect *cyclin E* mRNA was carried out (Figure 7.2). The pattern of *cyclin E* transcription in early homozygous *Su(DmcycE^{JP})2-2* embryos is similar to wild-type (data not shown), consistent with these cycles being regulated by maternally-derived

Figure 7.2 Mutations in *Su(DmcycE^{JP})2-2* affect *cyclin E* expression in trunk PNS cells.

Whole mount *in situ* hybridisation using an anti-sense DIG-labelled RNA probe to detect *cyclin E* transcript in wild-type (A, C) and *2-2^{38S4}* homozygous mutants (B, D). (A, B) stage 11 embryos showing *cyclin E* expression in proliferating CNS and PNS cells. While expression in the CNS is normal in *2-2^{38S4}* homozygotes, *cyclin E* transcripts are undetectable in trunk PNS cells of *2-2^{38S4}* homozygotes. *cyclin E* transcripts are still seen in head PNS cells (arrowhead). (C, D) stage 13 embryos showing a normal *cyclin E* pattern in the CNS, and the endo-replicating domains of the gut. Although homozygous *2-2^{38S4}* embryos are shown here, similar results were obtained with *2-2^{38S4}/Df(2R)trix*, and *2-2^{39S2}/Df(2R)trix*. Embryos are orientated with anterior to the left, dorsal side up.

Abbreviations used are anterior midgut (amg), posterior midgut (pmg), hindgut (hg), peripheral nervous system (PNS) and central nervous system (CNS).


wild-type
2-2^38S4/2-2^38S4
A
CNS
PNS
B
CNS
C
CNS
amg
pmg
hg
D
CNS
amg
pmg
hg

Cyclin E. Homozygous *Su(DmcycE^{JP})2-2* embryos showed appropriate downregulation of *cyclin E* transcription in the epidermis at G1 of cycle 17, and expression in the epidermal thoracic patches, which undergo a 17th cell cycle (data not shown). In addition, *cyclin E* expression in central nervous system (CNS) and the endoreplicating gut occured in the correct spatial and temporal manner (Figure 7.2; and data not shown). Surprisingly, *cyclin E* expression was not detected in the normally dividing peripheral nervous system (PNS) cells of the trunk region in stage 11 homozygous *Su(DmcycE^{JP})2-2* embryos (Figure 7.2B compared with 7.2A). Assuming PNS neurons are present, the *Su(DmcycE^{JP})2-2* gene product is required either directly or indirectly for *cyclin E* transcription in cells of the PNS. While the number of cells expressing *cyclin E* in the head PNS was reduced in homozygous *Su(DmcycE^{JP})2-2* embryos, it was not abolished. Transcription of *cyclin E* in head PNS cells is therefore, at least in part, under separate transcriptional control.

To determine whether the absence of *cyclin E* expression affects PNS S phases, BrdU-labelling was carried out on *Su(DmcycE^{JP})2-2* mutant embryos. Consistent with the absence of *cyclin E* transcripts in trunk PNS cells, no S phases were detected in these cells, while CNS and gut S phases were not affected (Figure 7.3; and data not shown). Consistent with fewer cells expressing *cyclin E* in the head PNS, the number of S phase cells was slightly reduced compared with wild-type. The reduction in the number of S phases during PNS divisions also affected the number of PNS neurons seen in *Su(DmcycE^{JP})2-2* mutant embryos, as detected by staining for the 22C10 neural antigen (Fujita *et al.*, 1982) (Figure 7.4B compared with 7.4A). Staining for the 22C10 antigen also confirmed that the absence of *cyclin E* transcription is not due to a general effect on PNS differentiation in *Su(DmcycE^{JP})2-2* mutants. The effect on the number of PNS neurons presumably affects the hatching of *Su(DmcycE^{JP})2-2* mutant embryos, as other mutations that affect PNS neuron number or morphology result in embryonic lethality (Kania *et al.*, 1995; Salzberg *et al.*, 1994).

7-5 Identification of the *Su(DmcycE^{JP})2-2* gene

To assess whether *Su(DmcycE^{JP})2-2* is novel, genes within the 51A cytological region were examined to determine whether any were consistent with the mutant phenotype and predicted function of *Su(DmcycE^{JP})2-2* (Table 7.4). Of the genes in the 51A1-5 region, seven were considered candidates, five *P* alleles, *Additional sex combs* (*Asx*) and *phyllopod* (*phyl*). The five *P* alleles in the region complemented *Su(DmcycE^{JP})2-2*, indicating that these mutations are not allelic. Although not an obvious candidate for *Su(DmcycE^{JP})2-2*, the Polycomb Group gene *Asx* was tested, as alleles of *Asx* have been shown to cause

overproliferation phenotypes (reviewed by Gateff *et al.*, 1996), which may be expected to result in suppression of the $DmcycE^{JP}$ rough eye phenotype. All three alleles of $Su(DmcycE^{JP})2\text{-}2$ complemented a null allele of *Asx*, demonstrating that this complementation group does not correspond to *Asx*.

Mutations in the *phyl* gene were also tested for allelism with $Su(DmcycE^{JP})2\text{-}2$. Phyl is a novel nuclear protein that acts downstream of *Ras*, *raf1* and *yan* in the development of the R1, R6 and R7 photoreceptors (Dickson *et al.*, 1995; Chang *et al.*, 1995). Embryos homozygous for null mutations in *phyl* have a reduced number of PNS neurons, as shown by staining for the 22C10 antigen (Chang *et al.*, 1995), a similar phenotype to that described above for $Su(DmcycE^{JP})2\text{-}2$ homozygous embryos (see Figure 7.4). A null allele of *phyllopod*, $phyl^{2245}$, failed to complement all three $Su(DmcycE^{JP})2\text{-}2$ alleles, indicating that $2\text{-}2^{28S2}$, $2\text{-}2^{38S4}$ and $2\text{-}2^{39S2}$ are likely to be alleles of *phyllopod* (Table 7.5).

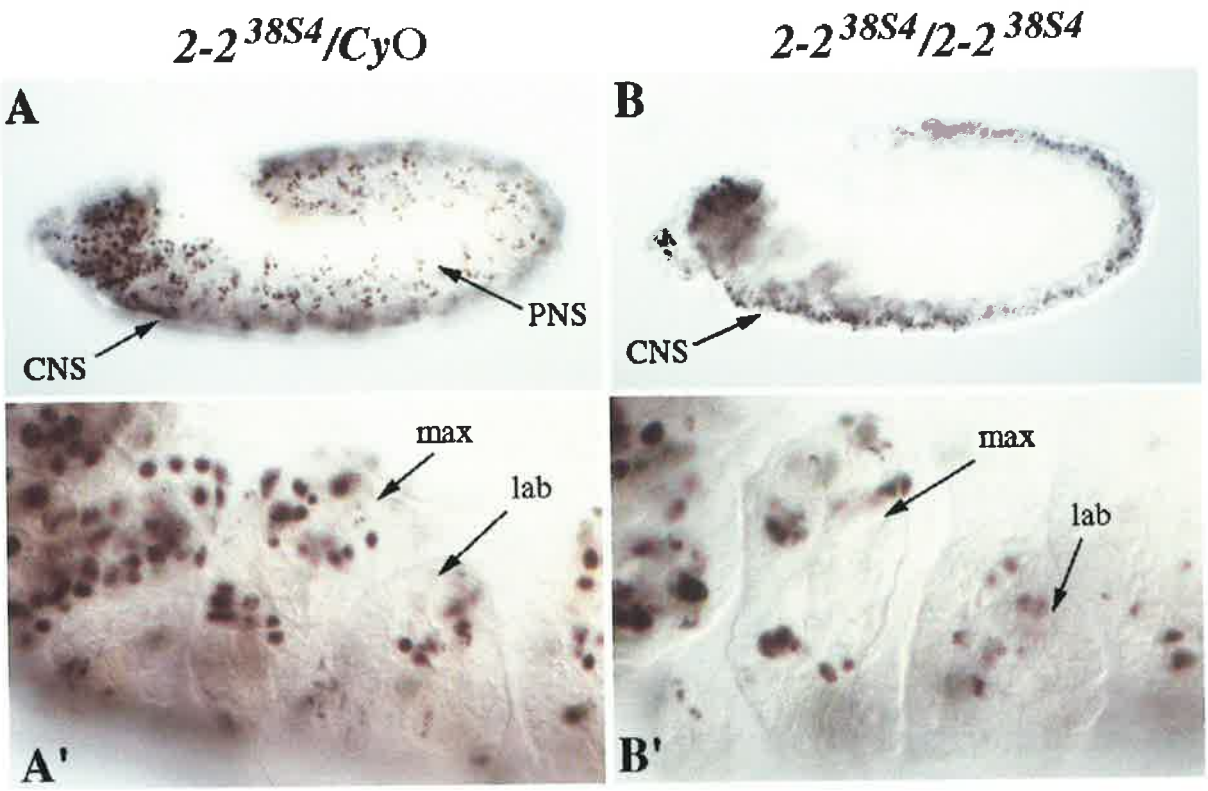
Figure 7.3 Mutations in *Su(DmcycE^{JP})2-2* affect trunk PNS S phases

BrdU labelling to reveal S phases from stage 11 (A) *2-2^{38S4}/CyO* heterozygous (same as wild-type) and (B) *2-2^{38S4}* homozygous mutant embryos, showing that S phases are detected in the CNS, but not PNS cells in *2-2^{38S4}* mutant embryos. A' and B' are enlargements of A and B, respectively. S phases are also slightly reduced in maxillary head segment PNS cells, but not affected in labial segment PNS cells of *2-2^{38S4}* homozygous embryos. Similar results were obtained with *2-2^{39S2}/Df(2R)trix* embryos. Anterior is to the left, dorsal side up.

Abbreviations used are peripheral nervous system (PNS) and central nervous system (CNS), labial head segment (lab) and maxillary head segment (max).

Figure 7.4 *Su(DmcycE^{JP})2-2* mutant embryos have a reduced number of PNS neurons

Neurons, detected using Mab 22C10, from (A) wild-type and (B) *2-2^{38S4}* homozygotes. A' and B' are enlargements of A and B, respectively. The number of PNS neurons in *2-2^{38S4}* homozygotes is reduced compared with wild-type and *Su(DmcycE^{JP})2-2* heterozygotes (not shown). Similar results were obtained with *2-2^{39S2}/Df(2R)trix* and *2-2^{39S2}/2-2^{38S4}* embryos. Anterior is to the left, dorsal side up for A and B. Anterior is down, dorsal side to the right in A' and B'.


2-2 38S4/CyO
2-2 38S4/2-2 38S4
A
B
PNS
CNS
CNS
max
lab
max
lab
A'
B'

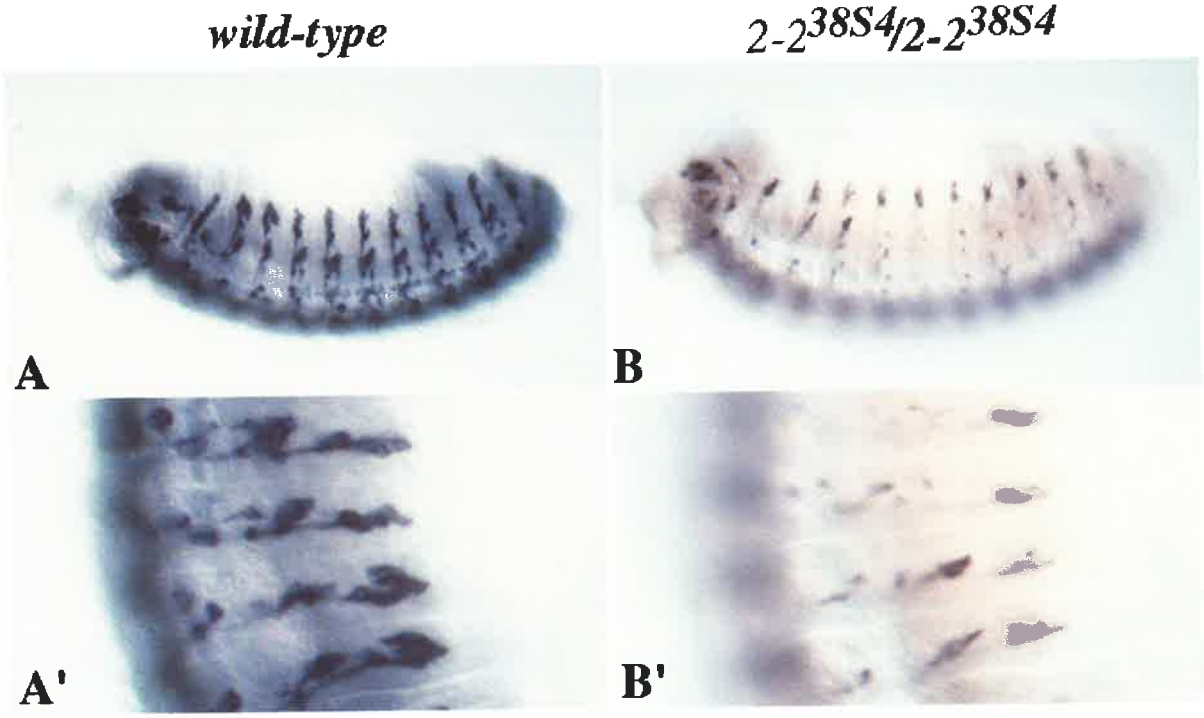

wild-type
2-2 38S4/2-2 38S4
A
B
A'
B'

Table 7.4 Genes in the 51A region

Cytological position	Gene	Known function or mutant phenotype
38B5-52D7	*l(2)DTS19*	Dominant temperature sensitive mutant. Pupal lethal.
38E3-51A1	*balding*	Homozygous adults have a bald spot on the thorax.
38E3-51A1	*l(2)S42*	Late pupal lethal mutation.
43E18-51A1	*Tyrosine 2*	Homozygous viable.
43E18-52D7	*Blackoid*	Homozygous viable. Semi-dominant body colour mutant.
43E18-52D7	*E(cactE^{10})7*	Regulator of dorsal/ventral axis.
43E18-52D7	*E(cactE^{10})81*	Same as above.
50F1-51B3	*mat(2)ea-A*	Homozygous female sterile.
51A1	*droopy*	Homozygous viable. Mutants have droopy wings.
51A1	*Lobe*	Homozygous viable eye mutation.
51A1-8	*smi51A*	Homozygous viable behavioural mutant.
51A1-B11	*Attacin-A*	Anti-microbial peptide.
51A1-F13	***l(2)k21105***	***P*-element allele.**
51A1-F13	*oho51*	Homozygous larval lethal.
51A2	***l(2)03563***	***P*-element allele.**
51A2	***l(2)06949***	***P*-element allele.**
51A2	***phyllopod***	***phyllopod* mutant embryos exhibit PNS defects similar to *Su(DmcycE^{JP})2-2*.**
51A2-60D1	*fs(2)TLM*	Female sterile mutation.
51A2-B3	***Asx***	**Some *Asx* alleles act as tumour suppressors.**
51A2-B6	*auk*	Homozygotes are pupal lethal.
51A2-51B4	*cpsf*	Required for addition of poly(A) tail to all mRNAs.
51A4-5	***l(2)02637***	***P* element allele.**
51A4-5	***l(2)k16805***	***P* element allele.**

Summary of the genes in the 51A cytological region. Genes considered to be candidates are indicated in bold. Gene abbreviations used are *overgrown hematopoietic organs* (*oho51*), *cleavage and polyadenylation factor (cpsf), maternal effect early arrest A* (*mat(2)ea-A*), *smell impaired 51A* (*smi51A*) and *Additional sex combs* (*Asx*). *E(cactE^{10})7* and *E(cactE^{10})81* are dominant enhancers of *cactus^{E10}*.

Table 7.5 *Su(DmcycE^{JP})2-2* fails to complement an allele of *phyllopod*

Su(DmcycE^{JP})2-2 Allele	*phy^{2245}*
2-2^{28S2}	0/98
2-2^{38S4}	0/109
2-2^{39S2}	0/197

All three alleles of *Su(DmcycE^{JP})2-2* fail to complement the null *phyllopod* allele, *phyl^{2245}*, indicating that *Su(DmcycE^{JP})2-2* is likely to be allelic to *phyl*.

7-6 Discussion and conclusions

The X-ray and EMS-induced dominant modifiers of the *DmcycE^{JP}* rough eye phenotype identified in the genetic interaction screen are expected to include regulators of *cyclin E* transcription and function. The characterisation of *Su(DmcycE^{JP})2-2* described in this chapter has demonstrated that this essential gene encodes a direct or indirect regulator of *cyclin E* transcription in the embryonic PNS. *Su(DmcycE^{JP})2-2* may also function in the transcriptional regulation of *cyclin E* in the eye disc, as *Su(DmcycE^{JP})2-2* did not dominantly enhance the *GMR-cyclin E* type I or II rough eye phenotypes.

Complementation tests revealed that *Su(DmcycE^{JP})2-2* is likely to be allelic to the previously identified gene, *phyllopod*. While all three alleles of *Su(DmcycE^{JP})2-2* fail to complement *phyl^{2245}*, this is not sufficient to demonstrate that *2-2^{28S2}*, *2-2^{38S4}* and *2-2^{39S2}* are alleles of *phyl*. Verification that *Su(DmcycE^{JP})2-2* corresponds to *phyl* will require characterisation of the molecular lesions associated with *2-2^{28S2}*, *2-2^{38S4}* and *2-2^{39S2}*. As both *2-2^{38S4}* and *2-2^{39S2}* result in recombination suppression, these alleles are likely to be chromosomal rearrangements. Genomic Southern analysis should therefore be used to determine whether these alleles have a molecular defect in the *phyllopod* locus. In addition, previously characterised *phyl* mutants should be recombined onto the *DmcycE^{JP}* chromosome to ensure that halving the dosage of *phyl* suppresses the *DmcycE^{JP}* rough eye phenotype.

7-6.1 The role of Phyllopod during larval development.

During eye development, *phyl* transcription is turned on in response to activation of the Ras pathway (Karim *et al.*, 1996). *phyl* expression is first detected in the MF of third larval instar imaginal discs, and is subsequently refined to the R1, R6 and R7 photoreceptors

(Chang *et al.*, 1995; Dickson *et al.*, 1995). *phyl* mutant clones in the eye lack the R1, R6 and R7 photoreceptors, demonstrating that Phyl is required for these cells to differentiate (Chang *et al.*, 1995; Dickson *et al.*, 1995). For R1, R6 and R7 photoreceptor development, Phyl, in association with Seven in absentia (Sina), is required to bind to, and target the two isoforms of Tramtrack (Ttk69 and Ttk88) for destruction by the ubiquitin/proteosome pathway (Li *et al.*, 1997; Tang *et al.*, 1997). Ttk69 and Ttk88 arise from alternatively spliced transcripts and have different carboxy Cys_2-His_2 zinc finger domains with distinct DNA binding specificities (Read *et al.*, 1990; Read and Manley, 1992), that act as transcriptional repressors (Brown *et al.*, 1991; Xiong and Montell, 1993; Lai *et al.*, 1996). During eye development, Ttk88 and Ttk69 are required to prevent photoreceptor differentiation, and Ttk69 is also required to repress cone cell fate (Li *et al.*, 1997). The targets of Ttk69 and Ttk88 repression during eye development are not known (Harrison and Travers, 1990). Phyl and Sina therefore promote the neural fate of R1, R6 and R7 by targeting inhibitors of photoreceptor development, Ttk88 and Ttk69, for destruction. Within the MF, *phyl* is clearly not required for the recruitment or differentiation of the pre-cluster photoreceptors, as R8, R2, R5, R3 and R4 develop normally in $phyl^-$ eye clones (Chang *et al.*, 1995). The function of Phyl in these cells is therefore not known.

As the function of Phyl within and immediately posterior to the MF is not known, it is not clear how halving the dosage of *phyl* acts to suppress the $DmcycE^{JP}$ rough eye phenotype. One possibility is that Phyl has distinct roles during R1, R6 and R7 photoreceptor differentiation compared with its role within and immediately posterior to the MF. Precedent for this has come from analysis of the epidermal growth factor (EGF) receptor and the downstream transcriptional repressor Yan (Baker and Rubin, 1992; Rogge *et al.*, 1995). Hypomorphic *yan* mutants have ectopic R7 photoreceptors, demonstrating that Yan is normally required to repress photoreceptor cell fate (Lai and Rubin, 1992; Tei *et al.*, 1992). In addition, clones of yan^- cells that span the MF show ectopic S phases (Rogge *et al.*, 1995). Depending on the cell type, Yan can therefore act as a negative regulator of proliferation or of differentiation. Similarly, the EGF receptor is required for photoreceptor differentiation (Freeman, 1996), and is also a regulator of entry into S phase within and immediately posterior to the MF, as the gain-of-function allele *Ellipse* results in ectopic S phases in the MF (Baker and Rubin, 1992). Phyl may therefore act as a negative regulator of cell proliferation within and immediately posterior to the MF during eye development. Whether Phyl acts as a negative regulator of proliferation can be tested in several ways. Firstly, some alleles of *phyl* survive until the third larval instar, allowing analysis of differentiating eye

imaginal discs. If Phyl is a negative regulator of the G1 to S phase transition within and immediately posterior to the MF, ectopic *cyclin E* expression and additional S phases would be expected in these cells. In addition, somatic clones spanning the MF could be generated using the FLP/FRT system (Xu and Rubin, 1993), to assess the effect of loss of Phyl on *cyclin E* transcription and entry into S phase.

What might be the mechanism of Phyl action? Although Phyl and Sina have been shown to lead to the ubiquitin-mediated degradation of Ttk69 and Ttk88 in presumptive R1, R6 and R7 photoreceptor cells (Tang *et al.*, 1997; Li *et al.*, 1997), the role of Phyl within and immediately posterior to the MF has not been examined. The most direct prediction for the action of Phyl within and immediately posterior to the MF is that Phyl and Sina are required for the degradation of Cyclin E or a positive regulator of *cyclin E* transcription. This transcriptional regulator is unlikely to be Ttk69 or Ttk88, as these proteins have only been demonstrated to be transcriptional repressors (Brown *et al.*, 1991; Xiong and Montell, 1993). Halving the dosage of *ttk* should, however, be tested for an effect on the $DmcycE^{JP}$ phenotype. Similarly, Phyl and Sina are unlikely to directly target Cyclin E for destruction. Halving the dosage of $Su(DmcycE^{JP})2\text{-}2$ did not enhance the *GMR-cyclin E* rough eye phenotype, indicating that $Su(DmcycE^{JP})2\text{-}2$ is likely to act upstream of *cyclin E* transcription, and not on Cyclin E stability. The identity of the Phyl/Sina target in this situation is therefore unknown.

Since the characterised role of Phyl requires Sina (Li *et al.*, 1997; Tang *et al.*, 1997), it may be expected that halving the dosage of *sina* would suppress the $DmcycE^{JP}$ rough eye phenotype. *sina* maps to 73D on the third chromosome (Harrison and Travers, 1990). *Df(3L)81K19*, which deletes 73A3 to 74F, suppresses $DmcycE^{JP}$ (Chapter 4), consistent with a model for regulation of *cyclin E* transcription involving both Phyl and Sina. Specific alleles of *sina* are available and should now be tested for their effect on $DmcycE^{JP}$. Additional evidence that Sina regulated protein degradation is involved in proliferation has arisen from analysis of a human homologue of Sina, Siah1. Siah1 may be an important effector of p53-inducible G1 arrest and tumour suppression (Matsuzawa *et al.*, 1998; Nemani *et al.*, 1996), linking Siah1-mediated destruction with negative regulation of G1 to S phase transition.

7-6.2 The role of Phyllopod during embryonic development.

Halving the dosage of $Su(DmcycE^{JP})2\text{-}2$ suppresses the $DmcycE^{JP}$ phenotype by increasing the number of S phases during eye development. This is consistent with the $Su(DmcycE^{JP})2\text{-}2$ gene product normally acting to negatively regulate the G1 to S phase

transition during eye development. This contrasts with the role of *Su(DmcycE^{JP})2-2* during embryonic development, where it positively regulates *cyclin E* transcription in the PNS. This difference is intriguing, and may be due to Phyl targeting different proteins for destruction in embryonic PNS and eye imaginal disc cells.

Unlike the role of *phyl* during the development of the R1, R6 and R7 photoreceptors, the role of *phyl* during embryogenesis is not well characterised. Null *phyl* mutants have a reduced number of PNS cells (Chang *et al.*, 1995). As *Su(DmcycE^{JP})2-2* mutants have no *cyclin E* expression in trunk PNS cells, Phyl (and Sina) may be required for the targeted degradation of a repressor of *cyclin E* transcription. In the absence of Phyl, this repressor would still be present in PNS cells, preventing *cyclin E* transcription and entry into S phase. This would then lead to a reduction in the number of PNS neurons. It is possible that this transcriptional repressor might be one of the two proteins encoded by the *ttk* locus, Ttk69 or Ttk88, as both of these proteins have been shown to act during embryonic PNS development (Guo *et al.*, 1995, 1996). *ttk* mutant embryos show a direct fate change of sensory organ precursor support cells into neurons and overexpression of *ttk* leads to neuronal cells adopting a support cell fate (Guo *et al.*, 1995). From these fate change phenotypes, it is unclear whether Ttk69 and/or Ttk88 is likely to represses *cyclin E* transcription in embryonic PNS cells.

cyclin E promoter dissection analysis has defined a region of 2.6kb, upstream of *cyclin E* type I transcription, as being required for transcription in trunk PNS and a subset of head PNS cells (Figure 7.5; Jones, 1997). This is based on analysis of the transcription directed by genomic DNA and promoter-*lacZ* transgenes. A 10.5kb genomic *cyclin E* transgene spanning 5.5kb upstream and 2.5kb downstream of the type I transcription unit is sufficient for *cyclin E* expression in trunk PNS and a subset of head PNS cells. This region was refined to a ~2.6kb region by examining the pattern of *lacZ* directed by two promoter-*lacZ* transgenes (Jones, 1997; Figure 7.5). While the 5.5kb construct resulted in *lacZ* expression in the same PNS pattern as the genomic DNA transgene, the 2.9kb construct did not drive any PNS expression. This indicated that the 2.6kb region was sufficient for trunk and a subset of head PNS expression. The *Su(DmcycE^{JP})2-2* gene product (Phyl) is therefore likely to act to regulate *cyclin E* transcription in trunk PNS cells via this 2.6 kb enhancer region upstream of the *cyclin E* type I transcript.

The BGDP has determined the sequence of the *cyclin E* genomic region. The 2.6 kb region involved in trunk PNS transcription was therefore analysed for transcription factor binding sites using 'TFSearch' (Akiyama, 1995). Significantly, five Ttk69 binding sites were

detected in this search, indicating that this repressor may bind directly to the *cyclin E* promoter region and repress transcription (Figure 7.5). It will therefore be interesting to test for direct binding of Ttk69 to the *cyclin E* upstream region by electrophoretic mobility shift assay (EMSA). The effect of overexpressing Ttk69 should also be examined, as this would be predicted to repress *cyclin E* transcription and S phases in embryonic PNS cells. In addition, homozygous *phyl* mutant embryos should be examined for their effect on Ttk69 protein stability. If Phyl is required for the degradation of Ttk69 in the embryonic PNS, Ttk69 would be expected to be stabilised, and therefore detected, in PNS cells.

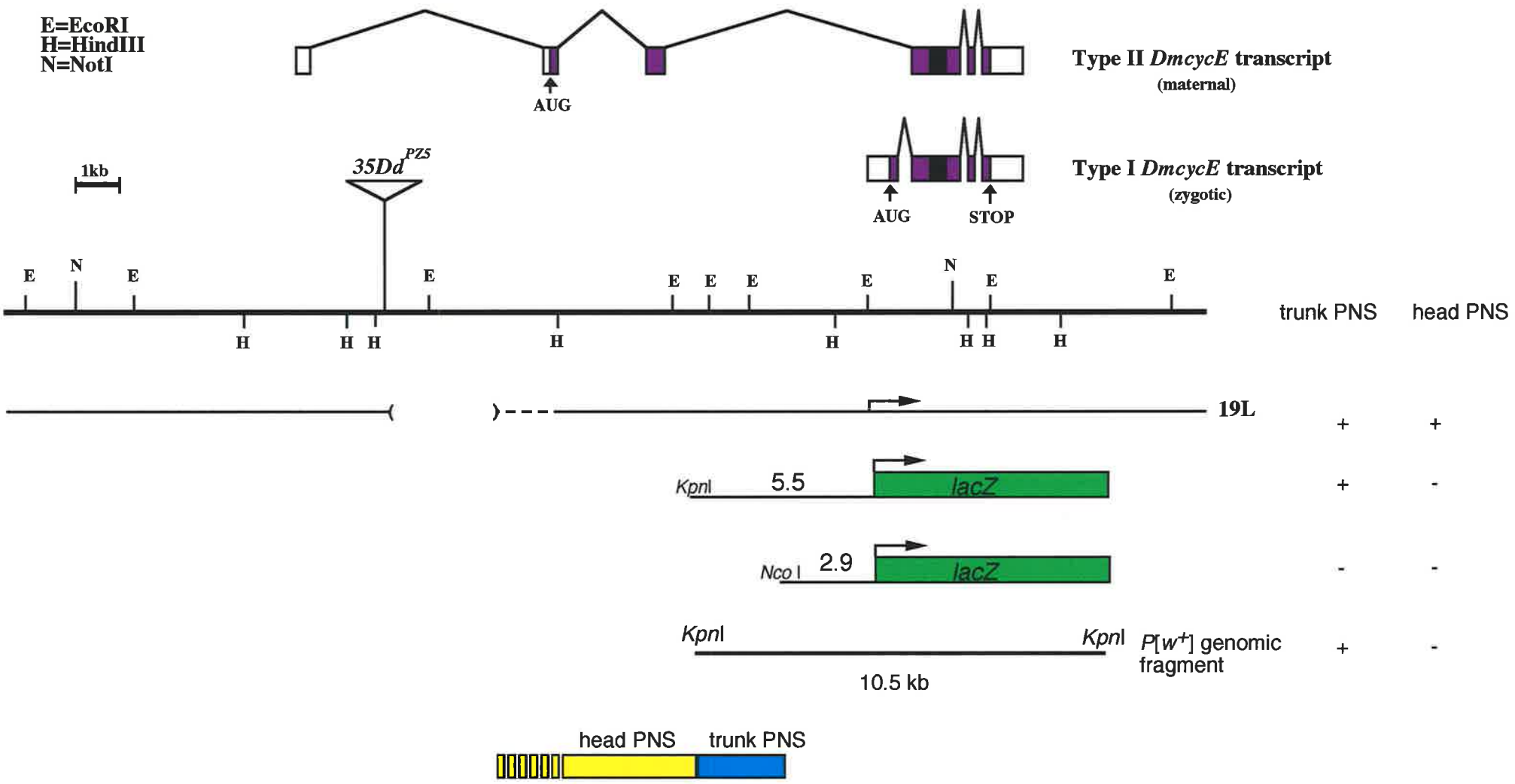
In summary, this chapter has described the characterisation of *Su(DmcycE^{JP})2-2*, which is likely to be an allele of *phyllopod*. Further characterisation of Phyl may demonstrate that this protein, in addition to its previously characterised role in photoreceptor development, is also a negative regulator of proliferation during eye development and a positive regulator in embryonic PNS cells. The identification of a previously characterised gene not necessarily predicted to modify the *DmcycE^{JP}* rough eye phenotype highlights the importance of a random mutagenesis to identify genes involved in Cyclin E-mediated entry into S phase.

Figure 7.5 *cyclin E* expression in the head and trunk PNS are controlled by separate enhancer regions

Schematic representation of the *cyclin E* genomic region showing the *cyclin E* type I and II transcripts, the position of the *PZ5 P* element used to generate deletions, a genomic DNA transgene, and two promoter-*lacZ* transgenes. Analysis of the *cyclin E* transcription pattern in embryos containing small deficiencies generated by imprecise excision of the *PZ5 P* element, from a 10.5 kb genomic DNA transgene or from the 5.5kb and 2.9kb promoter-*lacZ* contructs defined the boundaries of upstream sequences required for *cyclin E* expression in head and trunk PNS cells (Jones, 1997). 'Trunk' PNS in this case refers to all of the trunk PNS expression, in addition to a subset of head PNS expression. 'Head' PNS refers to the remaining head PNS expression. Adapted from Jones, (1997)

Within the 2.6kb region required for expression of *cyclin E* in trunk PNS cells, there are five Ttk69 binding sites (Read *et al.*, 1990; Read and Manley, 1992), as listed below. Underlined nucleotides differ from the consensus Ttk69 binding site.

Position within 2.6kb region	Sequence
Consensus	$G^{G}/_{C}TCCTG^{C}/_{G}$
459-466	GGTCCGGC
477-484	GCTCCTGC
982-987	GATCCTGC
2030-2037	GGTCCTTC
2482-2489	AGTCCTGC


E=EcoRI
H=HindIII
N=NotI
AUG
Type II DmcycE transcript
(maternal)
AUG
STOP
Type I DmcycE transcript
(zygotic)
1kb
35Dd PZ5
E N E E E E E E N E E
H H H H H H H
trunk PNS
head PNS
19L
+ +
KpnI 5.5 lacZ
+ -
NcoI 2.9 lacZ
- -
KpnI KpnI
10.5 kb
P[w+] genomic fragment
+ -
head PNS
trunk PNS

Chapter 8: Characterisation of *Su(DmcycE^{JP})2-1*

8-1 Introduction

Of the 10 second chromosome suppressor complementation groups with more than one allele identified in the genetic interaction screen described in Chapter 5, two were characterised further, *Su(DmcycE^{JP})2-2* and *Su(DmcycE^{JP})2-1*. This chapter describes the mutant phenotype characterisation and cytological location of *Su(DmcycE^{JP})2-1*, which has two X-ray generated alleles, *2-1^{23S9}* and *2-1^{27S3}* and two EMS alleles, *2-1^{E2S31}* and *2-1E6S2*.

8-2 *Su(DmcycE^{JP})2-1* acts by increasing the number of S phases during eye development

The four alleles of *Su(DmcycE^{JP})2-1*, *2-1^{23S9}*, *2-1^{27S3}*, *2-1^{E2S31}* and *2-1^{E6S4}*, are strong suppressors of the *DmcycE^{JP}* rough eye phenotype (Figure 8.1A-F). To determine whether this suppression was the result of an increase in the number of S phases during eye development, BrdU incorporation was carried out on *DmcycE^{JP}* eye discs heterozygous for *Su(DmcycE^{JP})2-1* alleles. Consistent with the suppression of *DmcycE^{JP}*, all four alleles of *Su(DmcycE^{JP})2-1* increased the number of S phases during eye development (Figure 8.1G-K), indicating that this gene is involved in negatively regulating the G1 to S phase transition during eye development.

8-3 Cytological position of *Su(DmcycE^{JP})2-1*

Preliminary genetic mapping indicated that *Su(DmcycE^{JP})2-1* mapped distal to *black* at cytological position 34D4-6 (data not shown). Crosses of *Su(DmcycE^{JP})2-1* alleles to deficiencies spanning from *black* to the telomere revealed that all four alleles of *Su(DmcycE^{JP})2-1* failed to complement two deficiencies that, based on their cytology, do not overlap. The first of these, *Df(2L)Prl*, removes 32F1-3 to 33F1-2, and although the proximal breakpoint of this deficiency has been mapped molecularly and breaks within the *pdm-1* gene (Yeo *et al.*, 1995), the distal breakpoint is based solely on cytology. Consistent with this deficiency removing *Su(DmcycE^{JP})2-1*, *Df(2L)Prl* suppressed the *DmcycE^{JP}* rough eye phenotype when recombined onto the *DmcycE^{JP}* chromosome (data not shown). The second deficiency that failed to complement *Su(DmcycE^{JP})2-1* was *Df(2L)J39*, which removes the cytological region 31D1-11 to 32D1-E5 (Clegg *et al.*, 1993). Neither of the breakpoints associated with this deficiency have been molecularly characterised, although the distal

breakpoint of *Df(2L)J39* has been mapped with respect to other *J* group deficiencies (Clegg *et al.*, 1993; Figure 8.2). To confirm that *Df(2L)Prl* and *Df(2L)J39* have at least one lethal complementation group in common, these two stocks were crossed together. Consistent with *Su(DmcycE^{JP})2-1* alleles failing to complement both stocks, no flies transheterozygous for *Df(2L)Prl* and *Df(2L)J39* were observed. The most obvious explanations for this are that either the *Df(2L)Prl* deficiency extends further distal than was previously thought or *Df(2L)J39* extends further proximal, or that one of the deficiencies has a second lesion within the other deficiency. To determine whether *Df(2L)Prl* deficiency extends further distal than 32F, crosses using *Df(2L)esc-P2-0* that removes 33A1-2 to 33B1-2 (Frei *et al.*, 1988), *Df(2L)esc-P3-0* that deletes 33A1-2 to 33E, *spalt-related* (*salr*) (de Celis *et al.*, 1996) and *spalt major* (*salm*) (kühnlein *et al.*, 1994), both of which map to 33A, were carried out (Figure 8.2). *Df(2L)Prl* failed to complement mutations in *salm*, and complemented mutations in *salr*, indicating that the distal breakpoint of *Df(2L)Prl* lies in the ~70 kb between these two genes (Figure 8.2). *Df(2L)esc-P2-0* and *Df(2L)esc-P3-0* failed to complement alleles of *salm* and *salr*, demonstrating that these deficiencies extend further distal than *Df(2L)Prl*. *Df(2L)esc-P2-0* and *Df(2L)esc-P3-0*, however, complement *Df(2L)J39*, suggesting that *Df(2L)J39* does not extend proximally past 33A and that *Df(2L)J39* and *Df(2L)Prl* are not simply overlapping deficiencies (Figure 8.2). *Df(2L)esc-P2-0* and *Df(2L)esc-P3-0* also complement *Su(DmcycE^{JP})2-1* alleles, suggesting that *Su(DmcycE^{JP})2-1* is not within the cytological region 33A1 to 33E1-10. As *Df(2L)prd1.7*, which removes 33B3 to 34A1-2, complements *Su(DmcycE^{JP})2-1* alleles, overlaps with *Df(2L)esc-P2-0* and extends further proximal than *Df(2L)Prl* (Yeo *et al.*, 1995), *Su(DmcycE^{JP})2-1* can not be within the cytological region removed by the *Df(2L)Prl* deficiency (32F1-3 to 33F1-2). Thus, the lethality of the transheterozygous combinations of *Df(2L)Prl*, *Df(2L)J39* and *Su(DmcycE^{JP})2-1* alleles is likely to be due to a second lesion on the *Df(2L)Prl* chromosome within the region removed by *Df(2L)J39*.

To refine the region defined by *Df(2L)J39*, *Su(DmcycE^{JP})2-1* alleles were crossed to five other deficiencies, *Df(2L)J2, Df(2L)J1, Df(2L)J77, Df(2L)J27,* and *Df(2L)J3*, for which the relative position of breakpoints is known (Clegg *et al.*, 1993; Stratmann and Lehner, 1996; Figure 8.2). All five deficiencies complemented *Su(DmcycE^{JP})2-1* alleles, therefore the critical region for *Su(DmcycE^{JP})2-1* is 32A1-2 to 32D1-E5, as defined by the distal breakpoint of *Df(2L)J2* (31B1-5 to 32A1-2) and the proximal breakpoint of *Df(2L)J39* (31D1-11 to 32D1-E5).

Figure 8.1 *Su(DmcycE^{JP})2-1* suppresses the *DmcycE^{JP}* rough eye phenotype by increasing the number of S phases during eye development

(A, B, C, D, E) Scanning electron micrographs of adult eyes from (A) *DmcycE^{JP}*, (B) *DmcycE^{JP}, 2-1^{23S9}/ DmcycE^{JP}*, +, (C) *DmcycE^{JP}, 2-1^{27S3}/ DmcycE^{JP}*, +, (D) *DmcycE^{JP}, 2-1^{E2S31}/ DmcycE^{JP}*, + and (E) *DmcycE^{JP}, 2-1^{E6S2}/ DmcycE^{JP}*, + showing suppression of the *DmcycE^{JP}* rough eye phenotype. (F, G, H, I, J) BrdU incorporation to reveal S phases from (F) *DmcycE^{JP}*, (G) *DmcycE^{JP}, 2-1^{23S9}/ DmcycE^{JP}*, +, (H) *DmcycE^{JP}, 2-1^{27S3}/ DmcycE^{JP}*, +, (I) *DmcycE^{JP}, 2-1^{E2S31}/ DmcycE^{JP}*, + and (J) *DmcycE^{JP}, 2-1^{E6S2}/ DmcycE^{JP}*, + third larval instar imaginal discs. Reducing the dose of any of the *Su(DmcycE^{JP})2-1* alleles increases the number of S phases during eye development, resulting in suppression of the *DmcycE^{JP}* rough eye phenotype. Anterior is to the right in all panels and dorsal is up in A, B, C, D, E and F. Bars in G, H, I, J and K indicate the MF.

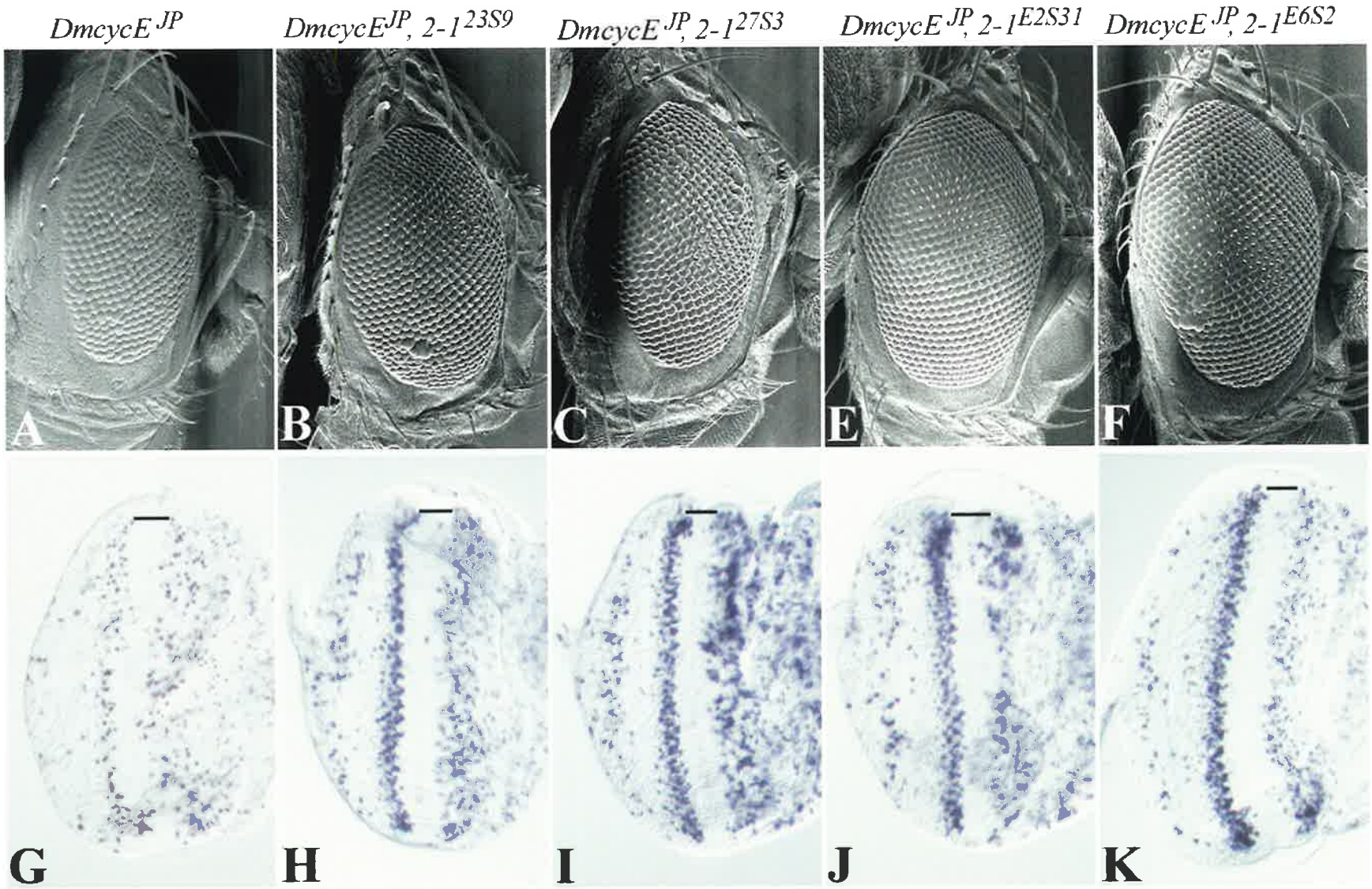
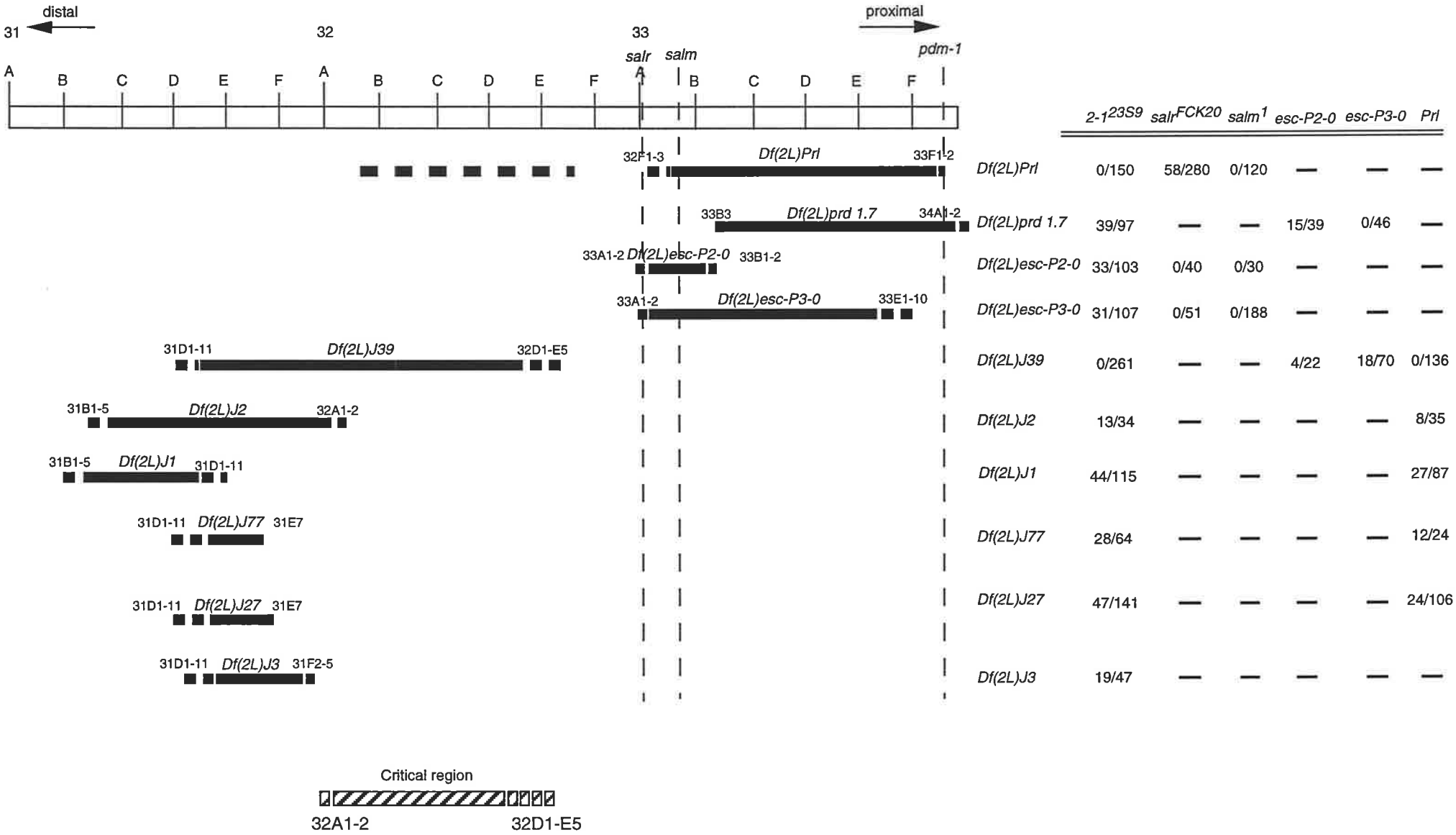

DmcycE JP
DmcycEJP, 2-123S9
DmcycE JP, 2-127S3
DmcycE JP, 2-1E2S31
DmcycE JP, 2-1E6S2
A
B
C
E
F
G
H
I
J
K

Figure 8.2 *Su(DmcycE^{JP})2-1* maps cytologically to 32A-E

The cytological position of *Su(DmcycE^{JP})2-1* was determined by crossing *Su(DmcycE^{JP})2-1* alleles to deficiencies. *Su(DmcycE^{JP})2-1* alleles fail to complement the non-overlapping deficiencies *Df(2L)Prl* and *Df(2L)J39*, but complement all other deficiencies shown. The *Df(2L)Prl* deficiency chromosome is likely to have at least one mutation/lesion within 32A-E in addition to the characterised deficiency that removes 32F1-3 to 33F1-2 (See text for details). Dashed regions at the extremes of deficiencies indicate breakpoint uncertainty regions. Complementation data on the right hand side of figure is presented as the number of transheterozygous flies/total number scored. Although complementation data are given for the *Su(DmcycE^{JP})2-1* allele *2-1^{23S9}*, similar results were obtained using the other three *Su(DmcycE^{JP})2-1* alleles. In addition, results obtained using the *salr* allele *salr^{FCK68}* and the *salm* allele *salm^{2}* were the same was those presented for *salr^{FCK20}* and *salm^{1}*, respectively.



	$2\text{-}1^{23S9}$	$salr^{FCK20}$	$salm^{1}$	esc-P2-0	esc-P3-0	Prl
Df(2L)Prl	0/150	58/280	0/120	—	—	—
Df(2L)prd 1.7	39/97	—	—	15/39	0/46	—
Df(2L)esc-P2-0	33/103	0/40	0/30	—	—	—
Df(2L)esc-P3-0	31/107	0/51	0/188	—	—	—
Df(2L)J39	0/261	—	—	4/22	18/70	0/136
Df(2L)J2	13/34	—	—	—	—	8/35
Df(2L)J1	44/115	—	—	—	—	27/87
Df(2L)J77	28/64	—	—	—	—	12/24
Df(2L)J27	47/141	—	—	—	—	24/106
Df(2L)J3	19/47	—	—	—	—	—

8-4 *Su(DmcycE^{JP})2-1* acts downstream of *cyclin E* transcription

To examine *Su(DmcycE^{JP})2-1* mutant phenotypes, all four alleles of this gene were recombined away from the *DmcycE^{JP}* mutation using the *al*, *b*, and *c* recessive second chromosome markers. Recombinants wild-type for *cyclin E*, but carrying *Su(DmcycE^{JP})2-1* were selected as non-*al*, *b*, and *c*, and stocks were generated. Homozygous lethal stocks were then crossed to *Su(DmcycE^{JP})2-1* alleles, *Df(2L)Prl* and *Df(2L)J39* to ensure the presence of the *Su(DmcycE^{JP})2-1* mutation. These stocks were then used for all subsequent analysis.

To determine whether *Su(DmcycE^{JP})2-1* acts downstream of *cyclin E* transcription, *Su(DmcycE^{JP})2-1* alleles were tested for their effect on the *GMR*-type I and II *cyclin E* rough eye phenotypes. Interestingly, halving the gene dose of *Su(DmcycE^{JP})2-1* mildly enhanced the *GMR-cyclin E* type I and type II eye phenotypes (data not shown), indicating that *Su(DmcycE^{JP})2-1* gene product acts on or downstream of *cyclin E* transcription. In addition, reducing the dose of *Su(DmcycE^{JP})2-1* suppressed the overexpression of human p21^{CIP1} rough eye phenotype. As described in Chapter 3 (Table 3.2), overexpression of human p21^{CIP1} in all cells posterior to the MF during eye development using the *GMR* driver results in a dramatic decrease in the number of cells entering S phase due to the inhibition of Cyclin E/Cdc2c kinase activity in these cells (de Nooij and Hariharan, 1995). The suppression of *GMR*-p21^{CIP1} by reducing the dose of *Su(DmcycE^{JP})2-1* indicates that *Su(DmcycE^{JP})2-1* acts downstream of p21^{CIP1} function (Figure 8.3). Halving the dose of *Su(DmcycE^{JP})2-1* did not, however, alter the *rux* rough eye phenotype (data not shown), so is likely to act in an independent pathway to Rux.

8-5 Zygotic mutant phenotype of *Su(DmcycE^{JP})2-1*

To determine the stage at which *Su(DmcycE^{JP})2-1* mutants are lethal, *Su(DmcycE^{JP})2-1* alleles were balanced using *Cy-Tb*. Non-tubby third instar larvae were observed in *2-1^{23S9}*, *2-1^{27S3}* and *2-1^{E2S31}* stocks balanced using *Cy-Tb*, while no non-tubby larvae were observed for *2-1^{E6S4}*. Larvae homozygous for *2-1^{23S9}* or *2-1^{E2S31}* failed to pupate and continued to grow, resulting in giant larvae that, at a low frequency developed melanotic masses (Figure 8.4). Third instar larvae homozygous for *2-1^{27S3}* were larger than wild-type, and delayed in pupariation. These pupae did not develop, indicating that this allele is lethal at the larval/pupal boundary and is less severe than *2-1^{23S9}* or *2-1^{E2S31}*. As no non-tubby third instar larvae were observed in the *2-1^{E6S4}* stock this may be a more severe allele, or have more than one lethal mutation. To distinguish between these possibilities, *Su(DmcycE^{JP})2-1* alleles and the two deficiencies known to remove *Su(DmcycE^{JP})2-1*, were

crossed together. All transheterozygous combinations of *Su(DmcycE^{JP})2-1* alleles and all alleles over *Df(2L)Prl* or *Df(2L)J39*, resulted in giant larvae that did not pupate (data not shown). The *2-1^{E6S4}* allele therefore has at least one other lethal mutation that was not recombined away when removing *DmcycE^{JP}* from the chromosome. Interestingly, the originally isolated stock of *2-1^{27S3}* also has other lethal mutation(s) as, unlike the original stocks of *2-1^{23S9}* or *2-1^{E2S31}*, no non-tubby larvae were observed when this stock was balanced using *Cy-Tb*. The original *2-1^{27S3}* chromosome therefore carried at least one other lethal mutation that was removed when *2-1^{27S3}* was recombined away from *DmcycE^{JP}*. Since *2-1^{E6S4}* has more than one lethal mutation, and *2-1^{27S3}* appears to be a hypomorphic allele of *Su(DmcycE^{JP})2-1*, *2-1^{23S9}* and *2-1^{E2S31}* were used for subsequent phenotypic analysis.

To determine whether any embryonic lethality was also associated with *Su(DmcycE^{JP})2-1* alleles, the number of hatched and unhatched embryos from balanced *2-1^{23S9}* and *2-1^{E2S31}* stocks was examined (Table 8.1). Approximately one quarter of the embryos from these stocks remained unhatched 24 hours after egg laying (AEL), demonstrating that these alleles are embryonic viable. The giant larval phenotype of homozygous *Su(DmcycE^{JP})2-1* alleles was therefore characterised further.

Table 8.1 *2-1^{23S9}* and *2-1^{E2S31}* homozygotes are embryonic viable

Allele	Number of unhatched embryos	Total number of embryos scored	% unhatched embryos
w^{1118}	10	353	3%
*2-1^{23S9}/Cy*O	270	884	30%
*2-1^{E2S31}/Cy*O	190	686	27%

To determine whether *Su(DmcycE^{JP})2-1* alleles were embryonic viable, *2-1^{23S9}* or *2-1^{E2S31}* flies were allowed to lay on grape agar plates and the number of hatched and unhatched embryos were counted after 24 hours. The number of unhatched embryos can be accounted for by those that are homozygous for the *Cy*O balancer chromosome.

Homozygous *2-1^{23S9}* or *2-1^{E2S31}* larvae were found to live up to 14 days AEL, while their heterozygous siblings and wild-type larvae pupate at approximately six days AEL. To examine the effect of this mutation on S phases in homozygous *Su(DmcycE^{JP})2-1* larvae, BrdU labelling was carried out (Figure 8.5). Comparison of wild-type and homozygous

Figure 8.3 Halving the gene dosage of *Su(DmcycE^{JP})2-1* suppresses the GMR-p21 rough eye phenotype

Scanning electron micrographs of adult eyes from (A) GMR-p21/+, (B) GMR-p21, +/+, *2-1^{23S9}* and (C) GMR-p21, +/+, *2-1^{E2S31}*, showing that halving the dosage of *Su(DmcycE^{JP})2-1* suppresses the GMR-p21 rough eye phenotype. The *Su(DmcycE^{JP})2-1* gene product therefore acts downstream of p21^{Cip1} function.

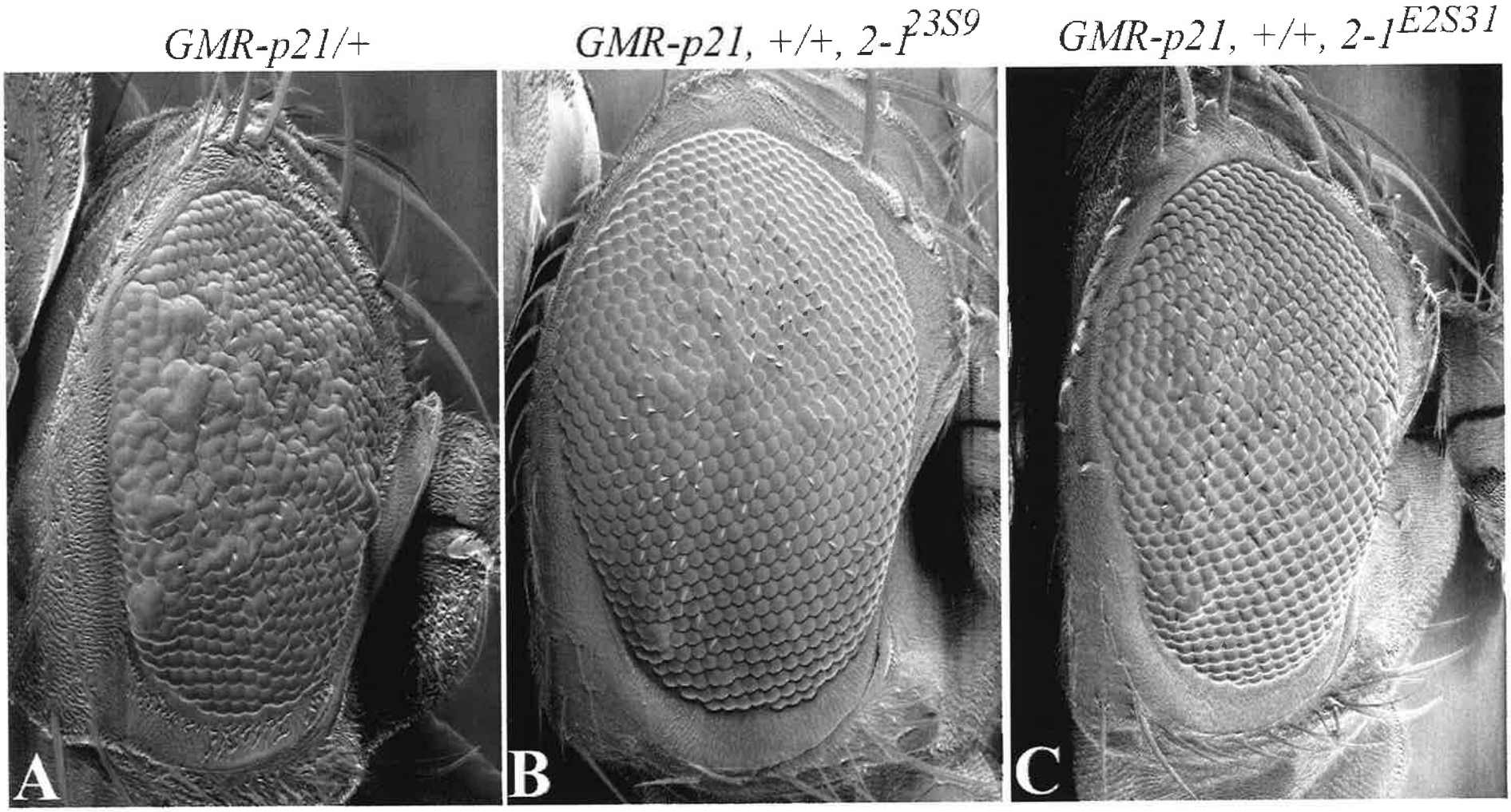

GMR-p21/+
GMR-p21, +/+, 2-1^23S9
GMR-p21, +/+, 2-1^E2S31
A
B
C

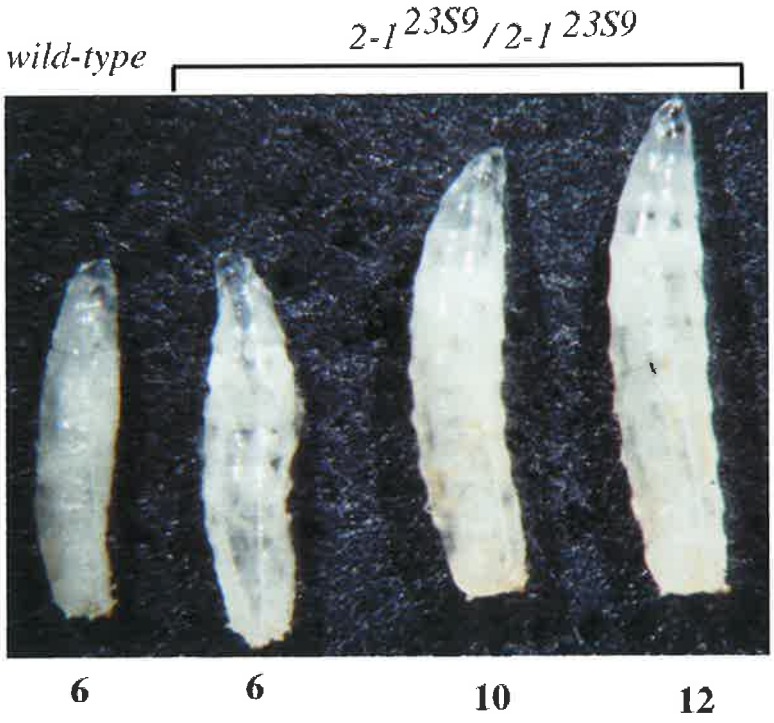
Figure 8.4 Homozygous *Su(DmcycE^{JP})2-1* larvae overgrow and fail to pupate

(A) Comparison of wild-type and *2-1^{23S9}* homozygous third instar larvae at approximately 6, 10 and 12 days AEL. While *Su(DmcycE^{JP})2-1* homozygotes are only slightly larger than wild-type at 6 days AEL, they continue to grow throughout their extended larval life.

(B) *Su(DmcycE^{JP})2-1* melanotic mass phenotype. At a low frequency, larvae homozygous for *2-1^{23S9}* or *2-1^{E2S31}* develop melanotic masses, as indicated by arrows.

Similar phenotypes were observed with *2-1^{E2S31}* homozygotes and *Su(DmcycE^{JP})2-1* transheterozygotes (data not shown).

A



B

Su(DmcycE^{JP})2-1 mutant larvae ~6 days AEL detected proliferative defects during larval brain and imaginal disc development. While S phases occur in wild-type larval optic lobes within the outer proliferative centre (OPC), inner proliferative centre (IPC) and lamina precursor cells (LPC), these regions are not easily discernible in *Su(DmcycE^{JP})2-1* mutant larvae (Figure 8.5B, D compared with A, C). Instead, S phases are detected throughout the optic lobes, and the large neuroblasts that are observed in S phase in wild-type larvae are not present in *Su(DmcycE^{JP})2-1* mutants. Significantly, *Su(DmcycE^{JP})2-1* mutants show a relatively normal pattern of S phases in the ventral ganglion, which at this stage show S phases in the thoracic ganglion region, but not in the abdominal ganglion region. Occasionally, a few S phases are observed in abdominal ventral ganglion cells in *Su(DmcycE^{JP})2-1* mutants, although this is not a consistent phenotype (Figure 8.5B). The optic lobes of *Su(DmcycE^{JP})2-1* mutant larvae continue to proliferate during their extended larval life, becoming very large (Figure 8.5F compared with E).

Imaginal discs are also affected in *Su(DmcycE^{JP})2-1* mutant larvae. Unlike the larval optic lobes of *Su(DmcycE^{JP})2-1* larvae which overgrow, imaginal discs are much smaller than wild-type and difficult to discern (Figure 8.6; data not shown). Eye discs, however, were able to be easily identified, as they are attached to the mouth hooks. While S phases are observed in eye discs of *Su(DmcycE^{JP})2-1* mutants throughout the third larval instar stage, these discs do not get larger. In addition, eye discs were not attached to the brain lobes in *Su(DmcycE^{JP})2-1* mutants, indicating that the optic nerve that would normally connect these two tissues is not present or very fragile. As halving the dosage of *Su(DmcycE^{JP})2-1* suppresses the *DmcycE^{JP}* rough eye phenotype by increasing the number of S phases, the small disc phenotype was surprising. This may be due to an increase in cell death, although this has not been investigated. Interestingly, the salivary glands of *Su(DmcycE^{JP})2-1* mutants are the same size as wild-type and endoreplicative cycles which occur during the polytenisation of this tissue terminate at the correct time in *Su(DmcycE^{JP})2-1* mutant larvae (Figure 8.6D compared with C; data not shown). *Su(DmcycE^{JP})2-1* mutants therefore have tissue specific proliferative defects during the third larval instar.

8-6 Analysis of maternal^{-}/zygotic^{-} *Su(DmcycE^{JP})2-1* embryos

To assess the role, if any, of maternally deposited *Su(DmcycE^{JP})2-1*, the FLP/FRT system originally described by Xu and Rubin (1993) was used to generate embryos lacking maternal and zygotic *Su(DmcycE^{JP})2-1* expression. This required recombining a FRT site,

which carries a neomycin resistance marker, at cytological position 40A onto the *2-1^{23S9}* and *2-1^{E2S31}* chromosomes. This was facilitated by the original recombination to remove *DmcycE^{JP}* from these two alleles, that resulted in the addition of the *b* recessive marker to these chromosomes. As *Su(DmcycE^{JP})2-1* maps to 32A-E, *b* maps cytologically to 34D4-6 and the FRT site is located at 40A, the FRT site was able to be recombined onto the *Su(DmcycE^{JP})2-1* mutant chromosome by selecting non-black flies that were resistant to neomycin. The presence of the *Su(DmcycE^{JP})2-1* mutation was then verified by crossing recombinants to other *Su(DmcycE^{JP})2-1* alleles, *Df(2L)Prl* and *Df(2L)J39*.

Germline clones giving rise to embryos lacking the maternal contribution of *Su(DmcycE^{JP})2-1* using *2-1^{23S9}* and *2-1^{E2S31}* were generated as described in Figure 8.7. Females carrying *Su(DmcycE^{JP})2-1* germline clones were crossed to *2-1^{23S9}/CyO wg-lacZ* or *2-1^{E2S31}/CyO wg-lacZ* males so that embryos lacking both maternal and zygotic *Su(DmcycE^{JP})2-1* expression could be distinguished from those carrying a paternally-derived wild-type copy of *Su(DmcycE^{JP})2-1* carried on the *Cy*O chromosome. Counting the number of unhatched and hatched embryos from this cross demonstrated that approximately half of these embryos hatch (Table 8.2). These larvae develop normally and give rise to curly winged adult flies, indicating that zygotic transcription of *Su(DmcycE^{JP})2-1* is sufficient for normal development. As the first 13 embryonic divisions occur in the absence of zygotic transcription, maternally deposited *Su(DmcycE^{JP})2-1* is not essential for these early divisions.

Table 8.2 Expression of *Su(DmcycE^{JP})2-1* is required for embryonic development

Strain	Number of unhatched embryos	Total number of embryos counted	% unhatched embryos
w^{1118}	54	500	10%
2-1^{E2S31} FRT40A/ovo^{D1} FRT40A x *Su(DmcycE^{JP})2-1^{23S9}*	845	1187	71%
2-1^{23S9} FRT40A/ovo^{D1} FRT40A x *Su(DmcycE^{JP})2-1^{23S9}*	948	1437	66%

To determine whether maternally-derived *Su(DmcycE^{JP})2-1* is required for embryonic development, embryos lacking maternal or maternal and zygotic *Su(DmcycE^{JP})2-1* were generated using the FLP/FRT system. Approximately half the embryos from these crosses did not hatch, indicating that embryos lacking maternal and zygotic *Su(DmcycE^{JP})2-1* die. The other half hatch and develop into adults.

Figure 8.5 *Su(DmcycE^{JP})2-1* mutants show overproliferation of the larval optic lobe

BrdU labelling to reveal S phases from (A, C, E) wild-type and (B, D, F) *2-1^{23S9}* homozygous third instar larvae. (A, B) Ventral view of optic lobes and ventral ganglion ~6 days AEL. While S phases of *2-1^{23S9}* homozygotes within the ventral ganglion appear normal, there are more S phases within the optic lobes which are disorganised. Bracketed regions in A and B in which S phases are observed, correspond to proliferating thoracic neural cells. Cells of the abdominal ventral ganglion have ceased proliferation at this stage, although occasionally a few cells are observed in S phase in *Su(DmcycE^{JP})2-1* mutants (arrowhead in B). (C, D) Lateral view of optic lobes ~6 days AEL. The distinctive domains of proliferation, in the OPC, IPC and LPC are not observed in *2-1^{23S9}* homozygotes. (E, F) Eye imaginal discs and brain lobes attached to mouth hooks from (E) ~6 days AEL wild-type and (F) ~13 days AEL *2-1^{23S9}* third instar larvae showing that the optic lobes of *2-1^{23S9}* homozygotes continue to proliferate and become dysmorphic. E and F are shown at the same magnification. Anterior is to the right in all panels. Similar results were obtained with *2-1^{E2S31}* homozygous larvae (not shown).

Abbreviations used are OPC (outer proliferative centre), IPC (inner proliferative centre), LPC (lamina precursor cells), BL (brain lobes), ED (eye disc).

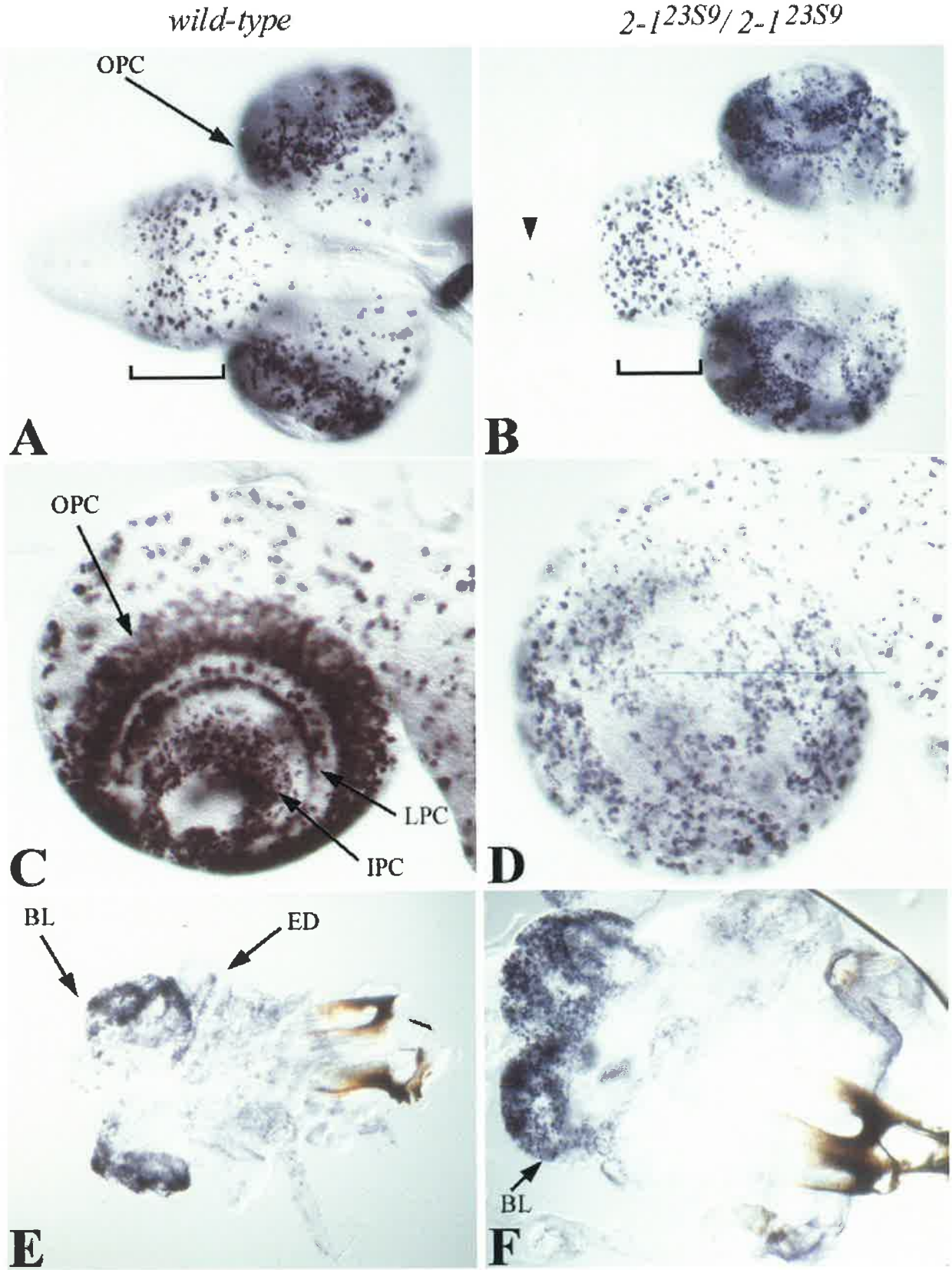
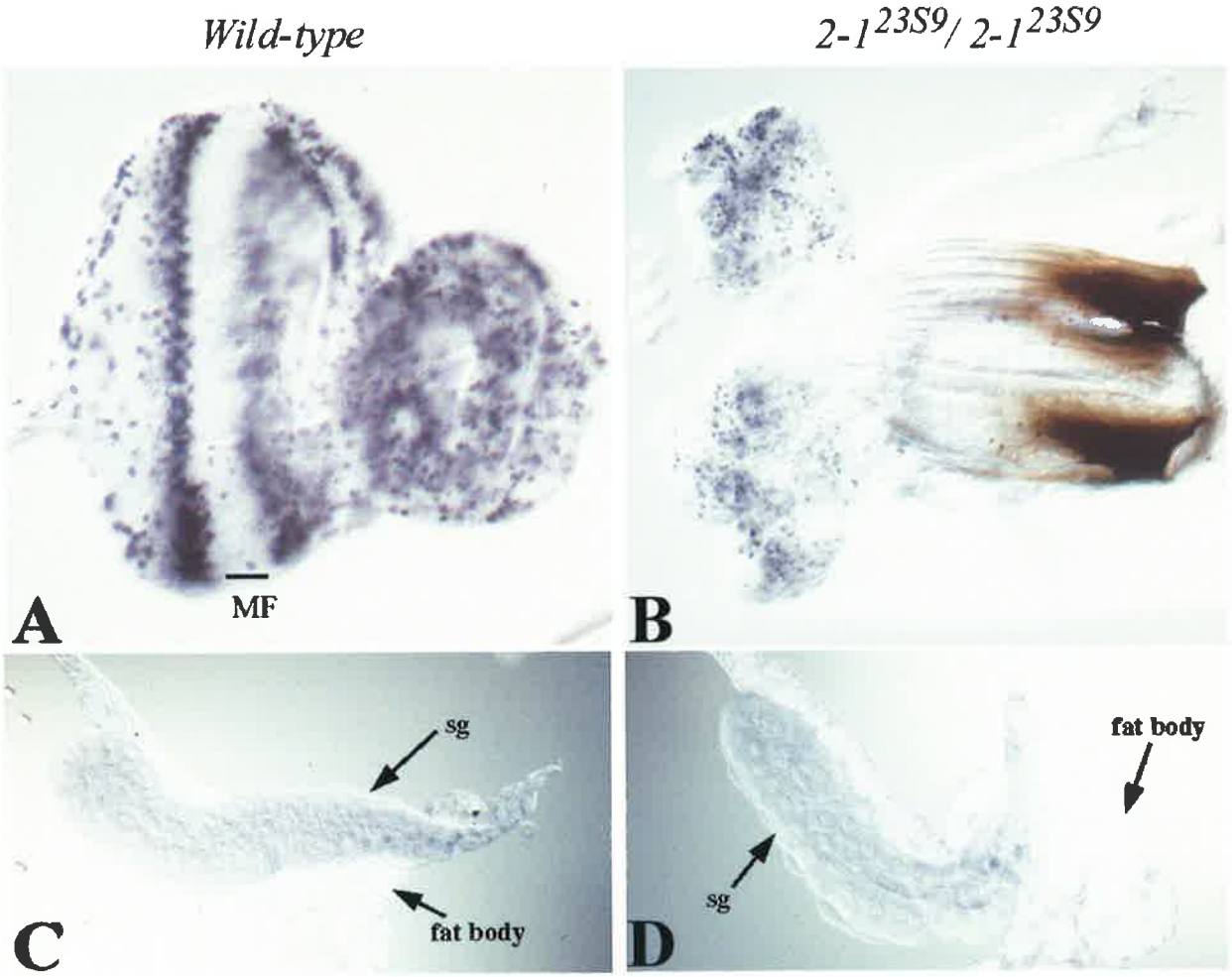

wild-type
2-1^23S9/ 2-1^23S9
OPC
A
B
OPC
LPC
IPC
C
D
BL
ED
E
BL
F

Figure 8.6 Eye imaginal discs and salivary glands of homozygous *Su(DmcycE^{JP})2-1* larvae. BrdU labelling to show S phases from (A, C) wild-type and (B, D) a *2-1^{23S9}* homozygote ~6 days AEL third instar larvae. (A, B) Eye-antennal imaginal disc from wild-type and a *2-1^{23S9}* homozygote showing that while S phases are observed in homozygous *2-1^{23S9}* eye-antennal discs, these discs are small and underdeveloped. A and B are shown at the same magnification. (C, D) salivary glands (sg) from wild-type and *2-1^{23S9}* homozygotes show a few BrdU labelled cells near the duct region. S phases terminate at the correct time in *2-1^{23S9}* homozygotes indicating that the *Su(DmcycE^{JP})2-1* gene product is not a negative regulator of proliferation in this tissue. Anterior is to the right in all panels. *2-1^{E2S31}* homozygotes show similar phenotypes (not shown).


Wild-type
2-1^23S9/ 2-1^23S9
MF
A
B
sg
fat body
C
fat body
sg
D

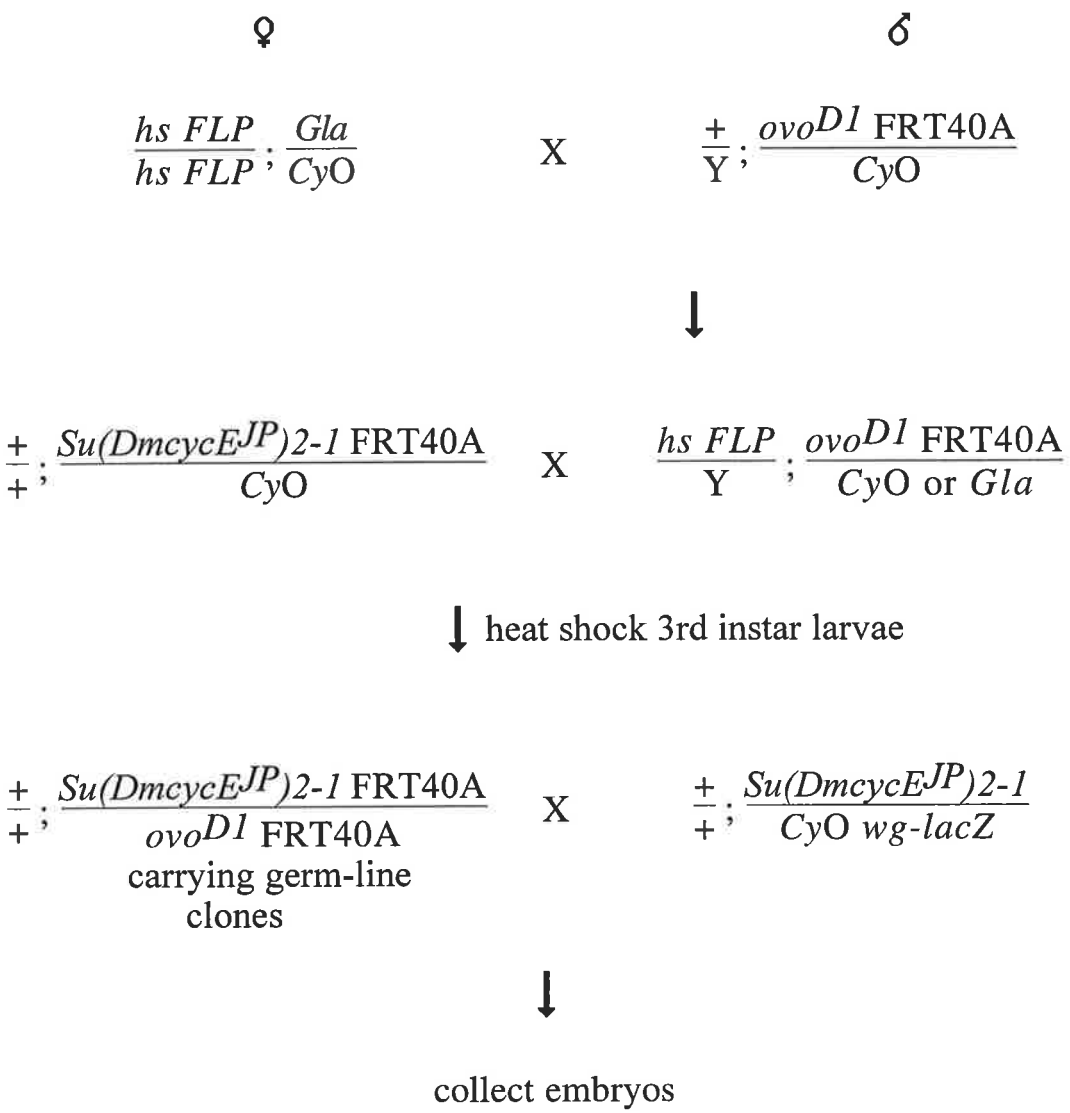


Figure 8.7 Generation of embyros lacking maternal and zygotic *Su(DmcycE^{JP})2-1*

Embryos deficient in both maternal and zygotic *Su(DmcycE^{JP})2-1* were generated as described in Xu and Rubin, (1993). Males carrying the dominant female sterile mutation *ovo^{D1}* were crossed to females carrying the Flipase (*FLP*) gene under the control of a heat shock promoter (*hs FLP*). From this cross, males carrying *hs FLP* and *ovo^{D1}* over *Cy*O or *Glazed* (*Gla*) were selected. These males were then crossed to a *Su(DmcycE^{JP})2-1* FRT40A stock and third instar larvae from this cross were heat shocked for 2 hours at 38°C to induce mitotic recombination at the FRT40A sites. Non *Cy*O (or *Gla*) females from this cross carry *Su(DmcycE^{JP})2-1* germline clones, and were crossed to *2-1^{23S9}* or *2-1^{E2S31}*/*Cy*O *wg-lacZ*. *Su(DmcycE^{JP})2-1* maternal^{-}/zygotic^{-} were distinguished from *Su(DmcycE^{JP})2-1* maternal^{-}/zygotic^{+} using the *wg-lacZ* marker on the *Cy*O chromosome carrying a wild-type copy of *Su(DmcycE^{JP})2-1*. No eggs were layed when third instar larvae from cross 2 were not heat shocked.

To determine whether embryos lacking maternal and zygotic *Su(DmcycE^{JP})2-1* expression have proliferative defects, BrdU incorporation to detect S phases was carried out. S phases occurred in the correct spatial and temporal pattern in embryos lacking maternal and zygotic *Su(DmcycE^{JP})2-1* (Figure 8.8). Furthermore, no ectopic S phases were observed, as S phases ceased in the epidermis, PNS and endoreplicating tissues at the appropriate time. Significantly, developmental processes do not occur normally in *Su(DmcycE^{JP})2-1* maternal^{-} /zygotic^{-} embryos (Figure 8.8). Up to and including stage 11 embryos, development appears to occur normally, with the germband extending. However, germband retraction does not occur properly (Figure 8.8D compared with E). Presumably as a consequence of germband retraction not occurring normally, dorsal closure does not occur, and these embryos do not hatch.

To further investigate the developmental defects associated with embryos lacking maternal and zygotic *Su(DmcycE^{JP})2-1*, these embryos were stained for expression of the patterning gene *engrailed* and with the monoclonal antibody 22C10 to examine neuronal development (Fujita *et al.*, 1982). Staining for Engrailed protein revealed that expression of this segment polarity gene is not initiated correctly, with the stripes of Engrailed expression being disorganised and four cells wide instead of two (Figure 8.9B, D compared with A, C). Nor is the expression of Engrailed maintained in the epidermis later in embryogenesis, although it is still present in the CNS (Figure 8.9F compared with E). Thus loss of *Su(DmcycE^{JP})2-1* expression indirectly or directly affects patterning. Staining with the 22C10 antibody to detect neurons demonstrated that *Su(DmcycE^{JP})2-1* maternal^{-}/zygotic^{-} embryos do not have any differentiated neurons (Figure 8.10B compared with A). Expression of *Su(DmcycE^{JP})2-1* during embryogenesis is therefore also required for correct determination or differentiation of embryonic neurons. Thus, while *Su(DmcycE^{JP})2-1* is not required for regulating proliferation during embryogenesis, it is required for other developmental processes.

8-7 Genes within the 32A-E cytological region

A summary of genes within the 32A-E region is presented in Table 8.3. As the cytological region defining *Su(DmcycE^{JP})2-1* is large, many genes have been mapped to this region. A total of 17 genes were considered candidates for *Su(DmcycE^{JP})2-1*, 15 *P* element alleles (some of which have recently been found to be allelic to known genes), *Ubiquitin conjugating enzyme D2* (*UbcD2*) and *Nitric oxide synthase* (*Nos*). All 15 *P* alleles in the region complemented *Su(DmcycE^{JP})2-1* alleles (data not shown), indicating that these are

alleles of *Su(DmcycE^{JP})2-1*. No mutations are available in the other two candidates, *UbcD2* and *Nos*, which are discussed below.

8-7.1 *UbcD2*

In mammals, Cyclin E is degraded by the Ubiquitin/proteosome pathway (Won and Reed, 1996; Clurman *et al.*, 1996). *Drosophila* UbcD2 ubiquitin conjugating enzyme may therefore be required for the degradation of Cyclin E. Halving the gene dosage of *UbcD2* would therefore be predicted to suppress the *DmcycE^{JP}* rough eye phenotype and enhance the *GMR-cyclin E* rough eye phenotypes. In addition, mutations in *UbcD2* would be expected to result in stabilised Cyclin E and ectopic S phases, consistent with the optic lobe phenotype seen in *Su(DmcycE^{JP})2-1* mutants. Whether a mutation in *UbcD2* would explain the mutant phenotype of *Su(DmcycE^{JP})2-1* maternal^{-}/zygotic^{-} embryos is less clear. UbcD2 is expected to be involved in the degradation of a large number of proteins, therefore the embryonic phenotype is difficult to predict. *UbcD2* was identified based on homology to mammalian Ubiquitin conjugating enzymes, and no mutants have been isolated (Matuschewski *et al.*, 1996). This gene was therefore not able to be tested directly for allelism with *Su(DmcycE^{JP})2-1*. To determine whether the *UbcD2* coding region is deleted or rearranged in either *2-1^{23S9}* or *2-1^{27S3}*, genomic Southern analysis using the *UbcD2* cDNA was carried out (Figure 8.11). No changes were observed in these two X-ray alleles, indicating that *Su(DmcycE^{JP})2-1* is unlikely to correspond to *UbcD2*. However, it is possible that these alleles have small lesions within the *UbcD2* coding region not detectable by Southern analysis, or have *UbcD2* promoter mutations.

8-7.2 Nitric oxide synthase (Nos)

Nitric oxide (NO) is an important signalling molecule synthesised from L-arginine by Nitric oxide synthase (Nos) (reviewed by Bicker, 1998). NO has been implicated in diverse biological functions including visual system processing, learning and memory, dilation of blood vessels, immune response and potentiation of synaptic transmission (reviewed by Bredt and Snyder, 1994; Bicker, 1998). As a free radical, NO is able to readily diffuse across cell membranes, and activates soluble guanylate cyclases, resulting in the production of cyclic GMP (cGMP). cGMP then affects cell physiology by acting on ion channels, protein kinases and phosphodiesterases (reviewed by Bicker, 1998).

By a cGMP independent mechanism, NO has also has been implicated as an antiproliferative agent in both mammalian tissue culture cells and *Drosophila* imaginal disc development (Peunova and Enikolopov, 1995; Kuzin *et al.*, 1996). Increasing levels of NO by

Figure 8.8 Expression of *Su(DmcycE^{JP})2-1* is required for proper embryonic development, and not regulation of S phases

S phases detected by BrdU labelling of (A, C, E, G) *2-1^{23S9}* maternal^{-}/zygotic^{+} and (B, D, F, H) *2-1^{23S9}* maternal^{-}/zygotic^{-} embryos. The pattern of S phases seen in *2-1^{23S9}* maternal^{-}/zygotic^{+} embryos is the same as wild-type (not shown). (A, B) Stage 11 wild-type and *2-1^{23S9}* maternal^{-}/zygotic^{-} embryos showing S phases in the CNS, PNS and thoracic patch cells in the correct spatial and temporal pattern. S phases in the epidermis are also downregulated appropriately (C, D) Stage 13 embryos showing S phases in the PNS have been downregulated normally and show the correct pattern of S phases in the CNS and endoreplicating gut. While germband retraction is almost complete in *Su(DmcycE^{JP})2-1* maternal^{-}/zygotic^{+} embryos, it has not begun in maternal^{-}/zygotic^{-} embryos. (E, F) Comparison of stage 15 embryos demonstrating that S phases are occurring in cells of the CNS and gut, although these appear disorganised due to the developmental abnormalities of *2-1^{23S9}* maternal^{-}/zygotic^{-} embryos (G, H) Stage 16 embryos showing that although germband retraction and dorsal closure have not occurred, S phases are still observed in the brain lobes and gut of *2-1^{23S9}* maternal^{-}/zygotic^{-} embryos. The brown staining in maternal^{-}/zygotic^{+} is from the *Cy*O *wg-lacZ* chromosome carrying the paternally derived wild-type copy of *Su(DmcycE^{JP})2-1*. Similar results were obtained using *2-1^{E2S31}* germline clones (not shown). All embryos are orientated anterior to the left, dorsal side up.

Abbreviations used are CNS (central nervous system), PNS (peripheral nervous system), tp (epidermal thoracic patch), (amg) anterior midgut, (pmg) posterior midgut, (cmg) central midgut.

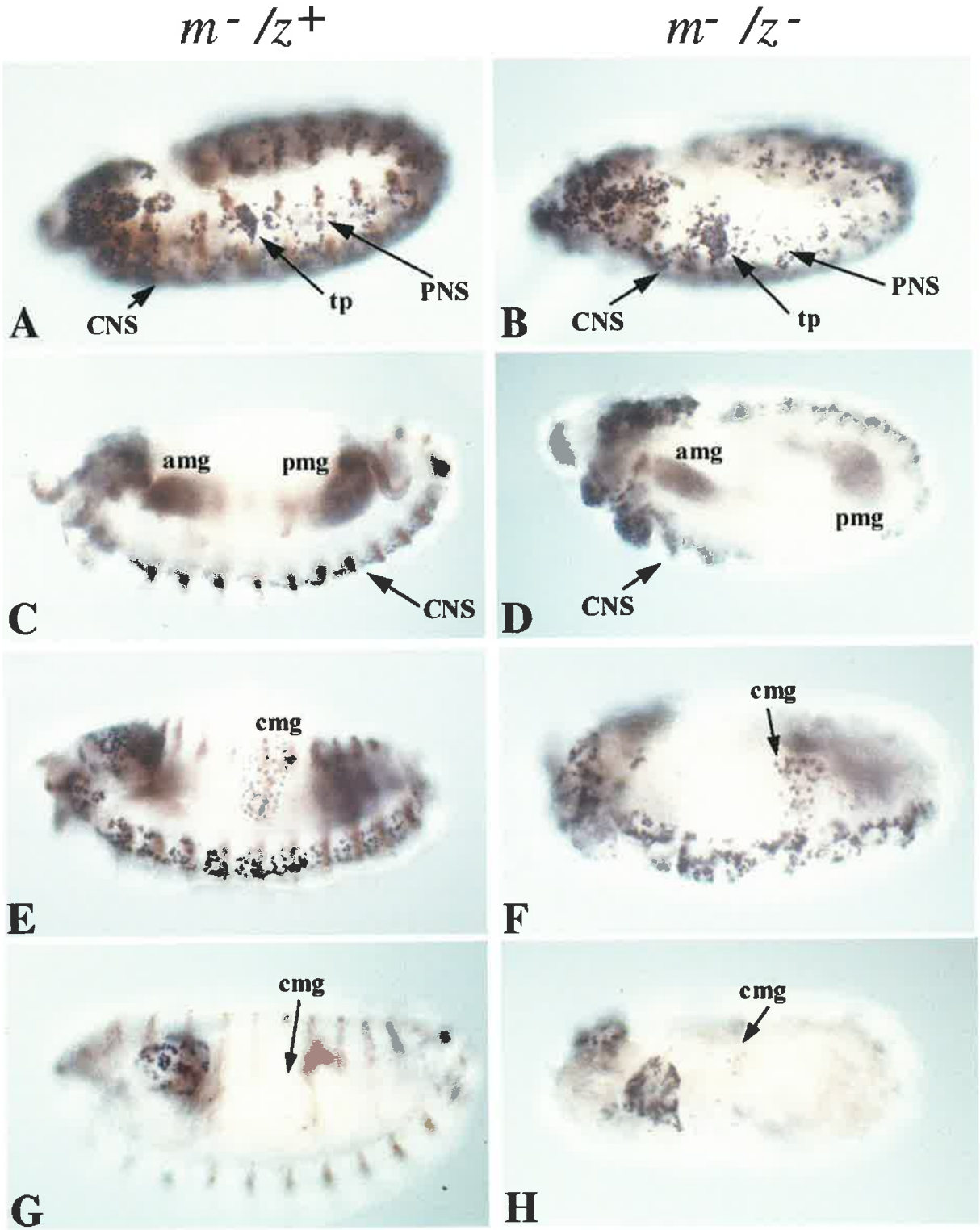

m- /z+
m- /z-
A
CNS
tp
PNS
B
CNS
tp
PNS
C
amg
pmg
CNS
D
amg
pmg
CNS
E
cmg
F
cmg
G
cmg
H
cmg

Figure 8.9 Segmentation is affected in *Su(DmcycE^{JP})2-1* maternal^{-}/zygotic^{-} embryos

Anti-Engrailed antibody staining of (A, C, E) *2-1^{23S9}* maternal^{-}/zygotic^{+} and (B, D, F) *2-1^{23S9}* maternal^{-}/zygotic^{-} embryos. (A, B) Stage 7 embryos showing that Engrailed expression is not initiated correctly. The stripes of Engrailed expression in *2-1^{23S9}* maternal^{-}/zygotic^{-} embryos during germband extension are four cells wide rather than two, and are disorganised. (C, D) Stage 11 embryos showing the disorganised and weaker nature of epidermal Engrailed expression in *2-1^{23S9}* maternal^{-}/zygotic^{-} embryos (E, F) By stage 14, Engrailed stripes of expression in the epidermis are not observed, indicating that Engrailed expression is not maintained correctly. The LacZ staining from the *wg-lacZ* balancer chromosome used to distinguish maternal^{-}/zygotic^{+} from maternal^{-}/zygotic^{-} embryos was carried out using a secondary antibody conjugated to the fluorophore FITC. Similar results were obtained using *2-1^{E2S31}* embryos (not shown). All embryos are orientated anterior to the left, dorsal side up.

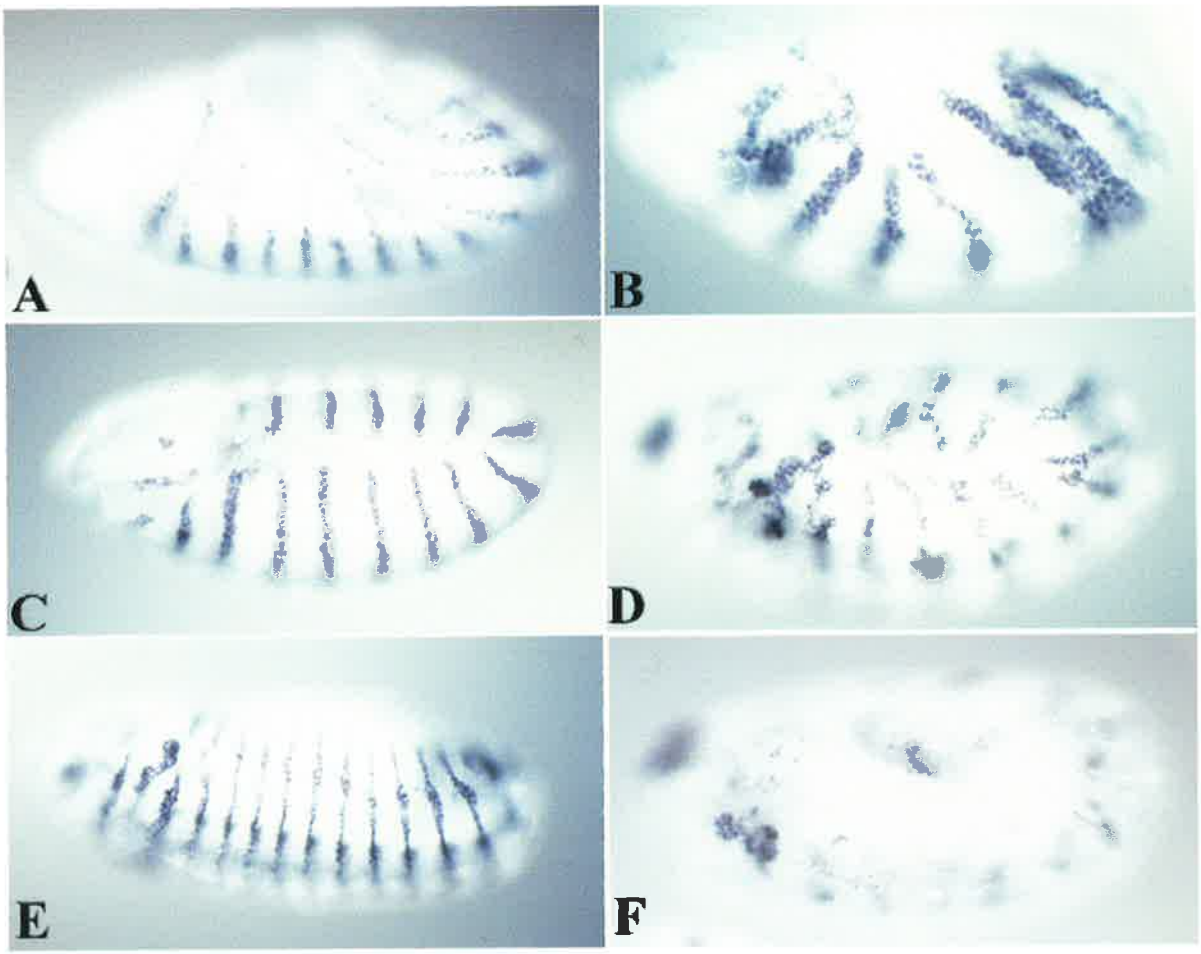
Figure 8.10 Neuronal development is affected in *Su(DmcycE^{JP})2-1* maternal^{-}/zygotic^{-} embryos

22C10 staining (blue) to detect neurons from (A) *2-1^{23S9}* maternal^{-}/zygotic^{+} and (B) *2-1^{23S9}* maternal^{-}/zygotic^{-} stage 16 embryos. The stereotypic pattern of neurons seen in maternal^{-}/zygotic^{+} embryos is not observed in maternal^{-}/zygotic^{-} embryos demonstrating that the *Su(DmcycE^{JP})2-1* gene product is required for neuronal specification and/or differentiation.

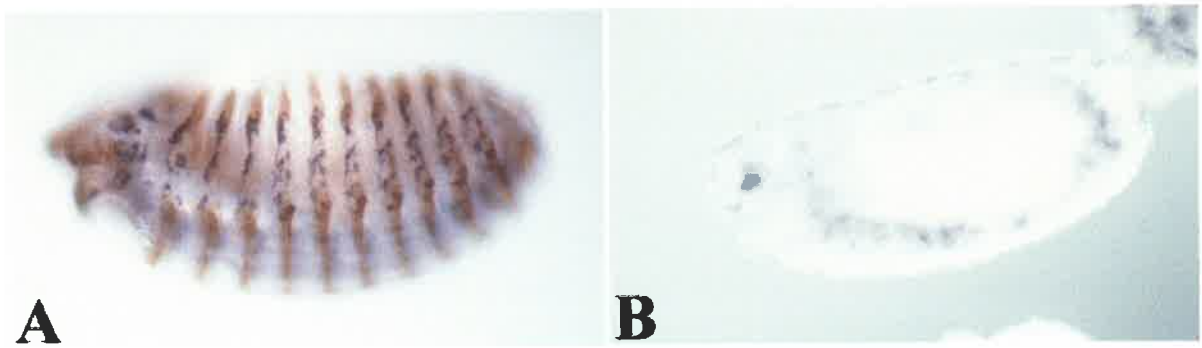
The brown staining in A is the *wg-lacZ* pattern used to distinguish maternal^{-}/zygotic^{+} from maternal^{-}/zygotic^{-} embryos. *2-1^{E2S31}* embryos showed a similar phenotype (not shown). Embryos are orientated anterior to the left, dorsal side up.

anti-En

m- /z+ m- /z-



22C10



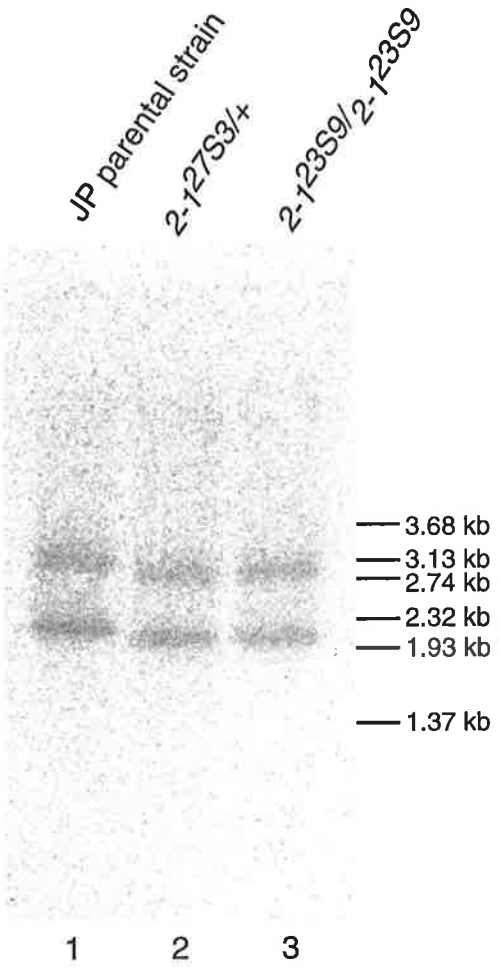


Figure 8.11 The *UbcD2* coding region is not altered in *2-1*^{27S3} or *2-1*^{23S9}

Genomic DNA from the homozygous *DmcycE*^{JP} parental strain (track 1), *2-1*^{27S3} / +, *DmcycE*^{JP} heterozygotes (track 2) and *2-1*^{23S9}/ *2-1*^{23S9} homozygotes (track 3) digested with *Hind*III, and probed with a *UbcD2* cDNA. The *UbcD2* coding region is not deleted or rearranged in either of the *Su(DmcycE*^{JP}*)2-1* X-ray generated alleles as the signal is not reduced in *2-1*^{27S3} / +, *DmcycE*^{JP} heterozygotes, and not altered in *2-1*^{23S9} homozygotes. In addition, digesting genomic DNA using *Eco*RI revealed that there were no changes within the *UbcD2* coding region I (not shown). Molecular weights are as indicated.

heat shock induction of the mouse NOS2 gene during larval development results in a decrease in the number of BrdU-labelled imaginal disc cells (Kuzin *et al.*, 1996). Conversely, decreasing Nos activity using the chemical inhibitor L-nitroarginine methyl ester (L-NAME) leads to an increase in the number of S phases in imaginal discs. Nos therefore acts as a negative regulator of the G1 to S phase transition during imaginal disc development. Halving the dosage of this gene would therefore be expected to suppress the $DmcycE^{JP}$ rough eye phenotype.

In addition to a role in negatively regulating proliferation during disc development, Nos activity has also been implicated in apoptosis (Kuzin *et al.*, 1996). While inhibition of Nos activity during the third larval instar has little effect on the adult eye, simultaneous inhibition of Nos activity and cell death during eye disc development results in a dramatic effect on adult eyes. This includes an increase in the number of ommatidia in the adult eye and additional pigment, cone and bristle cells. Thus, the overproliferation caused by decreasing Nos activity is masked by the induction of cell death, not only in eye discs, but all other discs examined. *Nos* mutants would therefore be expected to show overproliferation of imaginal discs, although the concomitant induction of cell death may result in small discs, consistent with larval phenotype seen in $Su(DmcycE^{JP})2\text{-}1$ mutants. The mechanism by which NO acts as a negative regulator of the G1 to S phase transition is not known, thus predictions on whether mutations in Nos would be expected to act downstream of *cyclin E* transcription cannot be made (Kuzin *et al.*, 1996).

The role of NO in optic lobe patterning during *Drosophila* pupal development has also been addressed (Gibbs and Truman, 1998). Nos and cGMP are required for the initial contact between retinal axons and their targets, with inhibition of Nos activity resulting in photoreceptor axons extending beyond their appropriate cell layer. The function of Nos during third larval instar optic lobe development has not, however, been examined. As NO has many varied roles, it would not be surprising that mutations in Nos would result in proliferative defects during larval development, and may only induce cell death in imaginal and not CNS tissue. This may lead to the overgrown brains and small disc phenotypes observed in $Su(DmcycE^{JP})2\text{-}1$ mutants. In addition, NO may not have a role in the negative regulation of proliferation during embryonic development, but affect other processes required for normal development. *Nos* is therefore a candidate for $Su(DmcycE^{JP})2\text{-}1$. As no mutations in *Nos* have been isolated, this candidate could not be tested genetically.

Table 8.3 Genes in the 32A-E region

Gene	Cytology	Known function/phenotype
denervated	31B-34A	Homozygous embryonic lethal
Inr-A	31D-33A	Transcription factor required for expression of *Adh*
dal	31F1-32D4	Female sterile. Centrosome separation fails
l(2)k02605	**31F3-4**	***P* element allele**
l(2)k09116	**31F4-5**	***P* element allele**
l(2)k10307	**31F4-5**	***P* element allele**
pie	32A	Homozygous viable mutation
Lrr47	32A	Expressed only in early embryos and female germline
Lipase 1	32A	Digestive lipase enzyme
Lipase 2	32A	Likely to be pseudogene
UbcD2	**32A-E**	**Ubiquitin conjugating enzyme**
RpS13	32A1-5	Heterozygotes have a medium-strong minute phenotype
l(2)k13206	**32A4-5**	***P* element allele**
Nos	**32B**	**Shown to be involved in regulating proliferation**
Ial	32B	Involved in centrosome cycle control
porin	32B3-4	Voltage dependent anion-selective channel in mitochondria
l(2)03788	**32C1-2**	***P* element allele**
l(2)k03107	**32C1-2**	***P* element allele**
l(2)k05812	**32C4-5**	***P* element allele**
l(2)k13811	**32C3-5**	***P* element allele**
Acp32CD	32C-D	Accessory gland specific peptide
ms(2)32D	32D	Male sterile mutation
zk	32D1-2	Nucleoporin. Allelic to *l(2)k07717 P* allele
Nup32D	32D1-2	Allelic to *l(2)01501 P* allele
hup	32D1-4	Required for oocyte positioning, and follicle cell fate
l(2)giant discs	32D1-4	Tumour suppressor. All imaginal discs overgrow
RpL9	32D1-5	Heterozygotes have short, thin bristles and rough eyes
sting	32D1-5	Male and female sterile
Cf7	32E	Chorion specific factor
Gyc32E	32E	Involved in transmission of light signal in photoreceptor
Vm32E	32E	Ovary specific protein
hrp	32E	Homozygous viable behavioural mutant
l(2)04008	**32E1-2**	***P* element allele**
l(2)04431	**32E1-2**	***P* element allele**
abrupt	32E1-2	Allelic to *l(2)k02807 P* element allele
l(2)04418	**33A1-2**	***P* element allele**
l(2)k05205	**33A1-2**	***P* element allele**

Summary of genes within the 32A-E cytological region. Abbreviations used are *pie* (*pineapple eye*), *daughterless-like* (*dal*), *Inverse-regulator-Alcohol dehydrogenase* (*Inr-A*), *Leucine rich repeat protein* (*Lrr47*), *Guanadylate cyclase* (*Gyc32E*), *Vitelline membrane protein* (*Vm32E*), *hyperpolarising receptor potential* (*hrp*), *IpII-aurora-like kinase* (*ial*), *Chorion factor 7* (*Cf7*), *zonder koten* (*zk*), *hold up* (*hup*), *Nitric oxide synthase* (*Nos*). Those considered candidates are indicated in bold. Those genes that have been cloned are underlined.

8-7.3 Analysis of genomic sequence in the 32A-E region

The BDGP has recently finished sequencing most of the 32A-E cytological region, with the longest contiguous cloned sequence (contig), *Gyc32E*, consisting of five P1 clones that span the approximate cytological region 32C to 32E (Figure 8.12). In addition, a P1 clone mapping to 32A has been sequenced, but does not overlap with the distal-most P1 of the *Gyc32E* contig. The total sequence available covers 386,929 bp and was analysed in two ways. Firstly, small overlapping regions were analysed using 'blastx' (Altschul *et al.*, 1997), which translates the nucleotide sequence in all six possible reading frames and compares this to the NCBI protein database. Genes identified in this way are therefore genes predicted based on homology of the conceptual translated product. To determine whether any ESTs were available for any of the predicted genes, and to localise any *P* element insertions in the region, the sequence available was analysed using the BDGP 'blastn' program, that compares the nucleotide sequence from the P1 clones to the *Drosophila* database that includes all nucleotide sequences such as ESTs and genomic sequence flanking *P* elements. The results of this analysis are summarised in Figure 8.12.

A total of 33 genes are predicted to be within this sequenced region. Seven of these have been previously mapped to the region, and are not considered candidates for *Su(DmcycE^{JP})2-1* (Figure 8.12; Table 8.3). Interestingly, nine cloned genes that map to the region, *Lrr47*, *Lipase I*, *UbcD2*, *RpS13*, *Nos*, *ial*, *Cf7*, *hrp* and *zk*, were not identified in this analysis (Table 8.3; Figure 8.12). These may fall within the gap in the sequence at 32B, or in the case of *Cf7*, may be outside the 32A-E region. 13 predicted genes have a corresponding EST, demonstrating that they are 'real' genes, although none of them are good candidates for *Su(DmcycE^{JP})2-1* (Table 8.4). In addition, two of these predicted genes, *replication factor-C* (*RF-C*) and *kinesin-like*, are likely to be affected by *P* element insertions. *l(2)13807* is inserted within the predicted *RF-C* gene, and is therefore likely to represent a *RF-C* mutant. Similarly, *l(2)04431* is inserted immediately upstream of the predicted *kinesin-like* gene, and is not an allele of the adjacent *abrupt* gene, indicating that this *P* insert may be affecting the *kinesin-like* gene. These *P* elements have been tested, and complement *Su(DmcycE^{JP})2-1* alleles, indicating that these genes do not correspond to *Su(DmcycE^{JP})2-1*. 8 ESTs identified within the sequence did not have significant homology to any known proteins when translated. As the sequence from ESTs is quite short, usually only ~500bp, it is not surprising that this may not be enough to detect any significant homology to known genes or proteins.

The remaining 9 predicted genes in the 32A-E region were identified based on homology, and do not have a corresponding EST (Table 8.5).

Table 8.4 Predicted genes that have a corresponding EST

Protein	Predicted function
Homology to yeast hypothetical protein at 32B	Similar to yeast hypothetical protein of unknown function
Tubulin-tyrosine ligase	Probably required for ATP dependent addition of tyrosine to α-tubulin (Ersfeld *et al.*, 1993)
hnRNP methyl transferase	Possible role in signal transduction or nuclear import (Gary and Clarke, 1998)
dUTPase	Likely to catalyse hydrolysis of dUTP, presumably to prevent uracil from being incorporated into DNA (Grafstrom *et al.*, 1978)
Homology to a human auto-antigen	Similar to human auto-antigen of unknown function (Garcia-Lozano *et al.*, 1997)
RalBP1 interacting protein	Human homologue binds to RALBP1, a GAP for the Ral, Rac and Cdc42 GTPases. Likely to be involved in regulation of actin cytoskeleton and/or exocytosis (Yamaguchi *et al.*, 1997; Ikeda *et al.*, 1998)
E1/E2 ATPase	Cation transport ATPase (Boldyrev and Quinn, 1994)
Homologous to yeast hypothetical protein at 32C	Similar to yeast hypothetical protein of unknown function
KIAA0197	Human cDNA of unknown function (Nagase *et al.*, 1996)
RF-C 38kDa subunit	Replication factor involved in initiation of DNA replication (O'Donnell *et al.*, 1993). Likely to be affected by *l(2)13807*
D-3-phosphoglycerate dehydrogenase	Likely to catalyse the first step in pathway of serine formation (Achouri *et al.*, 1997)
Neuronal Ca2+ β subunit	Calcium channel protein expressed in neural cells (Grabner *et al.*, 1994)
kinesin-like protein at 32E	Most similar to CENP-E kinetochore motor (Yen *et al.*, 1992). Likely to be affected by *l(2)04431 P* insert

Summary of the predicted function of genes within the 32A-E region for which ESTs are available. None of these genes are good candidates for *Su(DmcycE^{JP})2-1*. GAP = GTPase activating protein.

Figure 8.12 Analysis of genomic sequence in the 32A-E region

Summary of the genes predicted to be in the 32A-E region. Sequence available from the BDGP was translated in all six reading frames and compared to the protein database using blastx (Altschul *et al.*, 1997), to predict coding sequences based on homology to known proteins. In addition, expressed sequence tags (ESTs) were identified using the BDGP blastn program.

(A) P1 clone DS01895 does not overlap with the *Gyc32E* contig and maps by *in situ* hybridisation to the polytene band 32A.

(B) Sequence from DS1831, DS04313, DS06882, DS02889 and DS05505 overlap, forming contiguous sequence of the *Gyc32E* contig spanning the approximate cytological region 32C-E.

Included in this figure are already charactised *Drosophila* genes (dark blue), genes identified based on homology that do (red) or do not (light blue) have an EST available, and ESTs that do not have significant homology to any known genes or proteins (green).

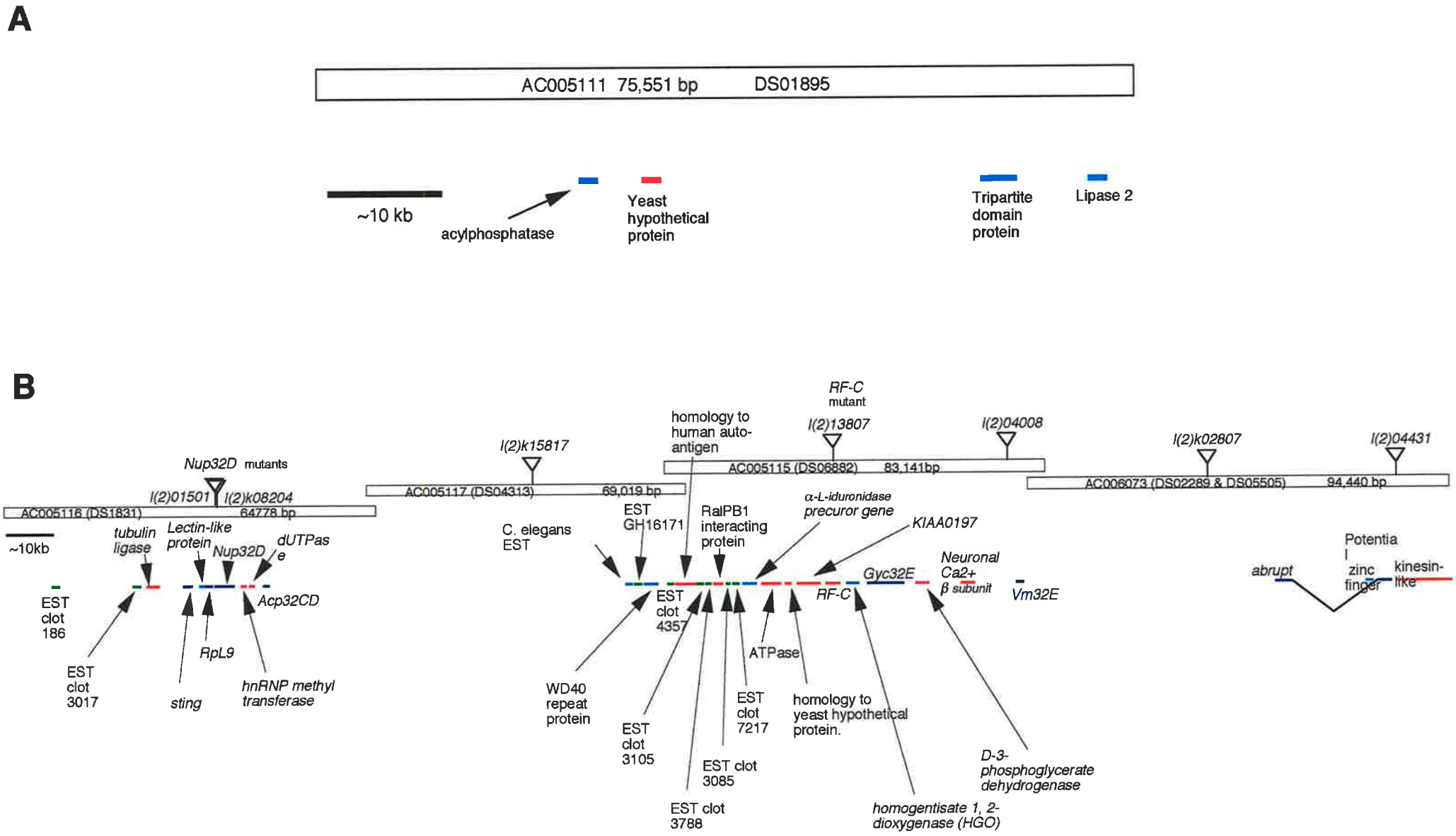

A
AC005111 75,551 bp DS01895
~10 kb
acylphosphatase
Yeast hypothetical protein
Tripartite domain protein
Lipase 2
B
Nup32D mutants
l(2)01501
l(2)k08204
AC005116 (DS1831) 64778 bp
~10kb
tubulin ligase
Lectin-like protein
Nup32D
dUTPase
EST clot 186
EST clot 3017
RpL9
sting
Acp32CD
hnRNP methyl transferase
l(2)k15817
AC005117 (DS04313) 69,019 bp
C. elegans EST
homology to human auto-antigen
RF-C mutant
l(2)13807
l(2)04008
AC005115 (DS06882) 83,141bp
EST GH16171
RalPB1 interacting protein
α-L-iduronidase precuror gene
KIAA0197
Gyc32E
Neuronal Ca2+ β subunit
Vm32E
RF-C
EST clot 4357
ATPase
WD40 repeat protein
EST clot 3105
EST clot 3085
EST clot 7217
homology to yeast hypothetical protein.
EST clot 3788
homogentisate 1, 2-dioxygenase (HGO)
D-3-phosphoglycerate dehydrogenase
l(2)k02807
l(2)04431
AC006073 (DS02289 & DS05505) 94,440 bp
abrupt
Potentia l zinc finger
kinesin-like

Table 8.5 Predicted genes with no corresponding EST

Predicted protein	Predicted function
Acylphosphatase	Likely to catalyse hydrolysis of organic acylphosphates (Thunnissen *et al.*, 1997)
Tripartite domain protein at 32A	**Similar to the promyelocytic leukemia tumour suppressor (Goddard *et al.*, 1991)**
Lectin-like protein	Similar to C-type lectins that are involved in immune response (Lohwasser *et al.*, 1999)
Homology to *C. elegans* EST.	No function predictions can be made.
WD repeat protein at 32C	**Similar to pRb binding protein, RbAP4648 (Neer *et al.*, 1994; Qian and Lee, 1995)**
α-L-iduronidase precursor	Lysosomal hydrolase (Scott *et al.*, 1991)
Homogentisate 1,2-dioxygenase	Likely to be involved in phenylalanine catabolism. Mutations in this gene are associated with Alkaptonuria in mammals (Granadino *et al.*, 1997)
Zinc finger protein at 32E	Only Zinc finger region identified. No function predictions can be made.

Summary of predicted genes in the 32A-E region for which no EST is available. A predicted function (if possible) is also given. Two of these genes, encoding a tripartite domain and WD40 repeat proteins (bold), are candidates for *Su(DmcycE^{JP})2-1* and are discussed in more detail in the text.

As this genomic sequence has only recently become available, no further work has been carried out to identify the *Su(DmcycE^{JP})2-1* gene. Of the genes identified in the 32A-E region, two candidates were identified, neither of which have a corresponding EST (Table 8.5). The first of these encodes a protein of the tripartite domain family that has a ring finger, a B box and a coiled-coil region (Henry *et al.*, 1998), identified within the P1 clone DS01895 (Figure 8.12; and data not shown). Mammalian members of this family include Midline1, which is affected in the genetic disease OPITZ syndrome (Quaderi *et al.*, 1997), estrogen-responsive finger protein efp (Inoue *et al.*, 1993), the ret proto-oncoprotein (reviewed by Mak and Ponder, 1996), the promyelocytic leukemia (PML) tumour suppressor (Goddard *et al.*, 1991) and the Leu5 tumour suppressor (Kapanadze *et al.*, 1998). It is not known precisely how these proteins function, although PML is known to associate with large complexes in the

nucleus termed nuclear bodies (Sternsdorf *et al.*, 1997). PML has also been shown to associate with pRb and inhibit pRb-mediated transactivation of the glucocorticoid receptor gene required for differentiation (Alcalay *et al.*, 1998), suggesting that PML may be involved in transcriptional regulation. Only one exon of this gene was identified in the blastx search of this region. This exon spans the ring finger motif and is most similar to the uncharacterised human gene D87458 and to *midline1*.

The second candidate for *Su(DmcycE^{JP})2-1* was identified in the P1 clone DS01895 and encodes a WD40 repeat protein with similarity to a pRb binding protein, RbAP4648 (Neer *et al.*, 1994; Qian and Lee, 1995). This protein is clearly different from RbAP4648, which has been characterised and maps to 88E8-9 (Tyler *et al.*, 1996). Given the similarity of the WD40 repeat protein to RbAP4648, this gene is a possible candidate for *Su(DmcycE^{JP})2-1*. In addition to the tripartite domain and WD40 repeat proteins, ESTs and proteins with no predicted function identified in the 32A-E region are also candidates for *Su(DmcycE^{JP})2-1*. Further work is therefore required to identify the *Su(DmcycE^{JP})2-1* gene.

8-8 Discussion and conclusions

This chapter has described the characterisation of a suppressor of *DmcycE^{JP}*, *Su(DmcycE^{JP})2-1*, identified in the genetic interaction screen described in Chapter 5. *Su(DmcycE^{JP})2-1* is likely to encode a negative regulator of the G1 to S phase transition during eye development, as halving the dosage of this gene increases the number of S phase cells in *DmcycE^{JP}* eye discs. In addition, genetic tests suggest that *Su(DmcycE^{JP})2-1* acts downstream of *cyclin E* transcription, and is required for cell cycle arrest induced by overexpression of human p21^{CIP1} during eye development. *Su(DmcycE^{JP})2-1* may therefore encode a protein that acts directly on Cyclin E by inhibiting Cyclin E/Cdc2c activity, or by positively regulating Cyclin E degradation. It is also possible that *Su(DmcycE^{JP})2-1* encodes a protein that indirectly modifies Cyclin E-associated kinase activity by affecting the presence or activity of an activator or inhibitor of Cyclin E/Cdc2c. Alternatively, *Su(DmcycE^{JP})2-1* may encode a downstream negative regulator of DNA replication (Figure 8.13). *Su(DmcycE^{JP})2-1* is expected to specifically affect Cyclin E, and not Cyclin A-associated kinase activity, as halving the gene dose of *Su(DmcycE^{JP})2-1* had no effect on the *rux* rough eye phenotype that results from ectopic Cyclin A activity. Further investigation and predictions for the function of *Su(DmcycE^{JP})2-1* gene product will be facilitated by the identification of the *Su(DmcycE^{JP})2-1* gene.

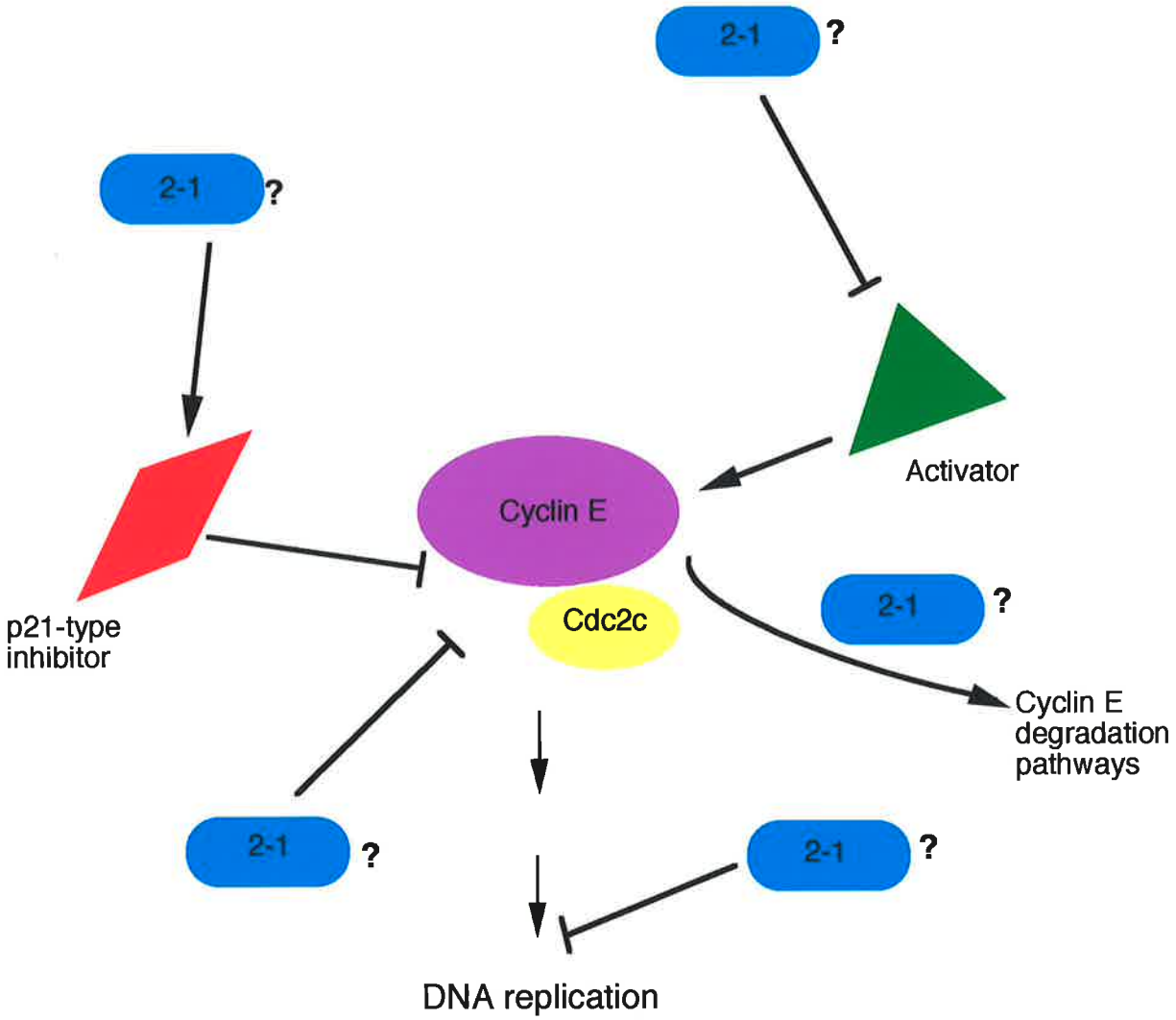


Figure 8.13 Possible mechanisms of action for Su(DmcycE^{JP})2-1

Halving the gene dosage of *Su(DmcycE^{JP})2-1* modifies the rough eye phenotypes generated by overexpression of *cyclin E* and human *p21*, indicating that *Su(DmcycE^{JP})2-1* acts downstream of *cyclin E* transcription. *Su(DmcycE^{JP})2-1* may therefore be involved in negative regulation of Cyclin E/Cdc2c function, required for Cyclin E degradation, or act as a downstream negative regulator of DNA replication.

Analysis of zygotic *Su(DmcycE^{JP})2-1* mutants demonstrated that this gene product is involved in negatively regulating proliferation during larval optic lobe but not ventral ganglion development. *Su(DmcycE^{JP})2-1* is also required for imaginal disc development, although, unlike the optic lobes of *Su(DmcycE^{JP})2-1* mutants which overgrow, discs are smaller than wild-type. One possible explanation for this small disc phenotype is that in the absence of the *Su(DmcycE^{JP})2-1* gene product, proliferation and cell death both occur. Consistent with this, one candidate gene in the critical region for *Su(DmcycE^{JP})2-1*, *Nos*, has been implicated in the regulation of cell death in imaginal cells (Kuzin *et al.*, 1996). The role of cell death in the small disc phenotype of homozygous *Su(DmcycE^{JP})2-1* larvae should be tested by staining imaginal discs with acridine orange, which labels apoptotic cells. The overproliferation phenotype of *Su(DmcycE^{JP})2-1* mutants suggests that Cyclin E protein levels may be elevated in the optic lobes and imaginal discs of mutant larvae. This should therefore be tested directly by staining homozygous *Su(DmcycE^{JP})2-1* tissues with Cyclin E antisera. Clonal analysis should also be used to investigate the effect of small numbers of *Su(DmcycE^{JP})2-1^{-}* cells on eye imaginal disc and optic lobe proliferation, cell death and differentiation.

Interestingly, *Su(DmcycE^{JP})2-1* is not required for regulation of endoreplication in salivary glands, as these S phases are not altered in *Su(DmcycE^{JP})2-1* mutants. Nor does *Su(DmcycE^{JP})2-1* appear to be involved in the regulation of proliferation during embryonic development, as S phases occur in the correct spatial and temporal manner in embryos lacking maternal and zygotic *Su(DmcycE^{JP})2-1* expression. Although not required to negatively regulate proliferation during embryogenesis, *Su(DmcycE^{JP})2-1* is required for normal developmental, as the patterning gene *engrailed* is not expressed appropriately and neuronal differentiation does not occur. *Su(DmcycE^{JP})2-1* therefore encodes a protein that functions differently in tissues at different stages of development, and is only required for regulation of proliferation during larval development. Determining the function of *Su(DmcycE^{JP})2-1* during embryonic development will require further experimentation which will be aided by the identification of the *Su(DmcycE^{JP})2-1* gene.

8-8.1 Toward identifying *Su(DmcycE^{JP})2-1*

Su(DmcycE^{JP})2-1 maps cytologically to 32A-E. Analysis of genes known to be within this region identified two candidates for *Su(DmcycE^{JP})2-1*, *UbcD2* and *Nos*, for which mutations have not been identified. In addition, analysis of genomic sequence available

for 32A and 32C-E identified genes encoding a WD40 repeat protein and a tripartite domain protein as candidates for *Su(DmcycE^{JP})2-1*.

It is unlikely that the gene encoding ubiquitin conjugating enzyme *UbcD2* corresponds to *Su(DmcycE^{JP})2-1*, as genomic Southern analysis demonstrated that the *UbcD2* coding region is not deleted or rearranged in the two *Su(DmcycE^{JP})2-1* X-ray alleles. However, it is possible that there are changes outside the coding region in *2-1^{23S9}* or *2-1^{27S3}*, or very small alterations within the *UbcD2* coding region, that affect *UbcD2* transcription or function. Confirmation that *Su(DmcycE^{JP})2-1* does not correspond to *UbcD2* will require genomic clones surrounding the *UbcD2* locus to be obtained and used for genomic Southern analysis. In addition, sequence analysis of the two *Su(DmcycE^{JP})2-1* EMS alleles and Northern analysis could be used to determine whether *Su(DmcycE^{JP})2-1* corresponds to *UbcD2*.

Three other candidates for *Su(DmcycE^{JP})2-1*, Nos, a tripartite domain protein, and a WD40 repeat protein remain to be tested. As *Nos* has been cloned (Regulski and Tully, 1995), genomic Southern analysis using the *Nos* coding region will determine whether this region is rearranged or deleted in *2-1^{23S9}* or *2-1^{27S3}*. In addition, anti-Nos antibodies have been generated (Regulski and Tully, 1995), which could be used to determine whether *Su(DmcycE^{JP})2-1* mutants lack detectable Nos protein. As previously isolated ESTs are not available for the genes encoding the tripartite domain protein and the WD40 repeat protein, cDNAs will need to be isolated and characterised before analysis of these genes in *Su(DmcycE^{JP})2-1* mutants can be carried out. The characterisation of these genes in *Su(DmcycE^{JP})2-1* X-ray and EMS generated mutants will determine whether any of these candidates corresponds to this interesting suppressor of *DmcycE^{JP}*.

Chapter 9: Conclusions and future studies

9-1 The *DmcycE^{JP}* rough eye phenotype

The research described in this thesis was undertaken with the aim of identifying genes involved in the regulation of *cyclin E* transcription and function. This was made possible by the isolation of a *cyclin E* allele, *DmcycE^{JP}*, that results in homozygous viable and fertile adults with rough eyes. Characterisation of the *DmcycE^{JP} P* element mutation demonstrated that this *cyclin E* allele is hypomorphic, with reduced Cyclin E protein levels leading to fewer cells entering S phase during eye development, resulting in a rough eye phenotype.

In addition to being sensitive to the gene dosage of *DmcycE*, the *DmcycE^{JP}* rough eye phenotype was also found to be sensitive to the dosage of the G1/S regulators *E2F*, *DP*, *RBF*, and *dap*. Interestingly, while *DmcycE^{JP}* was not modified by halving the gene dose of the G2/M regulator *cyclin B*, *DmcycE^{JP}* was sensitive to the dosage of *string*, *cyclin A*, and the Cyclin A regulators *Rux* and *Rca1*. Unlike other G1/S regulators tested, which acted by altering S phases in *DmcycE^{JP}* eye discs, halving the dosage of *string* or *cyclin A* enhanced the *DmcycE^{JP}* rough eye phenotype without reducing the number of S phase cells. Instead, the number of mitoses were reduced. Thus, Cyclin E/Cdc2c activity is implicated in the regulation of events that occur after the initiation of DNA replication. Determining whether Cyclin A has a role in the completion of S phase during eye development and whether regulation of Cyclin A function by Cyclin E/Cdc2c occurs by the direct phosphorylation and activation of String, requires further investigation.

This analysis demonstrated that *DmcycE^{JP}* is a dosage sensitive system that can be used to identify novel genes involved in the G1 to S phase transition. Two approaches were used to identify novel genes that interact with *cyclin E*. Firstly, chromosomal regions that interact with *DmcycE^{JP}* were identified using X and third chromosome deficiencies. Secondly, a random mutagenesis using the mutagens EMS and X-irradiation was carried out to generate specific alleles of *DmcycE^{JP}* modifiers.

9-2 X and third chromosome regions that interact with *DmcycE^{JP}*

In an attempt to identify novel regulators of *cyclin E* transcription and function, X and third chromosome deficiencies were tested for their effect on the *DmcycE^{JP}* rough eye phenotype. This analysis identified numerous chromosomal regions that enhanced or suppressed the *DmcycE^{JP}* phenotype and therefore removed genes potentially involved in *cyclin E* regulation. However, candidate gene(s) could not be assigned for many of the

chromosomal regions identified, highlighting the need for a random mutagenesis to generate specific alleles of modifiers that could be further characterised. It should be noted, however, that the genetic interaction screen described in Chapter 5 was not designed to identify modifiers of *DmcycE^{JP}* on the X chromosome. To further characterise X chromosomal regions that enhance or suppresse the *DmcycE^{JP}* rough eye phenotype, the region(s) responsible for the modified phenotype should be refined using overlapping deficiencies. All characterised and uncharacterised mutations within that region should then be tested for an effect on the *DmcycE^{JP}* phenotype. Additional candidates may also be revealed with the analysis of the completed sequence of the *Drosophila* genome.

9-3 The *DmcycE^{JP}* genetic interaction screen

9-3.1 Second chromosome suppressors

A total of 246 modifiers of *DmcycE^{JP}* rough eye phenotype were generated on the second and third chromosomes using the mutagens EMS and X-irradiation. Further characterisation of the 73 homozygous lethal second chromosome suppressors revealed that this represents 46 complementation groups. 10 of these complementation groups have multiple alleles, 7 of which were characterised with respect to several criteria (summarised in Table 9.1):

1. Do these genes suppress the *DmcycE^{JP}* phenotype by increasing the number of S phases during eye development?
2. Are any of these genes likely to act downstream of *cyclin E* transcription?
3. Where do the suppressors map cytologically?
4. Are these genes likely to be allelic to previously identified genes or mutations?

This analysis revealed that *Su(DmcycE^{JP})2-3* is likely to be allelic to the previously identified but uncharacterised mutation *l(2)36Fd* and that *Su(DmcycE^{JP})2-5* is allelic to a *P* element allele inserted into the *Drosophila* homologue of the *EB1* gene. In addition, candidate genes for which no mutations are currently available have been assigned for *Su(DmcycE^{JP})2-4* and *Su(DmcycE^{JP})2-6*, which should now be tested by genomic Southern analysis. No candidates were identified for *Su(DmcycE^{JP})2-7*. The sequence of this region is therefore needed to identify likely candidates for *Su(DmcycE^{JP})2-7*. More detailed mutant phenotype analysis was limited to *Su(DmcycE^{JP})2-2* and *Su(DmcycE^{JP})2-1* (see below). The remaining two lethal complementation groups with more than one allele, and the 36 single alleles, are yet to be characterised. These are expected to include components of negative

growth signalling pathways, negative regulators of Cyclin E/Cdc2c function and downstream negative regulators of DNA replication. The identification and characterisation of these suppressors will therefore aid in elucidating the mechanisms that negatively regulate the G1 to S phase transition during development.

9-3.2 *Su(DmcycE^{JP})2-2*

Su(DmcycE^{JP})2-2 is likely to be allelic to the previously identified gene *phyllopod* (*phyl*). During eye development, Phyl (in association with Sina) is required for the degradation of the Tramtrack transcriptional repressor during R1, R6 and R7 photoreceptor differentiation (Li *et al.*, 1997; Tang *et al.*, 1997). Although *phyl* is also known to be expressed in undifferentiated and precluster cells and immediately posterior to the MF, it has not been determined whether *phyl* has a function in these cells. The suppression of the *DmcycE^{JP}* rough eye phenotype by halving the dosage of *Su(DmcycE^{JP})2-2* indicates that Phyl is a negative regulator of the G1 to S phase transition immediately posterior to the MF. Whether Phyl is required to degrade Tramtrack in this context is not clear.

In contrast to the role of *Su(DmcycE^{JP})2-2* as a negative regulator of the G1 to S phase transition during eye development, *Su(DmcycE^{JP})2-2* acts as a positive regulator of *cyclin E* transcription during embryonic PNS divisions. Based on the role of Phyl in regulating the degradation of Ttk during photoreceptor differentiation, Phyl may be required to target the Ttk transcriptional repressor for degradation in embryonic PNS cells, allowing *cyclin E* transcription. Consistent with the 69kDa isoform of Ttk acting as a direct transcriptional repressor of *cyclin E*, five Ttk69 binding sites were identified in the enhancer region required for *cyclin E* expression during PNS divisions. How does *Su(DmcycE^{JP})2-2* act as a positive regulator of the G1 to S phase transition during embryonic development, but as a negative regulator during eye development? The answer to this is not clear. One possibility is that Phyl targets different proteins to degradation in these different developmental contexts. Further characterisation of the *Su(DmcycE^{JP})2-2* mutant phenotype, clonal analysis and investigation of the effects of overexpression of Ttk or Phyl during embryonic and eye development will enable this to be clarified.

9-3.3 *Su(DmcycE^{JP})2-1*

Unlike *Su(DmcycE^{JP})2-2*, which is likely to indirectly regulate *cyclin E* transcription, *Su(DmcycE^{JP})2-1* acts downstream of Cyclin E/Cdc2c complex formation in genetic tests. Mutations in *Su(DmcycE^{JP})2-1* result in third instar larvae that overgrow, fail to pupate and develop melanotic masses at a low frequency. Further analysis of this larval phenotype

demonstrated that *Su(DmcycE^{JP})2-1* is required to negatively regulate proliferation during larval optic lobe development and for normal imaginal disc development. *Su(DmcycE^{JP})2-1* does not, however, appear to be required for the regulation of the G1 to S phase transition during embryogenesis, but is necessary for correct tissue patterning. The identity of *Su(DmcycE^{JP})2-1* has not been determined, although four candidates mapping to the *Su(DmcycE^{JP})2-1* critical region and for which mutations are not currently available, should now be tested. Two of these, the ubiquitin conjugating enzyme UbcD2 potentially involved in regulating Cyclin E degradation, and Nitric oxide synthase (Nos) implicated in negatively regulating imaginal disc proliferation, are previously identified and cloned genes. The other two candidates, a tripartite domain protein and a WD40 repeat protein, were identified by analysing BDGP genomic sequence available for the 32A-E region, and are similar to proteins that have been implicated as tumour suppressors. The most likely candidate for *Su(DmcycE^{JP})2-1* is *Nos*, which is likely to regulate proliferation and apoptosis during imaginal disc development (Kuzin *et al.*, 1996), consistent with the *Su(DmcycE^{JP})2-1* zygotic mutant phenotype. In addition, as Nos activity has been implicated in a wide range of biological activities (reviewed by Bicker, 1998), mutations may therefore be expected to have pleiotrophic effects, consistent with the phenotype of embryos lacking *Su(DmcycE^{JP})2-1* expression. Identification of the *Su(DmcycE^{JP})2-1* gene will require molecular characterisation of the lesions associated with four *Su(DmcycE^{JP})2-1* alleles. The identification of *Su(DmcycE^{JP})2-1* will enable the function of this gene in the regulation of the G1 to S phase transition, and other processes, during development to be elucidated.

9-3.4 Characterisation of other modifiers of *DmcycE^{JP}*

This thesis has described the initial characterisation of homozygous lethal second chromosome suppressors of *DmcycE^{JP}*. While suppressor mutations mapping to the third chromosome are also being analysed (A. Brumby, J. Horsfield and H. Richardson, unpublished), enhancer mutations remain largely uncharacterised. These enhancers are expected to encode upstream positive regulators of *cyclin E* transcription, and downstream effectors of Cyclin E-mediated entry into S phase. One enhancer identified in the genetic interaction screen corresponds to a mutation in the Cyclin E-associated kinase, Cdc2c. Altering the dosage of *cdc2c* using this allele, *cdc2c^{JS}*, enhanced the *DmcycE^{JP}* rough eye phenotype by reducing the number of S phases during eye development, indicating that Cdc2c is required to positively regulate the G1 to S phase transition. In addition, characterisation of the mutant phenotype of *cdc2c^{JS}* revealed that this allele results in

lethality at the larval/pupal boundary, and that imaginal disc and brain lobe S phases were compromised. These results are consistent with the predicted role of Cdc2c as the kinase partner for Cyclin E. As sequence analysis has demonstrated that there are no mutations within the coding or intron regions of *cdc2c*^{JS}, further work is required to investigate the molecular basis of this mutation.

As described in the introduction, NPAT and the spliceosome associated protein SAP155 are likely to be targets of mammalian Cyclin E/Cdk2 activity (Seghezzi *et al.*, 1998; Zhao *et al.*, 1998). Mutations in homologues of these genes might therefore be expected among the enhancers of *DmcycE*^{JP}, the characterisation of which would allow their function in the G1 to S phase transition to be determined *in vivo*. In addition, alleles of genes identified in other genetic interaction screens are expected to be identified as modifiers of the *DmcycE*^{JP} rough eye phenotype. Alleles of genes identified as *DmcycE*^{JP} dosage sensitive interactors such as *E2F*, *cdc2c* and *dacapo* have also been isolated in a dominant genetic interaction screen carried out using an eye phenotype generated by the overexpression of *cyclin E* under the control of the *sevenless* promoter (*sev-cyclin E*) (C. Lehner, pers. com.). Alleles of the *DmcycE*^{JP} suppressors, *Brahma* and *Moira*, have also been identified in a genetic interaction screen for enhancers of overexpression of *E2F* and *DP* (*GMR-E2F/DP*) during eye development (Staehling-Hampton *et al.*, 1999). Thus, other modifiers identified in such screens may be expected among the uncharacterised *DmcycE*^{JP} modifiers. One gene identified as an enhancer of *sev-cyclin E* and *GMR-E2F/DP* is *polycephalon* (*poc*) (Staehling-Hampton *et al.*, 1999; C. Lehner, pers. com), which maps to 21A1-B6 on the second chromosome. The identification of loss of function *poc* alleles as enhancers of sev-*cyclin E* and *GMR-E2F/DP* has implicated Poc as a negative regulator of the G1 to S phase transition during eye development. How Poc acts to regulate proliferation in this context is unknown. No characterised suppressor of *DmcycE*^{JP} maps to 21A-B6, although only 7 second chromosome suppressor complementation groups have been assigned a cytological location. Further investigation of the mechanism by which *poc* acts to regulate the G1 to S phase transition during eye development awaits the cloning of this gene. Alleles of the downstream components of tyrosine kinase signalling cascades *pointed*, *Ras1* and *rolled* were also identified as enhancers of *GMR-E2F/DP* (Staehling-Hampton *et al.*, 1999), and may be expected among the suppressors of *DmcycE*^{JP}. Furthermore, alleles of the patterning genes *patched* and *hedgehog* have also been isolated as dominant modifiers of overexpression of human p21^{CIP1} eye phenotype (*GMR*-p21) (de Nooij, 1998), thus alleles of these genes may be expected as modifiers of *DmcycE*^{JP}. Novel genes identified in these screen would also be

expected to be identified in the *DmcycE*^{JP} genetic interaction screen. Characterisation of modifiers of the *DmcycE*^{JP} rough eye phenotype should therefore complement the characterisation of alleles identified as dominant modifiers of overexpression of *cyclin E*, *E2F/DP* or p21^{CIP1}.

9-4 Final conclusions

The work described in this thesis has indicated a novel role for Cyclin E in regulating the G2/M regulators, String and Cyclin A. In addition, the random mutagenesis undertaken has led to the isolation of novel regulators of the G1 to S phase transition during *Drosophila* embryonic and larval development and provided a basis for the discovery and characterisation of novel mechanisms in the regulation of the G1 to S phase transition during development. Due to the highly conserved nature of cell cycle regulators, G1/S regulators identified in *Drosophila* are likely to have comparable functions in mammalian systems.

Table 9.1 Summary of homozygous lethal second chromosome suppressors

Complementation Group	Alleles	Cytological location	Rescue S phases?	Stage lethal	GMR-*cyclin E* type I	GMR-*cyclin E* type II	*roughex*	Candidates
Su(DmcycE^{JP})2-1	*23S9, 27S3, E2S31, E6S2*	32A-E	yes	3rd larval instar	Enhanced	Enhanced	no effect	UbcD2, Nos, WD40 protein, Tripartite domain protein
Su(DmcycE^{JP})2-2	*28S2, 38S4, 39S2*	51A1-5	yes	Embryonic	no effect	no effect	no effect	Phyllopod
Su(DmcycE^{JP})2-3	*59S16, 65S12, l(2)36Fd*	36F7-37A1 to 37B2-8	yes	Pupal	no effect	no effect	no effect	l(2)36Fd
Su(DmcycE^{JP})2-4	*26S8, 57S6, 59S3*	41D2-E1 to 42A1-2	Yes	Before third larval instar	no effect	no effect	no effect	Act42A
Su(DmcycE^{JP})2-5	*42S11, 58S12, l(2)04524*	42B3-5 to 42C1-7	Yes	Pupal	no effect	no effect	no effect	EB1 homologue
Su(DmcycE^{JP})2-6	*41S1, 42S7, 42S13, 42S14, 65S4, 66S4, 67S7, E3S17, E3S18, E3S31*	57F5-6 to 58A1-2	Yes	Larval/pupal	no effect	no effect	no effect	HmgD, HmgZ, Su(var)57D58D, Su(tor)2-180
Su(DmcycE^{JP})2-7	*14S3, 19S3, 40S5, 42S3, 55S2, 57S1, 62S9, 64S10. 64S19, 65S19, 65S23, E10S15*	60E6	Yes	nd	no effect	no effect	no effect	None
Su(DmcycE^{JP})2-8	*E6S4, E6S19*	nd	Yes	nd	no effect	no effect	no effect	nd
Su(DmcycE^{JP})2-9	*25S11, E1S4*	nd	nd	nd	nd	nd	nd	nd
Su(DmcycE^{JP})2-10	*65S5, 65S13, E10S34*	nd	nd	nd	nd	nd	nd	nd

Summary of the characterisation of homozygous lethal second chromosome suppressors. Allele names, cytological location, the effect on *DmcycE^{JP}* S phases, stage of lethality, effect on overexpression of *cyclin E* or *roughex* (*rux^{3}*)rough eye phenotypes and any candidate genes for these second chromosome suppressors are given. Abbreviations used are Ubiquitin conjugating enzyme D2 (UbcD2), Nitric oxide synthase (Nos), High mobility groups protein-D or Z (HMG-D and HMG-Z), nd= not determined.

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Well, that's it.... Who's for beer?