TRANSCRIPTIONAL STUDIES OF BACTERIOPHAGE 186

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SUMMARY

This thesis describes an investigation of the control of transcription of bacteriophage 186 during lytic development following heat induction of a 186*cIts* prophage.

Preliminary characterization of transcripts as control or structural was based on filter hybridization to DNA from two phage, Hy5 and 186. A hybrid between the right 34.6% of 186 and the left 70% of the closely related phage P2 encodes the tail genes D and E (the exchange occurs in gene E) and all of the known control functions namely genes A and B, the origin of replication, *c*I, *c*II, *int*, *att* and *dho* the gene proposed to initiate the inhibition of host DNA synthesis. Early transcripts originated exclusively from this part of the 186 chromosome. During the late phase structural genes were also transcribed. Early transcription of A*am* phage was reduced and phage defective in either A or B function did not have normal late gene expression.

Attempts to analyse A*am* and B*am* transcripts by hybridization to Southern transfers of restriction endonuclease generated fragments of 186 DNA were unsuccessful, and the need to isolate pure fragments of known genetic function arose. Restriction fragments of 186 were cloned into the plasmid vector pBR322 and the genes encoded by each fragment were identified by marker rescue experiments. In this way the recombination and restriction maps were correlated to provide a physical map of the 186 chromosome.

Transcription of genes A, B, *c*I and *int*, *c*II and *dho*,

the cohesive ends (which had been cloned into λ) and the two late genes, W and F, cloned on separate fragments, was studied by filter hybridization to recombinant DNA. Transcripts of wild type phage and phage with a mutation in gene A or gene B were analysed. Genes A and B were both required, directly or indirectly, for the normal transition to the late phase; A protein was also needed for the transcription in the early region 87.0 to 93.8% but B*am* early transcription was identical to wild type. The dependence of phage directed transcription on protein synthesis and DNA replication was investigated by the addition of chloramphenicol to inhibit protein synthesis or by the use of a *dnaCts* host at a non-permissive temperature to inhibit phage DNA synthesis.

Transcripts made by the prophage were examined to test the hypothesis that B gene expression is constitutive in the prophage. No B gene transcripts were detected but RNA from the fragment coding for *c*I and also in the 87.0 to 93.8% region was found.

The DNA in the region 87.0 to 93.8% has two distinct functions; during lytic development transcription in this fragment is associated with DNA synthesis and is dependent on the A gene, while in the prophage state this fragment is active suggesting a role in maintaining stable lysogeny.

STATEMENT

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

E.J. Finnegan.

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ABBREVIATIONS

cfu	-	colony-forming units
c.p.m.	-	counts per minute
DNA	-	deoxyribonucleic acid
EDTA	-	ethylenediamine tetraacetate
EM	-	electron microscope
eop	-	efficiency of plating
EtBr	-	ethidium bromide
kb	-	kilobase (1,000 base-pairs)
moi	-	multiplicity of infection
mrf	-	marker rescue frequency
mRNA	-	messenger ribonucleic acid
PEG	-	polyethylene glycol
pfu	-	plaque-forming units
TCA	-	trichloroacetic acid
Tris	-	Tris(hydroxymethyl)aminomethane
UV	-	Ultraviolet light.

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SECTION I

GENERAL INTRODUCTION

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GENERAL INTRODUCTION

1. Phage 186

Phage 186 is a temperate bacteriophage of *E. coli* with a genome consisting of a single piece of nonpermuted double stranded DNA of molecular weight 19.7 x 10^6 daltons (Wang, 1967). The phage was isolated by Jacobs and Wollman (1956) who classified it with the 'non-inducible' phage; a later study proved that 186 is, in fact, inducible and that it shares the induction properties of the lambdoid phage (Woods, 1972; Woods and Egan, 1974).

However, 186 clearly does not belong to the same group as the other inducible phage which are closely related to λ; they are able to recombine, have similar morphology and cohesive ends which can anneal to form mixed dimers between λ and related inducible phage (Baldwin *et al.*, 1966; Yamagishi *et al.*, 1965). 186 differs not only in its cohesive ends but also in its size, morphology and ability to recombine with other phage in the lambdoid group.

2. Comparison of P2 and 186

P2 a truly non-inducible phage is the best characterized phage of this group. P2 and 186 show many similarities both in the DNA of their genomes and the morphology of the phage particle.

P2 and 186 have cohesive ends which differ in only two of the nineteen residues (Padmanabhan and Wu, 1972; Murray and Murray, 1973), and these can anneal to form mixed dimers (Mandel and Berg, 1968). Electron microscope heteroduplex studies have shown that the two phage share homologous DNA

sequences especially in that part of the genome coding for phage structural components (Skalka and Hanson, 1972; Younghusband and Inman, 1974). Contrasting with this is the non-homology, under the electron microscope, of the control sequences located on the right third of the genome.

The genetic distribution of functions is very similar (Hocking and Egan, 1979) both are morphologically similar under the electron microscope (Bertani and Bertani, 1971) and viable hybrids consisting of the structural section of the P2 genome combined with the 186 control genes have been formed by *in vivo* recombination (Bradley, Ong and Egan, 1975).

Although P2 and 186 have many properties in common there are, nevertheless, some differences, the most striking being the difference in inducibility. The agents which induce a λ prophage such as UV irradiation, mitomycin C and nalidixic acid also cause induction of the 186 prophage (Woods and Egan, 1974) while P2 is non-inducible (G. Bertani, 1968). Two factors contribute to the non-inducibility of P2; firstly the P2 repressor is not inactivated by UV irradiation (G. Bertani, 1968) and second, even when repression is lifted by subjecting a prophage with a temperature sensitive repressor to non-permissive temperatures, the prophage fails to excise (L.E. Bertani, 1968).

The inability of P2 and related phage to excise forms the basis for our interest in 186. It is hoped that a comparison of P2 and 186 will provide an insight into the reason for this difference and thus to an increased understanding in the events controlling integration and excision.

It appears that the inability of P2 to excise lies in the failure of the prophage to produce *int* (Bertani, 1970) which is needed for both integration and excision (Choe, 1969). Bertani (1970) has suggested that integration of P2 physically separates the *int* gene from its promoter, that is the operon is split by integration, and therefore that the phage *att* site must lie between *int* and its promoter. The direction of *int* transcription can be predicted from this model and from the mapping data which has placed *int* to the right of *att*. Leftward transcription of *int* would invalidate the model while the finding of a rightward *int* transcript would support the idea; proof would come with the relative locations of *att* and the *int* promoter.

A 186 prophage can excise, and since *int* is essential for excision, it is probable that 186 *int* is transcribed from the prophage and therefore that the control of *int* differs in the two phage.

Another difference between the two phage lies in the activation of late gene transcription. 186 is unaffected by a host mutation *gro*, which is unable to support P2 late transcription even though early development is unaffected (J.B. Egan, pers. commun.; Sunshine and Sauer, 1975). Furthermore, P4, a satellite phage cannot transactivate the structural genes of a 186 prophage, as it does from P2, although it can use 186 phage components during a mixed infection (E.R. Six, pers. commun.). The 186 control genes were able to activate late transcription in the hybrid phage even when the recombination occurred to the left of A, B and *ogr* the P2 genes required for P2 late transcription, effectively deleting these genes from the hybrid genome

(Hocking, 1977; Sunshine and Sauer, 1975). A comparison of late gene activation in P2 and 186 may increase the understanding of the molecular mechanism involved. This is of general interest because the α subunit of RNA polymerase is implicated in P2 late control.

3. Transcription Studies

DNA/RNA hybridization is a powerful technique for the analysis of phage directed RNA transcripts. The information gained can be increased by restricting transcription with mutant phage or by limiting growth conditions. In addition careful selection of DNA templates will allow the expression of defined tracts of DNA to be investigated.

(a) Deletion studies

Hybridization of phage RNA to deletion phage has provided information about the transcription of defined segments of the genome. Comparison between two parallel single step hybridizations to homologous phage DNA, one of which has a specified region of DNA deleted or substituted, provides information about RNA hybridizing to the region in question (Kourilsky *et al.*, 1971). This calculation may be associated with a large statistical error if the two hybridization values are large and the difference between them is relatively small. Judicious selection of phage with large deletions or substitutions makes this subtraction technique quite accurate.

Hybrid phage often provide a suitable basis for studying RNA from a segment of the genome. It is important that the exact location of the exchange and the extent of

semispecific homologous sequences are determined by heteroduplex analysis. Several well characterized λ.φ80 hybrids have been used in the study of λ transcripts (Bøvre and Szybalski, 1969).

The accuracy of transcription studies can be increased by multistep hybridization. In the first step, total RNA is hybridized to DNA from a suitable deletion phage. Phage specific RNA is then eluted and a second hybridization to separated strands of phage DNA determines the direction of transcripts not covered by the deletion. In this way defective deletions, that can only be maintained as a lysogen, can be used to select segment specific RNA by hybridization to the total DNA of the lysogen. Host RNA is subsequently removed by hybridization to purified phage DNA (Bøvre *et al.*, 1968).

A variation of the multistep procedure in which RNA is exhaustively hybridized to excess DNA containing a small deletion permits the isolation of RNA from the deleted region. The supernatant enriched for RNA corresponding to the small deleted region is then hybridized to homologous DNA in which this region is present as well as to a control which has the same deletion. Immunity specific RNA of λ and the λb2 region have been investigated in this way (Kourilsky *et al.*, 1971; Bøvre and Szybalski, 1969).

(b) Sheared DNA

The separation of sheared half molecules by Hg^{++}/caesium sulphate density gradient centrifugation (Wang *et al.*, 1965) provided another means to study phage

RNA from a relatively well defined portion of the genome. λ half molecules have been separated in this way (Hershey *et al.*, 1965). Hybridization to purified left and right halves of the P2 chromosome formed the basis of a study of P2 transcription reported by Geisselsoder *et al.* (1973).

(c) Isolated phage DNA strands

Phage DNA strands can be separated on poly rUG/ caesium chloride density gradients and then used in the hybridization of phage transcripts. The asymmetric distribution of P2 transcripts throughout the lytic cycle has been reported by Lindqvist and Bøvre (1972). Orientation of the two strands of λ with respect to the genetic map (Wu and Kaiser, 1967) has allowed the direction of transcription of each strand to be determined.

Two-step hybridization experiments in which separated DNA strands are used in the second step allows the direction of transcription of defined DNA segments to be determined (Bøvre *et al.*, 1971).

(d) Restriction fragments

The discovery of restriction endonucleases has provided the means to fragment DNA reproducibly into discrete pieces. Danna and Nathans (1971) first reported the location of restriction fragments on the SV40 genome, and since then restriction fragments have been used in the analysis of transcripts of a number of viruses, both eukaryote and prokaryote. Restriction fragments separated by agarose or polyacrylamide gel electrophoresis were recovered from the gel matrix and used in the hybridization of RNA

from SV40 (Khoury *et al.*, 1975; Sambrook *et al.*, 1973) and adenovirus (Petterson *et al.*, 1975).

Southern (1975) developed a technique for denaturing restriction fragments *in situ*, after electrophoresis, with the subsequent transfer of the fragments on to nitrocellulose. Known early mRNA from T7 infected cells has been hybridized to Southern transfers of *Hpa*I restriction fragments to identify T7 fragments coding for early genes (McAllister and Barrett, 1977). *Hpa*I fragments have also been used to study the appearance of late transcripts and *in vitro* transcripts of T7 DNA (McAllister and Wu, 1978; McAllister and McCarron, 1977).

Restriction fragments give the best resolution of all the DNA templates discussed for the hybridization of phage transcripts. In addition, any segment of DNA essential or otherwise can be isolated and used in hybridization assays without the need to select appropriate deletion phage. If no restriction cuts are located around the genes of interest, then overlapping fragments can be used in much the same way as overlapping deletion phage.

(i) Restriction enzymes

The existence of a restriction-modification system in bacterial cells was first recognised when it was observed that the ability of phage to plate on different cells was dependent on the strain in which the phage were propagated (Luria and Human, 1952; Bertani and Weigle, 1953).

Arber and Dussoix (1962) demonstrated

that following the replication of modified phage DNA in a non-modifying host, modification was retained only by phage possessing one or both parental DNA strands. Unmodified DNA was rapidly degraded to nucleotides in a restricting host. They concluded that restriction and modification act on the phage DNA and postulated that two distinct enzymatic processes were involved. The first required the recognition of a specific DNA sequence followed by double stranded DNA cleavage, while the second modified this recognition site to prevent restriction. This system provides the bacterial cell with an effective defense against invasion by foreign DNA while leaving the modified host DNA undamaged.

Digestion of unmodified DNA and the protection afforded by modification was demonstrated *in vitro* with an isolated enzyme fraction from *E. coli* K12 (Meselson and Yuan, 1968) and *E. coli* B (Linn and Arber, 1968).

The recognition of two distinct modes of restriction heralded further advances. Class I restriction endonucleases, such as those isolated from *E. coli* K and *E. coli* B recognise a specific nucleotide sequence and then cut the DNA at random (Murray *et al.*, 1973) leading to a heterogenous range of products. These enzymes characteristically require ATP, Mg^{++} and S-adenosylmethionine as cofactors for their activity (Lautenberger and Linn, 1972; Eskin and Linn, 1972).

In contrast, class II enzymes which in general require only Mg^{++} cleave DNA at discrete sequence specific sites. The first enzyme of this type was purified from *Haemophilus influenzae* serotype d in 1970 (Smith and Wilcox, 1970; Kelly and Smith, 1970). Since then more than 80 specific endonucleases have been described in both Gram-negative and Gram-positive bacteria, pointing to a widespread distribution throughout the bacterial world (see Roberts, 1976 for a review of this field). At least some of these enzymes are encoded on plasmids, for example, *Eco*RI and *Eco*RII (Arber and Morse, 1965; Bannister and Glover, 1970; Smith *et al.*, 1976). Although there is evidence for involvement in restriction-modification for some of these enzymes (Bron *et al.*, 1975; Yoshimori, 1971) the *in vivo* role of many is unknown. It is possible that such enzymes are involved in site specific recombinations (Chang and Cohen, 1977).

The value of class II restriction endonucleases was first realized by Danna and Nathans (1971) who utilized the enzyme purified from *Haemophilus influenzae* d to analyse SV40 DNA. The discrete fragments generated by these enzymes have been used in genome mapping, gene isolation, *in vitro* genetic recombination and sequence analysis of DNA from many different prokaryotic and more recently eukaryotic sources (see Nathans and Smith, 1974; and Roberts, 1976 for a review).

(ii) Cloning of restriction fragments

The potential of restriction fragments in the formation of hybrid DNA molecules between DNA from unrelated species was recognized, and two different techniques for cloning were developed.

Jackson *et al.* (1972) used terminal deoxynucleotide transferase to add single stranded homodeoxypolymeric extensions to the 3' termini of *Eco*RI restriction fragments. The addition of poly dA tails to one fragment (A) and poly dT tails to the second DNA (B) ensured the joining of A and B while preventing AA, BB or intramolecular unions. The vector in this experiment was SV40 digested with *Eco*RI and the *gal* operon of *E. coli*, isolated by an *Eco*RI digest of *λdvgal*, was inserted.

Mertz and Davis (1972) observed that *Eco*RI restriction resulted in a staggered cut thus generating short, complementary, cohesive ends. They found that *Eco*RI digested SV40 could be recyclized and that these molecules could be covalently joined by *E. coli* DNA ligase. The sequence that is cut by *Eco*RI was identified (Hedgpeth *et al.*, 1972) confirming that all *Eco*RI sites would produce identical cohesive ends. Any two DNA molecules containing an *Eco*RI restriction site can therefore be recombined at these sites by the sequential action of *Eco*RI and DNA ligase (Mertz and Davis, 1972; Hedgpeth *et al.*, 1972). In addition to the joining of molecules via hydrogen bonded cohesive ends, T4 DNA ligase catalyses the joining of native

DNA molecules by blunt end ligation (Sgaramella, 1972). Although the formation of hybrid molecules by the rejoining of restriction endonuclease generated cohesive ends does not inhibit AA, BB or intramolecular joining, and is thus less specific than the "tailing" method developed by Jackson *et al.* (1972), it is more readily accomplished and has the added advantage of easy dissection and recovery of the inserted DNA, by restriction of the hybrid with the enzyme(s) used in cloning.

The recombinant, formed by either method, is subsequently recovered by the transformation or transfection of *E. coli* (Mandel and Higa, 1970). Propagation of recombinants is possible only if the restructured molecule carries the functions necessary for autonomous replication within the cell. Phage and plasmids replicate autonomously and therefore are suitable vectors for the cloning of foreign DNA. Furthermore, the manipulation of phage λ and the construction *in vitro* of hybrid plasmids has permitted the rapid selection of recombinant molecules by an alteration to the phenotype conferred by the parental vector on the host.

Manipulation of λ has provided suitable vectors for the cloning of *Eco*RI (Murray and Murray, 1974), *Hin*dIII (Murray and Murray, 1975) and more recently for *Bam*HI and *Bgl*II, *Sal*I, *Sst*I, *Xho*I and soon for *Xma*I and *Sma*I (see Murray (1978) for a review of this field). Non-essential DNA has been

deleted to permit the inclusion of foreign DNA, while still keeping the recombinant molecule within the size range that can be accommodated by the λ head particle. Two types of λ vectors have been constructed:

(1) insertion vectors have a single restriction site into which the DNA to be cloned is inserted. Selection of recombinant phage is simplified when the single restriction site lies within the *c*I gene; uncut or rejoined parental molecules form turbid plaques, while the plaques of phage carrying inserted DNA are clear. Fragments of up to 9 to 10 kb can be cloned into insertion vectors (Murray, 1978).

(2) replacement vectors are phage that retain two restriction sites flanking a segment of DNA non-essential for λ development. Formation of a recombinant entails the replacement of this DNA with foreign DNA; the frequency of ligations producing parental molecules is reduced, compared to insertion vectors, because the left and right arms of the parental phage do not contain sufficient DNA to form viable phage without a third fragment. Replacement vectors therefore impose a minimum and maximum size (up to 24 kb for some Charon phage, Blattner *et al.*, 1977) on

the cloned DNA insert. Some include a central fragment that confers a distinctive phenotype on the host cell, thus allowing ready identification of the recombinant (Murray *et al.*, 1977; Blattner *et al.*, 1977). For example, the *Hin*dIII replacement vector used in this work codes for the *E. coli* mutant tRNA gene, *sup*F; parental phage were detected, by the suppression of a *lacZam* mutation in the bacterial host, as blue plaques on agar containing 5-bromo-3-chloro-2-indolyl-β-D-galactoside (XG), while recombinant plaques were colourless on XG agar plates.

Plasmid vectors have been constructed by combining certain features from different plasmids into a single molecule that has the following properties:

(1) a strong positive selective marker for the plasmid.

(2) a unique restriction site in which to clone an insert.

(3) relaxed mode of replication.

Initially the plasmids used were suitable for the cloning of fragments generated by a single endonuclease, for example, *Eco*RI (Hersfield *et al.*, 1974) *Sal*I or *Bam*HI (Hamer and Thomas, 1976).

Bolivar *et al.* (1977) have developed a multipurpose cloning vehicle pBR322. This is a relaxed replicating plasmid coding for the resistance genes to ampicillin and tetracycline; tetracycline resistance is destroyed by insertion at the unique *Hin*dIII, *Bam*HI or *Sal*I sites while the single *Pst*I site lies within the ampicillin gene, and in addition there is a unique *Eco*RI site. The recombinant plasmid can be easily distinguished from the rejoined or uncut parental molecules by the loss of resistance to either tetracycline or ampicillin, depending on the restriction enzyme used for cloning.

The choice of plasmid or phage vector is dependent on the DNA to be cloned as each system has some advantages.

The range of restriction enzymes for which suitable phage vectors are available has been more limited than for plasmids. New phage vectors are being developed but it is still true that for certain restriction enzymes or for fragments generated by mixed digestion plasmid vectors are more convenient. The use of synthetic linker molecules or blunt end ligation has also helped overcome the problem of the unavailability of a suitable vector. Terminal transferase tailing provides another means to join donor and vector DNA without the need for compatible cohesive ends but this method is currently restricted to plasmid vectors because the enzyme will also add homopolymer to the cohesive ends of λ.

The DNA that can be cloned into phage λ is restricted in size which can be advantageous for shotgun cloning of eukaryote genomes. Plasmid vectors impose no restrictions, either upper or lower, on the size of the inserted fragment, however, in practice smaller fragments are cloned more frequently than larger fragments (Collins, 1977; Collins *et al.*, 1976). Small DNA fragments are best cloned into small plasmid vectors because this permits the greatest amplification of the inserted DNA *per se*.

The recent use of *in vitro* packaging of DNA containing the λ cohesive ends has increased the efficiency with which recombinant molecules can be recovered (Hohn and Murray, 1977; Sternberg *et al.*, 1977).

Rapid screening methods for the identification of a particular recombinant have been developed for both plasmid and phage vectors. Grunstein and Hogness (1975) devised a technique for the screening of recombinants by the growing and lysing of colonies on nitrocellulose filters. The DNA was denatured *in situ* and a labelled probe (cDNA or RNA) for the DNA of interest was used in hybridization assays to select for recombinants carrying the DNA complementary to the probe. A similar assay for plaques has been described by Benton and Davis (1977). Miniscreening of plasmids also provides a rapid means for detecting recombinant molecules (Meyers *et al.*, 1976).

4. Transcriptional Controls

The development of phage λ is regulated by the sequential expression of groups of genes and is mediated by phage coded proteins. The proteins required for the expression of other phage genes have been identified and although it is known whether these proteins are positive or negative effectors their mode of action is not fully understood.

The expression of the two control proteins, N and *cro* immediately after λ infection does not rely on any phage coded products. N protein, a positive regulator needed for all subsequent transcription, acts as an antiterminator to allow chain elongation at t_L, t_R, and t_{R2} (Roberts, 1969; Lozeron *et al.*, 1976). There is evidence to suggest that N interacts with a specific site on the λ genome, not at the terminator sites where the effect of N is seen, but adjacent to or perhaps within the two promoters p_L and p_R at which transcription begins (Salstrom and Szybalski, 1978). Interaction between N and the β subunit of RNA polymerase is also indicated (Baumann and Friedman, 1976; Sternberg, 1976).

Late transcription is activated by the Q protein, a delayed early gene, which acts at a single site between genes Q and S. It has been proposed that while complementation testing shows that Q protein acts in *trans* (Campbell, 1961) it functions preferentially on the chromosome from which it was transcribed, that is *cis* (Echols *et al.*, 1976). The mechanism for late gene activation by Q protein is not known.

Two other positive regulators have been identified,

*c*II and *c*III, and these act together to stimulate transcription of the two proteins needed to establish the prophage state. Expression of *int*, which catalyses the site specific recombination needed for integration, and of *c*I, the phage repressor, is co-ordinately regulated, although two different sites of action are implicated (Katzir *et al.*, 1976; Chung and Echols, 1977). It has been suggested that *c*II/*c*III allow the elongation of λ *oop* RNA to cover *c*I (Honigman *et al.*, 1976) and that the activation of *int* transcription may be due to the antitermination of a 3S RNA in the *int* region of λ DNA (Honigman, Hu and Szybalski, cited by Chung and Echols, 1977). In addition to stimulating *int* and *c*I transcription it has been suggested that *c*II/*c*III act as negative regulators of late gene expression (Oppenheim *et al.*, 1977b).

Negative control of the genes required for the lytic cycle is mediated by *c*I, and the repressor binding sites, o_L and o_R, mapped adjacent to *c*I have been sequenced (Maniatis *et al.*, 1974; Maniatis *et al.*, 1975; Pirrotta, 1975). Each operator contains three adjacent repressor binding sites; the terminal sites o_{R1} and o_{L1} which are adjacent to the genes under their control have a higher binding affinity for the repressor than the other sites (Ptashne *et al.*, 1976). It has been shown that the repressor protein inhibits the binding of RNA polymerase at the early promoters p_L and p_R (Steinberg and Ptashne, 1971). The binding of repressor to o_{R1}, the site with the strongest binding affinity in the right operator, stimulates *c*I transcription as well as preventing rightward early transcription, and thus *c*I is a positive regulator of its own expression

(Meyer *et al.*, 1975).

A second negative control protein, *cro*, binds to the same three sites in o_R as *c*I although the binding affinities differ (Johnson *et al.*, 1978). It also acts near p_L to inhibit N synthesis (Herskowitz, 1973) but it has not yet been confirmed that the left *c*I and *cro* binding sites are coincident. *Cro* therefore prevents transcription from p_L and p_R as well as inhibiting repressor synthesis from p_{rm} (Oppenheim *et al.*, 1977a). It appears that like *c*I, *cro* also has a dual role, acting both as a repressor to turn off early transcription and as an activator of the synthesis of late proteins (Oppenheim *et al.*, 1977b).

It is probable that the interaction between the two control circuits, *c*I, *c*II and *c*III which favours lysogeny and inhibits late gene expression and *cro* which turns off the synthesis of *c*I, *c*II and *c*III and thereby favours lytic development, determines the outcome of any phage infection.

P2 is the best characterized phage from another major group, the non-inducible phage. Some control elements important in phage transcription have been identified and these differ in some respects from the control genes of phage λ.

Late transcription is normally dependent on DNA replication and the two phage replication genes, A and B, are required for late expression (Lindqvist and Bøvre, 1972; Geisselsoder *et al.*, 1973). It is not known if A and B are directly involved in the activation of late genes or if they are merely needed to provide a replicating template. The

identification of a host mutation (*gro*) that inhibits P2 late transcription allowed the isolation of mutants in a third phage gene (*ogr*) needed for the activation of late genes (Sunshine and Sauer, 1975). The *gro* mutation lies in the α subunit of RNA polymerase (Fujiki *et al.*, 1976) suggesting that P2 *ogr* may interact with this subunit and thus differ from λ N which interacts with the β subunit (Baumann and Friedman, 1976; Sternberg, 1976).

A phage repressor protein C has been identified and a single DNA binding site mapped by mutation (Lindahl, 1971). It is not known if other proteins analogous to λ *c*II and *c*III are required for gene C expression, nor if P2 has a λ *cro* protein. It is evident that the control of *int* must differ in P2 and λ because a P2 prophage does not produce *int* even after repression has been lifted (Bertani, 1970).

Thus λ and P2, which represent two major families of temperate coliphage (Bertani and Bertani, 1971), apparently show differences in their transcriptional control. An investigation of the control mechanisms of 186, which is classed as a member of the P2 family by morphology and yet shares the induction properties of the lambdoid phage, will be of value to the field of transcriptional control in a number of ways:

(1) a comparison of *int* control and thus the control of integration and excision of 186 and P2 should reveal the basis for the difference in excisability of the two phage.

(2) a comparison between the controls of P2 and 186 development should indicate whether 186 is more closely

related to the P2 or the lambdoid phage on this criterion.

(3) if 186 is like P2 and so differs in its transcriptional control from λ then a study of these mechanisms may increase the knowledge in the area of protein/DNA interactions which is relevant to both prokaryote and eukaryote gene control.

(4) there is increasing evidence for the integration of tumour virus DNA or the DNA equivalent of an RNA genome into the host genome (Doerfler, 1975). An understanding of the control of the prophage state in prokaryotes may increase the understanding of the factors important in the alteration of a cell containing a repressed tumour virus to the transformed state in which some viral genes are active.

5. Aim of This Work

The primary aim of the work presented in this thesis was to determine which genes are important in the control of transcription during 186 lytic development and in the maintenance of the prophage. A secondary aim was to construct a physical map by the correlation of the recombination and restriction maps for future use in studies of 186.

SECTION II

GENERAL MATERIALS AND METHODS

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GENERAL MATERIALS AND METHODS

A. MATERIALS

All bacterial and bacteriophage strains used in this work are described below.

1. Bacterial strains

The bacterial strains used in this study are described in Table 2.1 and Table 2.2.

2. Bacteriophage strains

Derivatives of phage 186, P2 and λ have been used in this study and are described below.

(a) Phage 186 strains

186*cIts*: a heat inducible mutant of 186 (Baldwin *et al.*, 1966).

186*cI*: a clear plaque mutant of 186 which fails to complement 186*cIts* (Huddleston, 1970).

186D*am*48: an amber mutant derived from wild type 186, obtained as a gift from A.D. Kaiser.

186*cIam*53*vir*1: a virulent phage, able to grow on a 186 lysogen, isolated as a spontaneous clear plaque mutant in a stock of the phage 186*cIam*53 (Woods, 1972).

186*am*1 to 186*am*47, 186*am*51, 186*am*52: amber mutants isolated by UV mutagenesis of 186*cIts* by W.H. Woods in this laboratory.

TABLE 2.1. Bacterial Strains

Collection number	Relevant phenotype	Genotype	Origin or reference
E. coli K12			
C600	Sup^-	F^- *thr leu thi lac*Y *ton*A *sup*E	Appleyard (1954).
W3350	Sup^+	F^- *gal*K *gal*T	Campbell (1965).
E251	Sup^+	F^- *gal*K *gal*T *str*748	This laboratory, by transduction of the str^r allele from CGSC 4214 into W3350.
E320	Sup^+	F^- *gal*K *gal*T *str*748 T_1^r T_5^r	This laboratory, a T_1, T_5 resistant derivative of E251.
E900	*dnaCts*	F^- *thy thi thr leu dnaCts*	This laboratory, by transduction of *dnaCts* from PC2 into E237.
E237	$dnaC^+$	F^- *thy thi thr leu*	This laboratory, a thy^- derivative of C600.
E393	*lacZam*	*ton*A *lacZam sup leu trp*A33 Sm^r	A *ton*A derivative of ED8538.
E. coli C			
E282	T_5^r	T_5^r T_6^s T_7^s $\phi 80^s$ $P2^s$	This laboratory, a T_5^r derivative of *E. coli* Cla.

TABLE 2.2. Lysogenic Derivatives of Strains Described in Table 2.1.

Collection number	Prophage	Derivative of:
E252	186*cIts*	E251
E263	186*cItsAam*5	E251
E264	186*cItsBam*17	E251
E321	186*cIts*	E320
E322	186*cItsAam*5	E320
E323	186*cItsBam*17	E320
E562	186*cItsAam*30	E320
E1148	186*cItsDam*48	E3350

186*am*57, 186*am*58, 186*am*60 to 186*am*67: amber mutants isolated by NNG mutagenesis of 186*cIts* by S.M. Hocking in this laboratory (Hocking, 1977).

186*del*2: a deletion phage of 186*cIts* isolated as a heat resistant strain by V.K. Dharmarajah in this laboratory (Dharmarajah, 1975).

(b) Phage P2 strains

P2*vir*1: a weak virulent phage (Bertani, 1960) that affects the expression of the C gene (Lindahl, 1969).

(c) P2.186 hybrid phage

Hy5: a P2.186 hybrid phage isolated in this laboratory by C. Bradley (Bradley *et al.*, 1975).

(d) Phage λ strains

λ: wild type λ (Lederberg, 1951).

λ762: a replacement vector for the *Hin*dIII restriction enzyme. Its genotype is (*sr*Iλ1-2)Δ[*shn*-λ3*sup*F](*att-red*)Δ*c*IΔ*nin*5*shn*λ6°.

λp186*s*RI3-1: a recombinant phage formed by the insertion of 186*s*RI3-1 fragment into λ641 (Hocking, 1977).

λppJF18: a recombinant phage formed by the insertion of pJF18 (Section V) into λ762 by E.J. Finnegan.

3. Plasmid vector

The plasmid vector used for the cloning of 186

restriction fragments described in this work was pBR322 (Bolivar *et al.*, 1977). The location of restriction sites on the genome of this vector is shown in Fig. 2.1.

4. Chemicals

The chemicals used in the methods described in Section II.B are detailed below.

CsCl: Harshaw Chemical Company, Cleveland, Ohio, U.S.A.

PEG 6000: Union Carbide Corporation, New York, U.S.A.

Phenol (Analar AR grade) was redistilled and stored under nitrogen in the dark at -15°C.

Bacto-trytone, Bacto-Agar, Yeast Extract, Nutrient Broth and Casamino Acids were obtained from Difco Laboratories, Detroit, U.S.A.

Tris buffers were prepared from Trizma base (Sigma Chemical Company) and the pH adjusted with HCl.

Scintillation fluid consisted of 3.5 gm 2,5-diphenyloxazole (PPO) and 0.35 gm 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) dissolved in 1 litre of toluene.

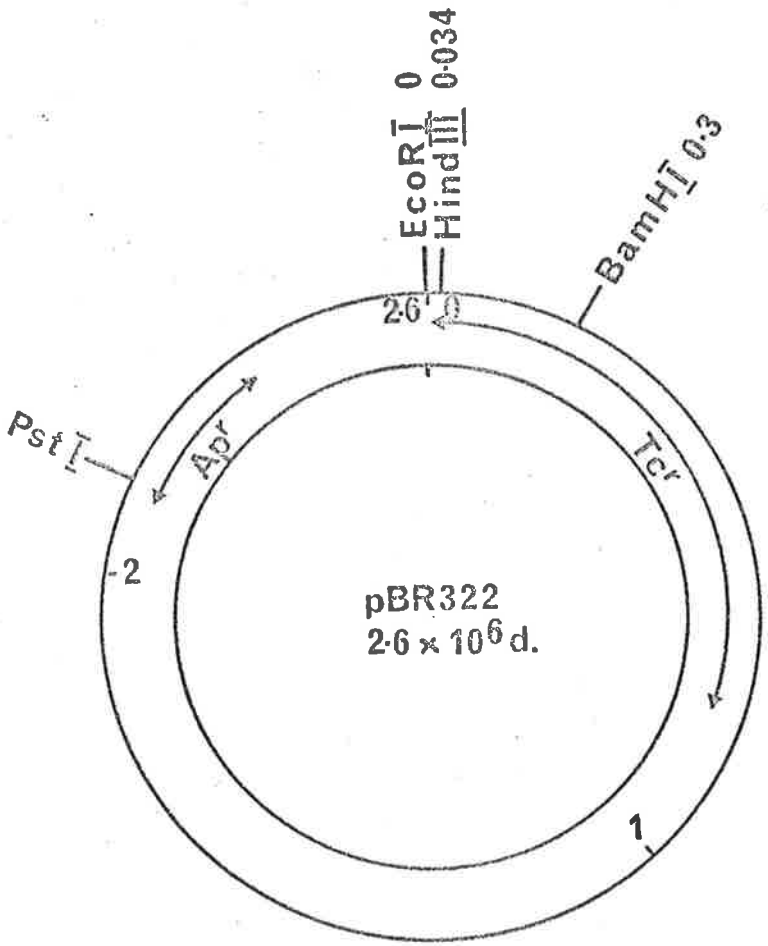
5. Liquid media and buffers

All media and solutions of chemicals were prepared in glass-distilled water and were sterilized by autoclaving for 25 minutes at a temperature of 120°C and a pressure of 15 lbs/inch^2. The components of the various media were as follows:

L broth: 1% Bacto-Tryptone, 0.5% Yeast Extract,

FIGURE 2.1. A Map of pBR322 Genome

The position of the unique restriction sites for *Eco*RI *Hin*dIII, *Bam*HI and *Pst*I are shown in relation to the tetracycline (Tc^R) and ampicillin (Ap^R) resistance genes on the pBR322 genome.


EcoRI 0
Hind III 0·034
BamHI 0·3
2·6
Pst I
Apr
Tcr
2
1
pBR322
2·6 × 10⁶ d.

1% NaCl.

LG broth: L broth supplemented with 0.1% glucose (the glucose was autoclaved separately as a 20% solution and added to sterile L broth).

LGC broth: LG broth to which 0.0024 M $CaCl_2$ was added (the $CaCl_2$ (0.4 M) was autoclaved separately and added to sterile broth).

LGM broth: LG broth to which 0.01 M $MgSO_4$ was added after autoclaving. A stock solution of 1 M $MgSO_4$ was autoclaved separately.

H-1: 0.1 M potassium phosphate buffer pH 7.0, 0.015 M $(NH_4)_2SO_4$, 0.001 M $MgSO_4$, 1.8×10^{-6} M $FeSO_4$. All components were autoclaved separately and added to sterile H_2O.

TM: 0.01 M Tris pH 7.1, 0.01 M $MgSO_4$.

TE: 0.01 M Tris pH 8.0, 0.001 M EDTA.

6. Solid media

Soft agar: 1% Bacto-Tryptone, 0.5% NaCl, 0.7% Bacto-Agar.

T plates: 1% Bacto-Tryptone, 0.5% NaCl, 1.2% Bacto-Agar.

L plates: 1% Bacto-Tryptone, 0.5% Yeast Extract, 1% NaCl, 1.5% Bacto-Agar.

LGC plates: 1% Bacto-Tryptone, 0.5% Yeast Extract, 1% NaCl, 0.0024 M $CaCl_2$, 1.5% Bacto-Agar.

Plates were prepared from 30 ml of the relevant mixture, dried with lids on at 37°C overnight and stored at 4°C until required.

B. GENERAL METHODS

1. Storage of bacteria and bacteriophage

Long term storage of bacterial stocks was either in freeze-dried ampoules maintained at 4°C or in 40% glycerol, frozen at -80°C. Short term storage was in 40% glycerol at -15°C. Glycerol stocks were prepared by the addition of an equal volume of sterile 80% glycerol to a fresh stationary phase bacterial culture.

Low titre bacteriophage stocks (approximately 10^{10} *pfu*/ml) were maintained in L broth, saturated with chloroform, at 4°C. High titre (approximately 10^{12} *pfu*/ml) were dialysed against TM and then stored at 4°C.

2. Growth of bacterial cultures

L broth was routinely used for growth of bacterial cultures.

A stationary phase bacterial culture was prepared by inoculating broth with a one in one hundred dilution of a glycerol stock stored at -15°C and incubating with aeration in a New Brunswick gyrotory water bath at 30°C or 37°C for approximately 16 hours. This culture was stored at 4°C and used for inoculating subsequent stationary phase cultures for two weeks before being discarded. A new culture was then prepared from the glycerol stock.

Indicator bacteria were prepared by inoculating broth with a thirty-fold dilution of a fresh stationary phase culture and incubating with aeration in a shaking water bath at 30°C or 37°C until the required cell density was

attained. The optical density (OD_{600}) was measured on a Gilford 300Ti spectrophotometer and an OD_{600} = 1.0 was found to correspond to approximately 4 x 10^8 *cfu*/ml. Indicator bacteria were chilled and stored on ice until required.

To determine the number of viable bacteria in a culture, 0.1 ml of an appropriate dilution in H-1 was spread on a T or LGC agar plate. This was incubated overnight at 30°C or 37°C and colonies scored.

3. Preparation of lysogens

Sup$^+$ lysogens of 186 *am* mutant phage were prepared by spotting phage (10^9 *pfu*/ml) on to a lawn of *sup*$^+$ bacteria and incubating at 30°C overnight. Bacteria from the centre of this spot were streaked for single colonies and then these were tested for immunity to superinfection by drawing across dried streaks of 186*c*I and 186*c*I*am*53*vir*1 (from stocks at 10^9 *pfu*/ml). Lysogenic colonies showing resistance to 186*c*I and sensitivity to 186*c*I*am*53*vir*1 were purified by an additional two single colony isolation steps, testing at each step for 186 immunity as described previously.

4. Preparation of T_1, T_5 resistant bacteria

T_1, T_5 resistant bacteria were prepared by spotting T_5 phage (10^9 *pfu*/ml) on to a lawn of the bacteria to be made resistant and incubating overnight at 37°C. Bacterial survivors from the centre of the spot were streaked for single colonies and then tested for resistance to T_5 by drawing across a dried streak of T_5 phage (10^9 *pfu*/ml).

Resistant bacteria were purified by an additional two single colony isolations and retested at each step. Resistance to T_5 infection usually confers resistance to T_1 infection and this was found to be true for the bacteria isolated in this way.

5. Titration of phage stocks

Phage were diluted in TM buffer and a 0.1 ml sample was added to 0.2 ml log phase indicator bacteria (6×10^8 *cfu*/ml) and then incubated at room temperature for 20 minutes to allow phage adsorption. Three ml of T soft agar (maintained at 47°C) was added and the contents of the tube gently mixed before being poured over an agar plate. When the agar had solidified the plates were inverted and incubated overnight at 30°C or 37°C. Plaques were scored the following day.

For assays of 186 and λ phage the bacteria were grown in L broth and T plates were used. Indicator bacteria were grown in LGC broth and LGC agar plates were used for assays of P2 and Hy5.

6. Preparation of phage stocks

(a) Low titre 186 stocks

Two methods have been routinely used for the preparation of 186 phage stocks and each usually gives phage titres of the order of 10^{10} *pfu*/ml.

(i) Heat induction

Stationary phase bacteria were diluted

thirty-fold into L broth and incubated at 30°C with aeration to an OD_{600} = 1.2. Cultures were then transferred to a 45°C water bath and shaken by hand for ten minutes. They were then incubated with aeration at 37°C until lysis was complete, or for four hours if lysis did not occur. Bacterial debris was removed by centrifugation (7,800 g, 4°C, 10 minutes), then the supernatant was titred and stored at 4°C over chloroform.

(ii) Liquid infection

186 phage carrying an unconditional clear plaque mutation were stocked in this way.

A single plaque of the phage to be stocked (removed from an agar plate with a pasteur pipette) was added to a flask containing 10 ml L broth. After 15 minutes 0.6 ml log phase bacteria (OD_{600} = 1.0) were added and the infected culture was incubated with aeration at 37°C until lysis was complete, or in the absence of lysis, for four hours. The culture was centrifuged (7,800 g, 4°C, 10 minutes) to remove bacterial debris and the titred supernatant was stored over chloroform at 4°C.

(b) Low titre P2 stocks

To 15 ml LGC broth 0.6 ml log phase bacteria (OD_{600} = 1.0) were added and the culture was incubated at 37°C for 15 minutes prior to infection with P2 phage leached from a single plaque into 1 ml LGC broth. When the culture had started to lyse, as determined by optical

density measurements, 0.3 ml 0.1 M EDTA was added (final concentration 0.002 M) and incubation continued until lysis was complete. Bacterial debris was removed by centrifugation (7,800 g, 4°C, 10 minutes) and the supernatant titred and stored over chloroform.

(c) Low titre λ stocks

(i) Plate stocks

An agar plug containing a single plaque was removed from a plate and added to 1 ml L broth and left at room temperature for 15 minutes to allow the phage to leach into the broth. 0.1 ml of this solution was plated with 0.2 ml log phase bacteria. The plates were incubated at 37°C for six to eight hours and then flooded with 3 ml TM when confluent lysis was apparent. The plates were transferred to 4°C and harvested after a further 16 hours. The phage solution was filtered through a millipore filter (pore size 0.45 μ) to remove bacteria and the filtrate was stored over chloroform at 4°C.

(ii) Liquid infection

Stationary phase bacteria were diluted thirty-fold into LGM broth and incubated at 37°C with aeration to OD_{600} = 1.0. The culture was then infected with phage, prepared in a plate stock, at an *moi* = 1.0. The infected culture was incubated at 37°C with aeration until lysis was complete. The lysate was centrifuged (7,800 g, 4°C, 10 minutes) to remove bacterial debris and the supernatant was

stored at 4°C over chloroform.

(d) High titre 186 stocks

High titre phage stocks were prepared by polyethylene glycol (PEG) precipitation of phage from low titre stocks then purified by CsCl density gradient centrifugation.

(i) Heat induction

500 ml low titre stock of 186*cI*ts phage were prepared by heat induction as described in Section II.B.6(a)(i) except that the culture was incubated at 47°C for 20 minutes instead of 10 minutes. The culture was incubated at 37°C for four hours after induction and then bacterial debris was removed by centrifugation (8,880 g, 4°C, 20 minutes). The supernatant was decanted and NaCl (solid) and PEG 6000 were added to a final concentration of 0.5 M and 10% (w/v) respectively. The solution was stored at 4°C for at least 16 hours and then the precipitate was collected by centrifugation (8,880 g, 4°C, 20 minutes), resuspended in a small volume of TM and purified by equilibrium centrifugation in CsCl (ρ = 1.43 gm/cm^3, 32,000 r.p.m., 24 hours, 4°C, Beckman Ti50 rotor). The phage were recovered by piercing the side of the tube, below the opaque phage band, with a syringe needle and withdrawing the band. The phage were then further purified by a second CsCl equilibrium gradient (as above).

(ii) Liquid infection

500 ml of low titre phage stock were

prepared as described in Section II.B.6(a)(ii), but the culture was infected with phage from a low titre stock (*moi* = 0.1) rather than from a single plaque. Four hours after infection bacterial debris was removed and the phage were purified as described in Section II.B.6(d)(i).

(e) High titre P2 stocks

Stationary phase bacteria were grown in 500 ml LGC broth at 37°C to OD_{600} = 0.8. The culture was then infected (*moi* = 0.1) with P2 phage from a low titre stock, and incubated at 37°C in a New Brunswick gyrotory air shaker. At the beginning of lysis, as determined by OD_{600} measurements, 9 ml 0.1 M EDTA was added. At the completion of lysis phage were purified by PEG 6000 precipitation and CsCl centrifugation (Section II.B.6(d)(i)).

(f) High titre λ stocks

500 ml of low titre λ stock was prepared as described in Section II.B.6(c)(ii) and then purified by PEG 6000 precipitation and CsCl equilibrium centrifugation (Section II.B.6(d)(i)). The initial density of the CsCl was increased to ρ = 1.46 gm/cm^3.

(g) High titre P2.186 hybrid phage stocks

Low titre phage stocks were prepared by induction of 500 ml culture of a C600 lysogen of Hy5 phage as described for 186 *am* phage except that the L broth was supplemented with 0.08 M $MgSO_4$. The phage were purified by PEG 6000 precipitation followed by equilibrium CsCl

gradients (ρ = 1.45 gm/cm^3).

7. Extraction of bacteriophage DNA

Freshly redistilled phenol was equilibrated with TE (78% phenol, 22% TE).

The high titre phage stock, diluted to 1 to 2 x 10^{12} *pfu*/ml was added to an equal volume of TE saturated phenol. The mixture was shaken gently and then the two phases were separated by centrifugation (3,000 g, 4°C, 10 minutes). The aqueous phase was withdrawn using a 'J' pasteur pipette and re-extracted until there was no protein visible at the interface. The phenol was washed with half volume of TE as above and two extractions were dialysed separately against 1 mM EDTA pH 8.0.

The concentration of DNA was determined by reading A_{260} on a Zeiss PMQII spectrophotometer. Purity of DNA prepared in this way was checked by measuring the absorbance over the range 230 to 340 mμ on a Beckman DK-2A spectrophotometer.

SECTION III

HYBRIDIZATION OF 186 mRNA TO Hy5 AND 186 DNA

SECTION III

HYBRIDIZATION OF 186 mRNA TO Hy5 AND 186 DNA

A. INTRODUCTION

Lytic development of a bacteriophage is a strictly controlled process ensuring that the maximum number of progeny are released from an infected cell. Phage development follows the general pattern of early transcription (prereplicative phase) then DNA replication followed by the expression of late genes (postreplicative phase). Mature phage particles are assembled and then the host cell lyses with release of progeny phage to complete the lytic cycle.

Phage λ provides the best understood example for control of development by sequential expression of genes at the transcriptional level (Herskowitz, 1973). Transcripts have been divided into three classes:

(1) immediate early during which genes N and *cro* are transcribed by host machinery immediately after infection/induction,

(2) N protein allows chain elongation of the immediate early transcripts and thus the expression of the phage recombination, replication and Q genes. This is termed the delayed early phase,

(3) the late stage of development is marked by the expression of the structural and lysis genes which are dependent on gene Q for their transcription.

The mechanism by which the lytic cycle is controlled

can be investigated by a study of phage gene transcripts. DNA-RNA hybridization can provide valuable information, the extent of which is increased by increasing the definition of DNA templates as well as manipulation of the infection conditions to alter the mRNA made.

A comparison between RNA made at different times during the wild type lytic cycle will reveal the temporal sequence of transcription only if there are at least two distinct DNA templates for hybridization, each of which derive from a different region of the phage genome. A series of overlapping deletion phage are ideal for the investigation of transcription of any genes which are covered by some but not by other deletions. In multi-step hybridization, step one enriches the supernatant for transcripts of genes included in the deletion, these can then be assayed by a second hybridization with DNA in which this region is present (Bøvre and Szybalski, 1971). Deletion phage are not always available, and so hybrids between the phage under study and a closely related phage may be used instead. One can then assay either for transcripts complementary to the homologous DNA contained in the hybrid or, by a second round of hybridization, for the transcripts read from the DNA which has been replaced in the hybrid phage.

RNA made by phage carrying a conditional mutation under non-permissive conditions may yield information about the role of the mutant gene product in normal transcription. The dependence of normal transcription on protein synthesis can be investigated by the addition of chloramphenicol to inhibit cellular protein synthesis prior to infection/induc-

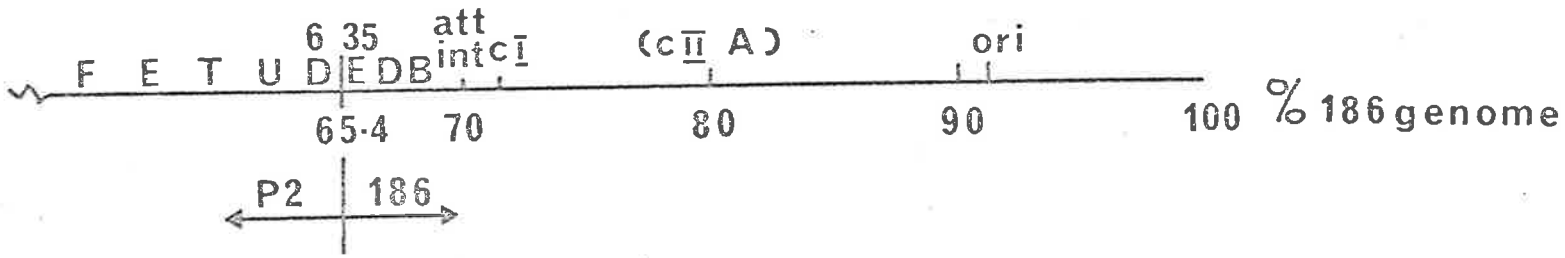
tion. Inhibition of phage DNA synthesis, by choosing phage defective in replication proteins, or by using a bacterial host which cannot provide functions essential to phage replication under certain culture conditions, will reveal which transcripts require DNA replication for their expression.

Only two 186 deletion phage have been isolated (Dharmarajah, 1975) and an E.M. heteroduplex study (R.M. O'Connor, pers. commun.) and restriction mapping (Saint, 1979) of the DNA have shown that the 2.1 kb deletions in 186*del*1 and 186*del*2 were identical. Therefore these phage were of limited use in study of 186 transcription, however, a series of 12 viable hybrids between 186 and the related phage P2 have been constructed *in vivo* (Bradley *et al.*, 1975). One of these, Hy5 (Fig. 3.1), in which the structural genes (0 to 65.4%) of 186 genome have been replaced by 0 to 70% of P2 DNA (Younghusband *et al.*, 1975), has been used in a preliminary characterization of the transcripts made following induction of 186*c*I*ts*, 186*c*I*ts*A*am* and 186*c*I*ts*B*am* prophage.

Inspection of the genetic map of 186 reveals that 65.4 to 100% of the genome (that is the portion which is present in Hy5) encodes the 'control functions' of the phage (Fig. 3.1). The A gene has been shown to be essential for DNA replication and, implicit in its poor complementation with all other cistrons, to be directly or indirectly required for the expression of all other essential genes (Hocking, 1977). Gene B has been described as a control gene, that is it codes for neither a head nor a tail function and, although DNA synthesis precedes more rapidly than for wild type phage, mature phage particles do not accumulate

FIGURE 3.1. The Genetic Map of Hy5

Hy5 contains 0 to 70% P2 chromosome encoding P2 structural genes Q through D and 65.4 to 100% 186 genome which codes for the tail genes D and E (the crossover occurs in gene E), and the control functions B, A, *c*I, *c*II, *int*, *att* and *ori*.


6 35
att
F E T U D E D B int cI
(cII A)
ori
65·4
70
80
90
100
% 186 genome
P2
186

nor does the host cell lyse (Hocking, 1977). Other 186 functions encoded by Hy5 are *int*, *att*, *c*I, *c*II, gene D, the most rightward tail gene, with the crossover point falling in gene E, another tail gene (Hocking, 1977).

Hybridization of 186 mRNA to Hy5 must represent transcripts homologous to the 186 portion of Hy5 and hence be read from the control genes or genes D and E. A comparison between hybridization to Hy5 and 186 DNA will allow a broad classification of transcripts into control (Hy5) and total (186). The difference between these values will give some indication of the proportion of total mRNA which is transcribed from the structural genes.

The percent of input counts hybridized in any assay can only be considered to reflect the rate of transcription if the infecting phage does not affect host RNA synthesis or if it shuts off host synthesis immediately after infection/induction. For this reason the effect of induction of 186*c*I*ts*, 186*c*I*ts*A*am*5 and 186*c*I*ts*B*am*17 prophage on host RNA synthesis was investigated.

The work presented in the section describes the preliminary characterization of the transcription pattern after heat induction of 186*c*I*ts*, and phage mutant in either gene A or B, by hybridization to Hy5 and 186 DNA.

B. MATERIALS

The following materials were purchased from the companies indicated:

[5,6,^{3}H] uridine: 1 mC/ml, 41.5 C/μmole, The Radiochemical

Centre, Amersham, U.K.

Bovine pancreatic ribonuclease Type II-A: Sigma Chemical Company, St. Louis, U.S.A. A stock solution at 1 mg/ml was immersed in a boiling water bath for ten minutes to inactivate deoxyribonuclease. Stock solutions were stored at 4°C.

Nitrocellulose (0.45 μ pore size): 32 cm x 64 cm sheets, from which 24 mm discs were punched, were obtained from Sartorius, 34 Gottingen, West Germany.

Nitrocellulose (0.45 μ pore size): 25 mm diameter discs of B-6 type filter were purchased from Schleicher and Schnell Co., Keene, New Hampshire, U.S.A.

Casamino Acids: Difco Laboratories, Detroit, U.S.A.

Hybridization buffer: Equal volumes of 2 x SSC and 2 x SSC saturated with phenol and adjusted to pH 7.5. Buffer was freshly prepared for each assay.

TES buffer: 0.15 M NaCl, 0.001 M Tris-HCl, 0.01 M EDTA, pH 7.7.

SSC: 0.15 M NaCl, 0.015 M Na_3citrate, pH 7.4.

TPG-CAA: based on a recipe given by Lindqvist (1971) was prepared as follows: To 900 ml water were added 0.5 gm NaCl, 8 gm KCl, 1.1 gm NH_4Cl, 12.1 gm Trizma base, 1 gm KH_2PO_4, 0.8 gm sodium pyruvate. The pH was adjusted to 7.4 with HCl, the volume brought to one L, and the solution autoclaved. To each 100 ml of this medium were added the following components

(sterilized individually) 0.1 ml 0.16 M Na_2SO_4, 0.1 ml 1 M $MgCl_2$, 0.1 ml 1 M $CaCl_2$, 0.1 ml 0.1 mg/ml $FeSO_4$, 1 ml 20% glucose, 4 ml 25% Casamino acids and 7 ml H_2O.

C. METHODS

In addition to the techniques described in Section II:

1. Latent period of 186*cIts* following heat induction

An overnight culture of E321 was diluted forty-fold into fresh TPG-CAA and grown with aeration at 30°C to a density of 2 x 10^8 *cfu*/ml. The culture was then transferred to a 38.5°C gyrotory bath and at the appropriate times after the temperature change 0.1 ml samples were withdrawn and added to 0.9 ml TM saturated with chloroform. At the end of the sampling period the chloroform was blown off and after dilution (where necessary) plaque forming units were assayed.

2. Incorporation of ^{3}H-uridine into TCA precipitable material

An overnight culture of E321 was diluted forty-fold into TPG-CAA and grown with aeration at 30°C to a density of 2 x 10^8 *cfu*/ml. Viable bacteria were assayed and then the culture was transferred to a shaking water bath at 38.5°C (t = 0).

At given times after the shift in temperature, ^{3}H-uridine was added (final concentration uridine 2.4 µM at 5 µC/ml) and incubated at 38.5°C with aeration. 50 µl aliquots withdrawn over a five minute period were precipi-

tated on to a GF/A filter by immersing it immediately in ice-cold 10% TCA. The filters were batch washed four times in ice-cold 10% TCA and then two times in ether. After drying, scintillation fluid was added and radioactivity was determined in a Packard scintillation spectrometer.

3. Total RNA synthesis following 186*cIts* heat induction

An overnight culture was diluted into TPG-CAA and grown, with aeration, at 30°C to a density of 2 x 10^8 *cfu*/ml. Viable bacteria, and where appropriate, infectious centres were assayed and then the culture was transferred to a 38.5°C bath with aeration (t = 0).

At five or ten minute intervals 0.2 ml samples were taken, added to a tube containing 50 µl ^{3}H-uridine (final concentration 2.18 µM, 1 µC/ml) and incubated without aeration for two minutes. The pulse was terminated by the removal of a 50 µl sample that was precipitated on to GF/A filter with ice-cold 10% TCA. The filters were washed as described previously.

4. Preparation of phage DNA filters for hybridization

Phage DNA was diluted in 1 mM EDTA pH 8.0 to a concentration of 2.5 µg/ml and dispensed (2 ml/tube) into glass screw capped tubes. The DNA was denatured by immersing the tubes in boiling water for three minutes and then rapidly chilled in an ice slurry.

Nitrocellulose filters were presoaked for at least 20 minutes and then mounted on to a New Brunswick filtration apparatus and washed with 10 ml 6 x SSC.

To increase the salt concentration to 6 x SSC ice-cold 10 x SSC (3 ml/2 ml DNA solution) was added, mixed and immediately applied to a filter, and allowed to pass through at a flow rate of 2 to 5 ml/minute. The filters were washed first with 10 ml 6 x SSC at the same flow rate, and then with a further 100 ml 6 x SSC (rapid filtration). Filters were air dried overnight prior to baking at 80°C *in vacuo* for three hours, and then stored in tightly capped vials.

5. Amount of DNA/filter

To determine how much DNA was required/filter for conditions of DNA excess, a constant volume of ^{3}H-RNA extract (see Section III.C.6) was hybridized to filters containing increasing quantities of DNA.

The filters for this experiment were prepared as described in Section III.C.4, but the volume of DNA solution and 10 x SSC were varied appropriately to give the required amount of DNA/filter.

6. Labelling and isolation of phage ^{3}H-RNA

An overnight culture of lysogenic bacteria was diluted forty-fold into TPG-CAA and grown, with aeration, at 30°C to a density of 2 x 10^{8} *cfu*/ml. When the required cell density was attained, the culture was dispensed (5 ml into a 50 ml flask) and transferred to a bath at 38.5°C and incubated with aeration (t = 0). The culture was assayed for viable bacteria at t = 0 and for infectious centres at t = 0 and t = 90 minutes.

Cultures were pulse labelled at the appropriate time after transfer by the addition of ^{3}H-uridine (final concentration 2.4 µM and 5 µC/ml or 25 µC/ml), with aeration, for two minutes. The pulse was terminated by the addition of NaN_3 (0.02 M) and by immediately pouring the culture over an equal volume of crushed ice; this was held on ice until all samples had been taken.

When the ice had melted the cells were pelleted 3,000 g, 4°C, 10 minutes, and then resuspended in 0.5 ml TES before being transferred to a clean tube containing 0.5 ml TES, 1% SDS. Cells were lysed by immersing the tube in boiling water for three minutes and then cooled at room temperature for five minutes.

RNA was isolated by three extractions with 1.5 ml water saturated phenol, preheated to 60°C. The phases were separated by centrifugation, 3,000 g, 10 minutes, 4°C, and the aqueous phase was withdrawn with a 'J' pasteur pipette. The salt concentration of the final aqueous phase was adjusted to 2 x SSC by the addition of 10 x SSC.

The total incorporation of ^{3}H-uridine in each extract was estimated by TCA precipitation of 10 µl aliquots on to GF/A filters. Filters were batch washed as described in Section III.C.2.

7. Filter hybridization

For each assay, 0.5 ml of freshly prepared hybridization buffer and 100 µl of ^{3}H-RNA extract (see Section III.C.6) were added to a vial containing the appropriate filters. The vials were tightly capped and incubated at 65 to 70°C

for 20 hours. To prevent non-specific annealing at intermediate temperatures the filters were rapidly chilled and then rinsed with 2 x SSC before being washed with 50 ml 2 x SSC through each side of the filter, mounted on a filtration apparatus.

To remove non-specifically bound RNA, filters were incubated in 2 x SSC containing pancreatic RNA'ase at 20 µg/ml for 90 minutes at room temperature. Filters were again rinsed with 2 x SSC before being washed with 50 ml 2 x SSC through each side of the filter. Finally, each filter was rinsed with 70% ethanol to aid drying. The dried filters were counted in toluene scintillation fluid using a Packard scintillation spectrometer.

D. RESULTS

1. Latent period of 186*c*I*ts* following heat induction

The latent period of infection, that is the elapsed time between the initiation of the lytic cycle and the appearance of progeny phage, is dependent on the growth conditions of the culture. Factors that affect the latent period are richness of media, cell density, bacterial host and, for heat induction, the rapidity in the change in temperature.

The latent period for 186*c*I*ts* heat induction was determined under the following conditions:

(1) shift from 30°C to 38.5°C

(2) E320 host cell

(3) growth medium TPG-CAA

(4) cell density at time of shift 2 x 10^8 *cfu*/ml.

Figure 3.2 shows that cell lysis, with the concurrent release of mature phage, began at about 30 minutes. The latent period (mid point of the burst) was 35 to 37 minutes with lysis being completed by 55 minutes.

These culture conditions have been maintained throughout the work described in this thesis, with the exception of the study using a *dnats* host strain. It was found that the effect of the *dnats* mutation on 186 burst size was more dramatic if infection with 186*cIts* rather than heat induction was used.

2. Incorporation of ^{3}H-uridine into TCA precipitable material

Incorporation of ^{3}H-uridine was followed over a five minute period as described in Section III.C.2. Although a two minute labelling time was used throughout this work it was deemed desirable that the incorporation of labelled precursor into RNA should be linear for more than two minutes to ensure that all mRNA made had equal specific activity. In this way a better estimate of the relative amounts of different RNA species could be obtained by hybridization assays.

Conditions chosen to give linear incorporation (see Fig. 3.3) were a cell density of 2 x 10^8 *cfu*/ml and uridine concentration of 2.4 μM.

3. Does 186 affect host RNA synthesis?

An estimate of the effect of 186 lytic development on

FIGURE 3.2. Latent Period for 186*cIts* Heat Induction

The latent period for 186*cIts* heat induction was determined under the following conditions:

(1) shift from 30°C to 38.5°C

(2) E320 host strain

(3) growth medium TPG-CAA

(4) cell density at time of shift 2 x 10^8 *cfu*/ml.

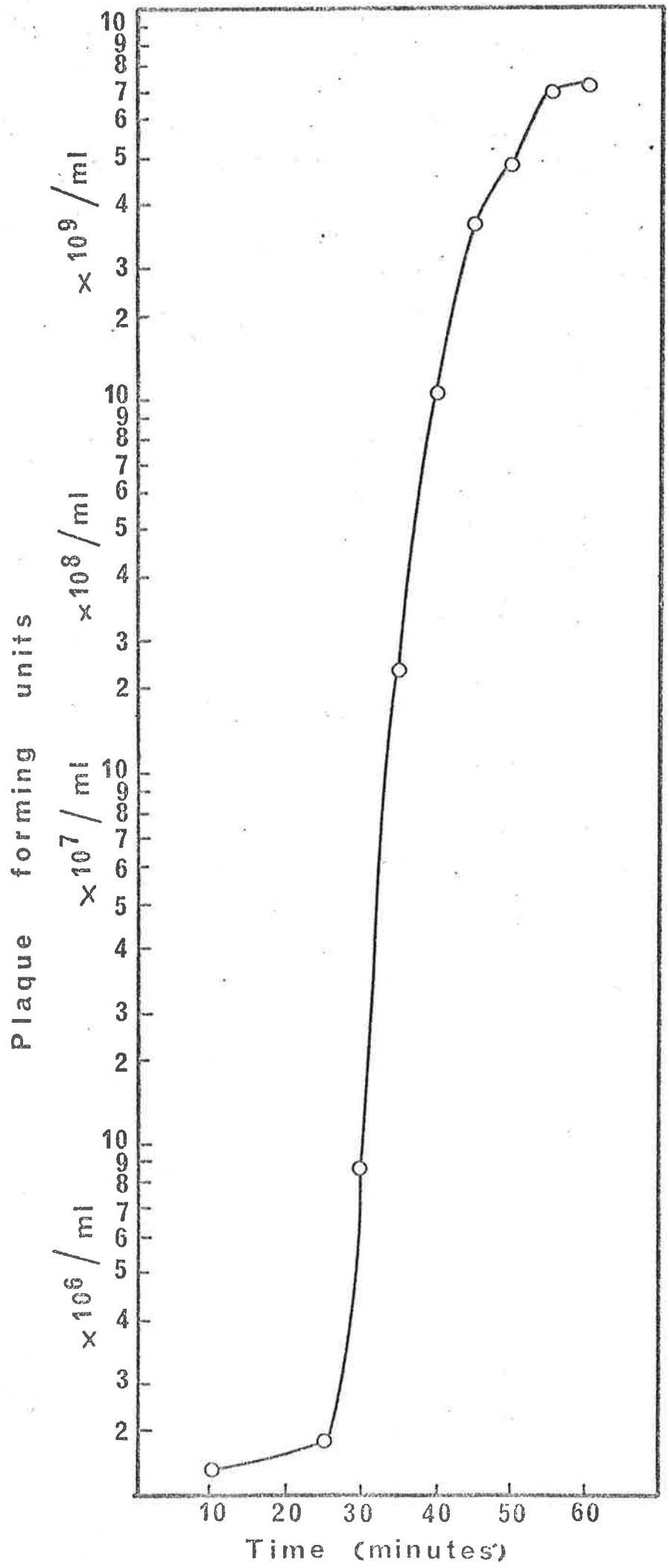
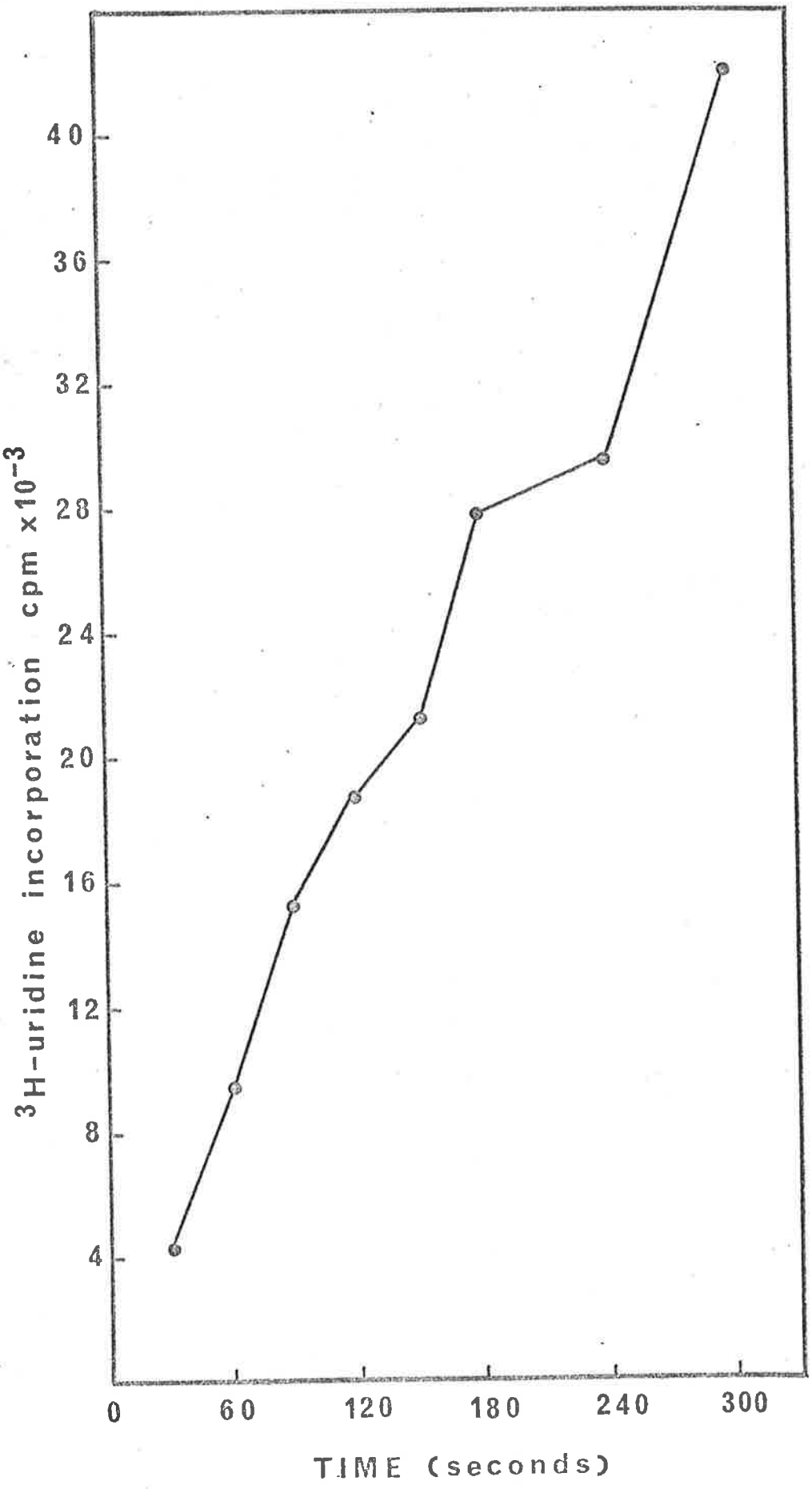

Plaque forming units
10 9 8 7 6 5 4 3 2 × 10⁹ / ml
10 9 8 7 6 5 4 3 2 × 10⁸ / ml
10 9 8 7 6 5 4 3 2 × 10⁷ / ml
10 9 8 7 6 5 4 3 2 × 10⁶ / ml
10 20 30 40 50 60
Time (minutes)

FIGURE 3.3. Incorporation of ^{3}H-uridine into TCA Precipitable Material

The cumulative incorporation of ^{3}H-uridine into TCA precipitable material was measured over a five minute interval at 38.5°C.

The final concentration of uridine was 2.4 µM at 5 µC/ml and the initial cell density was 2 x 10^{8} *cfu*/ml.


40
36
32
28
24
20
16
12
8
4
^{3}H-uridine incorporation cpm x10^{-3}
0
60
120
180
240
300
TIME (seconds)

host RNA synthesis can be obtained by examining the total incorporation of ^{3}H-uridine into TCA precipitable material.

A comparison of the rate of ^{3}H-uridine incorporation during a two minute pulse has repeatedly shown that RNA synthesis by lysogenic and non-lysogenic bacteria was identical for the first 30 minutes after induction of the prophage. This is illustrated by the data presented in Fig. 3.4. Total RNA synthesis of E251, a non-lysogen, increased exponentially during the 60 minute study as cell number increased; in contrast RNA synthesis of the lysogenic strain (E252) slowed and then declined as cell lysis occurred.

The effect on host RNA synthesis of a mutation in either gene A or gene B was also investigated. A comparison of RNA synthesis in E251, E252, E263 and E264 (E251 lysogens of 186*c*I*ts*A*am*5 and 186*c*I*ts*B*am*17 respectively) is illustrated in Fig. 3.5. The rapid decline in RNA synthesis observed at the time of lysis did not occur following induction of a 186*c*I*ts*A*am* or 186*c*I*ts*B*am* prophage, neither of which cause cell lysis. Instead the rate of total RNA synthesis remained constant after this time (cf E251 synthesis) indicating that these mutant phage did affect host metabolism in some way.

The effect of P2 infection on host RNA synthesis was also investigated. Unlike 186, P2 lytic development resulted in the inhibition of host RNA synthesis by ten minutes after infection even though cell lysis did not take place until 22 minutes (see Figs. 3.6 and 3.7).

FIGURE 3.4. The Effect of 186*cIts* Lytic Development on Host RNA Synthesis

The rate of total RNA synthesis was measured by the incorporation of ^{3}H-uridine (1 μC/ml, 2.4 μM) into TCA precipitable material in a two minute pulse.

At t = 0 the culture was transferred from 30°C to 38.5°C. Cell density at the time of the shift was 2 x 10^{8} *cfu*/ml.

○ E251.

● E251(186*cIts*).

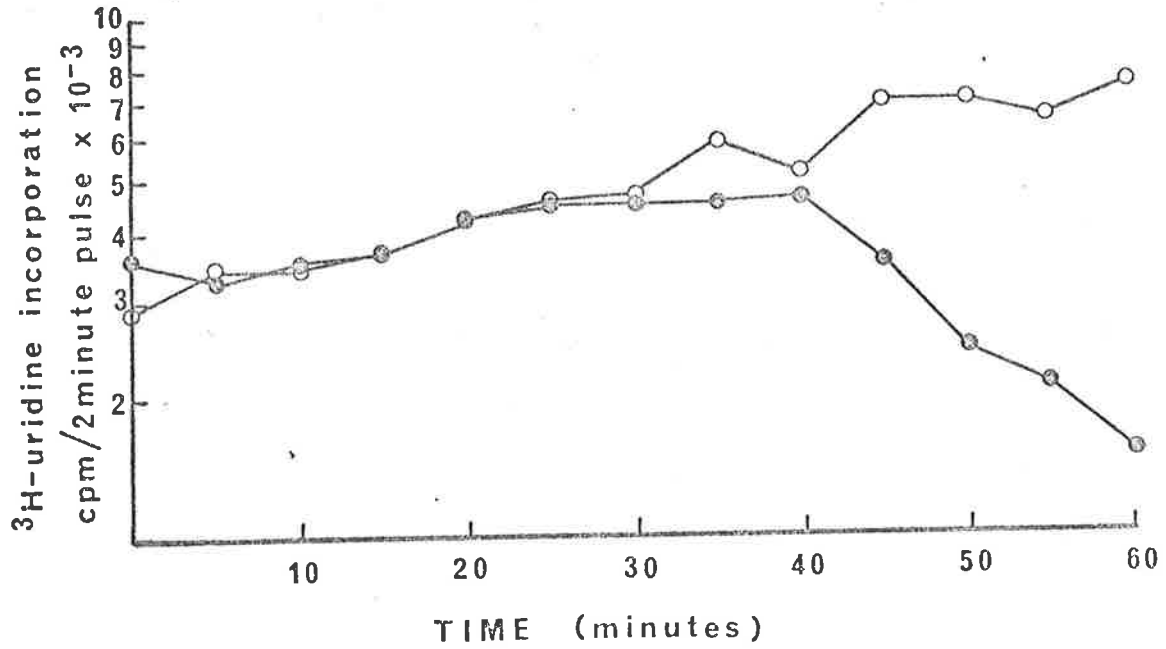

^{3}H-uridine incorporation
cpm/2minute pulse x 10^{-3}
10
9
8
7
6
5
4
3
2
10
20
30
40
50
60
TIME (minutes)

FIGURE 3.5. The Effect of 186*c*I*ts*A*am*5 and 186*c*I*ts*B*am*17 on Host RNA Synthesis

The rate of total RNA synthesis was measured by the incorporation of ^{3}H-uridine (1 μC/ml, 2.4 μM) into TCA precipitable material during a two minute pulse.

The culture was shifted from 30°C to 38.5°C at t = 0 when the cell density was 2 x 10^{8} *cfu*/ml.

○ E251.

■ E251(186*c*I*ts*).

● E251(186*c*I*ts*A*am*5).

□ E251(186*c*I*ts*B*am*17).

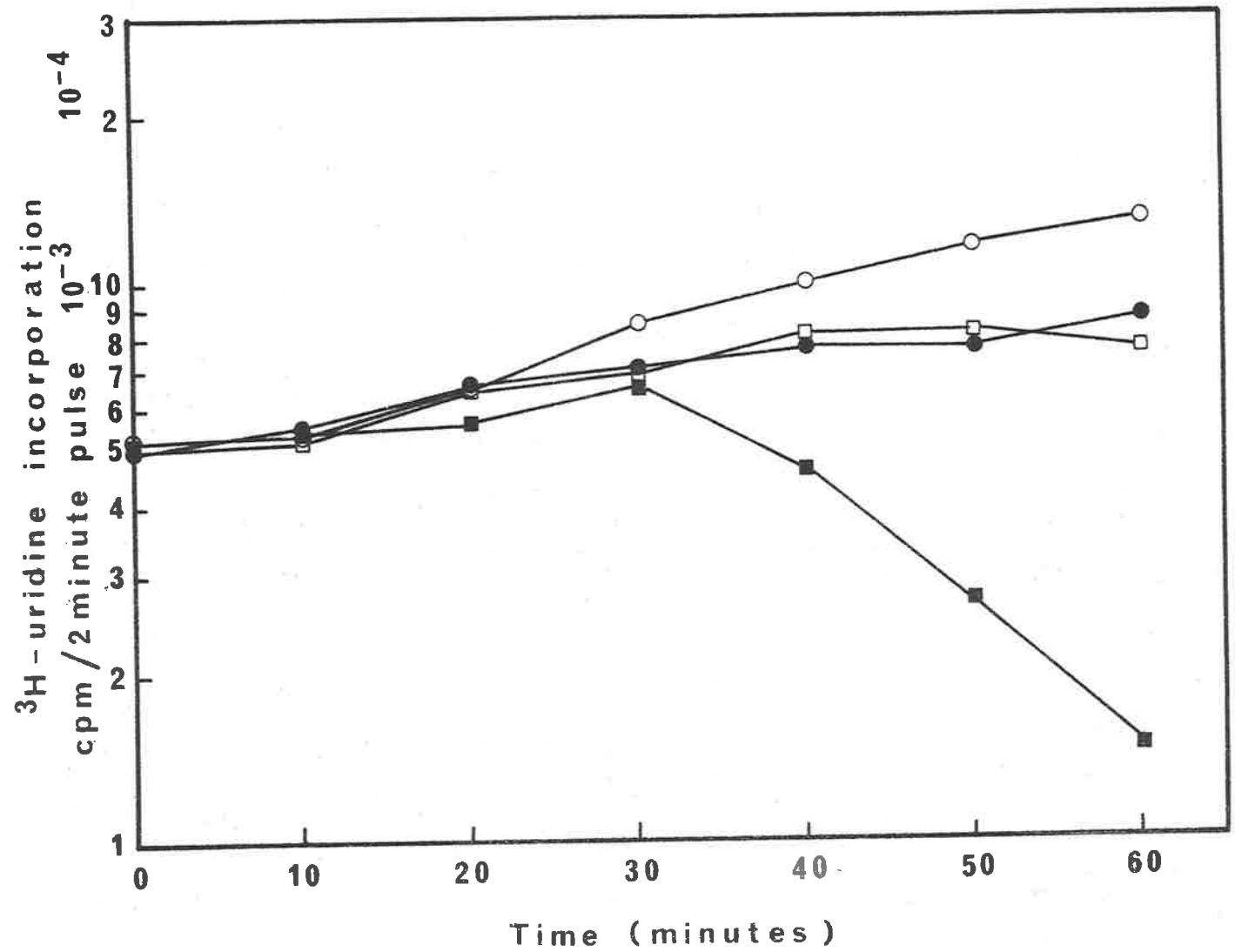

^{3}H-uridine incorporation
cpm/2minute pulse 10^{-3} 10^{-4}
3
2
10
9
8
7
6
5
4
3
2
1
0
10
20
30
40
50
60
Time (minutes)

FIGURE 3.6. Latent Period of P2*vir*1

The latent period for P2*vir*1 infection of E282 grown in TPG-CAA at 38.5°C. The cell density at the time of infection was 2 x 10^8 *cfu*/ml and the culture was inoculated at *moi* = 15. The addition of EDTA at the time of lysis would have prevented the decrease in phage titre observed by t = 40 minutes.

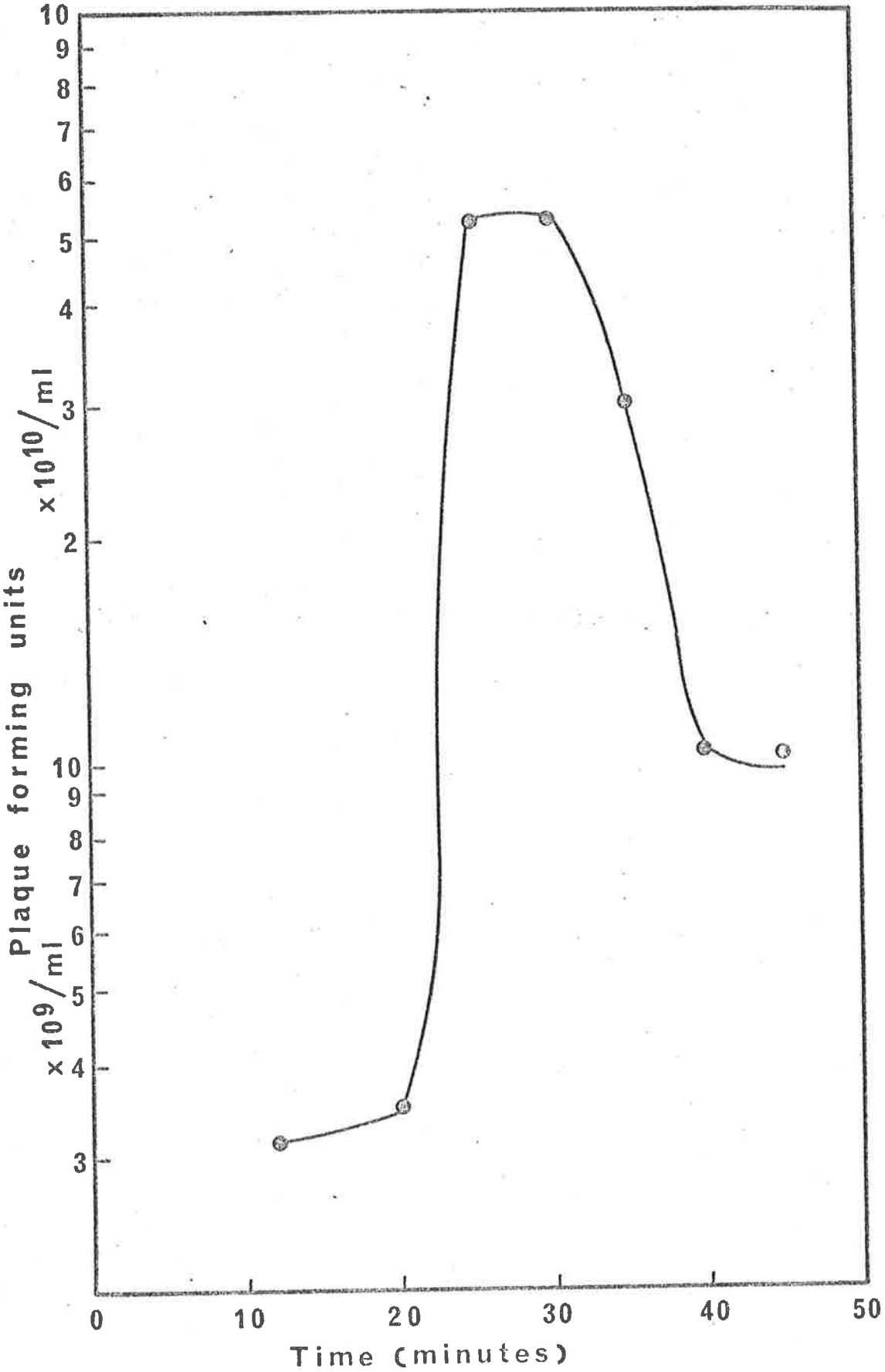

Plaque forming units
x 10^10/ml
x 10^9/ml
10
9
8
7
6
5
4
3
2
10
9
8
7
6
5
4
3
0
10
20
30
40
50
Time (minutes)

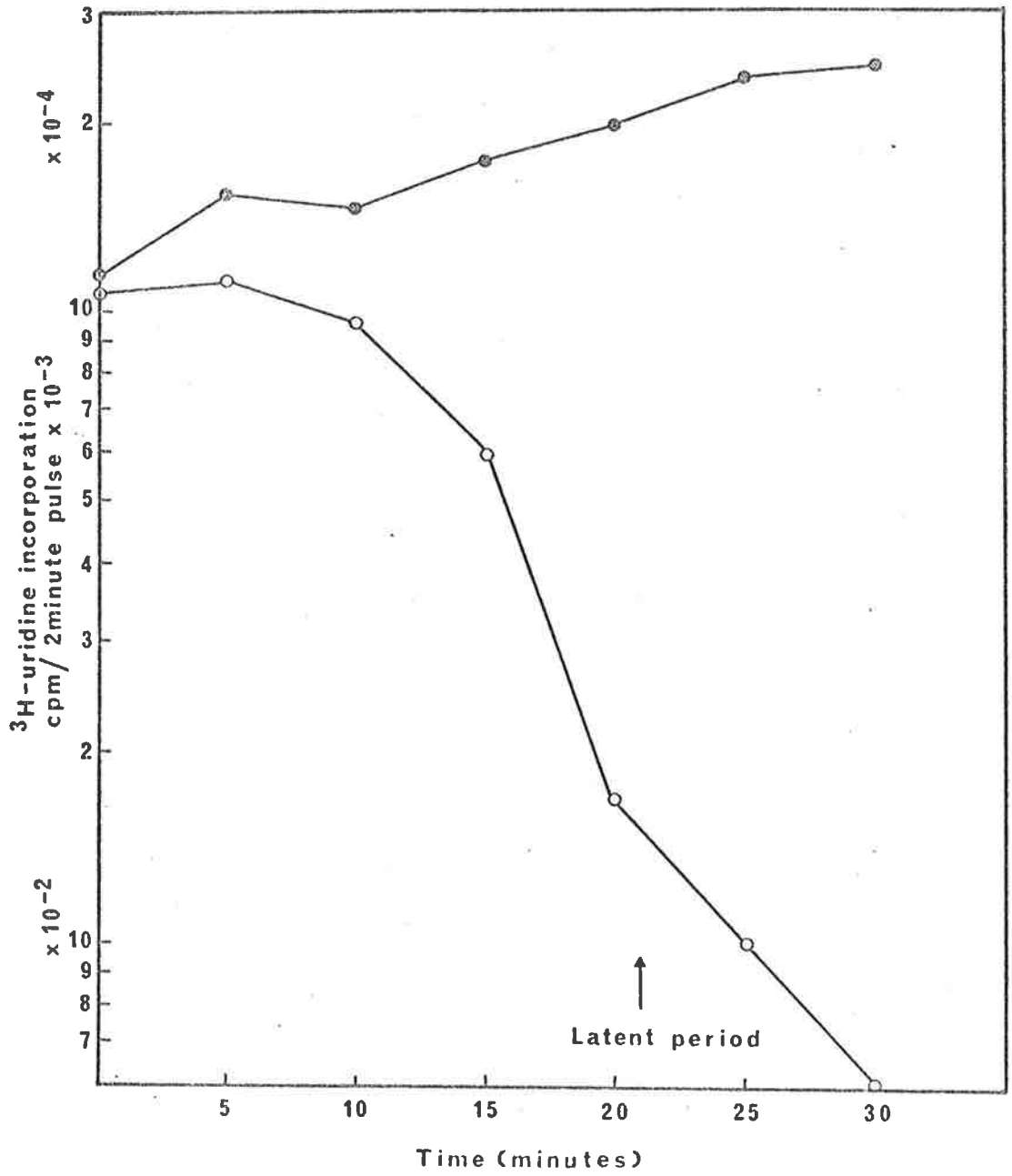
FIGURE 3.7. The Effect of P2*vir*1 Lytic Development on *E. coli* RNA Synthesis

The rate of total RNA synthesis was measured by the incorporation of ^{3}H-uridine (1 μC/ml, 2.4 μM) into TCA precipitable material during a two minute pulse.

E282 was grown in TPG-CAA at 38.5°C to a cell density of 2 x 10^{8} *cfu*/ml. The culture was divided in half and one half was infected with P2*vir*1 at *moi* 15 (t = 0); both cultures were incubated at 38.5°C during the 30 minute labelling period.

● E282.

O E282 infected with P2*vir*1.


3
2
x 10⁻⁴
10
9
8
7
6
5
4
3
2
x 10⁻²
10
9
8
7
³H-uridine incorporation
cpm/ 2minute pulse x 10⁻³
Latent period
5
10
15
20
25
30
Time (minutes)

4. Conditions of DNA excess

When the DNA immobilized on a filter is in excess all complementary RNA will be removed during hybridization and the percent of input counts bound will remain constant even if the amount of DNA/filter is increased. The time of maximum transcription of RNA complementary to the two different DNA templates (186 and Hy5) was determined. Conditions of DNA excess were then investigated by hybridizing a constant volume of this RNA extract to filters containing increasing amounts of DNA. Fig. 3.8 illustrates an experiment of this type. It is evident that for each DNA template 5 μg DNA/filter was more than sufficient to ensure that DNA was in excess. All filter hybridization described in this section have been done using filters containing 5 μg of DNA.

5. Time course of hybridization

A time course of hybridization was taken over a 27 hour period. Hybridization was stopped at various times by rapidly chilling the vial containing the filters; the filters were then removed, rinsed in 2 x SSC and stored at 4°C in 2 x SSC until the end of the sampling period. All filters were treated as described in Section III.C.7. The time course of hybridization to 186 DNA of two RNA extracts prepared at different times after induction is shown in Fig. 3.9. Hybridization was complete after 18 hours of incubation; all subsequent assays have been incubated for 20 hours.

FIGURE 3.8. Conditions of DNA Excess for Filter Hybridization

(a) Hybridization of a constant amount of 186*c*I*ts* ^{3}H-RNA extract to nitrocellulose filters containing different amounts of 186*c*I*ts* DNA.

- ● ^{3}H-RNA labelled at 30 to 32 minutes after induction.
- o ^{3}H-RNA labelled at 31 to 33 minutes after induction.

(b) Hybridization of a constant amount of 186*c*I*ts* ^{3}H-RNA extract to nitrocellulose filters containing different amounts of Hy5 DNA.

The ^{3}H-RNA was labelled at 30 to 32 minutes after heat induction.

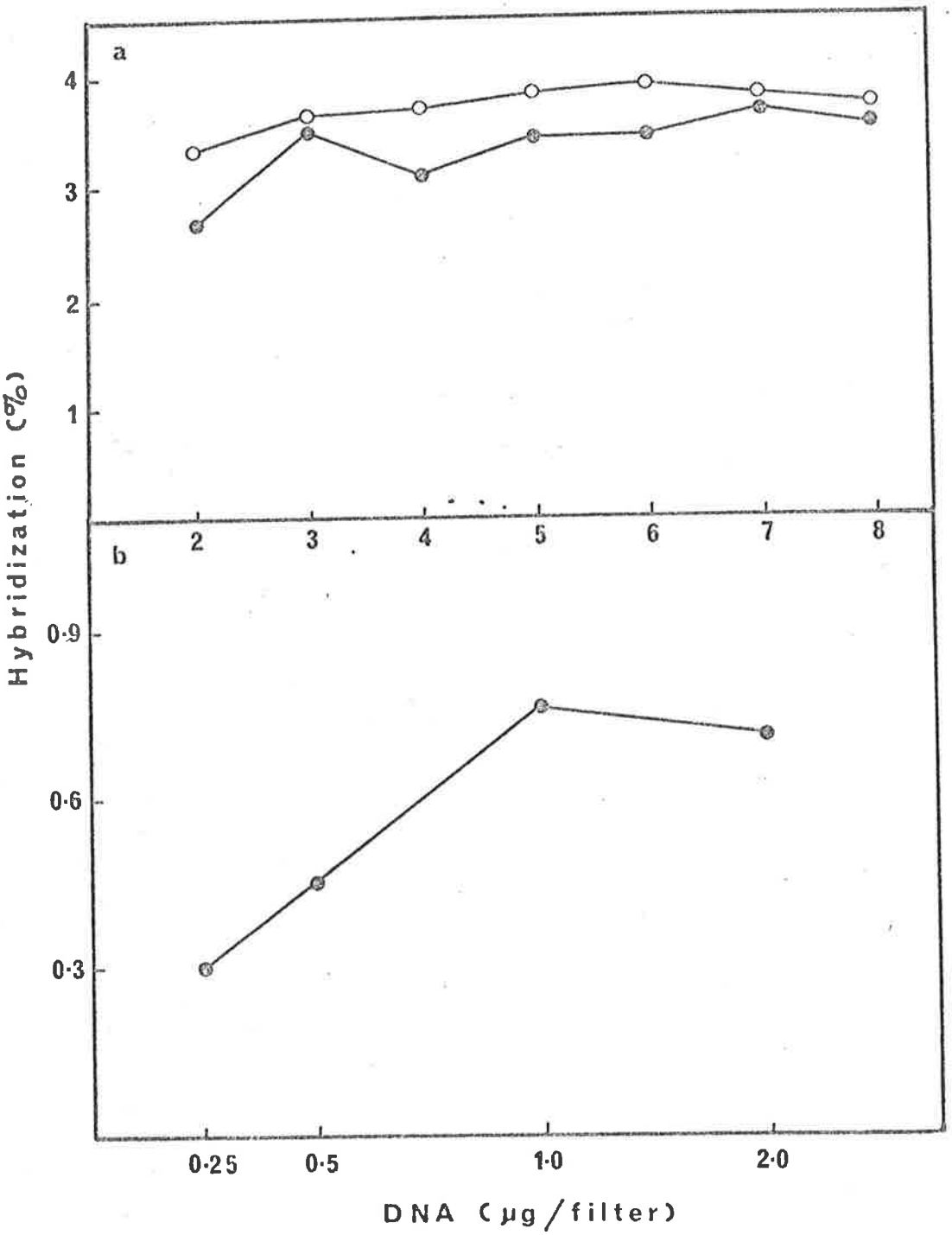
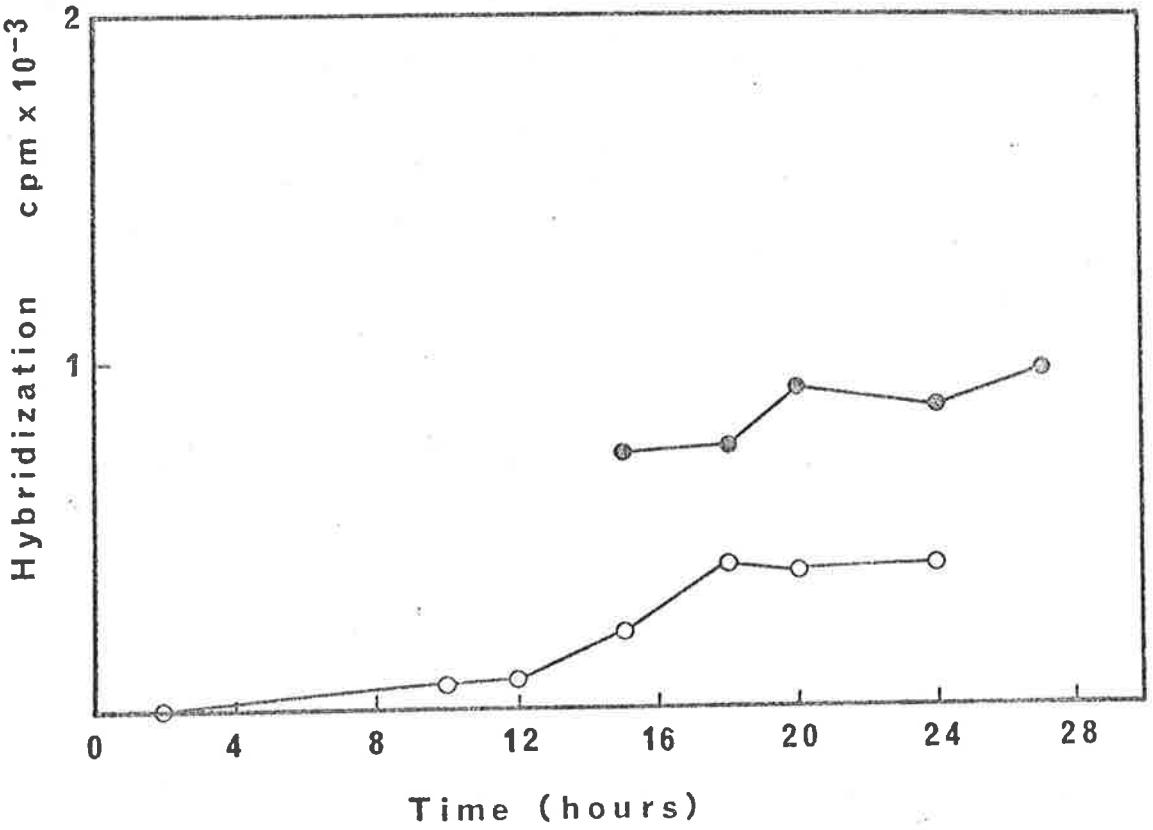

a
b
4
3
2
1
0·9
0·6
0·3
2
3
4
5
6
7
8
0·25
0·5
1·0
2·0
Hybridization (%)
DNA (µg/filter)

FIGURE 3.9. Time Course of Hybridization

The time course of hybridization reaction between 186*c*I*ts* ^{3}H-RNA and 186 DNA immobilized on nitrocellulose was measured over a 27 hour period.

- o ^{3}H-RNA labelled at 20 to 22 minutes after induction.
- ● ^{3}H-RNA labelled at 30 to 32 minutes after induction.


Hybridization cpm x 10^{-3}
2
1
0
4
8
12
16
20
24
28
Time (hours)

6. Non-specific hybridization

Non-specific hybridization between DNA bound to the filter and RNA present in the extract will lead to an overestimation of 186 transcription at any time. There are two sources of non-specific hybridization which may lead to inaccuracies of this kind.

(i) Cross hybridization between P2 DNA and 186 mRNA

Hy5 contains the structural genes of P2 (0 to 70% P2 genome) and the control genes of 186 (65.4 to 100%). Hybridization of 186 mRNA coding for 186 structural genes to the P2 region of Hy5 will lead to an overestimate of the transcription of 186 control genes. It is obvious from the results presented in Table 3.1 that cross hybridization between 186 mRNA and P2 DNA was small and remained constant throughout the lytic cycle.

(ii) Cross hybridization between host RNA and 186, Hy5 or P2 DNA

RNA was prepared from a culture of non-lysogenic bacteria as described in Section III.C.6 and was then hybridized with 186, Hy5 or P2 DNA filters. The results are presented in Table 3.2. The contribution of non-specifically bound host RNA to total hybridization was very small in all cases (for 186 and Hy5 $\leqslant 0.001\%$ and for P2 $\leqslant 0.005\%$) and so can be disregarded.

7. Transcription of 186*cIts*, 186*cItsAam*5 and 186*cItsBam*17

As mentioned in Section III.A, two phage have been used as a source of DNA templates for hybridization. This has

TABLE 3.1. Cross Hybridization of 186 ^{3}H-mRNA to P2*vir*1 DNA Immobilized on Nitrocellulose Filters

Time (minutes)	Input c.p.m.	% Hybridized to 186 DNA	% Hybridized to P2*vir*1 DNA
10-12	2.5×10^6	0.064	0.057
20-22	2.5×10^6	0.39	0.068
30-32	2.9×10^6	1.72	0.056

Each filter contained 5 µg phage DNA.

TABLE 3.2. Hybridization of *E. coli* ^{3}H-RNA to Phage DNA Immobilized on Nitrocellulose Filters

Source of RNA	Time (minutes)	Input c.p.m.	% Hybridized to		
			186 DNA	Hy5 DNA	P2*vir*1 DNA
W3350	10-12	1.2×10^6	0.001	0.001	0.005
W3350	30-32	1.6×10^6	0.001	0.001	0.005
W3350(186*cIts*)	10-12	2.4×10^6	0.31	0.24	-
W3350(186*cIts*)	30-32	1.6×10^6	3.33	0.89	-

Each filter contained 5 µg phage DNA.

permitted the classification of 186 mRNA into two broad categories.

Hy5 (see Fig. 3.1) is known to contain the 186 genes encoded on 65.4 to 100% of the 186 genome, that is the control genes A, B, *c*I and *c*II, the *int* gene and *att* site, as well as gene D and part of gene E, both of which are involved in tail formation. Cross hybridization between P2 DNA and 186 mRNA was insignificant (see Section III.C.6) and so one can conclude that any RNA hybridizing to Hy5 DNA immobilized on filters must be transcribed from the right most 35% of the 186 genome.

The second template used in these hybridization experiments was 186 DNA which will hybridize with all 186 coded mRNA. Hence, the difference between hybridization to the two DNA templates must represent transcripts complementary to the left 65% of the 186 genome and by implication must code for the phage structural genes.

The temporal pattern of 186 transcription is presented in Fig. 3.10. It was not until ten minutes after induction that transcription was detected, and, over the interval 10 to 20 minutes all transcripts were derived from the 'control' region of 186, that is 65.4 to 100%. Later in the lytic cycle (25 to 35 minutes) hybridization to 186 DNA increased rapidly. In comparison, hybridization to the Hy5 DNA increased slowly over the same period. This large difference in hybridization to the two filter types indicates that the structural genes of 186 were being expressed during 25 to 35 minutes of the lytic cycle. This pattern has been repeated in many experiments.

FIGURE 3.10. Transcription of 186*cIts* After Heat Induction of the Prophage

Hybridization of 186*cIts* ^{3}H-mRNA, labelled after heat induction of E251(186*cIts*), to 186 and Hy5 DNA immobilized on to nitrocellulose filters.

The conditions for heat induction and hybridization have been described in Section III.D.1 and Section III.C.7, respectively.

- o 186*cIts* mRNA hybridized to 186*cIts* DNA.
- ● 186*cIts* mRNA hybridized to Hy5 DNA.

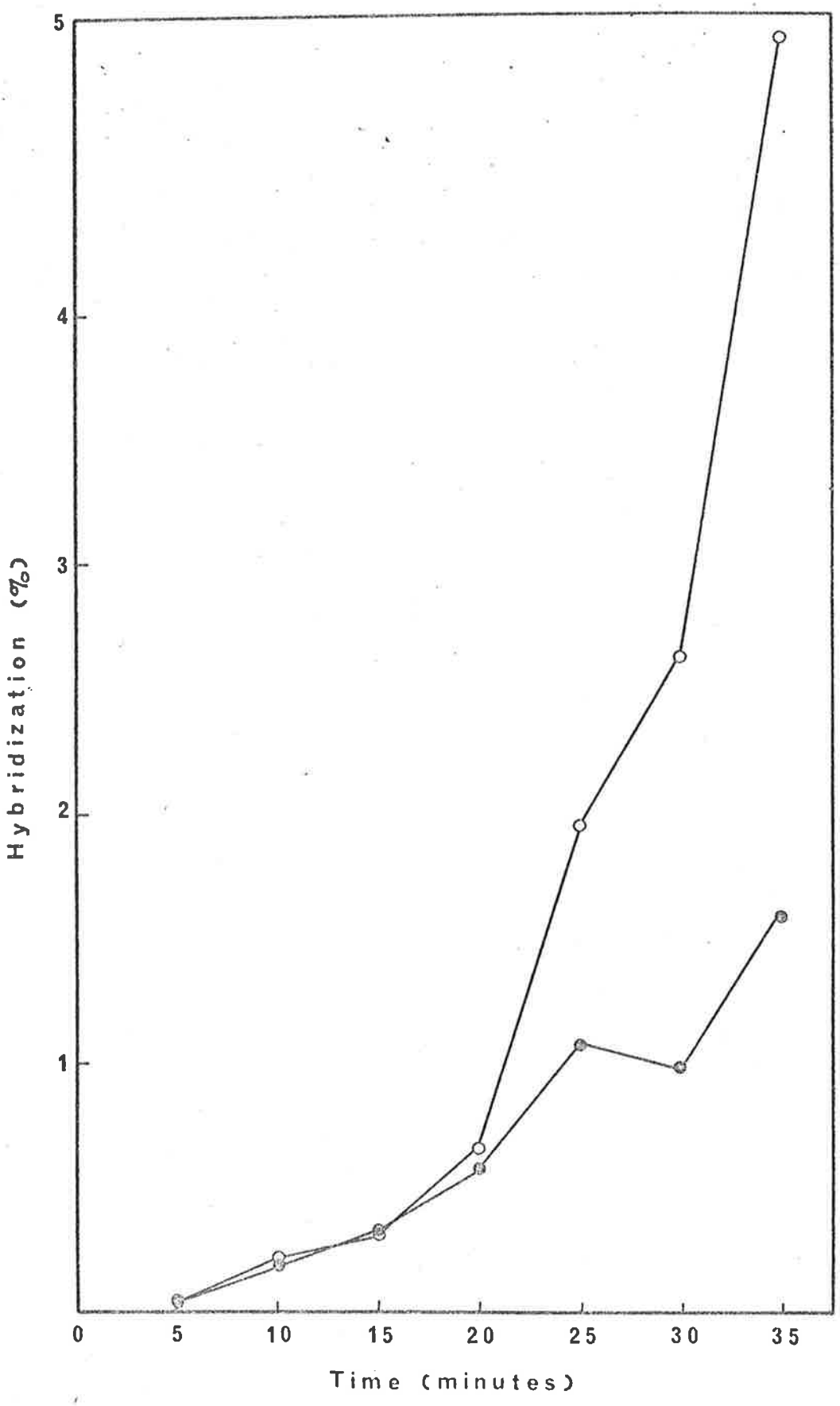

Hybridization (%)
5
4
3
2
1
0
5
10
15
20
25
30
35
Time (minutes)

Fig. 3.11 provides a comparison of the transcripts made following 186*cIts* and 186*cItsAam*5 heat induction. Transcription of 186*cItsAam*5 proceeded at a much slower rate than transcription of wild type phage, and it was confined to the 65.4 to 100% region of 186. One can conclude that late (structural) genes were not transcribed when the A function was absent.

Transcription of a 186*cItsBam*17 phage differed from that observed during 186*cItsAam*5 induction. Transcription of the control region of 186 proceeded at a rate similar to wild type transcription during the first 20 minutes of lytic development. During the second phase of the cycle, in which late genes were expressed by 186*cIts*, transcription of the control region was more rapid than for the wild type phage. It appears that although there was a small amount of hybridization to the 186 DNA filters that could not be attributed to the expression of the control genes, late gene expression was either completely inhibited or greatly reduced in the absence of B product. A comparison between 186*cIts* and 186*cItsBam*17 transcription is presented in Fig. 3.12.

E. DISCUSSION

1. 186 and host RNA synthesis

If phage lytic development does not affect host RNA synthesis or causes its immediate inhibition, then the proportion of labelled RNA which hybridizes with homologous phage DNA reflects the rate of phage directed transcription. Any effect on host RNA synthesis can be investigated by host

FIGURE 3.11. A Comparison of Transcription of 186*cIts* and 186*cItsAam*5

Hybridization of 186*cIts* or 186*cItsAam*5 ^{3}H-mRNA, labelled after heat induction of the prophage, to 186 and Hy5 DNA immobilized on to nitrocellulose filters.

The details for heat induction and hybridization have been described in Section III.D.1 and Section III.C.7, respectively.

- o 186*cIts* mRNA hybridized to 186*cIts* DNA.
- ■ 186*cIts* mRNA hybridized to Hy5 DNA.
- ● 186*cItsAam*5 mRNA hybridized to 186*cIts* DNA.
- □ 186*cItsAam*5 mRNA hybridized to Hy5 DNA.

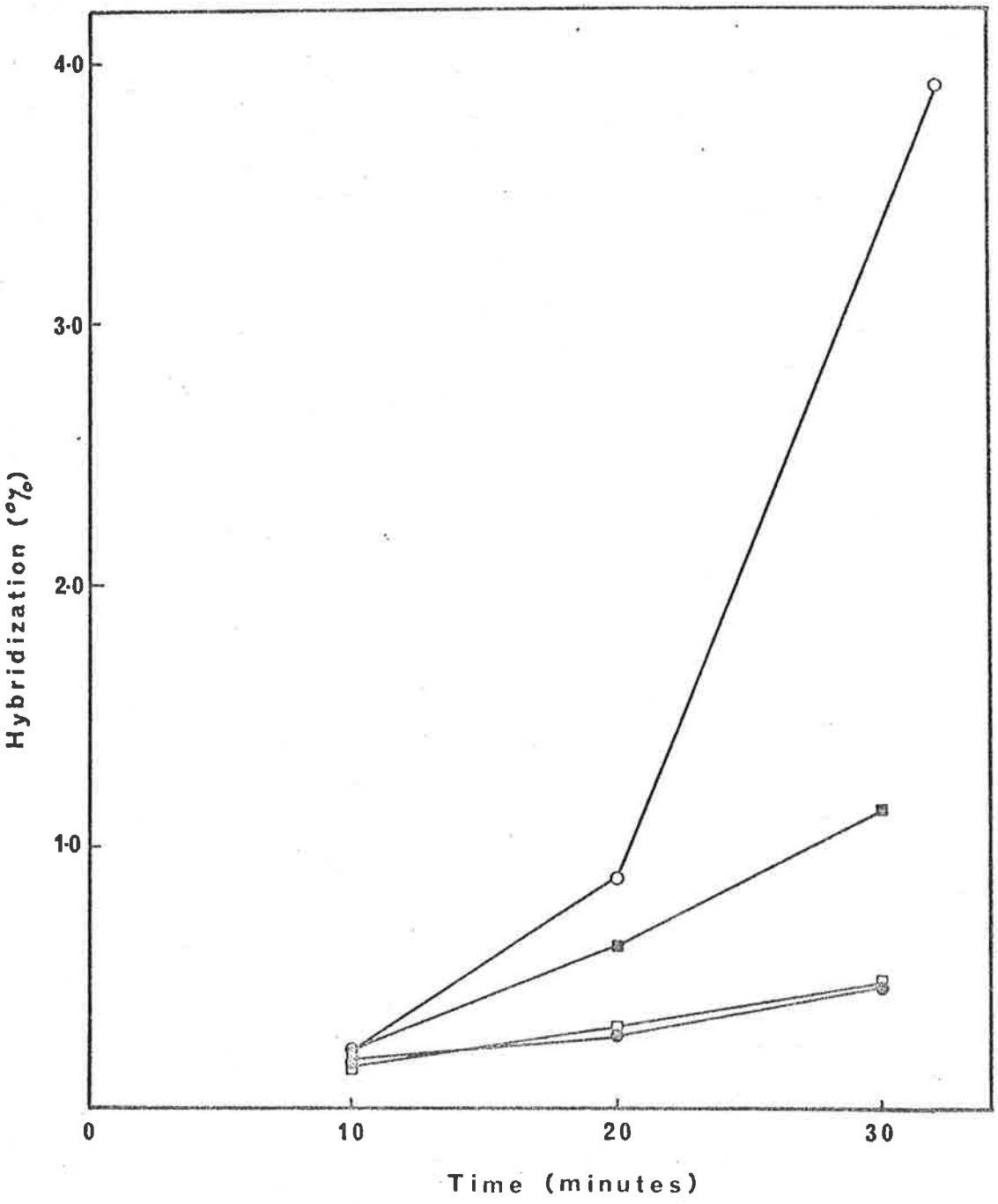
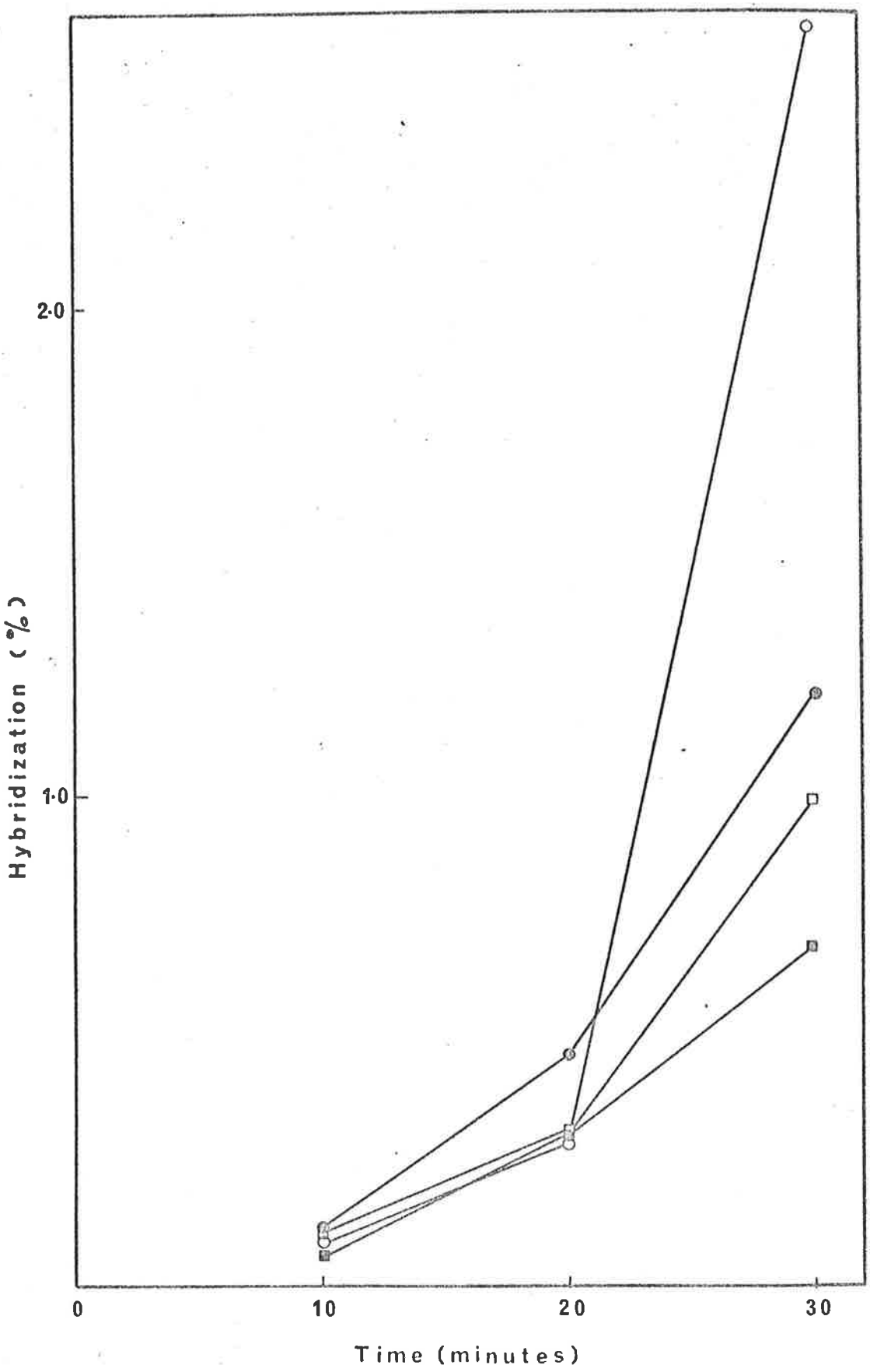

4·0
3·0
2·0
1·0
0
10
20
30
Hybridization (%)
Time (minutes)

FIGURE 3.12. A Comparison of Transcription of 186*cIts* and 186*cItsBam*17

Hybridization of 186*cIts* or 186*cItsBam*17 ^{3}H-mRNA, labelled after heat induction of the prophage, to 186 and Hy5 DNA immobilized on to nitrocellulose filters.

For details of heat induction and hybridization assays see Section III.D.1 and Section III.C.7, respectively.

- o 186*cIts* mRNA hybridized to 186*cIts* DNA.
- ■ 186*cIts* mRNA hybridized to Hy5 DNA.
- ● 186*cItsBam*17 mRNA hybridized to 186*cIts* DNA.
- □ 186*cItsBam*17 mRNA hybridized to Hy5 DNA.


2·0
1·0
Hybridization (%)
0
10
20
30
Time (minutes)

DNA/RNA hybridization or, if phage mRNA is only a minor component of the total cellular RNA, by measuring the incorporation of labelled precursor into TCA precipitable material.

It has been observed that infection of *E. coli* by the T-even phage (Cohen, 1948; Astrakan and Volkin, 1958; Brenner *et al.*, 1961; Nomura *et al.*, 1960) ϕX174 (Lindqvist and Sinsheimer, 1967; Ishiwa and Tessman, 1968) and infection by, or induction of λ prophage (Waites and Fry, 1964; Howes, 1965; Terzi and Levinthal, 1967) interfere with host metabolism, and in particular, inhibit cellular RNA synthesis. Inhibition is achieved in different ways, and although the mode is unclear it is apparent that for T-even phage the expression of phage genes is not needed. Inhibition is unaffected by UV irradiation prior to infection (Kaempfer and Magasanik, 1967) does not require viral protein synthesis (Nomura *et al.*, 1960) and can be mediated by phage ghosts as well as complete phage particles (Herriott, 1951; French and Siminovitch, cited by Cohen and Chang, 1970). A phage protein has been implicated for λ mediated inhibition by the observations that induction results in inhibition similar to that observed following infection (Cohen and Chang, 1970), and RNA synthesis of host cells treated with chloramphenicol prior to infection is almost identical to that of an uninfected control culture (Cohen and Chang, 1970). Inhibition is partially relieved by a mutation in gene N of the infecting phage indicating a role, either direct or indirect, for the N product in mediating this effect (Cohen and Chang, 1970). Lindqvist and Bøvre (1972), in a study of P2 transcription, concluded that P2 does not affect host RNA synthesis.

The effect of 186*cIts* heat induction on host RNA synthesis was investigated by measuring the incorporation of ^{3}H-uridine into TCA precipitable material. Incorporation of ^{3}H-uridine is linear over the two minute pulse and hence this is a true measure of the rate of RNA synthesis in the culture. RNA synthesis in lysogenic and non-lysogenic bacteria proceeded at the same rate for the first 30 minutes of the latent period (see Fig. 3.4). Cell lysis began at this time, and the decline in total RNA synthesis can be attributed to cell death at the end of the infective cycle. Clearly 186 does not affect host RNA synthesis and thus the percent of total RNA which hybridizes to 186 DNA is a good estimate of the rate of 186 directed RNA synthesis, for at least the first 30 minutes of the lytic cycle. After this time the estimate will be inflated because host synthesis is prevented by cell lysis.

Different mutations of phage λ have been shown to affect total cellular RNA synthesis by as much as three-fold during infection, and because phage RNA is only a small fraction of the total, it was suggested that these defective phage may be affecting host metabolism differently (Cohen and Hurwitz, 1967, 1968). For this reason, total RNA synthesis was also investigated following heat induction of 186 phage mutant in either of the control genes, A or B. Optical density measurements of bacterial cultures following heat induction of prophage with A*am* or B*am* mutations continued to increase at a rate similar to the non-lysogen for at least two hours (Hocking, 1977), and one might have expected that RNA synthesis would be similarly unaffected. For 30 minutes after induction the rate of RNA synthesis

did not differ significantly from that of a non-lysogenic host grown under identical conditions; however, after this time total RNA synthesis remained constant. 186 inhibits host DNA synthesis within ten minutes of the start of induction and phage mutant in either genes A or B retain this function (Hocking, 1977). Thus the plateau in RNA synthesis observed may be attributed to the inhibition of host DNA synthesis in contrast to the non-lysogen which will replicate and divide, providing more templates for RNA synthesis. The increase in optical density of cultures following induction of prophage with a defective A or B gene may be largely accounted for by increased cell size rather than cell number, although cell division may proceed at a reduced rate.

When the effect of P2 on net RNA synthesis was investigated, it was found to cause a dramatic decline in RNA synthesis after ten minutes, although the latent period extended over 22 minutes. Lindqvist and Bøvre (1972) studied the effect of P2 on host RNA synthesis by host DNA/RNA hybridization, and concluded that P2 did not affect host metabolism. The apparent contradiction may be explained by the fact that only 5 µg host DNA/filter was used for the hybridization assays; this may not have been in excess of host RNA as the maximum hybridization observed was only 4.1% of total counts.

A bacterial mutation, gro_{109}, which inhibits P2 late gene transcription has been defined; P2*ogr*, a mutant phage which overcomes the block imposed by gro_{109}, has been isolated, and it has been proposed that P2ogr^{+} may modify the gro^{+} product to allow transcription of P2 structural genes

(Sunshine and Sauer, 1975). This might be accompanied by a decrease in host transcription leading to the observed decline ten minutes after infection. The observation that 186 is not affected by the gro_{109} mutation, and that 186 does not inhibit cellular RNA synthesis, may indicate that no alteration to the host machinery is required for late gene expression.

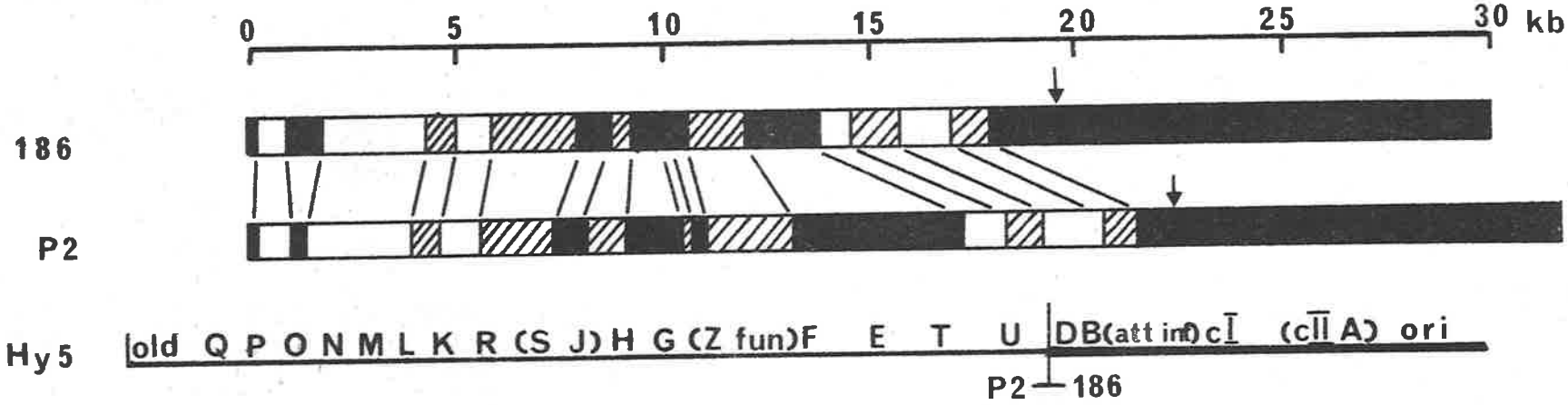
2. Non-specific hybridization

The work described in this section was undertaken to provide a preliminary analysis of 186 transcripts from different regions of the 186 genome. Twelve P2.186 hybrid phage have been isolated *in vivo*; these contain most of the P2 structural genes combined with 186 immunity, attachment site, origin of replication, control genes and a varying number of 186 tail genes (Bradley *et al.*, 1975; Hocking, 1977). The crossover point has been defined by genetic mapping and for some hybrids an electron microscope heteroduplex analysis has been carried out (Younghusband *et al.*, 1975). Hy5 was chosen because it has the smallest region of 186 DNA (65.4 to 100%) and codes for only one complete tail gene, D, with the crossover occurring in gene E, another tail gene (Hocking, 1977).

P2 and 186 are closely related phage and show 50% DNA homology by EM heteroduplex analysis (Younghusband and Inman, 1974). Homology and partial homology are limited to portions of the structural gene regions while the early genes and immunity regions are not homologous (see Fig. 3.13). A degree of cross hybridization between 186 mRNA and the P2 DNA segment of Hy5 was therefore expected. The results

FIGURE 3.13. A Comparison Between the Homology Maps for 186 and P2

The open areas represent regions of high homology, that is, were duplex at T_m-11°. Partially homologous regions (hatched areas) were single-stranded at T_m-11° but duplex at T_m-36°. Regions that were single-stranded at T_m-36° were considered to be non-homologous and are represented as filled areas in the figure (Younghusband and Inman, 1974).



↓ Hy5 crossover point

of an investigation of cross hybridization are presented in Table 3.1. Contrary to expectations based on the heteroduplex studies cross hybridization between P2 DNA and 186 mRNA was low. Transcripts of the non-homologous segments of DNA would not be expected to form stable RNA/DNA hybrids under these conditions, and this may also be true of the RNA derived from regions of partial homology. However, even the regions of apparent DNA homology seen in the presence of formamide must contain sufficient mismatching to prevent the formation of stable RNA/DNA hybrids under the conditions used in these experiments.

The amount of hybridization between 186 mRNA and P2*vir*1 DNA remained constant throughout the lytic cycle. There are two explanations of this observation:

(1) there was specific hybridization to P2 DNA of a class of 186 mRNA which is transcribed at a constant rate throughout the lytic cycle. As development proceeded this class of RNA represented a decreasing fraction of total 186 RNA.

(2) under the hybridization conditions used there was no specific hybridization of 186 mRNA to P2 DNA. The small but constant hybridization observed was due to non-specific hybridization of host and 186 RNA to P2 DNA.

The latter seems the more likely explanation; specific cross hybridization would be expected only when the structural genes of 186 which show DNA homology with P2 are being expressed, however, no increase in hybridization between 186 mRNA and P2 DNA was observed at late times

(Table 1, line 3). Early mRNA was present throughout the 186 lytic cycle (see Section VI) but this is read from DNA which is non-homologous to P2 DNA (see Fig. 3.13), and therefore should not show specific cross hybridization.

The difference in hybridization to Hy5 and 186 DNA templates can therefore be considered to reflect the expression of the structural genes encoded by 0 to 65.4% of the 186 genome.

Cross hybridization of cellular RNA with 186, P2 or Hy5 DNA was between 0.001% and 0.005% and can therefore be disregarded.

3. Transcription of 186*cIts*

The lytic cycle of 186 can be divided into two phases - early, during which the control genes are transcribed, and late, which is marked by the initiation of structural gene expression.

Induction of a 186*cIts* prophage by a temperature shift from 30°C to 38.5°C was followed by a delay of 5 to 10 minutes before transcription was detected. This delay is unique to induction because transcripts which did not appear until ten minutes after induction were present in significant amounts five minutes after infection (see Section VI). The lag may reflect the time taken for complete inactivation of the repressor and for excision of the prophage neither of which occur following infection.

The early phase of 186 lytic development after induction occupied the first 20 minutes of the latent period and

transcripts made during this time hybridized equally to 186 and Hy5 DNA. The early genes must lie solely on the 65.4 to 100% region of the 186 chromosome, that is, that part which is present in Hy5.

However, by the 25th minute it was evident that transcription of the structural genes had begun. Hybridization to 186 DNA increased rapidly during the final ten minutes of the latent period reflecting the rapid transcription of phage structural genes. The structural genes can thus be termed 'late' genes because their expression is initiated in the second (late) phase of the lytic cycle. Hybridization to Hy5 DNA increased slowly throughout the late phase, probably reflecting the transcription of genes E and D, 186 tail genes which map to the right of 65.4%, as well as the continued transcription of the early genes.

Replication of 186 DNA was first detected between 15 and 20 minutes after heat induction, although 186 replication earlier than this would have been masked by residual host DNA synthesis (Hocking, 1977) and thus it precedes the onset of the late phase by a few minutes. The rapid rate of late transcription mirrored the increasing rate of DNA synthesis which was maximal at 35 minutes then declined as cell lysis occurred. An increase in gene dosage may account for the slow increase in early gene transcription, rather than an increase in the rate of initiation/chromosome.

In a study of P2 transcription, Geisselsoder *et al.* (1973) demonstrated that early P2 mRNA also originated exclusively from the right half of the phage genome. As the P2 lytic cycle progressed, transcription from the left

half of the chromosome increased until towards the end of the cycle it contributed about two-thirds of P2 directed mRNA.

Thus 186 conforms to the pattern established for other phage, for example, λ, P2, μ with an early phase of transcription during which control genes and DNA replication proteins (gene A) are expressed. This is followed by a late phase concomitant with DNA synthesis (Herskowitz, 1973; Geisselsoder *et al.*, 1973; Wijffelman *et al.*, 1974).

It appears that no early genes are encoded by 0 to 65% of the genome because early RNA hybridized equally to both Hy5 and 186 DNA templates and this is in agreement with the genetic study of 186 (Hocking, 1977).

4. Transcription of 186*c*I*ts*A*am*5

The transcripts made following induction of a 186*c*I*ts*A*am*5 prophage hybridized equally to 186 and to Hy5 DNA and thus must be limited to the early genes, even at late times in the infective cycle. Not only was the extent of transcription reduced but the rate at which transcription proceeds was slower than for the corresponding transcription following wild type induction. Transcription could be limited if:

(1) all early transcripts were made but in a reduced amount. A protein is essential for 186 DNA replication (Hocking, 1978) and decreased early transcription may be due solely to the reduction in gene dosage caused by the absence of DNA synthesis.

(2) alternatively, only the A transcript was made and all other early genes belong to a separate class, delayed early, which require the A gene product for their expression. If this is the case then 186 may be analogous to λ in having two types of early genes. That the 186 A gene and λ N gene have a similar role seems unlikely because 186 gene A is thought to be *cis*-acting (it complements poorly with all other mutations) and is required for DNA synthesis (Hocking, 1977). In contrast λ N protein can act *trans* and there are two other proteins O and P which are involved in λ replication (Herskowitz, 1973). It is also possible that the A*am*5 mutation may exert a strong polar effect over a distal gene which has an 'N type' function in phage development; gene B, the only other control gene identified by mutation, is separated from the A gene by *int* and *c*I genes as well as the *att* site and therefore would not be affected in this way. No evidence has been obtained for any other control gene that might have this function.

That gene A is not the only gene transcribed following 186*c*I*ts*A*am*5 induction was indicated by the fact that host DNA synthesis was inhibited to the same degree by an A*am*5 mutant as by 186*c*I*ts* (Hocking, 1977). The mode of inhibition is unknown but it is likely that a phage protein is involved.

One can conclude then that the A gene must be directly or indirectly required for the expression of the late genes as there was no transition to the late phase of development following induction of a prophage defective in the A gene.

186 gene A is analogous to the P2 gene A - both act only in *cis* and are required for both DNA replication and late gene expression (Lindahl, 1974). Transcription during a non-permissive infection with P2A*am* has revealed that early transcripts are normal, both in amount and strand distribution. There was no increase in P2 specific RNA as infection proceeded, confirming that the A gene of P2 has a similar function to 186 A gene at the molecular as well as biological level (Lindqvist and Bøvre, 1972).

P2 has a second gene, B, which showed reduced DNA synthesis. As for the A mutant, non-permissive infection by P2B*am* had normal early transcription, however, it did show late transcription, though greatly reduced, with the same strand distribution as wild type RNA (Lindqvist and Bøvre, 1972). There is no evidence for a 186 gene with P2 B function.

5. Transcription of 186*c*I*ts*B*am*17

In contrast to A*am* phage the rate of transcription of B*am*17 following induction was similar to wild type until the onset of late transcription. The normal transition to the late phase was not observed at late times; transcription was predominantly from the early, that is Hy5, region of the genome but it occurred at a faster rate than for wild type phage. When DNA synthesis of 186*c*I*ts*B*am*17 was investigated it was found to far exceed that of wild type phage (Hocking, 1977) and hence the greater rate of transcription may be accounted for by the increased gene dosage. It is possible that the B product does play a negative role in the regulation of early transcription in normal phage development.

Hybridization to 186 DNA at late times was slightly greater than to Hy5 DNA which suggests that some late transcripts may be made (see Section VI) but the normal burst of late RNA synthesis did not occur. Therefore the B product must also be required directly or indirectly for normal late gene expression.

The B gene of 186 thus appears to be similar to the *ogr* gene of P2. Both genes are located between the tail genes and the phage *att* site and neither function is essential for DNA replication. Lytic development of P2*ogr* and of 186B*am* are characterized by normal early transcription and an absence of late mRNA. However, 186 development is not inhibited by the bacterial mutation *gro* (J.B. Egan, personal commun.) which prevents late gene expression in P2ogr^+ and therefore the mechanisms involved in the initiation of late gene transcription must differ. It has been proposed that the P2ogr^+ product may interact with the gro^+ product to allow transcription of P2 late genes (Sunshine and Sauer, 1975). The 186 B protein may be more like λ Q gene in its function. The λ Q gene is a positive regulator which activates late gene expression and, on the basis of phage complementation tests, acts in *trans*. Echols *et al.* (1976) have presented evidence that Q protein shows preferential *cis* action. 186 B protein functions in phage complementation tests, and therefore will act in *trans*, however, preferential *cis* action has not been investigated.

The question of the control of late gene expression in the viable P2-186 hybrid phage will be discussed in Sections VI and VII.

6. Summary

Filter hybridization of 186 mRNA labelled at different times during lytic development to Hy5 and 186 DNA has revealed at least two phases in the transcription of 186 following heat induction. The early phase was characterized by transcription of the control genes which are located on the 65.4 to 100% region of the chromosome. The transition to late transcription required both genes A and B, however, the mode of this transition is unclear. Gene A was required for DNA synthesis and in the absence of A protein early transcription occurred at a reduced level; DNA synthesis may be obligatory for late gene expression, and this may account for its absence in an A mutant. Phage replication takes place when B protein is defective and hence a more direct role in the late transition is implicated for the B gene.

Late gene transcription was observed by the 25th minute of the lytic cycle and thus was concurrent with DNA synthesis which began between 15 and 20 minutes and continued, at an ever increasing rate, until cell lysis.

Further elucidation of the role of the A and B genes in the control of transcription required better definition of the transcripts made in the absence of A and B proteins. This can be achieved by further fractionation of the 186 chromosome into discrete fragments followed by hybridization of the RNA made at different times during the lytic cycle. Restriction endonucleases are a powerful tool for the cleavage of DNA into discrete fragments which can be mapped with respect to the phage genome. A restriction map for 186 has been published by Saint and Egan (1979) and this has

SECTION IV

HYBRIDIZATION OF 186 mRNA TO 186 DNA DIGESTED WITH RESTRICTION ENDONUCLEASES

formed a basis for the work described in the succeeding sections.

SECTION IV

HYBRIDIZATION OF 186 mRNA TO 186 DNA DIGESTED WITH RESTRICTION ENDONUCLEASES

A. INTRODUCTION

Class II restriction endonucleases which cut DNA at sequence specific sites have proved to be invaluable in cleaving DNA to form reproducible, discrete fragments. The recognition site for class II enzymes is usually a palindromic sequence of four or six nucleotides, although two enzymes that recognise unique pentanucleotides have been isolated (Roberts, 1976). A given sequence of six nucleotides will occur, by chance, once in 4096 base pairs and so an enzyme that recognises a hexanucleotide will, on average, cut DNA every four kb. Smaller DNA fragments can be obtained by cleaving with an enzyme having a tetranucleotide recognition sequence that will occur approximately once every 250 base pairs. Enzymes with hexanucleotide recognition sequences were chosen to cleave 186 DNA which is 30 kb long and thus, on average, should be cut seven to eight times.

Agarose gel electrophoresis of restricted DNA permits the resolution into discrete fragments which can readily be visualized, after staining with ethidium bromide, by their fluorescence under UV irradiation (Sharp *et al.*, 1973). Southern (1975) developed a method for the transfer of DNA, denatured *in situ*, from an agarose gel to nitrocellulose filter paper, thus allowing hybridization to the individual restriction fragments.

Restriction fragments purified by electrophoresis have been used in the mapping of RNA transcripts of eukaryotic viruses, for example, SV40 (Khoury *et al.*, 1975; Sambrook *et al.*, 1973) and adenovirus (Pettersson *et al.*, 1975).

B. MATERIALS

The following materials were purchased from the companies indicated:

Agarose: Sigma Chemical Company, St. Louis, U.S.A.

Nitrocellulose (0.45 μ pore size): Sartorius, 34 Gottingen, West Germany.

Fuji Rx medical X-ray film: Fuji Photo Film Co. Ltd., Tokyo.

2475 recording film: Kodak, Melbourne, Australia.

Ethidium bromide (EtBr): Sigma Chemical Company, St. Louis, U.S.A. A stock solution at 10 mg/ml was prepared in sterile water and stored at 4°C in the absence of light.

The restriction enzymes *Eco*RI, *Bgl*II and *Hin*dIII prepared as described by Saint and Egan (1979) were generously donated by R.B. Saint, R.P. Harvey and L. Crocker respectively.

Gel electrophoresis buffer: 40 mM Tris-acetate pH 8.2, 20 mM Na-acetate, 1 mM EDTA.

Denaturing solution: 0.5 M NaOH, 0.75 M NaCl.

Neutralizing solution: 1 M Tris H-Cl pH 5.0, 1.5 M NaCl.

C. METHODS

1. Restriction endonuclease digestion

The enzymes used for this study were *Bgl*II, *Eco*RI and *Hin*dIII. Conditions for DNA restriction were as follows:

*Bgl*II digestions were carried out at 37°C in 10 mM Tris-HCl pH 7.5, 10 mM 2-mercaptoethanol and 10 mM $MgCl_2$. Digestion conditions for *Eco*RI were as for *Bgl*II with the addition of 100 mM NaCl to inhibit *Eco*RI* activity. *Hin*dIII digestion conditions were as for *Bgl*II with the addition of NaCl to 50 mM. Digestion with more than one enzyme was accomplished by sequential digestion using enzymes in the order *Bgl*II, *Hin*dIII and *Eco*RI with the addition of NaCl to the required concentration for each enzyme.

The reaction in each case was terminated by incubation at 65°C for five minutes.

2. Agarose gel electrophoresis

0.75% (w/v) or 1% (w/v) agarose in 40 mM Tris-acetate pH 8.2, 20 mM Na acetate and 1 mM EDTA was prepared by refluxing for ten minutes, and then cooled before being poured into an upright slab gel apparatus 20 cm x 20 cm x 0.2 cm and later 20 cm x 20 cm x 0.4 cm. The incubation mix was made 10% for glycerol before loading on to the gel, by the addition of one fifth volume of 50% glycerol containing bromophenol blue which acted as a tracker dye. The maximum amount of DNA loaded/cm of gel (that is /slot) was 2 µg for 0.2 cm gel and 10 µg for 0.4 cm gel. Electrophoresis was carried out at 10 mA (0.2 cm gel) or 20 mA (0.4 cm gel) for

15 hours at room temperature.

The gel was stained with EtBr (0.5 µg/ml) for 15 minutes (30 minutes for 0.4 cm gel) and then destained for a further 15 (30) minutes before being photographed under shortwave UV light using Kodak 2475 recording film and a red filter.

3. Transfer of DNA from a gel to nitrocellulose

The method used for transferring restricted DNA from an agarose slab gel to nitrocellulose filter was essentially that developed by Southern (1975).

The DNA was first denatured by bathing the gel in denaturing solution for 15 minutes (0.2 cm gel) or 30 minutes (0.4 cm gel) with occasional shaking. The gel was rinsed and the solution was replaced with neutralizing solution for a further 30 (45) minutes. Thick filter paper, wet with 10 x SSC, was placed over a glass surface with each end dipping into a reservoir of 10 x SSC beneath. The gel was placed on top of this, and perspex strips the same thickness as the gel were placed beside it leaving a gap of at least 3 mm between the two. A sheet of nitrocellulose, large enough to cover the entire gel and perspex strips, was wetted then carefully placed on top of the gel aligning tracks with those marked on the filter. The nitrocellulose was then covered with two sheets of moist, thick filter paper with the same dimensions as the nitrocellulose. Finally a wad of dry filter paper was put on top and held in place by a light weight. The air spaces between the gel and the perspex strips were cleared of all liquid to

prevent siphoning beside, rather than through, the gel. Transfer was allowed to proceed for at least two hours.

The nitrocellulose was then dried and baked *in vacuo* for three hours to immobilize the DNA.

4. Hybridization to transferred DNA

Strips of nitrocellulose 1 cm wide, corresponding approximately to the individual gel tracks, were cut from the filter to which the DNA had been transferred.

Individual filter strips were wet with sterile water then wound around the outside of a test tube. This was then placed inside a second close fitting tube and 1.5 ml hybridization buffer was added. The inner test tube was then removed, leaving the filter in place and the ^{3}H-RNA extract was added and thoroughly mixed. The inner tube was replaced; this raised the level of hybridization mix to cover the filter strips. A small volume of water was added to the inner tube to decrease the evaporation rate of the hybridization mix and the whole was covered with plastic and incubated for 20 hours at 65°C to 70°C.

The filter strips were removed, rinsed, and then incubated at 65°C to 70°C for at least four hours in 2 x SSC + 0.25% SDS, then 2 x SSC + 0.12% SDS and finally 2 x SSC. Strips were dipped in ethanol and dried, then each was cut into 2 mm slices perpendicular to the direction of electrophoresis, and the slices counted in a Packard scintillation spectrometer.

5. Autoradiography

After hybridization prefogged X-ray film was placed on top of the dried nitrocellulose strips which had been fixed, DNA side uppermost, to thick filter paper. This was then sandwiched between glass plates lined with thick filter paper, wrapped in foil and black plastic. The autoradiogram was allowed to expose at -80°C for six and a half weeks before developing.

D. RESULTS

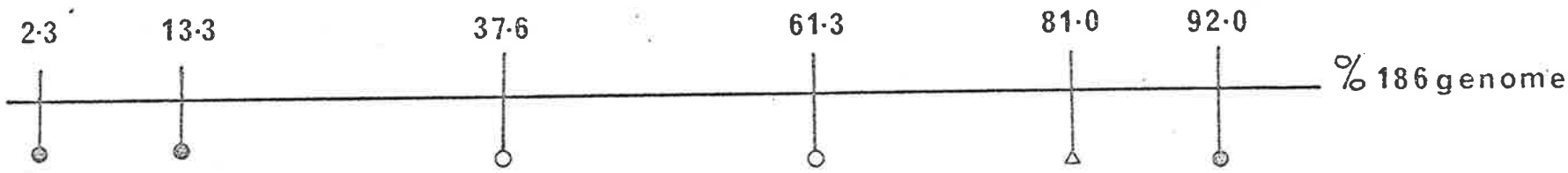
1. Hybridization of 186*c*I*ts* mRNA to 186 DNA digested with *Eco*RI and *Hin*dIII

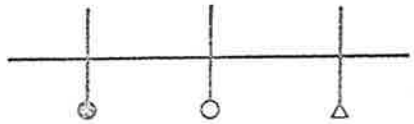
Restriction of 186 DNA with *Hin*dIII followed by *Eco*RI resulted in fragments of 9.2 kb, 7.3 kb, 7.1 kb, 3.3 kb, 3.1 kb (reannealed end fragments) and the end fragments of 2.4 kb and 0.7 kb respectively. The position of these fragments on the 186 chromosome is illustrated in Fig. 4.2.

186*c*I*ts* mRNA labelled at 15 to 17 minutes and 35 to 37 minutes after heat induction was hybridized to a transfer of 186 DNA that had been digested with *Hin*dIII and *Eco*RI as described in Section IV.C. The fragments were resolved by electrophoresis on a 0.2 cm 1% agarose gel, and therefore each filter strip contained a maximum of 2 µg DNA, assuming 100% transfer.

The distance from the top of the gel of each band was measured to permit correlation between the bands observed and any hybridization to the filters. This correlation was not exact for several reasons:

FIGURE 4.1. A Cleavage Map of 186*c*I*ts* DNA Showing the Position of the *Eco*RI, *Hin*dIII and *Bgl*II Restriction Sites

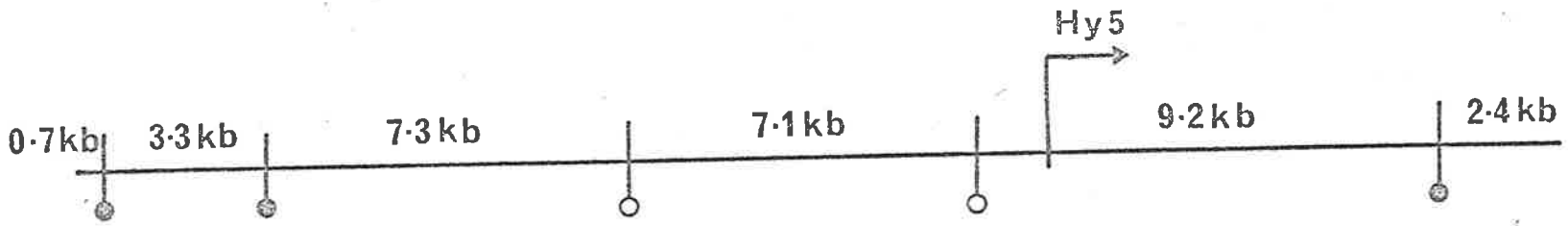


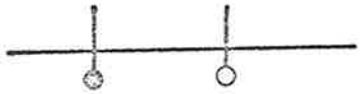


EcoRI HindIII BglII
Cleavage sites

FIGURE 4.2. The Fragments Generated by Digestion of 186*cIts* DNA with *Eco*RI and *Hin*dIII

The size (kb) and position of restriction fragments generated by double digestion of 186*cIts* DNA with *Eco*RI and *Hin*dIII are presented in the figure. The position of the Hy5 crossover point is shown in relation to the restriction sites.





EcoRI HindIII

Cleavage sites

(1) gels were sometimes stretched or compressed when setting up a transfer,

(2) filter strips were sometimes permanently mis-shapen after being coiled around a test tube for 20 hour hybridization assays, making it difficult to slice perpendicular to the direction of electrophoresis,

(3) cutting any filter into accurate 2 mm strips was difficult.

Fig. 4.3 presents the results obtained when filters were sliced and counted. RNA labelled between 15 and 17 minutes after induction hybridized weakly at a position on the filter corresponding with the position of the 9.2 kb fragment seen on the gel. Late (35 to 37 minutes) RNA hybridized to the 9.2 kb fragment as well as the 7.3 kb and 7.1 kb fragments which comigrated in 1% agarose. A minor peak of hybridization was also observed at a position corresponding to the 3.3 kb fragment.

An inspection of the restriction map for *Hin*dIII and *Eco*RI digestion of 186 DNA (Fig. 4.2) reveals that the 9.2 kb fragment covers the 61.3% to 92% region and so closely approximates the 186 DNA that is contained by Hy5. The analysis of 186*cIts* transcripts by a comparison of hybridization to Hy5 and 186 DNA (see Section III) led to the prediction that early mRNA, that is RNA made up to about 20 minutes after induction, originated exclusively from the rightmost 35% of the genome and therefore should hybridize only to the 9.2 kb and perhaps the 2.4 kb end fragment. On the same basis, late RNA (25 to 35 minutes) should

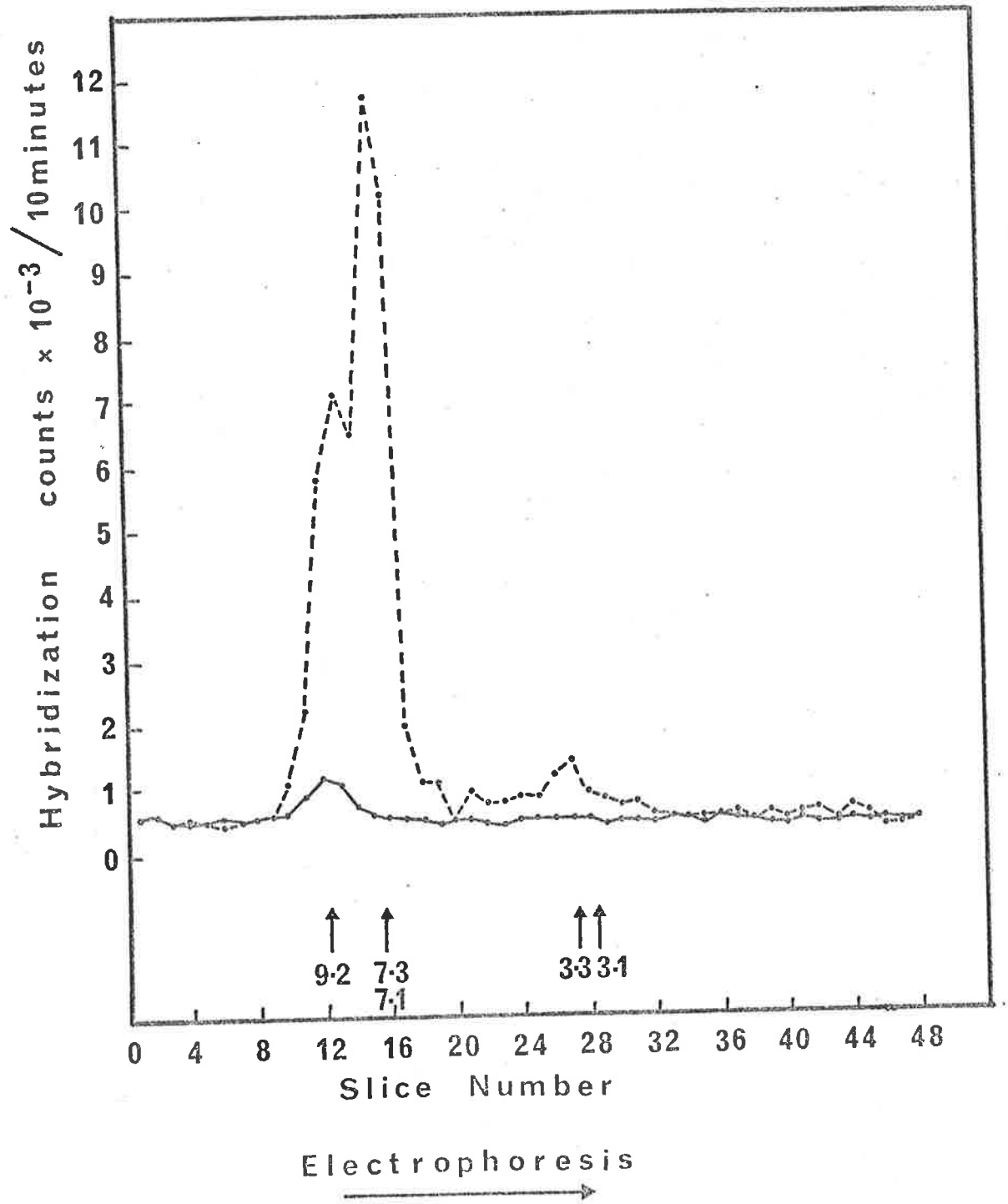
FIGURE 4.3. Hybridization of 186*cIts* mRNA to 186 DNA Digested with *Eco*RI and *Hin*dIII

186*cIts* DNA was digested with *Eco*RI and *Hin*dIII, electrophoresed through 1% agarose, then transferred to nitrocellulose. 186*cIts* ^{3}H-mRNA was hybridized to strips cut from the Southern transfer. After hybridization the strips were cut into 2 mm slices perpendicular to the direction of electrophoresis and these were then counted.

The profile of hybridization obtained in this way is presented in the figure. The approximate position of restriction fragments on the filter, corresponding to their observed position on the gel, is shown.

——— 186*cIts* mRNA labelled 15 to 17 minutes after induction.

------ 186*cIts* mRNA labelled 35 to 37 minutes after induction.


Hybridization counts × 10⁻³ / 10 minutes
0
1
2
3
4
5
6
7
8
9
10
11
12
9·2
7·3
7·1
3·3
3·1
0
4
8
12
16
20
24
28
32
36
40
44
48
Slice Number
Electrophoresis

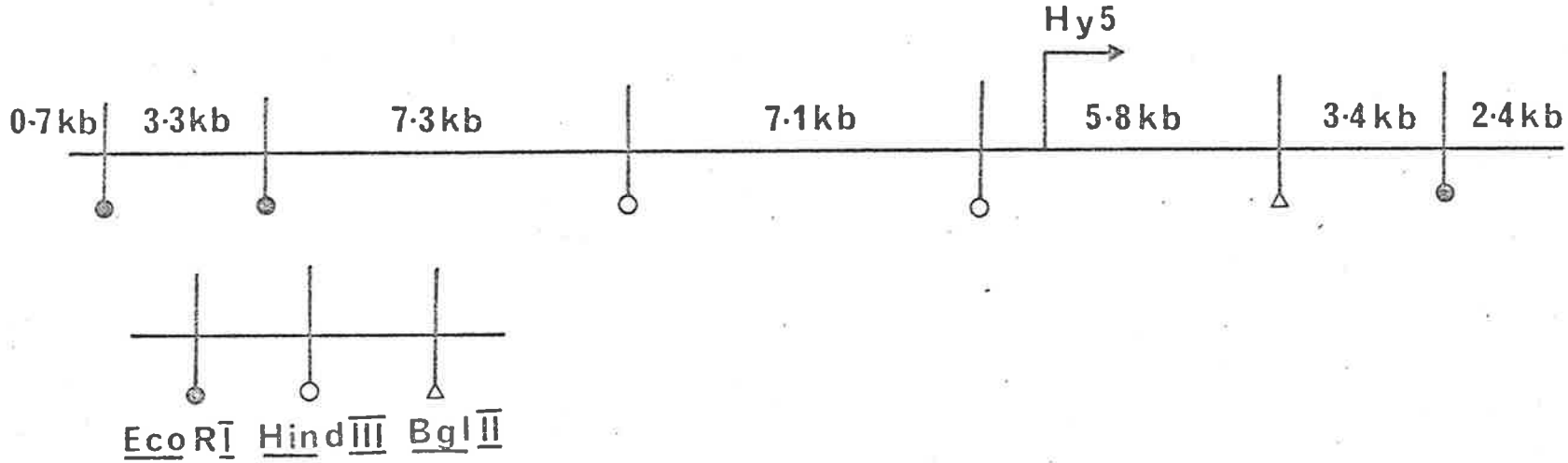
hybridize to all fragments generated by *Hin*dIII, *Eco*RI double digests, with the possible exception of the end fragments. The results obtained were therefore in agreement with predictions based on the results of the experiments reported in Section III. The results from an autoradiogram of a duplicate filter hybridized with 35 to 37 minute RNA are not presented. Lengthy exposure showed two very faint bands, corresponding to the position of the 9.2 kb and 7.3 kb, 7.1 kb restriction fragments. No further autoradiography was undertaken (see Section IV.E for a detailed discussion).

2. Hybridization to 186 DNA digested with *Eco*RI, *Hin*dIII and *Bgl*II

186 DNA digested sequentially with the three enzymes *Bgl*II, *Hin*dIII and *Eco*RI was cut into fragments of 7.3 kb, 7.1 kb, 5.8 kb, 3.4 kb, 3.3 kb, the end fragments of 2.4 kb and 0.7 kb and a 3.1 kb fragment formed when the two end fragments reannealed. The 9.2 kb *Hin*dIII/*Eco*RI restriction fragment was cut by *Bgl*II to yield fragments of 5.8 kb and 3.4 kb thus further fragmenting the early region of 186 (Fig. 4.4). A 0.75% agarose gel was used to try to resolve the 7.3 kb and 7.1 kb fragments, but without success; the 3.4 kb and 3.3 kb fragments were also very poorly resolved. To increase the amount of DNA present in the smaller restriction fragments 10 µg of DNA was loaded/cm on to a 0.4 cm gel. The gel was overloaded with respect to the larger fragments and there was evidence of trailing between the bands. There was also some evidence of *Eco*RI* activity.

FIGURE 4.4. The Fragments Generated by Digestion of 186*cIts* DNA with *Eco*RI, *Hin*dIII and *Bgl*II

The position of the Hy5 crossover point is shown in relation to the restriction fragments generated by digestion of 186*cIts* DNA with *Eco*RI, *Hin*dIII and *Bgl*II.


Hy5
0·7kb
3·3kb
7·3kb
7·1kb
5·8kb
3·4kb
2·4kb
EcoRI
HindIII
BglII
Cleavage sites

(a) Hybridization of 186*c*I*ts* late mRNA

186*c*I*ts* mRNA labelled in the late phase (30 to 32 minutes) of lytic development was hybridized to 186 DNA that had been restricted with the three enzymes *Bgl*II, *Hin*dIII and *Eco*RI. Hybridization was detected by cutting the filter into 2 mm slices and the results are plotted in Fig. 4.5. Although the positions of the gel bands and hybridization peaks did not coincide exactly the results suggested that the fragments 7.3 kb, 7.1 kb, 5.8 kb, 3.4 kb and 3.3 kb were being transcribed at the time of labelling. There was no hybridization corresponding exactly with the position of the 3.1 kb fragment but the high background over the lower region of the gel made it impossible to detect specific hybridization to the smaller fragments with confidence. Therefore using this technique it was not possible to determine whether RNA was transcribed from the 2.4 kb end fragment. This phenomenon was common to wild type A*am*5 and B*am*17 RNA and may have been due to hybridization to *Eco*RI* restriction fragments. The peak that would correlate with a fragment of greater than 7.3 kb has no corresponding band on the gel.

(b) Hybridization of 186*c*I*ts*B*am*17 RNA

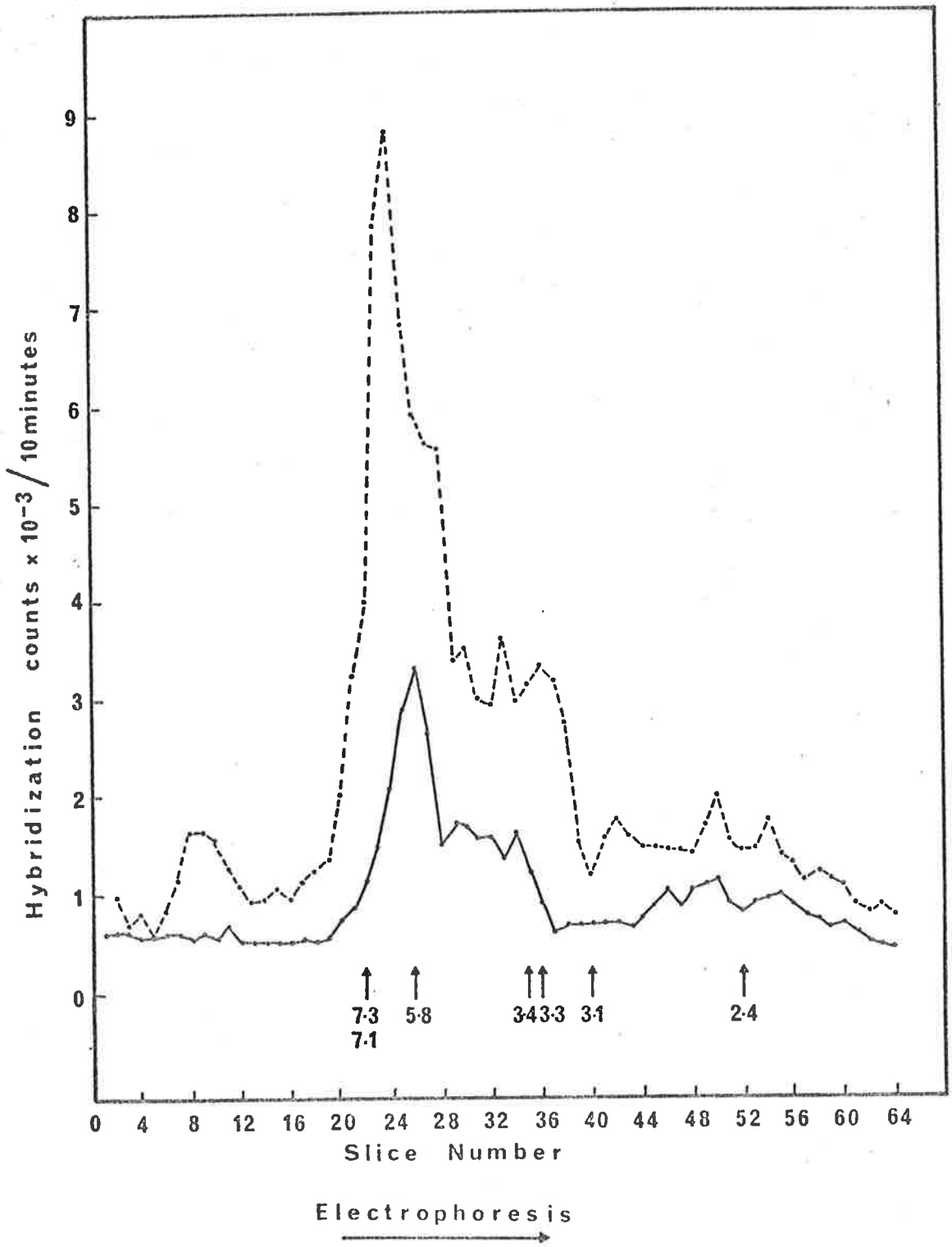
Hybridization of 186*c*I*ts*B*am*17 mRNA labelled at 30 to 32 minutes to 186 DNA digested with *Bgl*II, *Hin*dIII and *Eco*RI is illustrated by Fig. 4.5. When compared to the wild type profile it is obvious that the rate of transcription was slower and that the transcripts were generally confined to the region included in the 5.8 kb and 3.4 kb fragments with the possibility of a small amount of hybridization

FIGURE 4.5. A Comparison of Hybridization to the Restriction Fragments Generated by Digestion of 186*cIts* DNA with *Eco*RI, *Hin*dIII and *Bgl*II of mRNA Made After the Induction of 186*cIts* and 186*cIts*B*am*17 Prophage

186*cIts* DNA was restricted with *Eco*RI, *Hin*dIII and *Bgl*II to generate the fragments shown in Fig. 4.4. Details of the procedure used to obtain the profiles shown in this figure are outlined in the legend to Fig. 4.3. Electrophoresis was through 0.7% agarose.

------ 186*cIts* mRNA labelled 30 to 32 minutes after induction.

——— 186*cIts*B*am*17 mRNA labelled 30 to 32 minutes after induction.


Hybridization counts x 10⁻³ / 10 minutes
0
1
2
3
4
5
6
7
8
9
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64
Slice Number
7·3
7·1
5·8
3·4
3·3
3·1
2·4
Electrophoresis

to the 7.3/7.1 kb and 3.3 kb fragments. The majority of RNA hybridized with fragments corresponding closely to the early region of 186 defined in Hy5. The results presented in Section III.D.7 suggested a role for the B gene product in the transition to late gene expression observed in the final ten minutes of the lytic cycle. Hybridization of B*am*17 message to transferred restriction fragments supports the idea that late gene transcription requires active B protein.

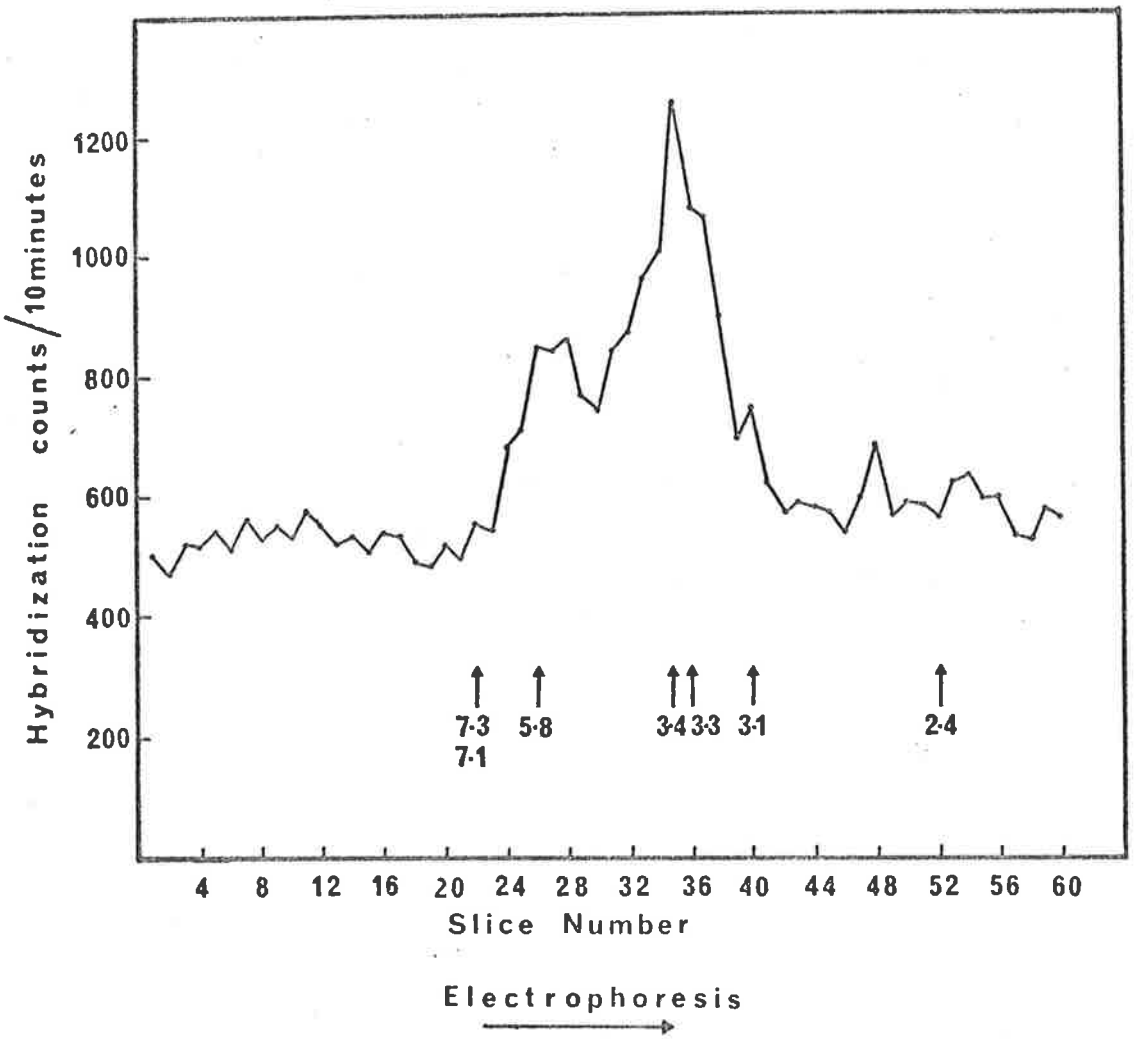
(c) Hybridization of 186*c*I*ts*A*am*5 mRNA

The profile presented in Fig. 4.6 is for the hybridization of 186*c*I*ts*A*am*5 mRNA labelled at 30 to 32 minutes after induction to 186 DNA restricted by *Bgl*II, *Hin*dIII and *Eco*RI.

The number of counts hybridized was lower than for either wild type RNA or B*am*17 phage RNA, in keeping with earlier observations (Section III.D.7). The region of the genome covered by the 7.3 kb and 7.1 kb restriction fragments was not transcribed 30 to 32 minutes after 186*c*I*ts*A*am*5 induction. This confirms the earlier observation that late transcription was absent when the A function was defective. In conflict with this result was the shoulder of hybridization corresponding to the 3.3 kb fragment which maps at 2.3 to 13.3% on the chromosome; one explanation is the poor resolution of the 3.4 kb and 3.3 kb fragments and this gains some support from the low level of hybridization to this fragment observed when B*am*17 RNA was tested. Another feature of the A*am*5 profile was the small peak at the 3.1 kb position, this was absent for both wild type and B*am*17 mRNA.

FIGURE 4.6. Hybridization of 186*c*I*ts*A*am*5 mRNA to 186*c*I*ts* DNA Fragments Generated by Restriction with *Eco*RI, *Hin*dIII and *Bgl*II

Details of the procedure used to obtain the hybridization profile are outlined in the legends to Fig. 4.3 and Fig. 4.5.


Hybridization counts/10minutes
1200
1000
800
600
400
200
7·3
7·1
5·8
3·4
3·3
3·1
2·4
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
Slice Number
Electrophoresis

E. DISCUSSION

1. Hybridization of 186*c*I*ts* RNA to restricted 186 DNA

Hybridization of 186*c*I*ts* RNA labelled at early or late times after heat induction, to 186 restriction fragments has confirmed the observations made when this RNA was hybridized with total 186 and Hy5 DNA. Genetic mapping placed the two known control genes, A and B, on the right 35% of the 186 chromosome (Hocking, 1977) and hybridization data presented in Section III showed that early mRNA was made exclusively from this region. Late mRNA originated from both 0 to 65.4% and 65.4 to 100% of the genome.

In this series of experiments restriction of 186 DNA with *Eco*RI and *Hin*dIII replaced the Hy5 region of 186 with a single restriction fragment of 9.2 kb extending from 61.3% to 92% 186. Early 186 mRNA hybridized exclusively with this fragment. Hybridization was low and it is possible that if transcripts from the smaller fragments were present then these were not seen because there was insufficient DNA to detect any hybridization. The fragments of 7.3 kb and 7.1 kb comigrated and therefore contained the DNA equivalent to a single fragment of 14.4 kb; if both were transcribed then the transcripts would certainly have been detected. Late mRNA was complementary to the 9.2 kb, 7.3 kb and/or 7.1 kb fragments as well as the 3.3 kb fragment.

Digestion with *Bgl*II in addition to *Hin*dIII and *Eco*RI divided the 186 early region into two fragments of 5.8 kb (61.3 to 81%) and 3.4 kb (81 to 92%) respectively. Transcription of both these fragments occurred during the late

phase of 186 lytic development.

2. Hybridization of 186*c*I*ts*A*am*5 RNA to restricted DNA

It was hoped that the separation of the early region of 186 DNA into three fragments 61.3 to 81% (5.8 kb), 81 to 92% (3.4 kb) and 92 to 100% (2.4 kb) would provide more information about the transcripts that were made by an A*am*5 phage. However, both the 5.8 kb and the 3.4 kb fragments were transcribed. Earlier results (Section III.D.7) gave rise to the prediction that:

(1) either all early transcripts were made but at a reduced level,

(2) or gene A, or another unidentified control gene, over which A*am*5 exerted a strongly polar effect, was required for the synthesis of the early genes not included in the A transcript.

The 3.4 kb fragment hybridized more RNA than the 5.8 kb fragment, but it should be remembered that the latter codes for at least two tail genes. Therefore although the second idea cannot be excluded the first explanation seems more likely.

It may be worth noting that unlike wild type or B*am* RNA extracts the A*am*5 extract may contain some RNA complementary to the 3.1 kb reannealed end fragment; the high background associated with lower region of the gel makes it impossible to detect specific hybridization to the 2.4 kb right end and so to confirm this result.

3. Hybridization of 186*cI*ts*Bam*17 RNA to restricted DNA

The results reported in Section III suggested that normal late transcription was prevented by the absence of a functional B protein. This cannot be attributed to the absence of DNA replication which exceeds wild type synthesis, and so it was proposed that the B gene may be directly required for late gene expression. Hybridization of *Bam*17 RNA to restricted 186 DNA supported this hypothesis. The early region, represented by 5.8 kb and 3.4 kb fragments, was transcribed but transcripts from the late genes were reduced to a minor shoulder on the slow side of the peak associated with the 5.8 kb fragment of wild type transcripts (Fig. 4.5).

The information obtained from these experiments was limited by a number of technical difficulties. These are outlined below:

(1) to ensure that transcripts of small restriction fragments would be detected a large quantity of DNA was loaded on to the gel. For example, assuming that 100% DNA was transferred and bound to the nitrocellulose, 10 µg 186 DNA must be loaded to give 1 µg of a 3.0 kb fragment. The problem then became one of overloading with respect to the larger fragments, with pronounced trailing between bands and a correspondingly high background.

(2) aligning the gel tracks with the tracks marked on the filter to which DNA was transferred, was difficult. This problem was minimized by loading samples into a single 15 cm slot. However, a new problem arose; the distribution

of DNA across the gel was not even and the fragments were represented unequally in different 1 cm strips.

(3) correlation between gel bands and the hybridization profile was not exact. Autoradiography of the filter strips would reduce this problem and the exposure time could be decreased by labelling mRNA with ^{32}P instead of tritium. This was not pursued because the results obtained would still have been limited by the other difficulties raised.

(4) many of the 186 restriction fragments are similar in size (see Fig. 5.1 for a more detailed restriction map) and therefore were poorly resolved by electrophoresis. It was impossible in some instances to determine which fragment was hybridizing RNA, for example, 7.3 kb and 7.1 kb or 3.4 kb and 3.3 kb fragments.

(5) a genetic map obtained by recombination experiments (Hocking, 1977) provided very little information about the physical position of the genes. Although the restriction fragments have been physically mapped with respect to the 186 chromosome (Saint and Egan, 1979) the genetic content of any particular fragment was unknown. Hybridization of RNA to individual restriction fragments would provide more information if the genes carried by each fragment were identified.

The best way to overcome the technical limitations that were encountered in this set of experiments is to look at the hybridization of RNA to pure restriction fragments of known genetic content. The simplest way to achieve this is to clone individual fragments into a vector that is

heterologous to 186. The genetic content of each fragment can be determined and hybridization can then be related to the transcription of the genes coded by each fragment. Cloning also provides the means to isolate large quantities of pure restriction fragment which are ideal for hybridization studies.

SECTION V

THE PHYSICAL MAP OF 186

SECTION V

THE PHYSICAL MAP OF 186

A. INTRODUCTION

The choice between a plasmid or a phage vector is dependent on the DNA to be cloned because each system has some advantages (see Section I). The plasmid pBR322 was chosen as the vector for this work for several reasons:

(1) the DNA fragments to be cloned were, for the most part, small (1 to 4 kb) and so by using a small plasmid vector greater amplification of the inserted DNA was obtained, for example, a 4.4 kb insert would represent 50% of the DNA of a pBR322 recombinant but only about 10% of a recombinant λ phage.

(2) a number of different restriction enzymes were used for this study, and therefore a vector that permitted the use of these enzymes alone or in combination was required; pBR322 has unique sites for *Pst*I, *Hin*dIII, *Bam*HI and *Eco*RI and is suitable for cloning fragments generated by double digestion with *Bam*HI/*Eco*RI or *Hin*dIII/*Bgl*II (since *Bam*HI and *Bgl*II generate the same cohesive ends).

(3) cloning into pBR322 allowed rapid identification of the genes encoded by any restriction fragment by marker rescue. Marker rescue from a recombinant λ phage is possible only after a lysogen (or, if the vector is defective in the *c*I gene, a double lysogen) has been isolated.

Two fragments cloned into pBR322 proved to be unstable and for one of these the entire plasmid (pJF18) was subse-

quently cloned into a λ replacement vector.

Marker rescue from restriction fragments was first described for ϕX174 by Edgell *et al.* (1972). In these experiments mutant, single stranded, circular DNA was annealed to a denatured wild type restriction fragment of replicative form DNA and the partial heteroduplex was used to infect *E. coli*. The host used was non-permissive for the mutation and so wild type phage were recovered only when the restriction fragment coded for the mutant gene. This technique has also been applied to temperature sensitive mutations of SV40 (Lai and Nathans, 1975) and amber mutations of fd, f1 and M13 (Seiburg and Schaller, 1975).

In the genetic mapping of 186 Hocking (1977) ordered many of the genes by the frequency of marker rescue obtained during the superinfection of a non-permissive host, lysogenic for 186*cItsam* or for a P2.186 hybrid prophage, with a virulent phage mutant in an essential gene. Rescue from a λ, λp186 double lysogen gave information about genes encoded on the 186 fragment inserted into the *Eco*RI site of a λ vector (Hocking, 1977). A further variation of marker rescue has made possible the location of genes on cloned restriction fragments. A sup^+ (non-permissive) cell carrying a recombinant plasmid was infected with an amber mutant phage; plaques were observed only if the restriction fragment encoded the gene mutant in the infecting phage and thus provided wild type function either by recombination or by complementation. The integration of the genetic and physical maps of T7 DNA has been achieved by recombination between cloned *Hpa*I restriction or random shear fragments of T7 DNA and a

mutant infecting phage (Campbell *et al.*, 1978).

Cloned restriction fragments of known genetic composition would be invaluable for the analysis of transcripts of the genes encoded by any fragment and therefore it was the aim of this section of work to correlate the genetic map of 186 (Hocking, 1977) illustrated in Fig. 5.1 with the restriction map constructed by Saint and Egan (1979) (Fig. 5.2) to provide a physical map of 186.

B. MATERIALS

The following materials were purchased from the companies indicated:

Low melting agarose: Marine Colloids Inc., Rockland, Maine, U.S.A.

T4 polynucleotide ligase: Miles Laboratories Inc., Elkhart, Indiana, U.S.A.

*Bam*HI restriction endonuclease: New England Biolabs. Inc., Beverly, Massachusetts, U.S.A.

DEAE-cellulose: Whatman, Maidstone, Kent, U.K.

Ampicillin: donated by Beecham Research Laboratories, Sefton, New South Wales, Australia.

Tetracycline: donated by Commonwealth Serum Laboratories, Melbourne, Australia.

Chloramphenicol BP micronised powder: a gift of Parke Davis and Co., Caringbah, New South Wales, Australia.

FIGURE 5.1. The Genetic Map of Coliphage 186

This map shows the order of the 186 genes determined by genetic recombination. The functions assigned to the genes are recorded below the map (Hocking, 1977). The position of *int* (70.3%) and *c*I (73.6%) genes have been determined by EM heteroduplex studies (Younghusband *et al.*, 1975). Gene E has been fixed at 65.4%, the crossover point in P2.186 Hy5.

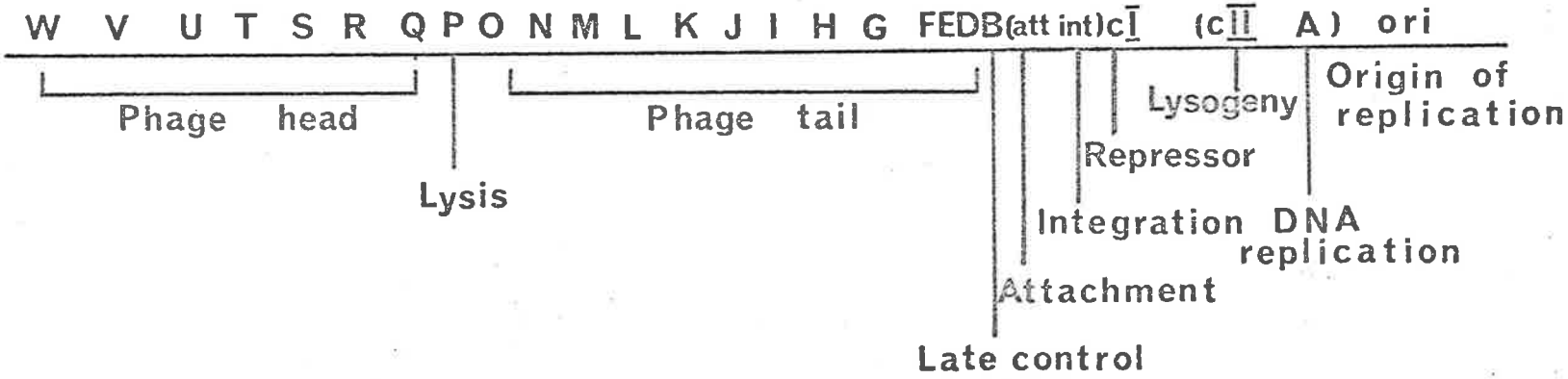
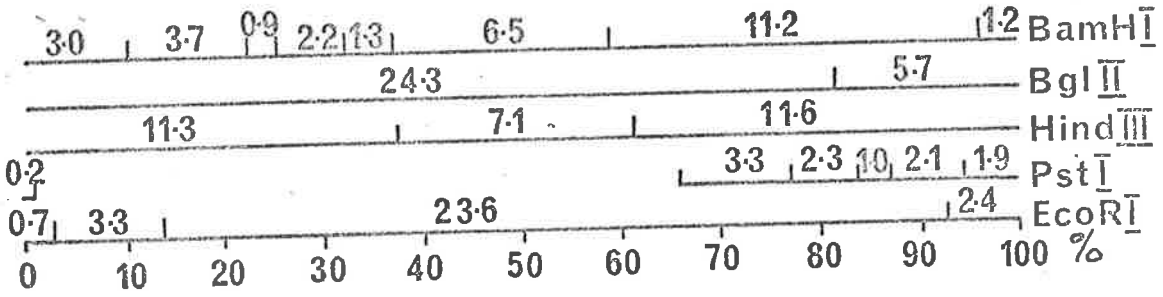

W V U T S R Q P O N M L K J I H G F E D B (att int) cI (cII A) ori
Phage head
Phage tail
Lysis
Late control
Attachment
Integration
Repressor
Lysogeny
DNA replication
Origin of replication

FIGURE 5.2. A Map of 186 Showing the Location of the Restriction Sites for *Eco*RI, *Hin*dIII, *Bgl*II, *Bam*HI and *Pst*I Endonucleases

The size of the fragments (kb) generated by digestion of 186*cIts* DNA with each enzyme is shown.


3·0
3·7
0·9
2·2
1·3
6·5
11·2
1·2
BamHI
24·3
5·7
BglII
11·3
7·1
11·6
HindIII
0·2
3·3
2·3
1·0
2·1
1·9
PstI
0·7
3·3
23·6
2·4
EcoRI
0
10
20
30
40
50
60
70
80
90
100
%

Streptomycin sulphate: Sigma Chemical Company, St. Louis, U.S.A.

Lysozyme: Sigma Chemical Company, St. Louis, U.S.A.

Selective agar plates were L agar plates containing 20 µg/ml tetracycline or 25 µg/ml ampicillin respectively.

XG agar plates: T plate agar with the addition/L of 10 ml 0.1 M isopropyl-β-D-thiogalactopyranoside and 0.4 gm 5-bromo-4-chloro-3-indolyl-β-D-galactoside dissolved in 10 ml dimethyl formamide.

Denhardt solution: 0.02% Bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll.

Triton solution: 1 ml 10% (v/v) Triton X-100, 12.5 ml 0.5 M EDTA, 5 ml 1 M Tris-HCl pH 8.0, 80 ml H_2O.

Hybridization buffer: 2 x SSC, 0.5% SDS.

The *sup*$^+$ bacterial strain used in transformation experiments was W3350 (Section II.A.1).

C. METHODS

1. Cloning of 186 restriction fragments into pBR322

(a) Restriction endonuclease digestion

The digestion conditions used for *Bgl*II, *Eco*RI and *Hin*dIII were those described in Section IV.C.1 In addition *Bam*HI digestions were carried out at 37°C in 10 mM Tris-HCl pH 7.5, 10 mM 2-mercaptoethanol and 10 mM $MgCl_2$. The conditions for *Pst*I digestions were as for *Bam*HI with the addition of NaCl to 50 mM. *Pst*I digestion was carried

out at 30°C because the enzyme was reported to be unstable at 37°C (Smith *et al.*, 1976).

(b) Ligation

Donor and vector DNA were mixed in a donor:vector ratio of 10:1 and ligation was carried out in 660 mM Tris-HCl pH 7.5, 10 mM EDTA pH 9.0, 100 mM $MgCl_2$, 100 mM dithiothreitol, 1 mM ATP with 0.5 units of T4 polynucleotide ligase. The reaction mix was incubated at 10°C for six hours and then transferred to 4°C for at least 15 hours.

(c) Transformation

Stationary phase bacteria were diluted one hundred-fold into L broth and grown to a cell density of 2 to 4 x 10^8 *cfu*/ml. The culture was chilled for 20 minutes and then the cells were washed once in one half volume of cold 0.1 M $MgCl_2$ and finally resuspended in one-tenth original volume of cold 0.1 M $CaCl_2$. The cells were left on ice for 60 to 90 minutes. Aliquots of ligation mix containing 0.1 µg or 0.01 µg of vector DNA were diluted with 100 mM Tris pH 7.6 to a final volume of 0.1 ml, and 0.2 ml competent cells were added. This mixture was chilled for 30 minutes, then heat shocked at 42°C for two minutes and chilled for a further 30 minutes. The cells were held at room temperature for 30 minutes and then incubated at 37°C for 30 minutes after the addition of 0.5 ml L broth, before being plated on to L + tetracycline (20 µg/ml) or L + ampicillin (25 µg/ml) plates (3 plates/0.2 ml cells). Plates were incubated at 37°C overnight and then recombinants were selected by screening for sensitivity to the second anti-

biotic (ampicillin or tetracycline respectively).

The transformation efficiency of competent W3350 cells for unrestricted pBR322 was 5 x 10^5 to 10^6 *cfu*/µg DNA. In general the efficiency was reduced one hundred-fold by restriction and was increased approximately ten-fold by ligation. The number of hybrid recombinant plasmids produced varied with the restriction enzyme, and small DNA fragments were cloned more frequently than larger fragments.

Cells carrying recombinant plasmids were purified by single colony isolation and plasmid DNA was prepared by the procedure described in Section V.C.5. The recombinant plasmid was analysed by restriction with the enzyme used in its cloning followed by agarose gel electrophoresis. The genetic content of cloned fragments was determined by marker rescue (Section V.C.4).

(d) Transfection

Competent cells for transfection were prepared (see Section V.C.1(c)) and 0.2 ml of cells were added to 0.1 ml 100 mM Tris pH 7.6 containing ligated DNA. This mixture was chilled for 30 minutes, then heat shocked at 42°C for two minutes and chilled for a further 30 minutes. The cells were held at room temperature for 30 minutes and then plated on to the appropriate agar plates to permit the selection of recombinant phage.

2. Purification of DNA from low melting agarose

The cloning of particular fragments of 186 was achieved via the purification and subsequent isolation of

the restriction fragment from low melting agarose. Plasmids cloned in this way were pJF18 (*Pst*I digestion of Hy5 DNA) and pJF23 (*Bam*HI digestion of 186).

The donor DNA was digested with the appropriate restriction endonuclease and then the fragments were resolved by electrophoresis on a 1% low melting agarose gel (Section V.C.3). The DNA was stained as described (Section IV.C.2) and the band representing the fragment of interest was cut from the gel. The agarose was melted at 70°C and then the DNA was purified by passage through an hydroxy-apatite column equilibrated at 70°C with 12 mM $NaPO_4$, 1 mM EDTA pH 7.0. The column was extensively washed with this buffer to remove the agarose and then the DNA was eluted with 0.4 M $NaPO_4$, 1 mM EDTA pH 7.0. After dialysis to remove phosphate the DNA was ethanol precipitated and dissolved in 50 µl 10 mM Tris-HCl pH 7.5 for ligation with pBR322.

The recovery of transforming ability of 1 µg of pBR322 from low melting agarose was only 10%. The desired recombinants were at a very low frequency, approximately 1 in 600 and 1 in 800 for pJF18 and pJF23 respectively.

3. Agarose gel electrophoresis

The procedure for agarose gel electrophoresis used in the analysis of recombinant plasmids was essentially that described in Section IV.C.2. The plasmids were electrophoresed with pBR322 and 186 (or where more appropriate Hy5), digested with the same restriction enzyme, in adjacent tracks to permit the identification of the inserted 186 fragment.

Gels of 0.2 cm were used with 0.5 µg DNA loaded/cm track.

Low melting agarose gels used in the purification of restriction fragments were 20 cm x 20 cm x 0.2 cm and were 1% agarose. These gels were run at 4°C at an angle to prevent the gel collapsing.

4. Marker rescue

Marker rescue experiments were performed in one of two ways.

(a) Spot testing

Spot testing allowed the rapid screening of a large number of recombinant plasmids for the presence of a number of 186 essential genes. In this way 12 different genes could be tested on each plate.

A suspension of the *sup*$^+$ bacteria hosting a recombinant plasmid was made by taking a loopful of the culture from a selective plate into 0.2 ml selective broth. After the addition of 3 ml molten agar a lawn was poured on to a selective plate. When the agar had set, a single drop of each solution containing about 10^7 *pfu*/ml of 186 amber mutant phage was spotted, to form a grid. When the spots were dry the plate was incubated at 37°C overnight. A control plate with a *sup*$^+$ bacteria hosting pBR322 forming the lawn was prepared in a similar way.

Rescue was scored by the appearance of individual plaques or a cleared area coincident with the spot. The background reversion frequency was monitored by the use of the control plate.

Positive spot testing was then confirmed by assaying for the recovery of wild type function (Section V.C.4(b)). The other alleles from the same gene as well as the adjacent genes were also tested to define the end points of the fragments.

(b) Assay testing

To 0.2 ml log phase *sup*$^+$ bacteria carrying the recombinant plasmid of interest 0.1 ml 186 amber mutant phage (10^7 *pfu*/ml) were added and allowed to adsorb before plating on tryptone agar. Plates were incubated at 37°C overnight and the number of plaques observed were compared to the number obtained when the same phage was assayed on the *sup*$^+$ bacteria lacking the plasmid. A positive result was recorded only if the frequency of marker rescue was at least one hundred-fold above the background reversion frequency.

5. Plasmid DNA preparation

Stationary phase bacteria carrying the appropriate plasmid were diluted one hundred-fold into L broth, containing 25 µg/ml ampicillin or 20 µg/ml tetracycline and grown to a density of about 3×10^8 *cfu*/ml. Chloramphenicol was added to 150 µg/ml and the cultures were incubated for another 15 hours. Amplification by the addition of chloramphenicol was subsequently discarded in favour of growing the cultures to stationary phase (overnight at 37°C) because this gave improved yields and minimized the problems experienced with unstable plasmids (see Appendix I(b)).

The cells derived from a one litre culture were washed in 500 ml cold TE, resuspended in 15 ml iced 25% sucrose in 50 mM Tris-HCl pH 8.0 and lysed as follows: 5 ml lysozyme (10 mg/ml) were added, the mix swirled on ice for five minutes, a further 5 ml 0.5 M EDTA pH 8.5 added, gently mixed for five minutes on ice, and finally 12.5 ml iced Triton solution added and the lysate swirled for ten minutes on ice. The lysate was cleared by a 30 minute centrifugation (60 minute for stationary cultures) at 30,000 r.p.m. in a Ti50 rotor, and the supernatant was carefully removed. The volume was measured (X ml) and a CsCl gradient was prepared by the addition of X gm CsCl and 0.X ml ethidium bromide to the supernatant. The gradient was centrifuged to equilibrium (50 to 60 hours) in a Ti50 rotor at 40,000 r.p.m. and 20°C.

The lower plasmid band, visible under UV light, was retrieved by side puncture of the tube. The dye was removed by extraction against iso-amyl alcohol and the DNA was dialysed against 1 mM EDTA pH 8.0 and stored at 4°C.

6. Hybridization to recombinant clones represented by single plaques

(a) Filter preparation

Plaques that appeared to be colourless (that is, recombinant) on XG agar plates were stabbed into a lawn of *lacZam* bacteria on another XG plate and into a bacterial lawn on an L plate to confirm that the phage were recombinant and that 186 DNA had been inserted. A negative control of λ vector was included on each grid. After growth the

L plate for the hybridization assay was put at 4°C for 30 minutes to harden the agar. A dry nitrocellulose filter, the size of the dish, was placed on the agar allowing no air bubbles to remain between the filter and the agar. Phage were allowed to adsorb to the filter for one minute. The phage and DNA were denatured and fixed *in situ* by dipping the filter into 0.1 N NaOH, 1.5 M NaCl for one minute and then the filter was neutralized in 0.2 M Tris-HCl pH 7.5, 1.5 NaCl for one minute. Filters were blotted then baked at 80°C *in vacuo* for three hours (Benton and Davis, 1977).

(b) Hybridization and autoradiography

Filters were incubated for six hours in Denhardt solution at 65°C to 70°C and blotted dry prior to hybridization with randomly primed ^{32}P-186 cDNA (gift of R.B. Saint). Hybridization was set up by dipping the filter into a solution of hybridization buffer containing the probe, the volume of which was sufficient to wet a dry filter with an area equal to that used (13 µl solution/cm^2 filter). The filter was placed in a petri dish, covered with moist Whatman 3 mm paper held in place with microscope slides then overlayed with paraffin oil. The filter was incubated at 65°C to 70°C for 20 hours and then rinsed with 2 x SSC before washing in 2 x SSC + 0.25% SDS, then 2 x SSC + 0.12% SDS and finally 2 x SSC at 65°C to 70°C for at least four hours in each solution.

The filter was dried and an autoradiogram was set up as described in Section IV.C.5 but without prefogging the X-ray film.

7. Preparation of *Pst*I restriction endonuclease

*Pst*I restriction endonuclease from *Providencia stuartii* was purified by the method of Smith *et al.* (1976) but with some alterations.

Twenty grams of cells were washed twice with 175 ml of buffer containing 0.01 M Tris-HCl pH 7.8, 0.03 M NaCl, resuspended in 175 ml of buffer containing 0.033 M Tris-HCl pH 7.8, 0.003 M EDTA, 20% (w/v) sucrose and stirred vigorously at room temperature for 20 minutes. After centrifugation at 16,000 g for 10 minutes the supernatant was discarded and excess sucrose was removed from the pellet with a cotton swab. The pellet was resuspended in 100 ml ice cold 5×10^{-4} M $MgCl_2$, stirred vigorously at 4°C for 10 minutes and centrifuged as before. The supernatant was adjusted to 0.01 M $NaPO_4$ pH 7.0, 0.001 M EDTA, 0.001 M 2-mercaptoethanol and 0.15% Triton X-100 (Buffer A) by the addition of a concentrated stock solution. Streptomycin sulphate (1 ml 10% (w/v) $SmSO_4$/1500 OD units) was added and stirred for 20 minutes at 4°C then centrifuged as before to precipitate nucleic acids. The supernatant was loaded on to a DEAE-cellulose column (1.5 cm x 20 cm) and the enzyme was eluted with a 500 ml gradient of 0 to 0.5 M NaCl in Buffer A.

Fractions were assayed for endonuclease activity by digestion of λ DNA followed by electrophoresis. Active fractions were pooled and dialysed overnight against Buffer A plus 50% glycerol (v/v). The enzyme was stored at -20°C.

D. RESULTS

1. Physical map for the late functions of 186

186 has six sites in the late region for *Bam*HI restriction endonuclease (Fig. 5.3) and each *Bam*HI fragment was cloned in the plasmid vector pBR322 (Fig. 5.4). Marker rescue experiments, in which the wild type allele was recovered from cloned 186 DNA by a superinfecting mutant phage, identified the genes encoded within each fragment. A single mutant allele of each gene was chosen to represent that gene for these experiments. Then when the terminal genes of each fragment had been identified, every available allele of the terminal genes was tested to define more accurately the fragment end points. The results were tabulated (Table 5.1) and the following limits can be assigned to the positions of certain genes: W, 0 to 10.0%; U through R, 10.0 to 22.3%; Q, 22.3 to 25.3%; P through N, 25.3 to 32.7%; M through L*am*2, 32.7 to 37.0%; L*am*2 through G, 37.0 to 58.7%; F through A, 58.7 to 96.0%.

Recovery of all alleles was observed and, with the exception of L*am*2, no allele appeared on more than one fragment. This allele was recovered from both pJF5 and pJF11, which suggested that the mutation was located within the recognition sequence of the *Bam*HI restriction endonuclease. It may be worth noting that the recovery of this allele from pJF5 was ten-fold higher than from pJF11, even though the inserted 186 DNA in pJF5 is five times smaller. The remaining five *Bam*HI sites appeared to fall between genes.

Genes W through R were mapped more accurately by the

FIGURE 5.3. A Cleavage Map of the 186 Genome Showing the Location of the *Bam*HI Restriction Sites

The position of the sites is given as % 186 genome where 1% represents 296 b.p. The fragments cloned to give the plasmids pJF5, pJF6, pJF8, pJF10, pJF11 and pJF23 are indicated below the map.

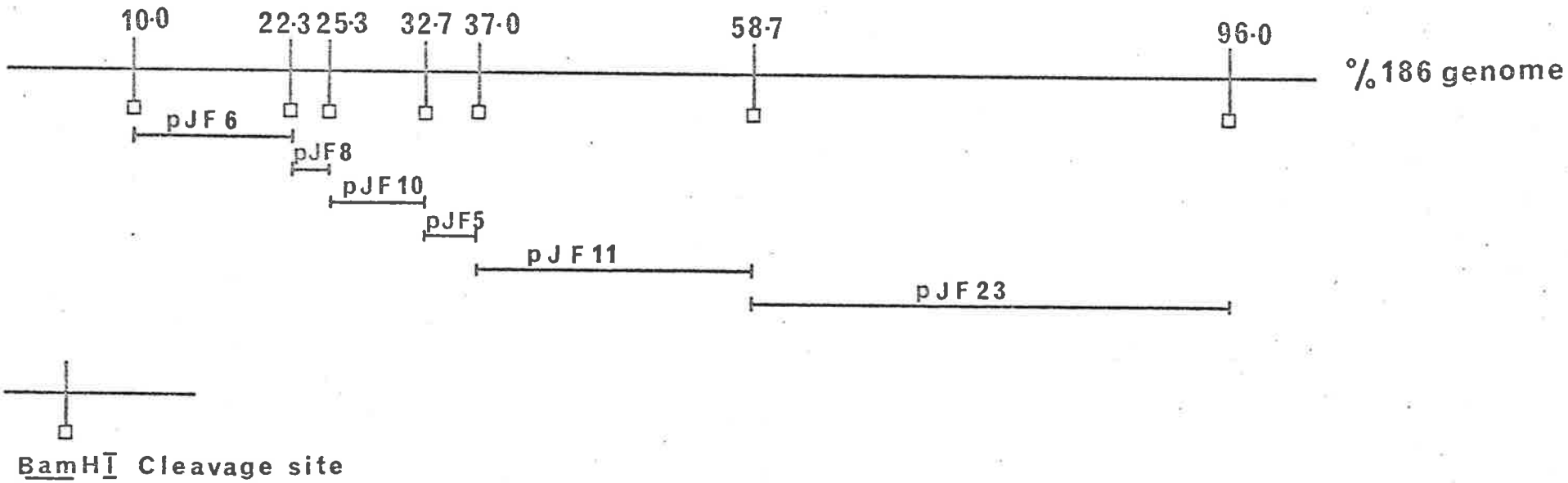

10·0
22·3
25·3
32·7
37·0
58·7
96·0
% 186 genome
pJF 6
pJF8
pJF 10
pJF5
pJF 11
pJF 23
BamHI Cleavage site

FIGURE 5.4. Digestion by *Bam*HI of Plasmids Carrying *Bam*HI Restriction Fragments of 186

In Figures 5.4, 5.6 and 5.8 molecular weight estimations were taken from Saint and Egan, (1979). Digested 186 and Hy5 DNA did not undergo end denaturation before electrophoresis; the sizes of the end fragments of 186 and Hy5 are underlined and the fragment size of the reannealed end pieces is in brackets.

(a) *Bam*HI digestion of: 186 DNA, track A; pJF6 DNA, track B; pJF8 DNA, track C; pJF10 DNA, track D; pBR322 DNA, track E.

(b) *Bam*HI digestion of: 186 DNA, track F; pJF23 DNA, track G; pJF11 DNA, track H; pJF5 DNA, track I; pBR322 DNA, track J.

Note the heavy band in track G, migrating slightly faster than the 3.7 kb fragment of 186. This represents the plasmid resulting from the breakdown of pJF23 during preparation (see Appendix I(b)).

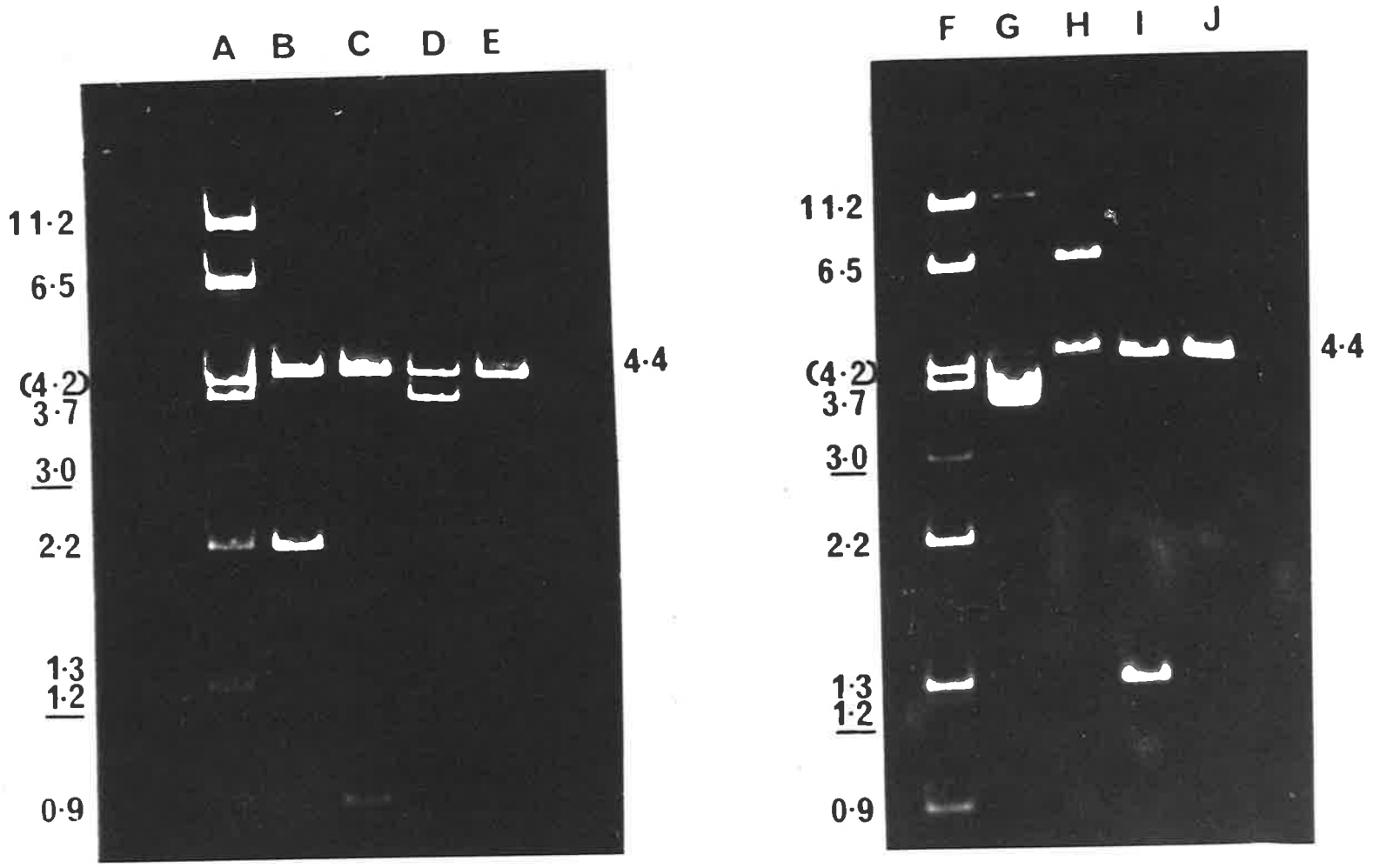

A B C D E
11·2
6·5
(4·2)
3·7
3·0
2·2
1·3
1·2
0·9
4·4
F G H I J
11·2
6·5
(4·2)
3·7
3·0
2·2
1·3
1·2
0·9
4·4

TABLE 5.1. Marker Rescue Experiments from *Bam*HI Restriction Fragments Cloned into pBR322

A single allele has been chosen to represent each gene; alleles used were:-

W*am*15	P*am*45	N*am*50
V*am*38	O*am*62	G*am*9
U*am*37	N*am*47	F*am*20
T*am*8	M*am*19	E*am*7
S*am*4	L*am*21	D*am*48
R*am*6	K*am*42	B*am*57
Q*am*49	J*am*41	A*am*12
	I*am*40	

Plasmid	Gene																						
	W	V	U	T	S	R	Q	P	O	N	M	*Lam*21	*Lam*2	K	J	I	H	G	F	E	D	B	A
pJF6	--[a]	+[a]	+	+	+	+[a]	--[a]	-	-	-	-	-	O	-	-	-	-	-	-	-	-	-	-
pJF8	--	-	-	-	-	-[a]	++[a]	------[a]	-	-	-	-	O	-	-	-	-	-	-	-	-	-	-
pJF10	-	-	-	-	-	-	--[a]	++++++[a]	+	+[a]	---[a]	-	O	-	-	-	-	-	-	-	-	-	-
pJF5	-	-	-	-	-	-	-	-	-	-[a]	+++[a]	+	+[a]	---[a]	-	-	-	-	-	-	-	-	-
pJF11	-	-	-	-	-	-	-	-	-	-	-	-	+[a]	+++[a]	+	+	+	++++[a]	-[a]	-	-	-	-
pJF23	-	-	-	-	-	-	-	-	-	-	-	-	O	-	-	-	-	----[a]	+[a]	+	+	+	+

[a]Every allele of the terminal genes has been tested.

In the Table, + represents marker rescue of at least 100-fold over the background reversion rate.

- represents no increase above background.

O not tested.

use of the *Eco*RI restriction sites (Fig. 5.5). The *Eco*RI fragment including the cohesive ends (92.0 to 2.3%) had previously been cloned into a λ vector and shown to encode no known 186 genes (Hocking, 1977). *Bam*HI/*Eco*RI double restriction fragments were cloned into pBR322 (Figs. 5.5 and 5.6) and the gene content determined by marker rescue. The data presented in Table 5.2 permitted the following placements: W, 2.3 to 10.0%; V, 10.0 to 13.3%; U through R, 13.3 to 22.3%.

Finally the *Hin*dIII fragment (37.6 to 61.3%) was cloned (pJF22) and analysed by marker rescue (Fig. 5.5, Table 5.3). Inability to rescue allele L*am*2 from this fragment confirmed the positioning of the *Hin*dIII site (37.6%) to the right of the nearby *Bam*HI site (37.0%). F*am*20, which could not be recovered from pJF22, was therefore to the right of 61.3%, whereas all the known alleles of the adjacent gene G were to the left of the *Bam*HI site at 58.7%.

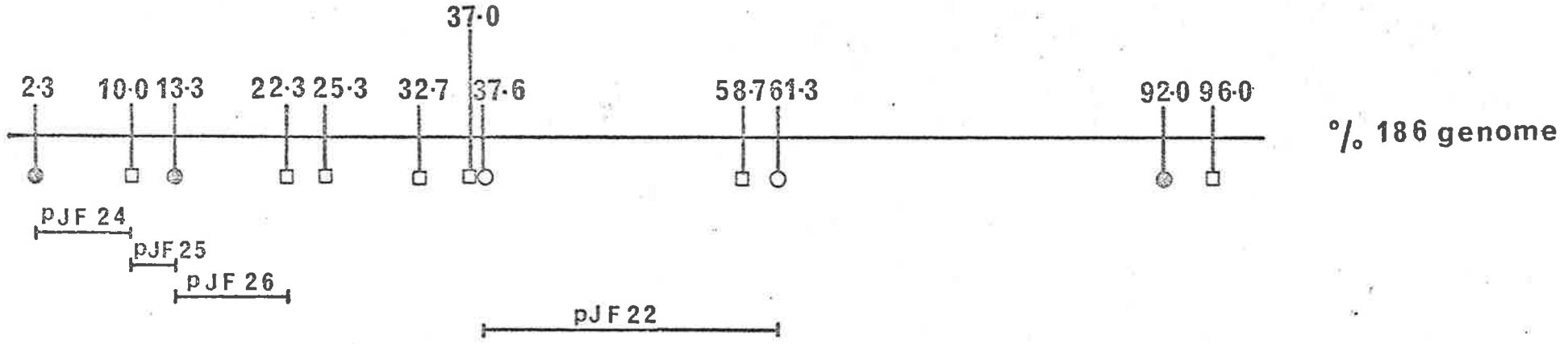
2. Physical map of the early functions of 186

*Bam*HI has only one site (96.0%) to the right of its site at 58.7% and therefore it was of limited use in the physical mapping of 186 early functions. However, *Pst*I, *Bgl*II and *Eco*RI, each of which has restriction sites within this region, proved most useful for this work.

Recombination mapping had placed gene A in the 30% of the genome to the right of the *att* site (Fig. 5.1; Hocking, 1977). Initially gene A was located on the 81.0 to 92.0% *Bgl*II/*Eco*RI fragment carried by pJF13; a finer analysis was made using the *Pst*I fragments depicted in Figs. 5.7 and 5.8.

FIGURE 5.5. A Cleavage Map of the 186 Genome Showing the Location of *Bam*HI, *Eco*RI and *Hin*dIII Restriction Sites

The position of the sites is given as % 186 genome. The fragments generated by double digestion with *Bam*HI and *Eco*RI were cloned to give the plasmids pJF24, pJF25 and pJF26 which are indicated below the map. The *Hin*dIII fragment cloned in pJF22 is also shown.


37·0
2·3
10·0
13·3
22·3
25·3
32·7
37·6
58·7
61·3
92·0
96·0
% 186 genome
pJF 24
pJF 25
pJF 26
pJF 22

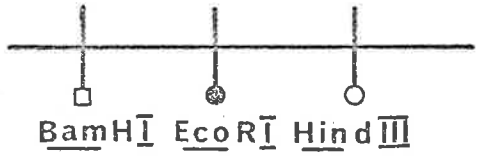

BamHI EcoRI HindIII
Cleavage sites

FIGURE 5.6. Double Digestion by *Bam*HI and *Eco*RI of the Plasmid Clones pJF24, pJF25 and pJF26

Track A: pBR322 DNA.

Track B: pJF24 DNA.

Track C: pJF25 DNA.

Track D: pJF26 DNA.

Track E: 186 DNA.

186 DNA was not end denatured prior to electrophoresis.

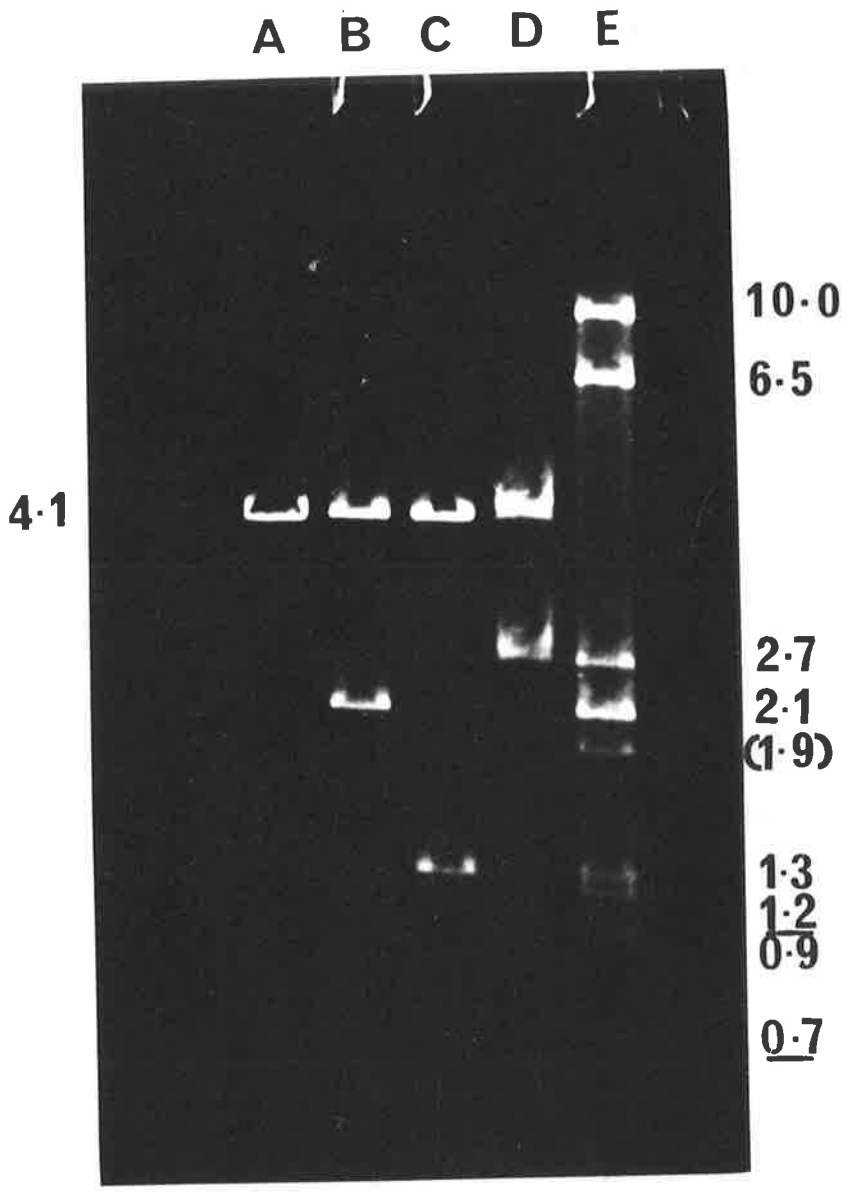

A
B
C
D
E
10·0
6·5
4·1
2·7
2·1
(1·9)
1·3
1·2
0·9
0·7

TABLE 5.2. Marker Rescue from Cloned *Bam*HI/*Eco*RI Restriction Fragments

\+ Represents marker rescue of at least 100-fold over the background revision rate.

\- Represents background level.

Plasmid	Gene							
	W	V	U	T	S	R	G	P
pJF24	+++	-	-	-	-	-	-	-
pJF25	---	+	---	-	-	-	-	-
pJF26	-	-	+++	+	+	+	--	-

TABLE 5.3. A Comparison of Marker Rescue from pJF22 and pJF11

pJF22 carries of *Hin*dIII restriction fragment 37.6 - 61.3% of 186 genome; pJF11 carries the B*am*HI restriction fragment for the same region 37.0 - 58.7%.

\+ Represents marker rescue of at least 100-fold above revision frequency.

\- Represent background revision frequency.

Plasmid	Gene								
	M	L*am*21	L*am*2	K	J	I	H	G	F
pJF11	-	-	+	+	+	+	+	+++	-
pJF22	-	-	-	+	+	+	+	+++	-

FIGURE 5.7. A Cleavage Map of the Region 65 to 100% of the 186 Genome Showing the Location of the *Pst*I, *Eco*RI and *Bgl*II Restriction Sites

The position of the sites is given as % 186 genome. The fragments cloned to give the plasmids pJF13, pJF15, pJF16, pJF17, pJF18 are indicated below the map.

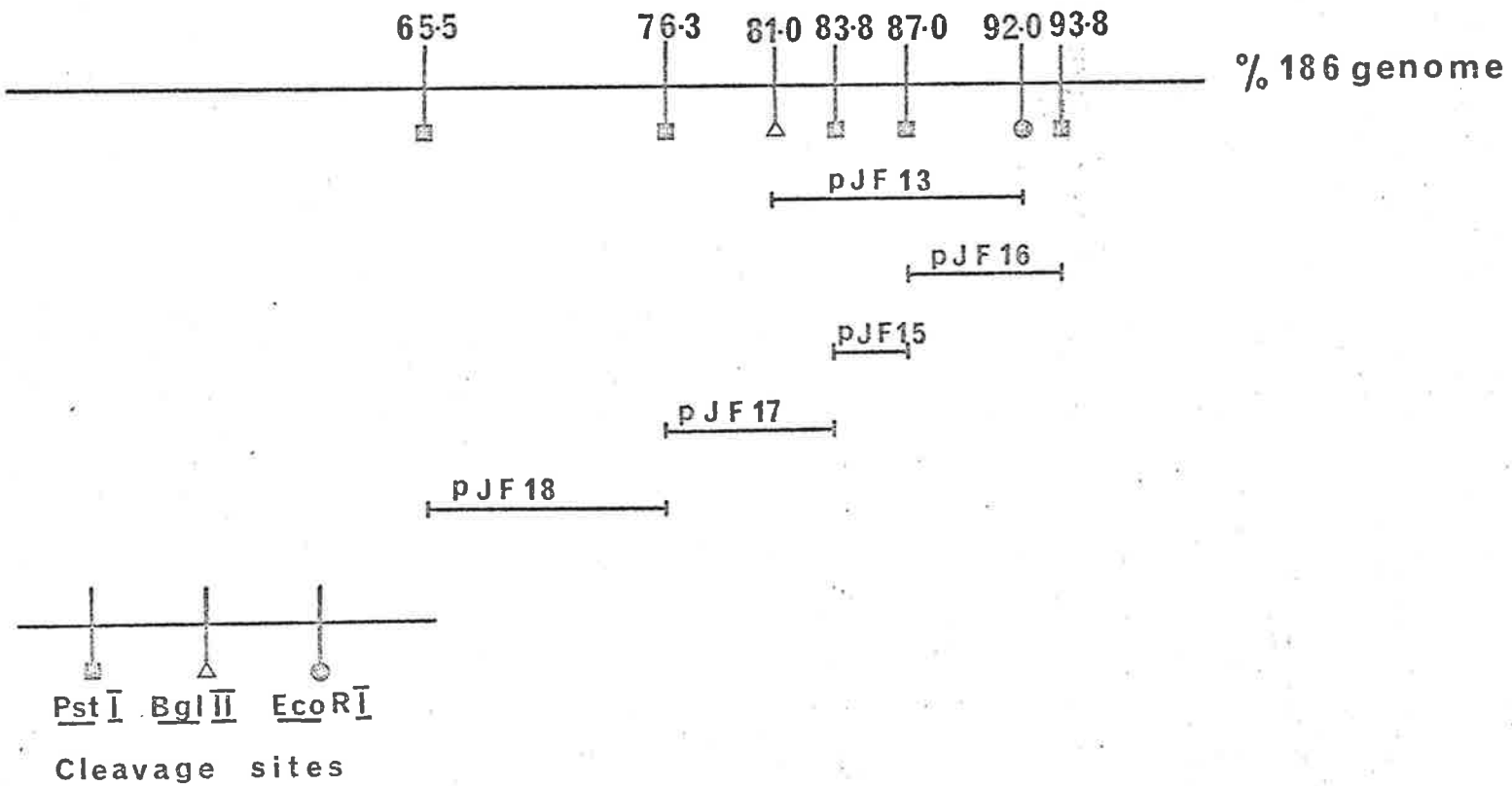

65·5
76·3
81·0
83·8
87·0
92·0
93·8
% 186 genome
pJF 13
pJF 16
pJF15
pJF 17
pJF 18
Pst I
Bgl II
EcoR I
Cleavage sites

FIGURE 5.8. Digestion by *Pst*I of the Plasmid Clones pJF15, pJF16, pJF17 and pJF18

Track A: pBR322 DNA.

Track B: pJF18 DNA.

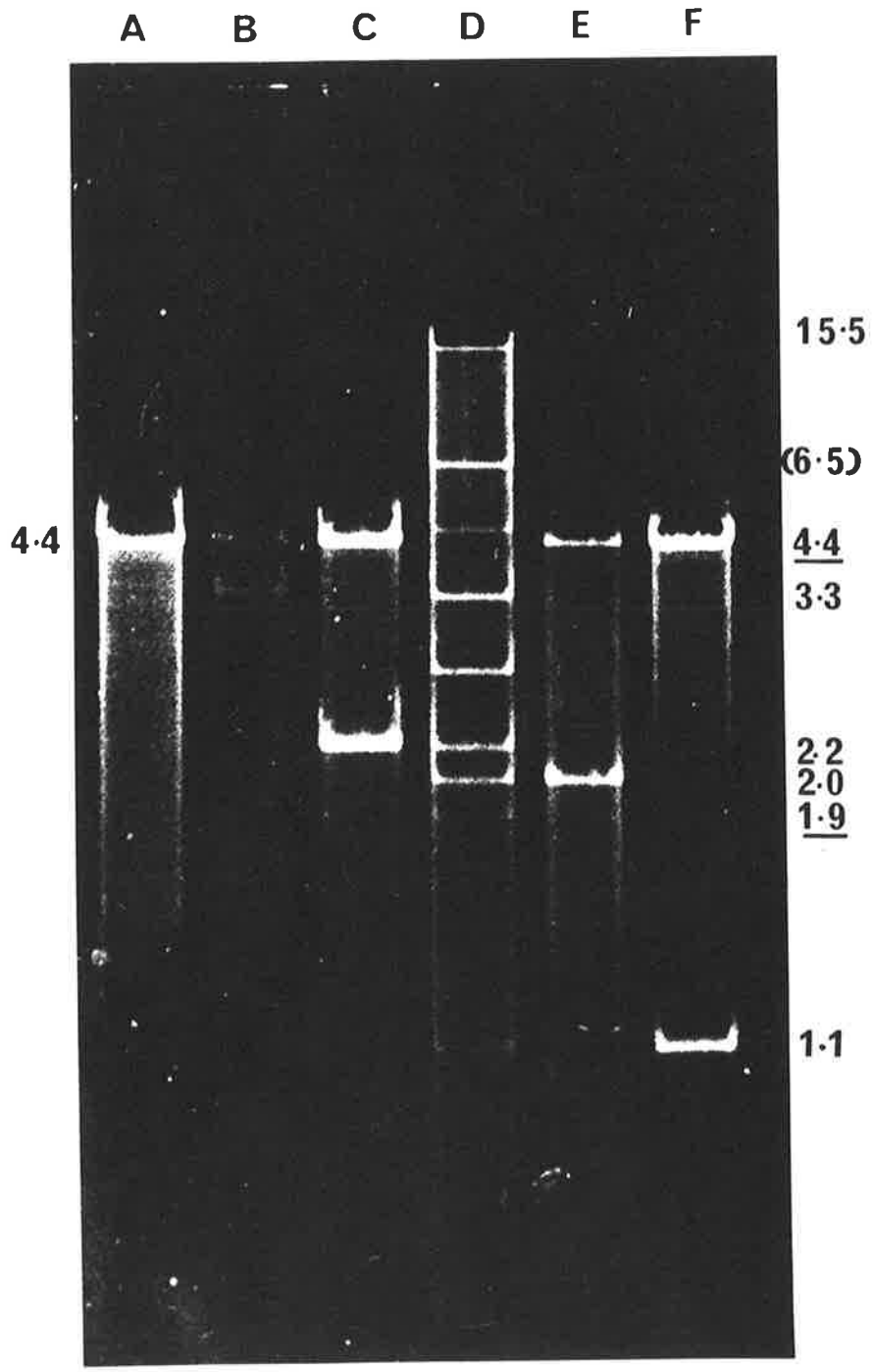
Track C: pJF17 DNA.

Track D: Hy5 DNA.

Track E: pJF16 DNA.

Track F: pJF15 DNA.

The end fragments of Hy5 (underlined) of 1.9 kb and 4.4 kb are derived from the 186 and the P2 regions of the hybrid, respectively.


A
B
C
D
E
F
4·4
15·5
(6·5)
4·4
3·3
2·2
2·0
1·9
1·1

Marker rescue experiments (Table 5.4) indicated that gene A spanned the restriction fragment in pJF15 (83.8 to 87.0%), with A*am*43 to the left of 83.8% located on pJF17 (76.3 to 83.8%), and with A*am*24 and A*am*33 to the right of 87.0% located on pJF16 (87.0 to 93.8%).

The remaining *Pst*I fragment (65.5 to 76.3%) coded for two of the four alleles of the tail gene D, fixing gene D at 65.5%. The *c*I repressor gene has been physically mapped by EM heteroduplex analysis at 73.6% and the *int* gene at 70.3% (Younghusband *et al.*, 1975); both genes were therefore expected to reside on the *Pst*I fragment (65.5 to 76.3%) cloned into pJF18. Marker rescue confirmed that gene B, genetically mapped between D and *att* (Hocking, 1977), was also contained on the *Pst*I fragment in pJF18.

3. Cloning of pJF18 into λ

The *Pst*I fragment (65.5 to 76.3%) cloned into pJF18 was found to be unstable during DNA preparation (see Appendix I(b) for a discussion) and so the entire plasmid was cloned into the λ *Hin*dIII replacement vector 762. Parental 762 phage, plated on a *lacZam* indicator lawn on XG agar plates, form blue plaques because the DNA flanked by the two *Hin*dIII restriction sites codes for the *E. coli supF* gene which suppresses the *lacZam* mutation. Recombinant phage, in which this DNA has been replaced, are detected as colourless plaques.

Colourless plaques were stabbed on to two plates; the first, an XG agar plate with a *lacZam* bacterial lawn, to confirm that the phage were recombinant (Fig. 5.9) and the

TABLE 5.4. Marker Rescue from the *Bgl*II/*Eco*RI (pJF13) and the *Pst*I Restriction Fragments Cloned into pBR322

Plasmid	Gene	D				B	A							
	Allele	48	26	23	14	57	43	5	11	12	13	30	24	33
pJF13		-	0	0	0	-	+	+	+	+	+	+	+	+
pJF16		-	0	0	0	-	-	-	-	-	-	-	+	+
pJF15		-	0	0	0	-	-	+	+	+	+	+	-	-
pJF17		-	0	0	0	-	+	-	-	-	-	-	-	-
pJF18		-	-	+	+	+	-	-	-	-	-	-	-	-

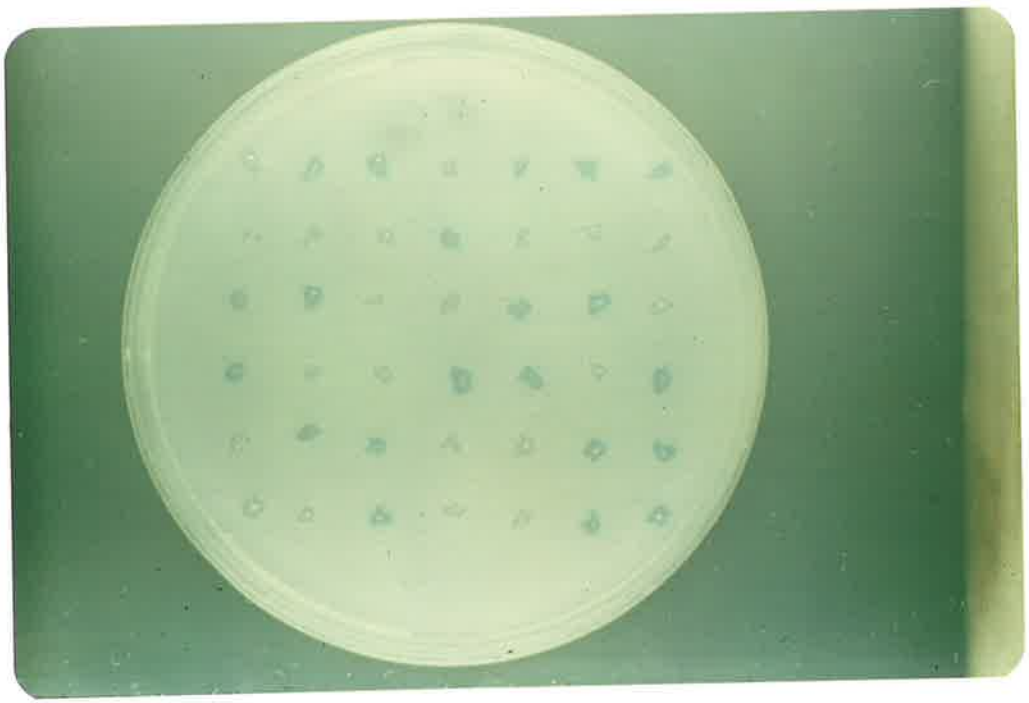
+ represents marker rescue of at least 100-fold above background reversion frequency.

- represents background.

0 not tested.

FIGURE 5.9. Selection of Recombinant λ Phage

Plaques that appeared to be colourless on an XG agar plate were stabbed into a lawn of *lacZam* indicator bacteria on a second XG agar plate. Recombinant λ phage produce colourless plaques while parental phage form blue plaques.



second, a replica, for use in a hybridization assay to determine whether the insertion was 186 DNA. DNA was prepared from a recombinant phage which gave a positive result when hybridized to 186 ^{32}P-cDNA (Fig. 5.10) and, following digestion with *Hin*dIII, it was analysed by electrophoresis (Fig. 5.11).

The plasmid pJF18 cloned in a λ *Hin*dIII replacement vector was stable and for this reason all transcription analysis was done with λppJF18 rather than pJF18 itself.

4. Cloning of pJF30 and pJF35

The *Pst*I fragment cloned into pJF18 codes for the functions *c*I, *int*, *att*, B and part of gene D. To analyse the transcription of gene B a smaller fragment was needed. A deletion of 186, 186*del*2, has been isolated and an EM heteroduplex study (R.M. O'Connor, pers. commun.) and a restriction analysis (Saint, 1979; Finnegan, unpub. obs.) revealed that this phage has a 2.1 kb deletion from 67.9 to 74.9% and a 0.4 kb insertion in the *Pst*I/*Bgl*II fragment 76.3 to 81.0% which inactivated the operator site (Fig. 5.12).

Deletion of 67.9 to 74.9% covers the *int* (70.3%), part, if not all, *c*I gene and possibly the *att* site. The B gene must lie to the left of 67.9% because 186*del*2 is a viable phage. The *Pst*I fragment (65.5 to 76.3%) of 186*del*2 that is equivalent to the fragment cloned in pJF18 would therefore be a suitable DNA template for studying the transcription of gene B. In addition to the B gene it will code for part of the gene D (a tail gene) and perhaps part of *c*I gene.

FIGURE 5.10. Hybridization of 186*cIts* ^{32}P-cDNA to Recombinant λ Phage

The presence of 186 DNA in recombinant phage formed by cloning pJF18 into λ762 was detected by the hybridization procedure developed by Benton and Davis (1977). All plaques in rows 1 through 5 and the first two plaques in row 6 were colourless when tested on a *lacZam* indicator lawn on an XG agar plate. The last three plaques in row 6 contained parental λ762 phage and thus constituted a negative control. Although the plaque in row 2, column 3, was colourless it did not contain 186 DNA.

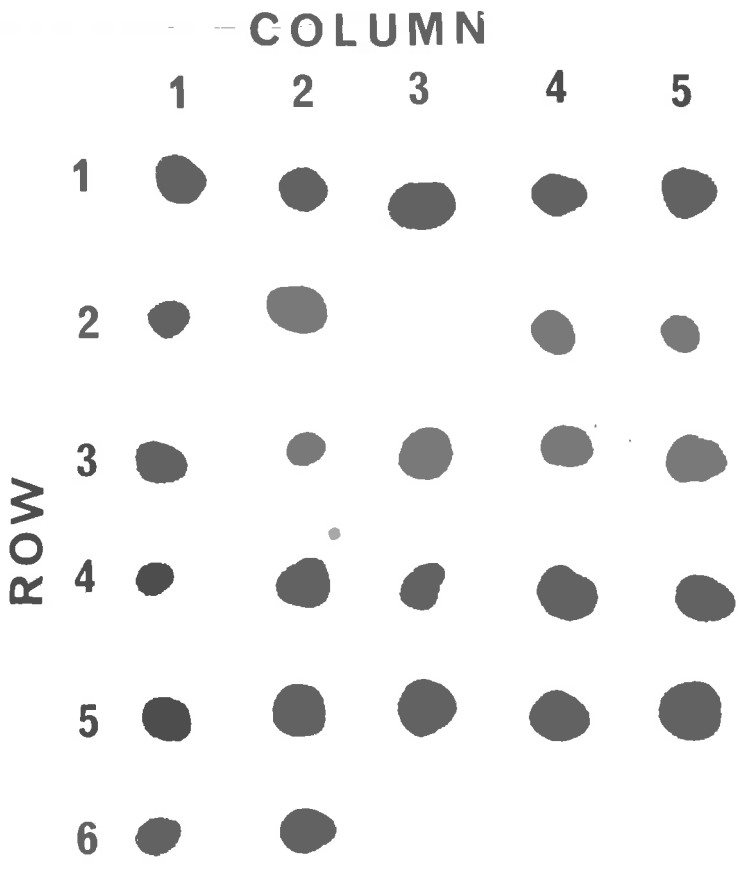

COLUMN
1
2
3
4
5
ROW
1
2
3
4
5
6

FIGURE 5.11. Digestion of λppJF18 with *Hin*dIII

Track A. λppJF18.

Track B. pJF18.

Track C. λ762.


A
B
C

*Pst*I has at least 22 cleavage sites on λ86 DNA (Saint and Egan, 1979). Therefore the approach used in cloning the *Pst*I fragment (65.5 to 76.3%) was to clone the *Hin*dIII/*Bgl*II fragment (61.3 to 81.0%) into pBR322 (pJF32) and then, after *Pst*I digestion of pJF32, to reclone these fragments into *Pst*I digested pBR322. The fragment 65.5 to 76.3% deleted from 67.9 to 74.9% was cloned into pJF35 (Fig. 5.12). Two other plasmids were also isolated; pJF36 codes for gene E as well as gene D*am*48 and D*am*26 while the second, pJF30 codes only for gene F. The exact location of these *Pst*I fragments has not been determined because the *Pst*I restriction sites to the left of 65.5% have not been mapped (Saint and Egan, 1979).

E. DISCUSSION

1. Physical map of λ86

The physical map derived from marker rescue analysis of fragments generated by *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I and *Bgl*II restriction endonucleases is depicted in Fig. 5.13. The left 23 kb (0 to 75%) codes for 23 known genes although their distribution is not uniform. The regions 10.0 to 37.0% and 61.3 to 73.6% are heavily occupied while the 0 to 10.0% and 37.0 to 61.3% are less well represented.

In contrast to the relative saturation of coding potential of the left hand 75% of the genome, the right hand 25% has only one known essential gene, gene A. It is probable that a second gene, the *c*II gene, needed to establish lysogeny (Huddleston, 1970) is located within this region, and perhaps a third gene, the hypothetical *dho* gene (see below

FIGURE 5.12. A Cleavage Map of 60 to 100% of the 186*del*2 Genome Showing the Position of *Hin*dIII, *Pst*I and *Bgl*II Restriction Sites

The position of the 2.1 kb deletion has been determined by EM heteroduplex analysis of 186*del*2 (R.M. O'Connor, pers. commun.); the 0.4 kb insertion has been mapped between 76.3 and 81.0% by restriction analysis of 186*del*2 (Saint, 1979; E.J. Finnegan, unpublished observations). The *Pst*I restriction sites to the left of 65.5% have not been mapped.

The fragments cloned to give the plasmids pJF30, pJF32, pJF35 and pJF36 are indicated below the map.

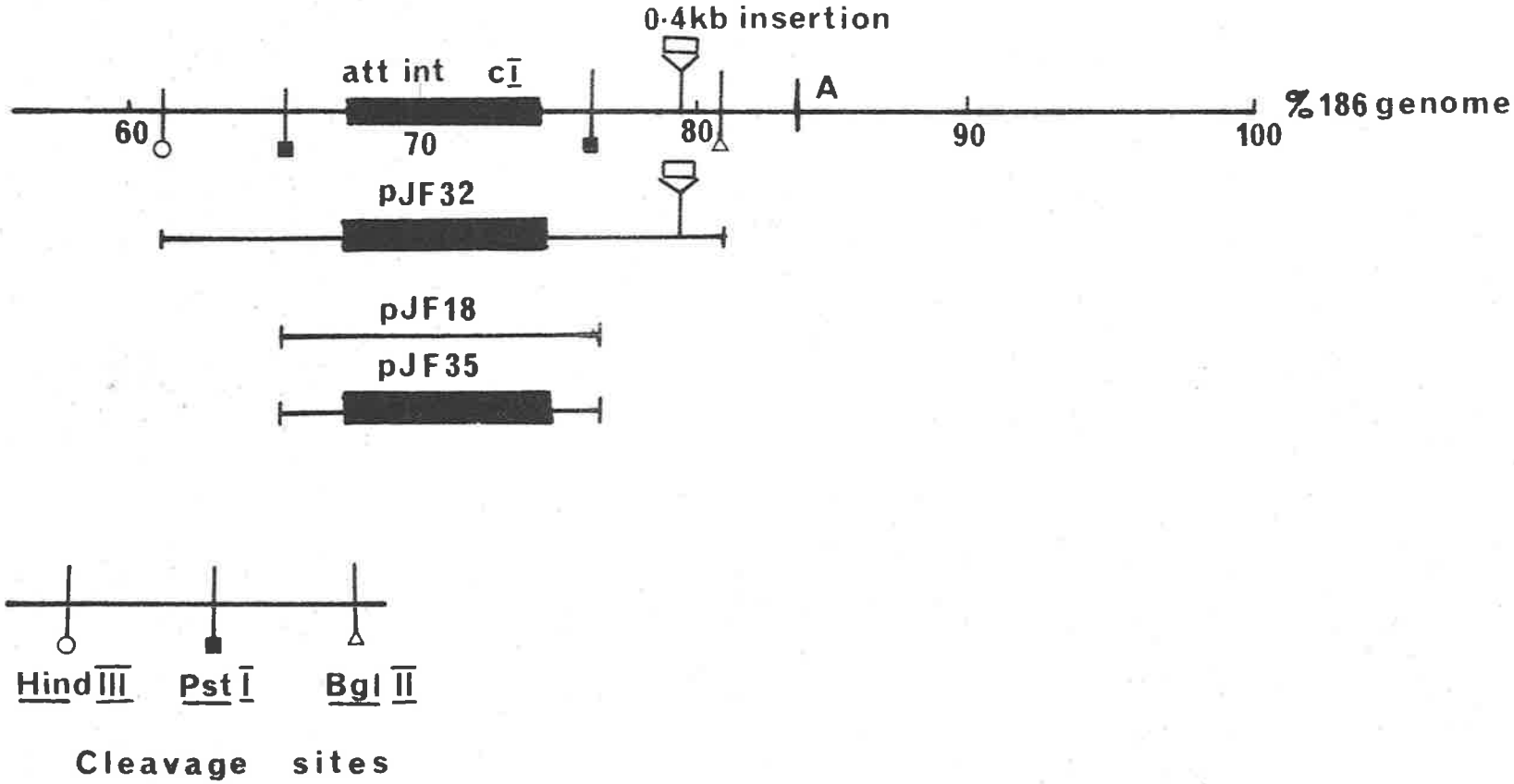
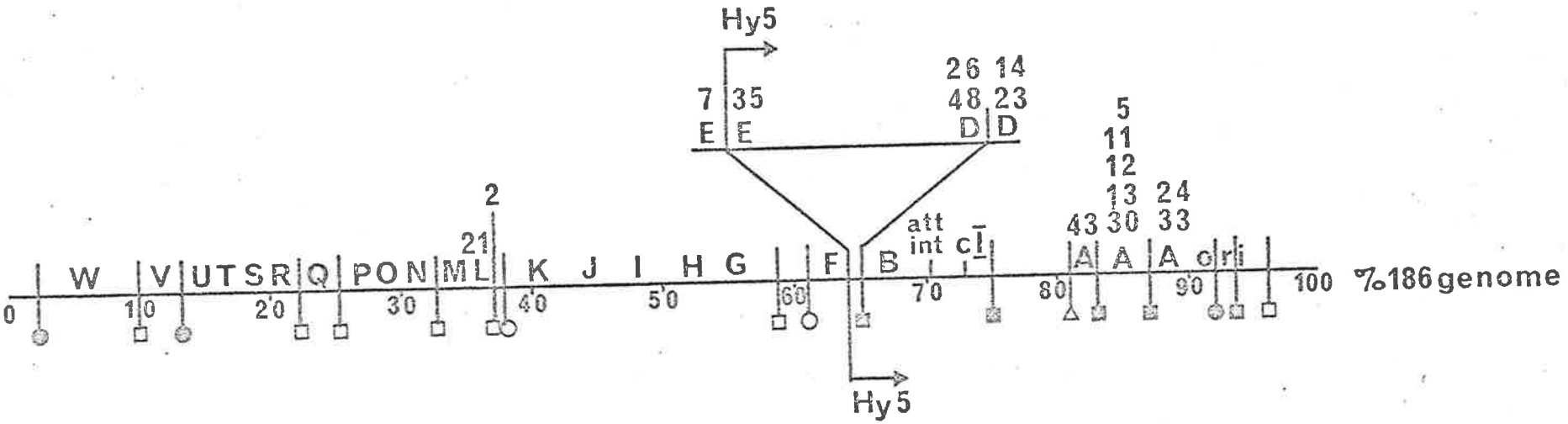
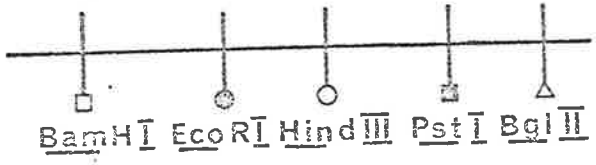

0·4kb insertion
att int cI
A
60
70
80
90
100
% 186 genome
pJF32
pJF18
pJF35
Hind III
Pst I
Bgl II
Cleavage sites

FIGURE 5.13. The Physical Map of Coliphage 186

This map shows the position of 186 genes determined by an analysis of cloned restriction fragments generated by the enzymes *Bam*HI, *Eco*RI, *Pst*I, *Bgl*II and *Hin*dIII. As mentioned in the text, the positions of gene E, *int*, *c*I and *ori* have been determined by electron microscopy.

The genes have been equally spaced within the limits defined by the cleavage sites.





Cleavage sites

for further discussion). It seems likely that more genes within this region remain to be discovered.

Another function that has been physically mapped within the 75 to 100% region of the genome is the origin of replication (*ori*) placed at 92.9 ± 1.8% from the left end (Chattoraj and Inman, 1973). The position of *ori* relative to gene A, which is essential for DNA replication, is of particular interest because of the similarities to the A genes of P2 and ϕX174 The A gene of ϕX174 is both *cis*-acting and required for DNA replication (Sinsheimer, 1968); moreover, ϕX174 gene A catalyses a single strand cleavage at the origin of replication which is located within this gene (Francke and Ray, 1972; Weisbeek and van Arkel, 1976; Eisenberg *et al.*, 1977). This may be a general feature of *cis*-acting proteins required for DNA replication and hence it would be of interest to determine the position of 186 gene A relative to *ori*.

P2 gene A also catalyses a single strand cleavage at *ori* (Geisselsoder, 1976) and it is possible that this gene overlaps *ori* mapped at 89.0 ± 1.1% by Schnös and Inman (1971); however, the position of A has not been determined. The published data for 186 indicated that the left-most position of *ori* is 91.9% which is more than 2 kb from the left-most A allele, A*am*43 at 83.8%. Only if the A protein is more than 85,000 daltons could *ori* lie within this gene. Purification of the A protein may help clarify this point.

The existence of the *dho* gene, encoding a function to turn off host DNA synthesis, was postulated to explain the immediate inhibition of host DNA synthesis seen after heat

induction of a 186*cIts* prophage (Hocking, 1977). Inhibition also occurred following the induction of a prophage defective in A or B function, implying that if *dho* is a phage coded function, then it must be an early gene depending on neither A nor B for its expression. Host DNA synthesis was not inhibited by a 186 prophage and therefore *dho* must be under repressor control.

The frequency of mutation to virulence in 186 suggests that there is a single site of *c*I action controlling functions essential to lytic development which has been mapped to the right of *att* (Hocking, 1977). 186*del*I, a non-virulent phage, and the virulent phage 186*del*2 have the same deletion, as determined by the limits of a heteroduplex analysis confirming that it is probably the insertion to the right of 76.3% which inactivated the repressor binding site in 186*del*2 thus supporting the idea of a single essential operator site in 186. Therefore it seems likely that *dho* and gene A may be transcribed from a single promoter that is under repressor control. An obvious location for *dho* would be between *c*I (73.6%) and gene A (left-most allele at about 83.8%) with the promoter and operator sites between *c*I and *dho*.

2. Recloning of pJF18

Two independent isolates of pJF18 were made, one by the purification of the *Pst*I fragment from low melting agarose and the second by 'shot-gun' cloning of *Pst*I digested Hy5 DNA. Both isolates were unstable during the preparation of pJF18 DNA (see Appendix I(b)) which rendered impossible the purification of large amounts of the cloned fragment that were necessary for a transcription analysis. There-

fore pJF18 was cloned into a virulent λ vector.

Cloning of DNA into a virulent phage obviates the need for the maintenance of a long term stable relationship between the host and vector. The instability observed during preparation of pJF18 DNA may have been due to the expression of some function encoded by the insert which resulted in plasmid breakdown (see Appendix I(b)) and so pJF18 was cloned into a λ *Hin*dIII replacement vector.

There were two reasons for cloning the entire plasmid rather than the 186 fragment alone:

(1) the fragment cloned in pJF18 was generated by *Pst*I digestion and at the time of cloning there was no λ *Pst*I vector available. Cloning by the addition of homo-deoxypolymeric tails cannot be employed with a phage vector because the naturally occurring cohesive ends are also subject to tailing.

(2) a replacement vector imposes a minimum as well as a maximum size limit on the inserted DNA. It was hoped that by using the replacement vector 762 the whole plasmid would be inserted and maintained rather than a smaller breakdown plasmid which would approach the minimum size necessary for phage packaging.

The recombinant λppJF18 has proved to be stable and has been used for the work presented in Section VI.

3. Expression of 186 functions from cloned fragments

Does marker rescue result from recombination between the infecting phage and the 186 DNA within the plasmid or is

it due to the expression of wild type product from the cloned DNA which can then be utilized by the infecting phage, that is, by complementation?

Some genes were split by a restriction cut, for example, gene A which contains two *Pst*I restriction sites, and yet alleles from each side of the cut were recovered from the adjacent, cloned fragments (pJF17, pJF15 and pJF16 each resuce gene A alleles). This surely is an example of marker rescue by recombination as neither pJF17, pJF15 nor pJF16 code for the complete A gene. Rescue from pJF13 which has the complete A gene occurs at a similar frequency.

The frequency of recovery of wild type function (*mrf*)* from cloned fragments was compared to the frequency of recombination observed when a 186*cIts* prophage replaced the recombinant plasmid for marker rescue. This frequency was at least ten to one hundred-fold lower when the wild type allele was recovered from cloned DNA implying that rescue was via recombination rather than complementation. This was true of all the essential genes with the exception of gene B. Marker rescue of gene B from a 186*cIts* prophage was at a high frequency (*mrf* = 0.2 cf 0.001 $\leqslant$*mrf* $\leqslant$0.007 for other genes) and it was proposed that complementation was occurring (Hocking, 1977). Similarly the recovery of B function from the cloned restriction fragment coding for gene B was high (*mrf* = 0.88) and thus indicative of complementation rather than recombination.

Although the infecting phage must activate the expression of its own late genes, it appears that it was unable to provide the function necessary to transactivate late genes

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* $mrf = \frac{pfu \text{ on } sup^+ \text{ (plasmid)}}{pfu \text{ on } sup}$

encoded on a cloned restriction fragment. Several polarity groups were identified by Hocking (1977) and pJF11 includes a fragment coding for genes from three separate polarity groups. The central group, represented by genes H, I and J, must be complete and, if each group is associated with its own promoter, then pJF11 must contain the promoter for these genes. The frequency of recovery of these markers was ten-fold higher than the level expected for recombination but ten-fold lower than B gene complementation, suggesting that if normal transcription is initiated at a promoter adjacent to these genes then to obtain full expression some other physical requirement, for example, concurrent DNA replication, has not been met. No other cloned fragment encodes a complete polarity group and thus other genes may have been separated from their promoters, or the promoter destroyed by a *Bam*HI restriction site. Polarity studies of P2 indicated that late transcription must proceed in both directions and thus P2 must have at least two late promoters (Lindqvist, 1971). In contrast phage λ has a single promoter from which late transcription is initiated (Herskowitz, 1973).

Contrasting to the absence of late gene expression, genes from the early region, including B and the non-essential functions *cI*, *int* and *dho* are active. Evidence for the expression of the non-essential genes is presented and discussed in Appendix I.

4. Restriction fragments for transcription mapping

A physical map of 186 was elucidated to permit the selection of the restriction fragments required for a trans-

criptional analysis of the phage. Any study of transcripts must revolve around the control genes; in what order are they transcribed? how do their products interact? how is late expression controlled? A preliminary investigation of control gene transcription was possible using Hy5 DNA as a template for hybridization, and now by choosing the appropriate restriction fragments transcription of individual genes within that region can be examined.

The fragments cloned into pJF13, pJF15, pJF16 and pJF17 (Fig. 5.7) will all give some information about gene A transcription. pJF15 codes for five of the eight alleles of gene A and furthermore, because the two adjacent fragments each code for A alleles, pJF15 cannot code for any other 186 function. The adjacent fragment (87.0 to 93.8%) cloned in pJF16 codes for the two right-most alleles of gene A, and in addition may cover the *ori* site which was located at 92.9 $\pm$ 1.8%. The *Pst*I fragment to the left of the pJF15 insert was cloned in pJF17; this fragment encodes A*am*43 and is the most probable location for the non-essential functions *c*II and *dho*. The entire A gene was cloned into pJF13 but the *Bgl*II/*Eco*RI fragment extends beyond the limits of the A gene and therefore some other functions may be included, in part, on this fragment, for example *c*II, *dho* and *ori*.

The other major control gene, gene B, was cloned on the *Pst*I fragment in pJF18, however, this fragment also codes for the repressor gene *c*I, *int*, *att* and two alleles of gene D, a tail gene. 186*del*2 has a deletion extending from 67.9 to 74.9% which covers *int* (mapped at 70.3%), part if not all *c*I (placed at 73.6%) and probably the *att* site. Gene B must

be to the left of 67.9% because 186*del2* is a viable phage. The *Pst*I fragment (65.5 to 76.3%) from 186*del2* was cloned in the plasmid pJF35 and thus provides the best estimate of gene B transcription. A comparison between hybridization to pJF18 and pJF35 DNA will give an indication of transcription of the *c*I and *int* genes.

The cohesive ends (92.0 to 2.3%) were cloned as an *Eco*RI fragment into a phage, λpl86*s*RI3-1, and do not code for any known essential gene (Hocking, 1977); however, this region may cover *ori* located at 92.9 ± 1.8%.

To monitor late gene expression two clones were chosen: pJF24 encodes gene W the left-most head gene, and pJF30 codes for gene F, a tail gene mapping near the right hand end of the clustered late genes.

In conclusion then, the control region of 186 has been conveniently cut by restriction enzymes and the cloning of the fragments generated in this way will facilitate a study of the transcription of gene A, gene B, *c*I and *int*, the blank region between *c*I and gene A, as well as the cohesive ends. Two clones representing the late genes were also selected to follow the expression of the structural genes of the phage. The work presented in the subsequent section is a study of 186 transcription based on the hybridization of mRNA to cloned 186 restriction fragments.

SECTION VI

HYBRIDIZATION OF 186 mRNA TO CLONED RESTRICTION FRAGMENTS OF 186 DNA

SECTION VI

HYBRIDIZATION OF 186 mRNA TO CLONED RESTRICTION FRAGMENTS OF 186 DNA

A. INTRODUCTION

Transcription of many different viruses has been studied by the hybridization of RNA from infected cells to restriction endonuclease generated fragments of viral DNA. Detailed transcription maps of SV40 (Khoury *et al.*, 1973; Sambrook *et al.*, 1973; Weinberg *et al.*, 1974) and adenovirus (Petterson *et al.*, 1976) were prepared by liquid or filter hybridization to restriction fragments that had been eluted from the gel matrix after electrophoresis.

McAllister and Barrett (1977) transferred the restriction fragments of T7 DNA to nitrocellulose after electrophoresis and hybridized known early mRNA to the fragments in order to identify the regions coding for early genes thereby correlating genetic and physical maps.

An attempt to determine which DNA restriction fragments were active at different stages of the 186 lytic cycle by hybridization of mRNA to Southern transfers of 186 restriction digests proved unsuccessful for a number of reasons (Section IV). To overcome the technical difficulties encountered, restriction fragments of 186 were cloned in a heterologous vector (pBR322 or λ) and phage RNA was hybridized to filters containing the hybrid DNA.

The restriction fragments selected to study 186 transcription cover the control genes encoded by 65.5 to 100% of

the genome, that is the Hy5 region, as well as the cohesive ends and two structural genes, cloned on separate fragments. Genes A, W and F have been cloned on discrete fragments while *att*, *int* and *c*I, and *dho* and *c*II were probably located on the same fragments. The cohesive ends were cloned as an *Eco*RI restriction fragment that codes for no known functions, and gene B was cloned on a fragment which also includes two alleles of gene D.

To maximise the information obtained by hybridization to cloned restriction fragments of 186 DNA phage mRNA was prepared under a number of different conditions. The importance of genes A and B to normal phage transcription can be investigated by extracting RNA from phage mutant in either the A or B gene.

Chloramphenicol inhibits bacterial and phage protein synthesis. Infection or induction in the presence of chloramphenicol will reveal any transcripts that require a phage coded protein for their expression. Delayed early transcripts of phage λ were not made in the presence of high levels of chloramphenicol indicating the need for a phage coded protein (Kourilsky *et al.*, 1968; Gros *et al.*, 1969; Takada, 1975). Transcription of 186 induction and infection was investigated to see if early mRNA also consisted of two distinct classes: those that are independent of phage products and a delayed early class which require a phage protein.

Inhibition of DNA synthesis might prevent phage transcription if:

(1) transcription of a class of genes will only proceed from a replicating template.

(2) transcription is associated with DNA replication *per se*.

It has been found that transcription of λ *oop* RNA requires O, P, *ori*, at least the β subunit of RNA polymerase and host functions *dna*G and *dna*B, although *dna*E which is also essential for λ replication does not influence *oop* transcription (Hayes and Szybalski, 1973).

Replication of 186 was inhibited by using a 186*c*I*ts*A*am* prophage or by the infection of a host carrying a *dna*C*ts* mutation at a non-permissive temperature (Hooper, 1973) and the RNA made was analysed.

The work presented in this section extends the preliminary characterization of 186 mRNA by hybridization to Hy5 and 186 DNA described in Section III and by hybridization to Southern transfers of 186 restriction digests outlined in Section IV. Cloned 186 restriction fragments were used in the hybridization of RNA from 186*c*I*ts*, 186*c*I*ts*A*am* and 186*c*I*ts*B*am* phage as well as 186*c*I*ts* RNA transcribed in the absence of protein synthesis or when DNA synthesis was inhibited.

B. MATERIALS

The following materials were used for the experiments described in this section:

L[4,5-^{3}H] leucine: (40-60 Ci/mmol) Radiochemical Centre, Amersham.

Hybridization buffer: equal volumes of 2 x SSC and 2 x SSC saturated with phenol, adjusted to pH 7.5. Buffer was freshly prepared for each assay.

TPG: was similar to TPG-CAA described in Section III.B, but without the addition of Casamino acids.

C. METHODS

1. Filter hybridization with cloned DNA

DNA filters were prepared as described in Section III.C.4, but the amount of DNA/filter was adjusted so that each 24 mm disc contained 2 µg of the cloned 186 fragment (Table 6.1). Control filters to monitor non-specific hybridization to the vector contained the amount of DNA equal to the maximum of vector DNA associated with 2 µg of the 186 insert. Hybridization to control filters would therefore measure the maximum level of cross reaction. The parental phage λ762 carries *E. coli supF* gene, and therefore will specifically hybridize *E. coli* tRNA; this is replaced by the cloned DNA and so the recombinant phage will not hybridize *E. coli* tRNA. Therefore wild type λ replaced λ762 as the phage vector control.

After the addition of DNA the filters were cut in half and half filters, containing 1 µg of cloned DNA were used in hybridization experiments. Filters were placed in three separate vials for hybridization to avoid competition for mRNA species between overlapping or adjacent fragments. Table 6.2 shows the arrangement of filters in the vials. Each vial contained a total of 1.5×10^6 c.p.m. ^{3}H-mRNA extract in a total volume of 1.5 ml hybridization buffer.

TABLE 6.1. Size of 186 Restriction Fragments Inserted into Recombinant Plasmids and the Amount of Plasmid Used in Filter Hybridization

Plasmid	Size of insertion (kb)	Total size of recombinant plasmid (kb)	Amount DNA/filter (µg)
pJF24	2.3	6.4	5.6
pJF30	approx. 1.4	5.8	8.2
pJF35	1.2	5.6	8.5
λppJF18	3.3	37.7	22.5
pJF17.2	2.3	6.7	5.8
pJF15	1.0	5.4	10.8
pJF16	2.1	6.5	6.2
pJF13	3.2	7.3	4.4
λp186*s*RI3-1	3.1	39.1	25
pBR322	-	4.4	8.8
λ	-	45	20

TABLE 6.2. Arrangement of Filters in Vials for Hybridization

Vial	1	2	3
FILTER	pJF35	pJF16	λppJF18
	λp186*s*RI3-1	pJF17	pJF13
	Blank	pJF30	pBR322
	pJF15	λ	pJF24

After hybridization filters were batch washed as described in Section V.C.4 and then counted in a Packard scintillation spectrometer.

2. Incorporation of ^{3}H-leucine into TCA precipitable material

An overnight culture of E321 was diluted forty-fold into TPG and grown with aeration at 30°C to a density of 2 x 10^{8} *cfu*/ml. A 0.4 ml sample was added to a tube containing ^{3}H-leucine (final leucine concentration of 0.1 µg/ml) and incubated at 38.5°C. At 30 second intervals a 50 µl aliquot was taken and immediately precipitated on to a GF/A filter by immersing it in ice-cold 10% TCA. Filters were batch washed as described in Section III.C.2.

3. Chloramphenicol inhibition of protein synthesis

An overnight culture of E321 was diluted forty-fold into TPG and grown with aeration at 30°C to a cell density of 2 x 10^{8} *cfu*/ml. Chloramphenicol was added (0 µg, 100 µg, 200 µg or 400 µg/ml) and the culture was returned to 30°C for a further five, ten or fifteen minutes. A 200 µl sample was withdrawn and protein synthesis at 38.5°C was measured over a one minute interval by the addition of ^{3}H-leucine. A 50 µl aliquot was precipitated and counted as described in Section VI.C.2.

4. Isolation of chloramphenicol mRNA

(a) Heat induction

An overnight culture of E321 was diluted forty-fold into TPG-CAA and grown with aeration at 30°C to a cell

density of 2 x 10^8 *cfu*/ml. Chloramphenicol was added at 200 µg/ml and the culture was incubated at 30°C for a further ten minutes before being transferred to a 38.5°C shaking bath (t = 0). RNA was labelled and isolated by the method described in Section III.C.6.

(b) Infection

An overnight culture of E320 was diluted forty-fold into TPG-CAA and grown with aeration at 38.5°C to a cell density of 2 x 10^8 *cfu*/ml. Chloramphenicol was added at 200 µg/ml and the culture was incubated at 38.5°C for a further ten minutes. At this time (t = 0) the culture was infected with 186*cIts* at a multiplicity of infection (*moi*) 5 to 10 and mRNA was prepared as described in Section III.C.6.

5. Burst size of 186*cIts* following infection of *dnaCts* host

An overnight culture of E990 was diluted forty-fold and grown at 30°C with aeration to a cell density of 2 x 10^8 *cfu*/ml. The culture was transferred to 41.5°C shaking bath and incubated for 15 minutes. The culture was then inoculated (t = 0) with 186*cIts* *moi* of 5 to 10 and plaque forming units were assayed through chloroform at t = 0, t = 45 minutes and t = 90 minutes.

The latent period and burst size following 186*cIts* infection at 41.5°C was determined for an isogenic *dnaC*$^+$ host.

6. Preparation of mRNA in the absence of DNA synthesis

An overnight culture of E990 was diluted forty-fold

and grown at 30°C with aeration to a cell density of 2 x 10^8 *cfu*/ml. The culture was shifted to a 41.5°C bath and incubated for 15 minutes with aeration. Then the culture was inoculated with 186*cIts* at a *moi* of 5 to 10 (t = 0). RNA was labelled and isolated as described in Section III.C.6.

D. RESULTS

1. Hybridization to cloned restriction fragments of 186 DNA

Although the results presented in the subsequent sections are from single experiments all the observations have been confirmed by duplicate experiments, with the exception of 186*cIts* infection of E320 in the presence of chloramphenicol which has been performed only once.

The total amount of DNA/filter varied for the different plasmid clones but in each case the equivalent of 1 µg of the inserted DNA was present on the filter. The size of the cloned fragments differed and therefore a direct comparison between the counts hybridizing to different plasmid clones does not necessarily reflect a difference in transcriptional activity, for example, if the entire insert was active then a fragment of 2 kb would hybridize approximately twice the counts hybridized to a 1 kb 186 fragment. Hybridization can be standardized per kb of 186 DNA, but if only a part of the cloned fragment was transcribed then standardization will make this fragment appear less active.

A direct comparison of hybridization can be made between overlapping fragments. Hybridization to λppJF18 DNA but not to pJF35 must reflect transcription from the

DNA covered by the deletion 67.9 to 74.9% (see Fig. 5.12), and, conversely, if hybridization to λppJF18 and pJF35 is identical then 67.9 to 74.9% of the genome must be inactive and the transcripts must originate in the 65.5 to 67.9% plus 74.9 to 76.3% regions. The immunity region, encoding *att*, *int* and *cI*, is approximately equivalent to 67.9 to 74.9% while the DNA in pJF35 which codes for gene B, two alleles of gene D (a late gene) and perhaps the rightward end of *cI* provides the best indication of B gene activity. Only at late times would gene D transcripts be expected to contribute to hybridization to this fragment. Fragments coding for overlapping regions of the genome are also cloned in pJF13 and pJF15 plus pJF13 and pJF16.

The location, on the 186 genome, of the cloned fragments and the genes encoded by each are shown in Fig. 6.1. For ease of reference a 'lift-out' version of this figure is provided inside the back cover of this volume.

2. Transcription of the prophage

Generally transcription of 186 has been studied following heat induction of the prophage, therefore the transcripts made by the prophage were investigated as these were of interest *per se* and to provide the baseline for transcripts appearing soon after induction.

RNA prepared from a cell carrying an uninduced prophage was hybridized to filters of cloned 186 restriction fragments and the results are presented in Table 6.3. Transcripts were detected in the regions 67.9 to 74.9% and 87.0 to 93.8% that encode *int*, *att* and *cI*, and *Aam*24, *Aam*33

FIGURE 6.1. The Genes Encoded by Cloned Restriction Fragments and Their Location on the 186 Chromosome

The cloned restriction fragments used in hybridization experiments are shown below the map.

The genes encoded by each fragment are indicated above the map. *C*II has been located to the right of *att* by genetic recombination but its position relative to gene A has not been determined (Hocking, 1977). For simplicity, it has been assumed that *c*II lies to the left of gene A and is encoded on pJF17.2 although this has not been confirmed by genetic complementation.

A lift-out version of this figure is supplied inside the back cover of this volume.

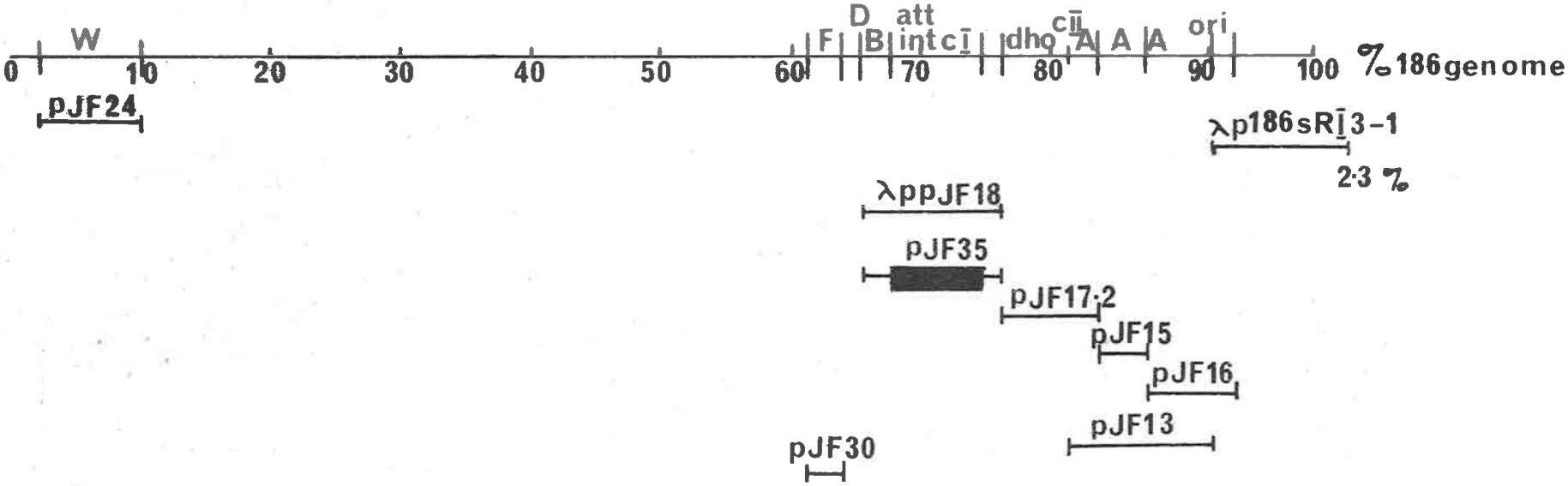

W
D
att
F
B
int
cI
dho
cII
A
A
A
ori
0
10
20
30
40
50
60
70
80
90
100
% 186 genome
pJF24
λp186sRI3-1
2·3 %
λppJF18
pJF35
pJF17·2
pJF15
pJF16
pJF13
pJF30

TABLE 6.3. Hybridization of RNA From an Uninduced Lysogen to Filters Containing Cloned 186 DNA

Filter	Blank	pBR322	λ	pJF24	pJF30	pJF35	λppJF18	pJF17.2	pJF15	pJF16	pJF13	λp186 *s*RI3-1
Uninduced lysogen	59.2	62	58	62	67.5	77	134	73	80	145.5	93.5	80

The figures in the table represent counts per minute hybridized from a total input of 1.5×10^6 c.p.m.

and *ori* respectively.

3. Transcription of 186*cIts* following heat induction

Messenger RNA made following heat induction of 186*cIts* was hybridized to cloned restriction fragments of 186 DNA and the results are presented in Table 6.4 and Fig. 6.2.

The first transcripts that were made following heat induction of a 186*cIts* prophage were those hybridizing to the DNA cloned in pJF16 (87.0 to 93.8%) and the region included in λppJF18 but not pJF35, that is the immunity region. These fragments were also active in the prophage state and therefore may be a carryover of transcripts made by the prophage. Ten minutes after induction all fragments from the early region (65.4 to 100%) with the exception of the cohesive ends, were being transcribed.

The rate of transcription from the fragments cloned in pJF35 and in λppJF18 increased rapidly over the first 25 minutes of the latent period; the rate had slowed by 30 minutes but rose again towards the end of the lytic cycle. In contrast, transcription from the fragments cloned in pJF17.2, pJF15 and pJF13 increased to a maximum at 30 minutes and the rate then declined. Transcription from the remaining early fragment, 87.0 to 93.8% cloned in pJF16, proceeded at a low, approximately constant rate throughout the lytic cycle.

When the transcriptional activity of fragments from the early region was compared, after standardization to hybridization/kb insert, the fragments in pJF35, pJF17.2, pJF15 and λppJF18 appeared to be equally active for the

TABLE 6.4. Filter Hybridization of 186*cIts* mRNA Following Heat Induction to Cloned Restriction Fragments of 186 DNA

Time (minutes)	FILTER											
	Blank	pBR322	λ	pJF24	pJF30	pJF35	λppJF18	pJF17.2	pJF15	pJF16	pJF13	λp186 *s*RI3-1
5-7	72	76	69.5	89.5	84.5	112.5	210.5	99.5	80	204	109	114.5
10-12	87	76	74	65	75	267.5	524	302.5	147.5	189.5	288.5	105.5
15-17	65.5	88.5	87.5	103	87.5	635.5	1215	1142.5	523.5	302	896	122.5
20-22	68.5	116	98	112.5	126	1018.5	2050.5	1276.5	672	240.5	1192.5	121
25-27	71	105	76.5	291	516	1987.5	3487.5	2207	808.5	363	1736.5	234
30-32	76	142.5	86.5	285.5	522.5	1177.5	2363	2604	1150.5	416.5	2027	398.5
35-37	42.5	94	74.5	710.5	1284	1948	3632	1499	768.5	329	1405.5	332.5

The figures in the table represent counts per minute hybridized.

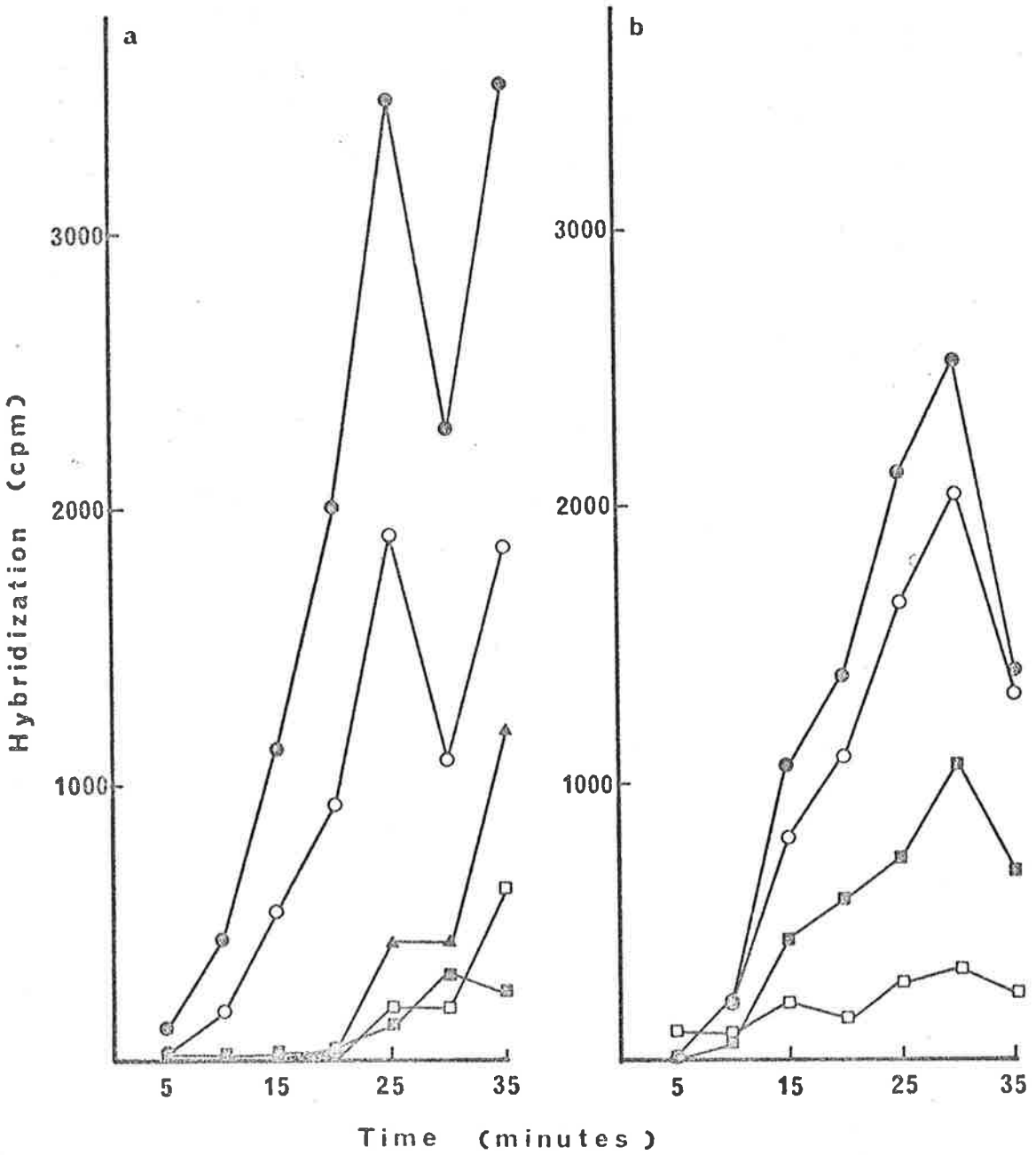
The total input was 1.5×10^6 c.p.m.

FIGURE 6.2. Hybridization of 186*cIts* mRNA to Cloned Restriction Fragments

The average background of counts hybridized to filters containing pBR322 DNA, λ DNA or no DNA (83 c.p.m.) has been subtracted from total counts hybridizing to filters containing recombinant DNA.

(a)
- ● λppJF18 DNA
- ○ pJF35 DNA
- ▲ pJF30 DNA
- □ pJF24 DNA
- ■ λp186*s*RI3-1 DNA

(b)
- ● pJF17.2 DNA
- ○ pJF13 DNA
- ■ pJF15 DNA
- □ pJF16 DNA.


a
b
3000
2000
1000
Hybridization (cpm)
5
15
25
35
Time (minutes)

initial 20 minutes after induction (Fig. 6.3). The fragment in pJF16 was much less active and pJF13, which overlaps pJF16 and pJF15, showed intermediate activity.

The transition to the late phase occurred between 20 and 25 minutes, by which time transcripts from the fragments cloned in pJF24, pJF30 and λpl86*s*RI3-1 were seen.

4. Transcription of 186*c*I*ts*A*am*30 following induction

The results of the hybridization of 186*c*I*ts*A*am*30 RNA to cloned restriction fragments of the 186 chromosome are tabulated in Table 6.5. A comparison between wild type and 186*c*I*ts*A*am*30 transcripts from different regions is presented in Fig. 6.4. A*am*5 used in the experiments described in Sections III and IV was replaced by A*am*30 for this study because it was the least leaky of the available A*am* mutations.

As seen in Sections III and IV transcription of an A*am* mutant phage was reduced when compared to the wild type. The transition to the late phase, normally observed 25 minutes after wild type induction, did not occur when the prophage was defective in gene A. By 35 minutes after induction a low level of transcripts from the regions coding for the late genes W and F, as well as the fragment containing the cohesive ends, were detected.

The only transcripts that were unaffected by a mutation in the A gene were the A gene transcripts *per se* (Fig. 6.4(b)), and the rate of transcription from pJF15 was the same following induction of 186*c*I*ts* and 186*c*I*ts*A*am*30. The rate of transcription from the fragments in λppJF18 and pJF17.2 was decreased between two and three-fold while the inhibition of

FIGURE 6.3. Hybridization of 186*cI*ts mRNA per kb 186 DNA

Hybridization of 186*cI*ts mRNA to cloned restriction fragments has been standardized/kb 186 DNA by dividing the counts hybridizing to a plasmid clone by the size of the 186 DNA inserted into that plasmid.

The average background (83 c.p.m.) was subtracted before standardization.

(a) □ pJF35 DNA ■ pJF15 DNA

▲ λppJF18 DNA ○ pJF16 DNA

(b) ■ pJF15 DNA

△ pJF17.2 DNA

● pJF13 DNA

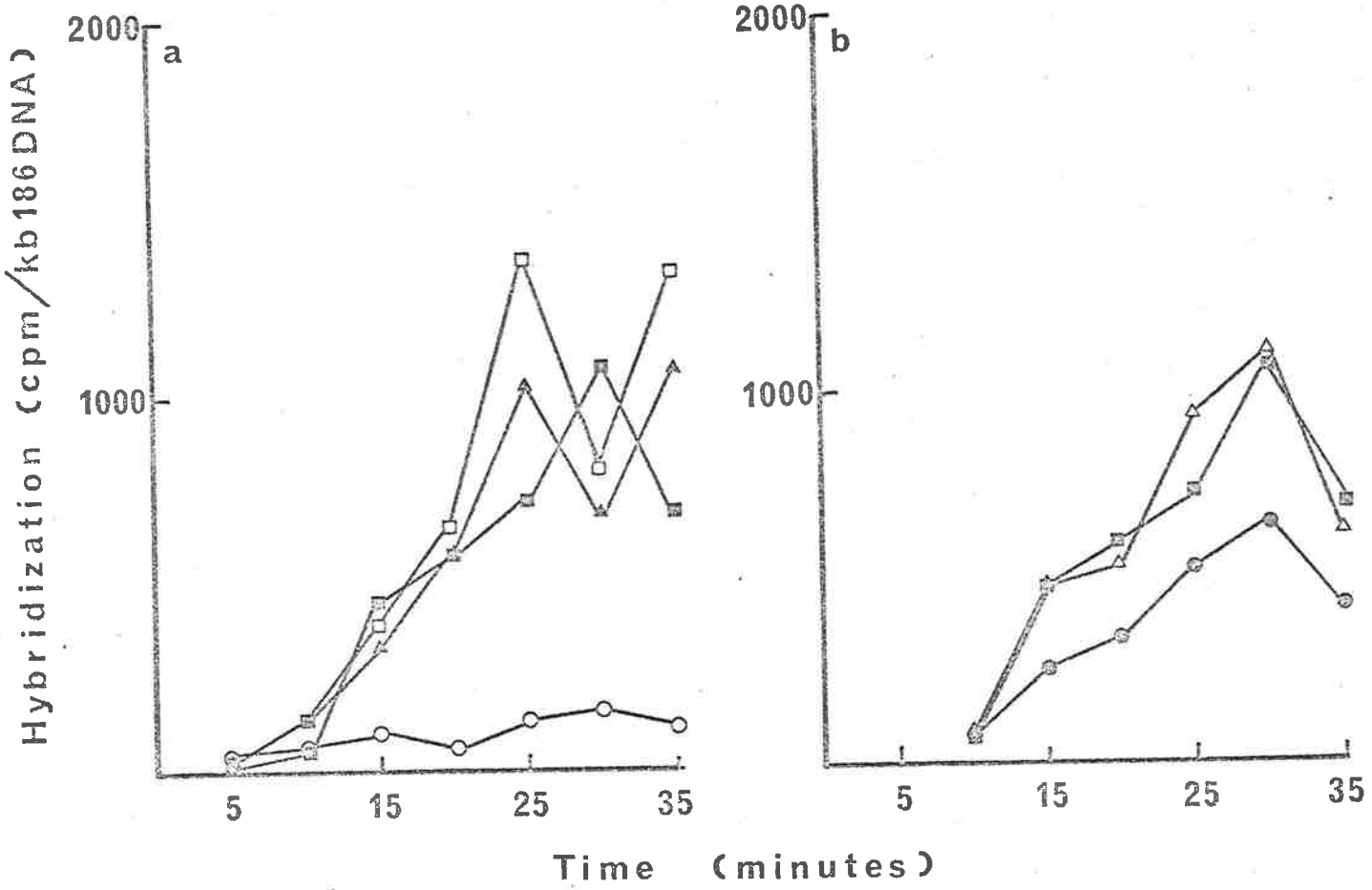

Hybridization (cpm/kb 186 DNA)
2000
1000
a
b
5
15
25
35
Time (minutes)

TABLE 6.5. Filter Hybridization of 186*c*I*ts*A*am*30 RNA Following Heat Induction to Cloned 186 Restriction Fragments

Time (minutes)	FILTER											
	Blank	pBR322	λ	pJF24	pJF30	pJF35	λppJF18	pJF17.2	pJF15	pJF16	pJF13	λpl86 *s*RI3-1
5-7	82	93.5	86.5	93	96.5	120.5	187.5	101.5	146	129.5	146.5	106
15-17	112	84	86	83	89.5	268	777	480.5	373.5	168	622.5	118.5
25-27	78	91	81	98.5	91	296.5	1057.5	821.5	651	153.5	963.5	97.5
35-37	80.5	73.5	88	141	138	420.5	1589	973	809	162.5	1408	124

The figures in the table represent counts per minute hybridized. The total input was 1.5×10^6 c.p.m.

FIGURE 6.4. A Comparison of the mRNA Made Following 186*cIts* and 186*cItsAam*30 Heat Induction by Hybridization to Cloned 186 DNA

The average background of counts hybridized to filters containing pBR322 DNA, λ DNA or no DNA has been subtracted from total counts hybridizing to filters containing recombinant DNA. The background was 83 c.p.m. and 86 c.p.m. for wild type and 186*cItsAam*30 RNA respectively.

(a) --●-- 186*cIts* RNA hybridized to λppJF18 DNA

--○-- 186*cItsAam*30 RNA hybridized to λppJF18 DNA

—●— 186*cIts* RNA hybridized to pJF35 DNA

—○— 186*cItsAam*30 RNA hybridized to pJF35 DNA

(b) --●-- 186*cIts* RNA hybridized to pJF17.2 DNA

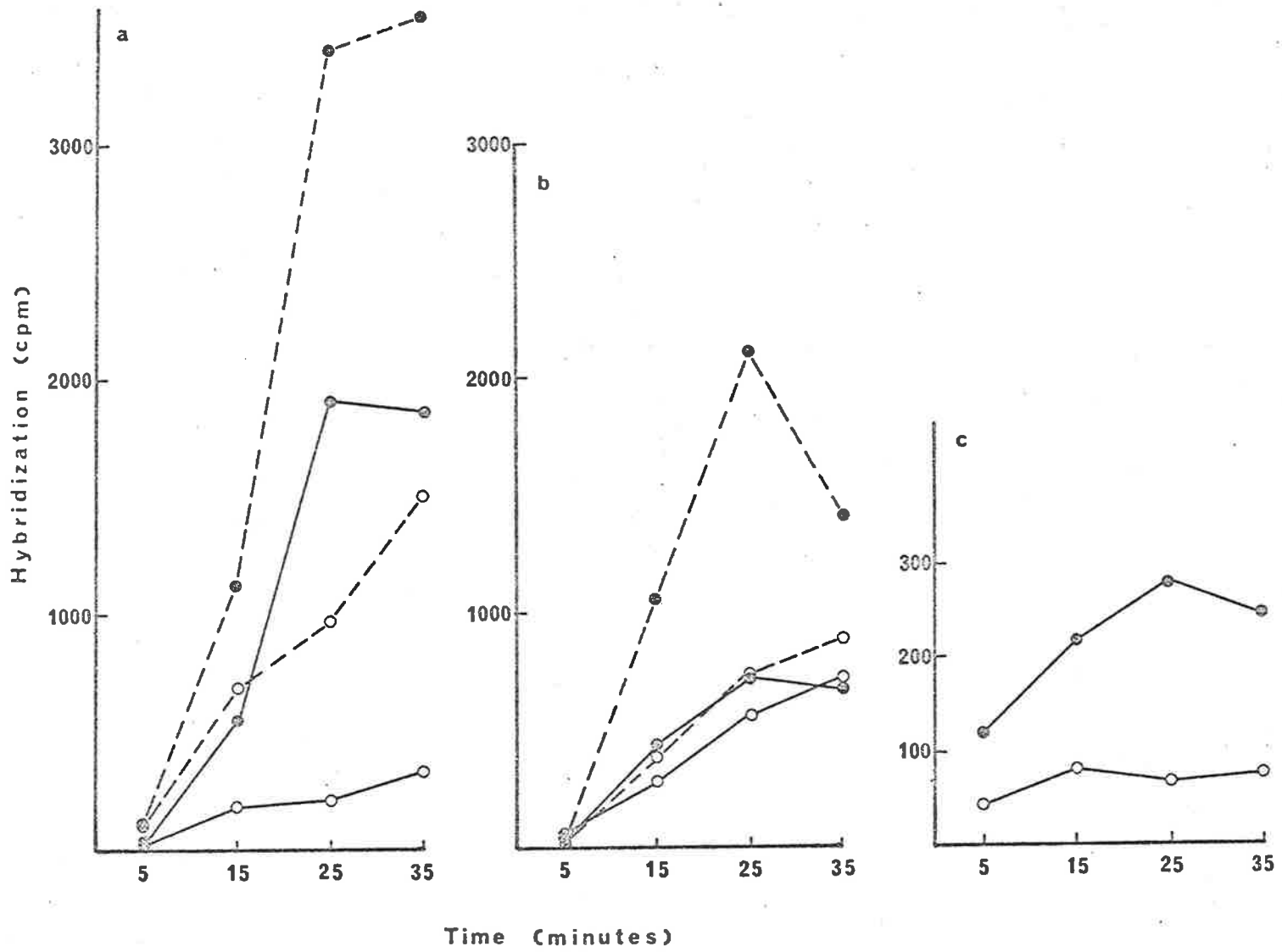
--○-- 186*cItsAam*30 RNA hybridized to pJF17.2 DNA

—●— 186*cIts* RNA hybridized to pJF15 DNA

—○— 186*cItsAam*30 RNA hybridized to pJF15 DNA

(c) —●— 186*cIts* RNA hybridized to pJF16 DNA

—○— 186*cItsAam*30 RNA hybridized to pJF16 DNA.


a
b
c
3000
2000
1000
300
200
100
5
15
25
35
Hybridization (cpm)
Time (minutes)

transcription from pJF35 and pJF16 was more severe.

5. Transcription of 186*cI*ts*Bam*17 following induction

To investigate the role of the B gene in transcription a 186*cI*ts*Bam*17 prophage was induced and the mRNA made at different times following induction was hybridized to cloned DNA; the results are presented in Table 6.6. A comparison between the transcripts made during wild type and B*am*17 phage development is presented in Fig. 6.5.

Transcription from the fragments of the early region was identical following induction of a 186*cI*ts* and a 186*cI*ts*Bam*17 prophage. Twentyfive minutes after induction the effect of the B*am*17 mutation was evident. When compared to wild type early, transcripts were made at a much higher level late in the lytic cycle. No structural gene transcripts were detected even after 35 minutes, although by this time a low level of transcription from the cohesive ends was observed.

6. Inhibition of bacterial protein synthesis by chloramphenicol

The effect of different concentrations of chloramphenicol on bacterial protein synthesis was investigated, and the results are presented in Table 6.7. After ten minutes preincubation in the presence of chloramphenicol, protein synthesis was completely inhibited, as estimated by the incorporation of ^{3}H-leucine above background.

All preparations of 186 RNA were made after preincubation for ten minutes in the presence of 200 µg/ml

TABLE 6.6. Filter Hybridization of 186*c*I*ts*B*am*17 RNA Following Heat Induction to Cloned 186 Restriction Fragments

Time (minutes)	F I L T E R											
	Blank	pBR322	λ	pJF24	pJF30	pJF35	λppJF18	pJF17.2	pJF15	pJF16	pJF13	λp186 *s*RI3-1
5-7	77	86.5	78.5	91	77.5	129.5	349.5	93.5	98.5	165	105	124
15-17	74	80.5	86	85	97.5	611.5	1617.5	1072	499	227.5	876.5	136.5
25-27	71	89	82.5	106.5	85	1329	3736.5	1776.5	791.5	249	1440.5	140
35-37	81	126	92.5	190	129	5512.5	14789	2885.5	1497	410	2296.5	307

The figures in the table represent counts per minute hybridized. The total input was 1.5×10^6 c.p.m.

FIGURE 6.5. A Comparison of the mRNA Made Following 186*cIts* and 186*cItsBam*17 Heat Induction by Hybridization to Cloned 186 DNA

The average background of counts hybridized to filters containing pBR322 DNA, λ DNA or no DNA has been subtracted from total counts hybridizing to filters containing recombinant DNA. The background was 83 c.p.m. and 85 c.p.m. for wild type and 186*cItsBam*17 RNA respectively.

(a) --●-- 186*cIts* RNA hybridized to λppJF18 DNA

--○-- 186*cItsBam*17 RNA hybridized to λppJF18 DNA

—●— 186*cIts* RNA hybridized to pJF35 DNA

—○— 186*cItsBam*17 RNA hybridized to pJF35 DNA

(b) --●-- 186*cIts* RNA hybridized to pJF17.2 DNA

--○-- 186*cItsBam*17 RNA hybridized to pJF17.2 DNA

—●— 186*cIts* RNA hybridized to pJF15 DNA

—○— 186*cItsBam*17 RNA hybridized to pJF15 DNA

(c) —●— 186*cIts* RNA hybridized to pJF16 DNA

—○— 186*cItsBam*17 RNA hybridized to pJF16 DNA.

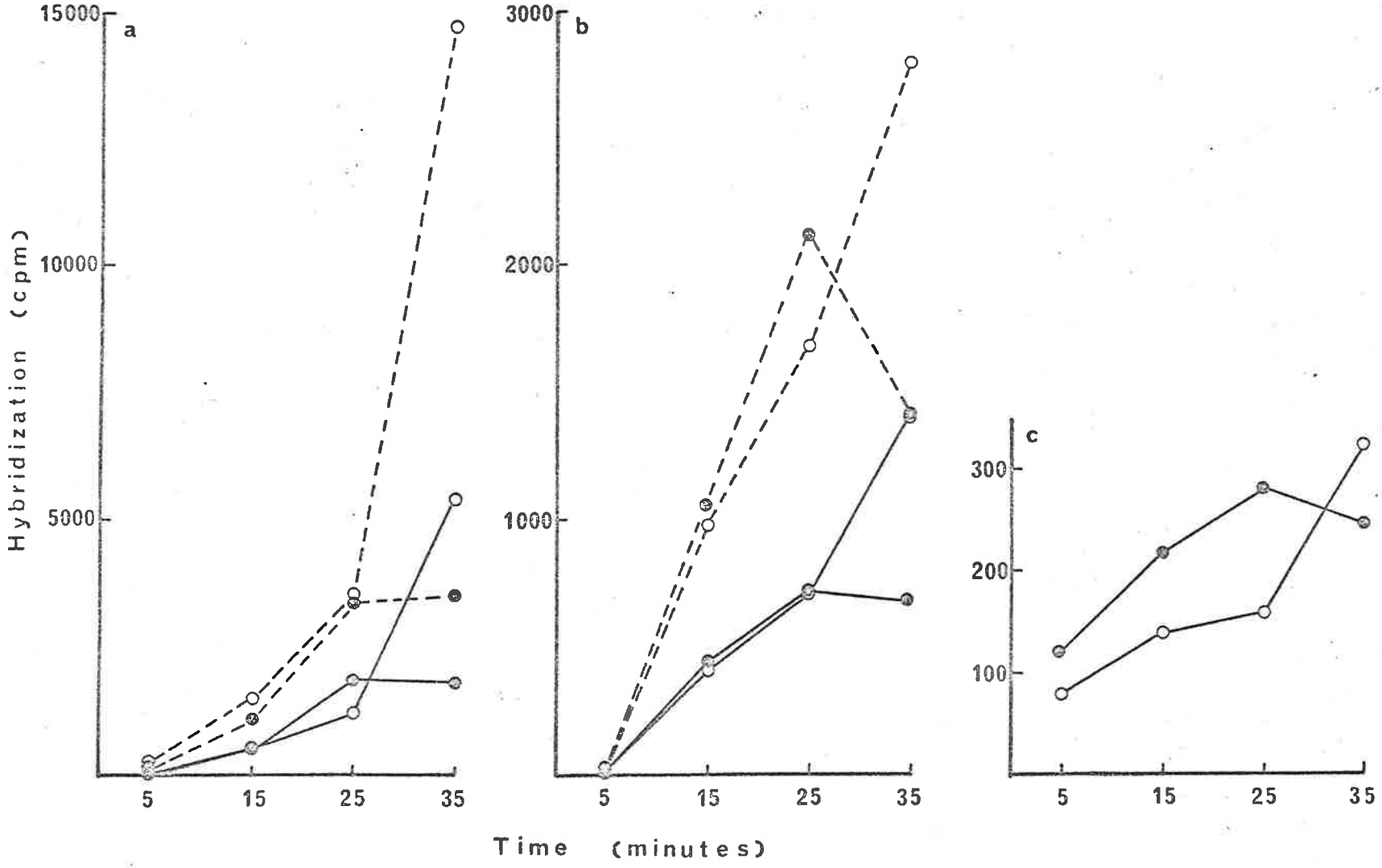

a
b
c
15000
10000
5000
3000
2000
1000
300
200
100
5
15
25
35
Hybridization (cpm)
Time (minutes)

TABLE 6.7. The Effect on Bacterial Protein Synthesis of Different Concentrations of Chloramphenicol

A bacterial culture (2 x 10^8 *cfu*/ml) was preincubated at 30°C in the presenc of 0, 100, 200 or 400 µg/ml chloramphenicol for five, ten or fifteen minutes before protein synthesis was estimated by the incorporation of ^{3}H-leucine into TCA precipitable material during a one minute pulse at 38.5°C.

Preincubation with antibiotic (minutes)	Chloramphenicol (µg/ml)			
	0	100	200	400
5	8854	640	617	521
10	14953	717	476	407
15	17588	858	566	533

The values in the table are ^{3}H-leucine (c.p.m.) in TCA precipitable material/50 µl aliquot. The background reading was 691 c.p.m.

chloramphenicol.

7. Transcription in the presence of chloramphenicol

Transcription following the induction of a 186*cIts* prophage in the presence of chloramphenicol was investigated and the results are presented in Table 6.8. The chloramphenicol and 186*cIts*A*am*30 transcripts were compared (Fig. 6.6) because it appears that gene A may be the first essential gene to be transcribed as all other early transcripts were decreased by an A*am*30 mutation. In addition to this, transcription from an *Aam* phage should be equivalent to transcription from a single template since the A protein is essential for phage replication.

With the exception of the fragment cloned in pJF35, the rate of transcription from all early fragments was more sensitive to chloramphenicol than to a mutation in gene A. Transcription of the B gene (pJF35) was comparable following induction of a 186*cIts* prophage in the presence of chloramphenicol and a 186*cIts*A*am*30 prophage.

Although repression of the prophage was lifted by a shift to a non-permissive temperature prophage excision would have been prevented by chloramphenicol. Transcription of a phage in the vegetative rather than the prophage state was investigated by 186*cIts* infection of the non-lysogenic host E320 which had been pre-incubated in the presence of chloramphenicol. The results are presented in Table 6.8(b) and a comparison with transcription following induction is presented in Fig. 6.6.

Hybridization to pJF35 and λppJF18 was identical

TABLE 6.8. Filter Hybridization of 186*cIts* RNA, Made in the Presence of Chloramphenicol, to Cloned 186 Restriction Fragments

Time (minutes)	FILTER											
	Blank	pBR322	λ	pJF24	pJF30	pJF35	λppJF18	pJF17.2	pJF15	pJF16	pJF13	λp186 *s*RI3-1
(a)												
5-7	97.5	75	127.5	96.5	82	102	170.5	82.5	90	106.5	114	148.5
15-17	90	99	167.5	102.5	101.5	155	225	115.5	100	79.5	106.5	182
25-27	61.5	81	153	106.5	87	372.5	404.5	217	131	91	181.5	272
(b)												
5-7	73.5	85	101.5	84.5	103	282	286	112.5	87.5	72.5	144	153.5
15-17	66.5	84	129.5	101.5	96.5	577	669.5	266	116	92	198	270
25-27	69	81	-	77	137	695	671.5	350.5	122	83.5	203	295

(a) 186*cIts* RNA made following induction in the presence of chloramphenicol.

(b) 186*cIts* RNA made following infection in the presence of chloramphenicol.

The values in the table are c.p.m. hybridized to recombinant DNA. The total input was 1.5×10^6 c.p.m.

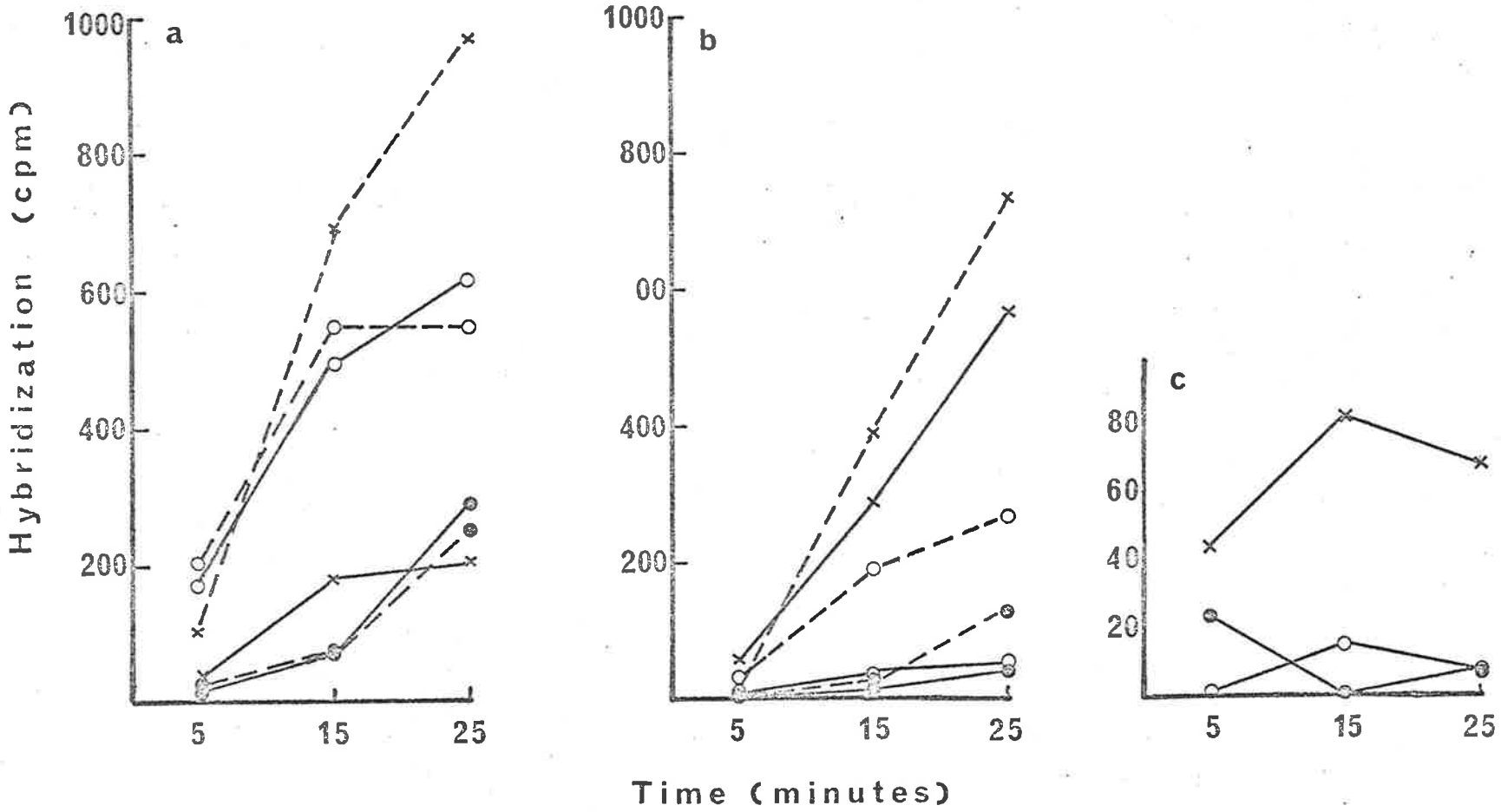
FIGURE 6.6. A Comparison of mRNA Made Following 186*cIts* Induction and Infection in the Presence of Chloramphenicol with 186*cItsAam*30 RNA Made After Induction

The average background of counts hybridized to filters containing pBR322 DNA, λ DNA or no DNA has been subtracted from the total counts hybridizing to filters containing recombinant DNA. The background for 186*cItsAam*30 RNA was 86 c.p.m. For 186*cIts* RNA made in the presence of chloramphenicol the background for pBR322 clones and λ clones was computed separately and was 84 c.p.m. and 149 c.p.m. or 77 c.p.m. and 116 c.p.m. respectively after induction and infection.

(a) --×-- 186*cItsAam*30 RNA hybridized to λppJF18 DNA
--●-- 186*cIts* chloramphenicol RNA made after induction hybridized to λppJF18 DNA
--○-- 186*cIts* chloramphenicol RNA made after infection hybridized to λppJF18 DNA
—×— 186*cItsAam*30 RNA hybridized to pJF35 DNA
—●— 186*cIts* chloramphenicol RNA made after induction hybridized to pJF35 DNA
—○— 186*cIts* chloramphenicol RNA made after infection hybridized to pJF35 DNA

(b) --×-- 186*cItsAam*30 RNA hybridized to pJF17.2 DNA
--●-- 186*cIts* chloramphenicol RNA made after induction hybridized to pJF17.2 DNA
--○-- 186*cIts* chloramphenicol RNA made after infection hybridized to pJF17.2 DNA
—×— 186*cItsAam*30 RNA hybridized to pJF15 DNA
—●— 186*cIts* chloramphenicol RNA made after induction hybridized to pJF15 DNA
—○— 186*cIts* chloramphenicol RNA made after infection hybridized to pJF15 DNA

(c) —×— 186*cItsAam*30 RNA hybridized to pJF16 DNA
—○— 186*cIts* chloramphenicol RNA made after induction hybridized to pJF16 DNA
—●— 186*cIts* chloramphenicol RNA made after infection hybridized to pJF16 DNA.


Hybridization (cpm)
1000
800
600
400
200
a
5
15
25
b
1000
800
00
400
200
5
15
25
c
80
60
40
20
5
15
25
Time (minutes)

following both induction and infection in the presence of chloramphenicol and thus one can conclude that the region 67.9 to 74.9% was inactive in the absence of phage protein synthesis. The relative increase in the rate of transcription in pJF17.2 after infection compared with induction may reflect an increased gene dosage in infection. Moreover, the rate after both infection and induction was lower than that seen in an *Aam* phage suggesting that transcription from a part of the pJF17.2 fragment is dependent on phage protein synthesis. Transcription from the fragments in pJF15 and pJF16 remained the same following infection (compare with induction) in the presence of chloramphenicol suggesting that all transcription in these fragments was protein dependent.

8. Burst size of 186*cIts* in the absence of DNA synthesis

Bacteriophage 186 uses many host functions for the replication of its DNA, and among these are the initiation functions *dna*A and *dna*C (Hooper, 1973). A bacterial host with a temperature sensitive mutation, *dna*C*ts*, should not propagate 186 if grown at non-permissive temperatures. A *dna*C*ts* strain (E990) was grown at 41.5°C for 15 minutes to inactivate the *dna*C protein prior to infection with 186*cIts*. Phage production by E990, and an isogenic wild type host E237, infected with 186*cIts* at 41.5°C was compared and the results presented in Table 6.9.

When the bacterial host carried a *dna*C*ts* mutation the number of free phage decreased with time, probably due to the phage adsorption, but no subsequent increase was observed even by 90 minutes after infection. When wild type cells

TABLE 6.9. Production of 186*cIts* Phage After Infection of *dnaC* and *dnaCts* Bacteria at 41.5°C

A culture of *dnaC* or *dnaCts* bacteria (2×10^8 *cfu*/ml) incubated at 41.5°C for 15 minutes were infected with 186*cIts* (*moi* five to ten) and the free phage produced at 41.5°C were assayed at t = 0, t = 45 and t = 90 minutes after infection.

Host		Minutes after infection 0	45	90
dnaCts	a	9.3×10^8	2.4×10^8	1.5×10^8
	b	6.3×10^8	2.2×10^8	-
dnaC	a	7.2×10^8	3.0×10^9	5.2×10^9
	b	7.9×10^8	1.9×10^9	-

Phage were assayed through chloroform and thus the values in the table represent free phage (*pfu*/ml).

a and b are duplicate experiments.

were infected with 186*cIts* at 41.5°C the number of free phage also decreased over the initial 32 minutes of the infection, however, after this time progeny phage were released. Fig. 6.7 illustrates that the latent period of this infection was 42 to 43 minutes and that there was a ten-fold increase in free phage by 55 minutes.

Although no progeny phage were released by an E990 host at 41.5°C total absence of 186 DNA synthesis was not confirmed by 186 DNA/DNA hybridization experiments. RNA prepared under these conditions was subject to this limitation.

9. DNA synthesis and 186 transcription

The effect of the inhibition of DNA synthesis on 186 transcription was studied and the results are presented in Table 6.10. Interpretation of these results is subject to the limitations that:

(1) inhibition of phage DNA synthesis has been inferred from the failure of E990 to host 186*cIts* infection at 41.5°C. Phage DNA replication should be assayed by DNA/DNA hybridization because it may still proceed but at a very slow rate.

(2) all previous studies were made following the heat induction of a 186*cIts* prophage. To allow time for the inactivation of the *dnaCts* protein phage infection rather than induction was used. The transcripts made in the absence of DNA synthesis should therefore be compared to those

FIGURE 6.7. Latent Period and Burst Size of 186*cIts* Infection of *dnaC* and *dnaCts* Host at 41.5°C

Cultures of *dnaC* (E237) and *dnaCts* (E990) bacteria at 2×10^8 *cfu*/ml were incubated at 41.5°C for 15 minutes prior to infection with 186*cIts* at a *moi* of five to ten. Incubation was at 41.5°C throughout the infection.

The latent period of E237 infection was 42 to 43 minutes and the burst size was ten. No increase in free phage was observed after E990 infection.

o E237 infection

■ E990 infection.

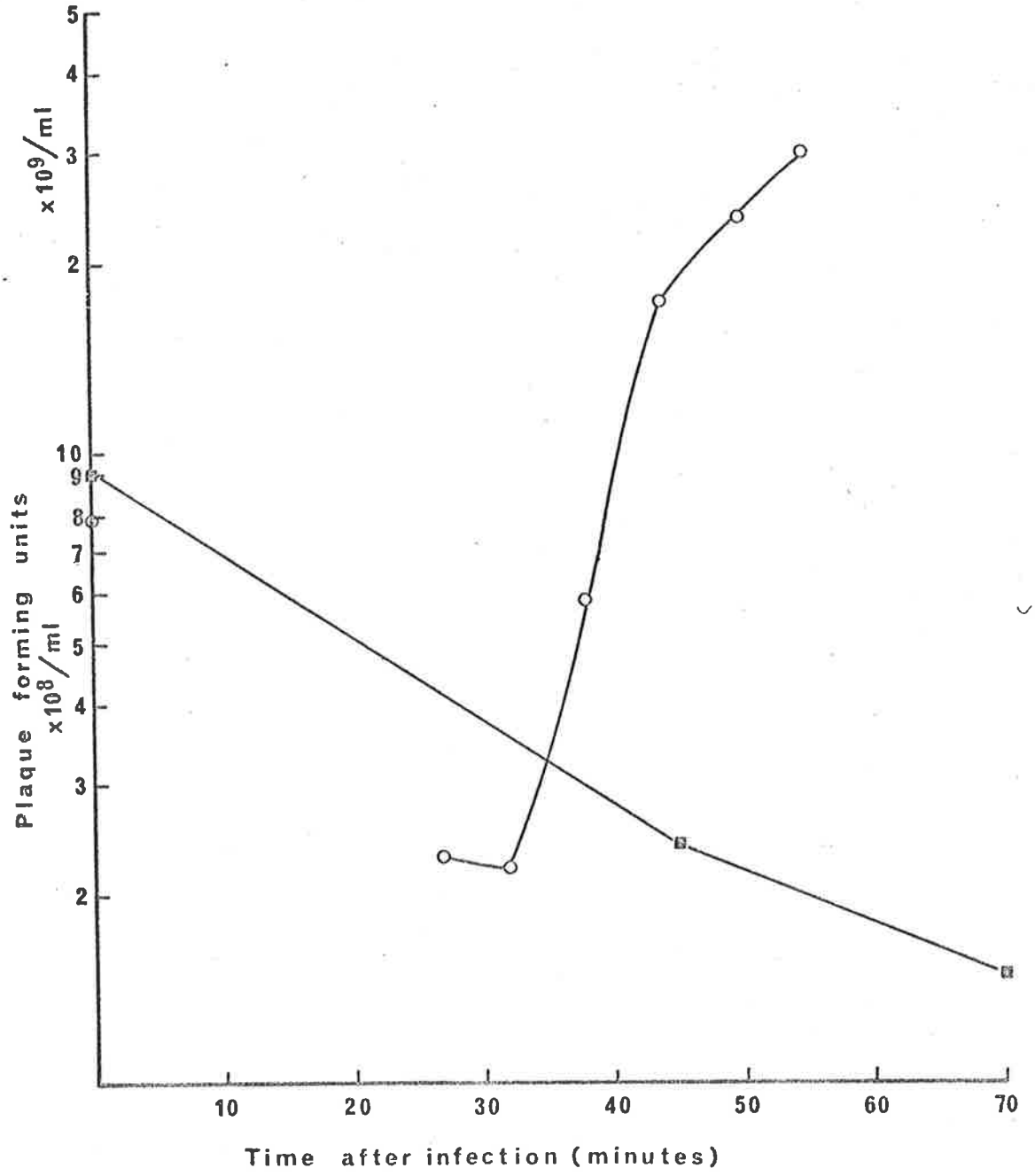

Plaque forming units
x10⁸/ml
x10⁹/ml
5
4
3
2
10
9
8
7
6
5
4
3
2
10
20
30
40
50
60
70
Time after infection (minutes)

TABLE 6.10. Filter Hybridization of 186*c*I*ts* RNA Made Following Infection of *dna*C*ts* Host at 41.5°C to Cloned Fragments of 186 DNA

Time (minutes)	FILTER											
	Blank	pBR322	λ	pJF24	pJF30	pJF35	λppJF18	pJF17.2	pJF15	pJF16	pJF13	λp186 *s*RI3-1
5-7	69	80.5	127	72	130	571	513	818	355	85.5	629.5	165.5
15-17	58.5	74	88	92.5	127	643.5	1137	1000	373.5	97.5	717.5	160
25-27	61.5	116	114	215	322	1326	2371	2287.5	915.5	170	1611	238

The values in the table represent counts per minute hybridized to each filter. The total input was 1.5×10^6 c.p.m.

made after wild type infection rather than induction; however, it is likely that the sequence of transcription following prophage excision is very similar, if not identical, to the sequence of events following infection. The actual time of appearance of transcripts will probably differ because following the temperature shift for induction prophage excision must occur before lytic development can proceed.

The rate of transcription from the early fragments (pJF35, λppJF18, pJF17.2, pJF13 and pJF15) was higher 5 to 7 minutes after infection than it was after induction. Transcription of the immunity region was not observed until 15 minutes after infection. When DNA synthesis was inhibited by the *dnaCts* mutation transcription from the fragment cloned in pJF16 was not detected until 25 minutes when an extremely low level of transcription was seen.

The transition to the late phase of development was seen by 25 minutes after infection when some transcription from pJF24, pJF30 and λp186*s*RI3-1 fragments was detected.

E. DISCUSSION

Fig. 6.1 illustrates that the cloning of 186 restriction fragments has permitted the transcription of individual phage control genes to be investigated. Gene A transcription can be followed by measuring hybridization to pJF15; *c*II and *dho* are cloned together in pJF17.2. The overlapping fragments in pJF35 and λppJF18 allow the transcription of the immunity region (present only in λppJF18) to be

been described (Bertani, 1960). It has been shown that the Z gene, which maps between two tail genes (Lindahl, 1969) does not control *int* expression, nor does it regulate the surrounding tail genes (Bertani, 1976). The C gene of a Z mutant phage is active, but it has been suggested that Z may be needed for the constitutive expression of the C gene in an established lysogen. The Z function is not immunity specific because P2$Hy_1 dis$, which differs from P2 in its immunity region, can complement a P2Z mutant (Bertani, 1976). The fragment (87.0 to 93.8%) cloned in pJF16 may encode a gene, analogous to P2 gene Z, that is needed for stable lysogeny.

B function can be recovered by complementation from the prophage and it was suggested that either the B product was made consitutively by a 186 prophage or that B expression was activated by a superinfecting phage (Hocking, 1977). No RNA complementary to the fragment cloned in pJF35 was detected in the RNA isolated from an uninduced lysogen indicating that the B gene was not expressed constitutively by the prophage. It is unlikely that B is under direct repressor control since it can be transactivated from the prophage (Section V.E.3).

In summary, two regions of the 186 genome were transcribed by the prophage; these were the immunity region and the fragment 87.0 to 93.8% which may encode a function that is essential for lysogeny (*c*III or *c*IV) in addition to *ori* and part of the A gene. The B gene was not constitutively expressed by the prophage.

distinguished from B gene transcription, the best estimate of which is gained by hybridization to pJF35. The best estimate of transcription around *ori* can be guaged from hybridization to pJF16 although this fragment also encodes two alleles of gene A. Within the limits of any fragment untranscribed tracts of DNA cannot be detected nor can the switching of transcription from one gene to another encoded by the same fragment be distinguished.

1. Transcription of the prophage

What functions are necessary to maintain the prophage state? Mutation in the *c*I gene results in a clear plaque morphology suggesting that the *c*I protein acts as the 186 phage repressor (Huddleston, 1970). Transcripts of the immunity region (67.9 to 74.9%) were present in an uninduced lysogen indicating that at least part of this region was expressed constitutively by the prophage. The *int* gene is included on this fragment and may be under direct repressor control, alternatively if it is transcribed by the prophage then the *c*I protein must exert an epistatic block to prevent phage excision.

Transcription in the 87.0 to 93.8% region cloned in pJF16 was also detected in the uninduced lysogen. Two alleles of gene A and *ori* are encoded by this fragment which is large enough (2 kb) to include an additional function that is essential for the maintenance of the prophage. Two other 186 genes, *c*III and *c*IV, that play a part in the lysogenic response have been identified but have not been fully characterized (Huddleston, 1970). A second P2 gene, Z, required for the maintenance of a stable prophage, has

2. Transcription following prophage induction

Induction following the transfer of a culture from a bath at 30°C to one at 38.5°C was not an instantaneous occurrance. One to two minutes elapsed before the temperature of the medium had equilibrated to the higher temperature at which the repressor was inactivated. The RNA transcribed five to seven minutes after the shift was from the same fragments that were active in the prophage state and it is most likely that the transcripts made by the prophage were still expressed early after induction. In addition functions needed for prophage excision may be active at this time. These transcripts may originate from the same fragments but cover different genes.

The immunity region codes for *int* which is required for excisive recombination (Bradley *et al.*, 1975) and therefore would be transcribed before excision. Although no 186 protein specific to excision has been identified the immunity region has sufficient coding potential to include such a function (Section VII). Both λ and P2 have proteins that are required solely for prophage excision and which map close to the *int* and phage repressor genes (Guaneros and Echols, 1970; Lindahl and Sunshine, 1972). Transcripts in the 87.0 to 93.8% region have no known role in prophage excision.

Ten minutes after the temperature shift, transcripts from each of the fragments between 65.5 and 93.8% were present, although the extent of transcription within each was not determined. The early genes encoded by fragments in pJF13, pJF15 and pJF17.2 (A, *c*II and *dho*) were trans-

cribed maximally at 30 minutes; transcription then decreased suggesting that these genes may be subject to negative control late in the lytic cycle. Transcription of fragments in pJF35 and λppJF18 decreased at 30 to 32 minutes and then increased over the final five minutes of the latent period. The upsurge in transcription of pJF35 and λppJF18 may be due to increased activity of the late genes (in particular gene D) seen during the final ten minutes of the cycle. The late phase began between 20 and 25 minutes after induction, with the appearance of transcripts from the cohesive ends and genes W and F, and continued until cell lysis.

The pattern of transcription from the fragment cloned in pJF16 (87.0 to 93.8%) was unique. The activity of the DNA in this region was low and approximately constant throughout the entire latent period (Fig. 6.2 and Fig. 6.3).

3. The role of the A gene in transcription

The experiments described in Sections III and IV showed that early transcription of an A*am* phage was reduced compared to wild type, but it was not possible to determine whether the extent of transcription was reduced or whether all classes of early transcripts were made at a decreased rate. Hybridization of 186*cI*ts*A*am*30 RNA to cloned restriction fragments revealed that, with the exception of gene A transcripts, all other early mRNA was reduced about two to three-fold by a mutation in the A gene. A comparison between the kinetics of the rate of increase in transcription for each 'early fragment' for A*am* and wild type phage induction (Fig. 6.4), showed that transcription in two regions cloned in pJF35 and pJF16 was preferentially inhibited by a

mutation in gene A (reduced nine-fold and six-fold respectively).

Throughout the sampling period the expression of the fragment 87.0 to 93.8% (pJF16) was very low, and could probably be attributed to the transcription of two alleles of gene A which map on this fragment. The A protein is essential for DNA replication (Hocking, 1977) and the finding that transcription from the region which encodes *ori* (87.0 to 93.8%) was inhibited by A*am*30 suggests that the A protein is essential for transcription around *ori*. A preliminary study of 186 transcription when phage DNA synthesis was inhibited also indicated that transcription of this region was associated with phage replication (Section VI.D.9). Transcription associated with replication is discussed in Section VI.E.7.

Transcription from the fragment cloned in pJF35 was also more sensitive to mutation in gene A than other early transcripts (nine-fold reduction in rate compared to a general reduction of two to three-fold). This suggests that a functional A protein is required for efficient transcription of gene B. A prediction that follows is that gene A transcripts will appear before B transcription begins. The time interval between RNA samples taken during wild type development was too long to allow confirmation of this idea, as neither gene was transcribed at five to seven minutes and both were active by 10 to 12 minutes. Phage mutant in gene A complement poorly with all other 186 mutants and it has been suggested that, like the DNA replication genes of P2 and ϕX174, 186 gene A acts in *cis* (Hocking, 1977). If A protein is needed directly for efficient B transcription

then the site of A function is probably not adjacent to gene A, since B is separated from gene A by the genes *c*II, *dho*, *c*I, *int* and *att*. If A acts near B then it is likely that A may activate B transcription in *trans*. This idea is supported by the complementation of a B*am* infecting phage by a 186*c*I*ts* or 186*c*I*ts*A*am*5 prophage (Finnegan, unpublished observation) and by the restriction fragment encoding B, cloned in pJF18 (see Section V.E.3). Gene B was not transcribed constitutively by the prophage (Section VI.E.1) and thus the infecting phage must *trans*-activate B expression. Why do A and B mutant phage show poor complementation if A can activate B in *trans*? The answer to this question must await further studies on B gene control.

The two to three-fold reduction in other early transcripts in an A*am* induction may be due to a gene dosage effect in the absence of DNA synthesis although one would not anticipate a large reduction in early transcripts because 186 DNA synthesis was not detected until 15 to 20 minutes after induction.

Although in the experiments reported in Section III no transition to the late phase was observed by 30 to 32 minutes after infection a very low level of late transcription was detected by 35 minutes when RNA was hybridized to cloned restriction fragments.

Therefore, not only is the A gene essential for DNA replication, but also for the efficient transcription from the fragments cloned in pJF16 (coding for *ori*) and pJF35 (B gene). Very little late transcription occurred from an A*am* phage indicating that the A protein is essential for

the transition to the late phase.

4. B protein and late transcription

The pattern of early transcription following induction of a B*am* prophage closely resembled that of the wild type phage, although the rate of increase of transcription from the pJF16 fragment was slower for the initial 25 minutes after induction. By this time the effect of the B*am* mutation was evident as no transition to late transcription occurred. Instead, the rate of transcription from all early fragments increased dramatically (Fig. 6.5); this may be due to the failure to shut down early promoters mediated by the B product *per se* or another late gene under B control, or it may reflect the increased number of templates since replication of B*am* phage is far in excess of normal (Hocking, 1977). The first alternate seems more likely because in wild type induction early transcription declined at a time when the rate of late transcription was increasing suggesting a switch from early to late expression.

Transcription of the cohesive ends, cloned in a λ vector, was detected by 35 minutes after induction, but even at this time there was no evidence of expression from the two late genes W and F. Preliminary experiments in which RNA was hybridized to Hy5 and 186 DNA indicated that at late times some transcripts originating outside the Hy5 portion of the genome were made by a B*am*17 phage (Section III.D.7). The only active region to the left of the Hy5 crossover point was from 0 to 2.3% (a fragment including the left cohesive end) although any transcripts from 10.0% to about 61% would not have been detected by the fragments

selected for these experiments.

Therefore, although early development appeared to be normal when the B protein was defective, late gene transcription did not follow. Late in the lytic cycle early transcription far exceeded wild type. Furthermore, the low transcription of the cohesive ends in the absence of late gene expression might indicate that the B protein allows the elongation of this transcript to cover the late genes.

5. Protein synthesis and transcription

Bacterial and therefore phage protein synthesis can be inhibited by the addition of chloramphenicol, and this permits the identification of any transcripts which depend on a phage coded protein for their expression. It was found that the λ delayed early functions were not transcribed following induction or infection in the presence of chloramphenicol (Kourilsky *et al.*, 1968; Gros *et al.*, 1969; Takada, 1975); the level of antibiotic needed to inhibit λ N protein synthesis was found to be much higher than the level required to inhibit bacterial protein synthesis (Skalka *et al.*, 1967).

Several predictions about 186 transcription in the absence of protein synthesis, based on the data discussed in the preceding paragraphs, can be made:

(1) transcription of gene B is probably dependent on A function and thus should be inhibited by chloramphenicol.

(2) late transcription depends on both A and B proteins and therefore will not proceed.

(3) DNA replication will be inhibited because this requires a functional A gene; transcription of the fragment in pJF16 was associated with replication and required A protein, therefore in the absence of protein synthesis this fragment will not be transcribed.

It was anticipated that transcription of 186*cIts* in the presence of chloramphenicol would resemble A*am* phage transcription because the A protein appears to lie on the first essential transcript. A comparison between the transcripts made under these two conditions is presented in Fig. 6.6.

Transcription of 186*cIts* after induction in the presence of chloramphenicol revealed that in addition to the two regions predicted on the basis of A dependence (cloned in pJF35 and pJF16) all other early transcripts were sensitive to the antibiotic. Chloramphenicol would prevent prophage excision and so to investigate transcription of a vegetative chromosome 186*cIts* infection of a non-lysogen pre-incubated with antibiotic was studied. Hybridization to cloned restriction fragments was compared following induction and infection in the presence of chloramphenicol (Fig. 6.6). It was clear that all early transcripts were sensitive to chloramphenicol although after infection transcription from pJF35, λppJF18 and pJF17.2 was greater than that following induction, perhaps reflecting increased gene dosage after infection.

(a) Transcription from the fragments known to require A function

After induction, transcription from the fragment cloned in pJF35 was similar to the rate of transcription from this region by an A*am* phage. Although the rate was increased by infection relative to induction, this was probably due to an increased gene dosage and therefore this observation is compatible with the requirement for A protein for the efficient expression of gene B.

The rate of transcription from pJF16, which also requires A protein for activity, was comparable following induction of an A*am*30 prophage and of 186*c*I*ts* in the presence of chloramphenicol confirming that the activity of this fragment during lytic development was phage protein dependent.

(b) Transcription from pJF17.2 and pJF15

Surprisingly, transcription from the pJF17.2 fragment was also sensitive to chloramphenicol. Some transcription was observed although not until 25 minutes after indcution. Transcription from a vegetative phage (after infection) was more rapid than after induction but even this was two to three-fold lower than the activity of the same region in an A*am* phage. This suggests that only a part of the transcript from pJF17.2 was made in the absence of a phage coded protein. I propose that the transcript made from this region in the presence of chloramphenicol codes for a protein which is needed for all subsequent 186 transcription. The two known genes encoded by pJF17.2 are *c*II and *dho*; could either of these be the positive regulator

for transcription of the remainder of this fragment?

It is unlikely that *c*II controls the transcription of this fragment because a 186*c*II phage has a clear plaque morphology indicating that *c*II is essential for lysogeny but not for lytic development.

Could *dho* activate phage transcription in addition to inhibiting host DNA synthesis? It has been proposed that *dho* is located on the *Pst*I fragment cloned in pJF17.2 and that the promoter for *dho* is located to the left of the *Pst*I restriction site at 76.3% (Appendix I). The major phage operator, mutation of which will cause virulence, has been placed between 76.3 and 81.0% by the location of an insert which destroys this operator in 186*del*2, a virulent phage. If *dho* is essential for the expression of all other phage genes then the insertion in 186*del*2 will only produce a virulent phenotype if it is located in the operator of *dho*. However, it has been shown that the detrimental effect of the cloned *dho* gene (in pJF32) was controlled by the growth of the plasmid in a cell lysogenic for 186, and therefore even though this plasmid was derived from the virulent phage 186*del*2, the operator for the *dho* gene was functional (Appendix I). *Dho* cannot be under control of the major phage operator and therefore one can conclude that *dho* does not activate phage transcription.

I propose that another phage gene, X, is required to activate transcription from the remainder of the pJF17.2 fragment. Gene X must be to the right of the essential phage operator located between 76.3 and 81.0% and must be a positive effector for delayed early transcription in 186.

Genes included in the delayed early class would probably be *c*II, A, and B which is regulated by A protein. If X is an essential gene, as suggested by studies in the presence of chloramphenicol, then it is surprising that no X*am* mutations were recovered during phage mutagenesis. This situation is not unprecedented since the existence of P2 *ogr* was recognised only by the isolation of a host mutation that inhibited P2 late transcription. Mutations in *ogr* were subsequently isolated in an *E. coli gro* host (Sunshine and Sauer, 1975).

The mode of X action is unknown but it may be analogous to λ gene N which is an antiterminator of early transcription (Lozeron *et al.*, 1976).

In a study of T4 chloramphenicol RNA it was noted that incorporation of ^{3}H-uridine into phage specific RNA was reduced by about two-fold (Young, 1975). Approximately 25% of early T4 mRNA was present at about the same concentration in the presence or absence of antibiotic, but the remaining species were made in reduced amounts or were not transcribed. New early mRNA species continued to appear until at least 15 minutes after infection while in the absence of chloramphenicol all species of early mRNA were made by five minutes. Young proposed that chain elongation was slower in the presence of chloramphenicol and found that nearly all active RNA coding for polypeptides larger than 25,000 daltons was preferentially degraded or its synthesis inhibited. The effect of chloramphenicol differed from the artificial 'polar effect' of the antibiotic on polycistronic *trp* mRNA (Morse, 1970) because it was unaffected by growth in a SuA host (Young, 1975).

The apparent protein dependence of some transcripts from pJF17.2 may be an artefact produced by chloramphenicol and may be similar to the effect of the antibiotic on long T4 transcripts. This could be tested by a study of *in vitro* transcripts of 186 DNA. If the X gene product is required for all subsequent transcription then the majority of *in vitro* transcripts should be short and hybridize to only a part of pJF17.2. On the other hand if the effect of chloramphenicol is artefactual then *in vitro* transcripts should hybridize to pJF17.2 and probably to pJF15.

Transcription from the fragment in pJF15 (that is the A gene) was completely inhibited by chloramphenicol suggesting that the activity of this fragment was also dependent on a phage protein. The most likely explanation is that gene A is included on the same transcript as the remainder of pJF17.2 and thus that A expression is regulated by protein X.

(c) Transcription of the immunity region

Hybridization to λppJF18 was identical with pJF35 indicating that the immunity region (67.9 to 74.9%) must depend on a phage protein to initiate transcription.

The establishment of repression in phage λ requires the products of two genes, *c*II and *c*III, in addition to the *c*I gene. It has been proposed that *c*II and *c*III act as positive regulators of *c*I transcription from p_{re}. Once repression has been established the *c*I protein inhibits transcription of *c*II and *c*III and controls its own expression from a second promoter p_{rm} (Reichardt and Kaiser, 1971).

A second gene, *c*II, required for lysogeny of 186 has been identified (Huddleston, 1970) and mapped to the right of *att* (Hocking, 1977). 186 *c*II may be analogous to λ *c*II or *c*III proteins and may therefore be required to initiate the transcription of 186 *c*I. In the presence of chloramphenicol *c*II would probably not be transcribed nor translated and so *c*I transcription would not ensue. The *int* gene is also encoded in the immunity region (67.9 to 74.9%) and must require a phage protein for its expression. *Int* and *c*I may be controlled by the same positive effector (*c*II) and so be co-ordinately regulated, though transcription need not be from the same promoter (compare to λ, Section I.6).

(d) Transcription of the cohesive ends

A low level of transcription of the cohesive ends, comparable to that seen after wild type induction was detected late after induction or infection in the presence of chloramphenicol suggesting that this transcript was independent of phage protein synthesis.

(e) Summary of chloramphenicol RNA

Inhibition of protein synthesis by chloramphenicol has revealed that all 186 early transcription was sensitive to the antibiotic. The immunity region, 67.9 to 74.9% of 186 genome, was not transcribed following induction or infection in the presence of chloramphenicol, and it was suggested that *c*II is a positive regulator of *c*I and *int* expression. The *c*II protein is known to be essential in the formation of 186 lysogens and maps to the right of

att on a region of DNA that was transcribed at a slow rate in the presence of chloramphenicol.

Transcription of only part of pJF17.2 was insensitive to chloramphenicol and a protein, X, was proposed as the positive effector of transcription from the remainder of pJF17.2 as well as pJF15. It has been proposed that X maps to the right of the essential promoter located between 76.3 and 81.0% and that it regulates rightward transcription from a site adjacent to gene X. No mutations have been assigned to this gene.

6. DNA synthesis and transcription around *ori*

An outstanding feature of transcripts made when DNA synthesis was inhibited either by using a phage defective in the A gene or a host which under certain conditions cannot support phage DNA synthesis, was the lack of transcripts from the region 87.0 to 93.8%. The functions that have been assigned to this DNA are two alleles of gene A (A*am*24, A*am*33) and *ori* which has been mapped physically at 92.9 $\pm$ 1.8% (Chattoraj and Inman, 1973).

Transcription around *ori* has been investigated for phage λ and although the situation is not clear the evidence suggests that transcription is an essential part of phage replication. A small leftward transcript, *oop*, which arises in the *ori*, O, P region has been isolated from both a lysogen, where it comprises about 2% of phage RNA, and from phage in the lytic cycle (Hayes and Szybalski, 1973). A dual role has been suggested for *oop* RNA by Honigman *et al.* (1976). They proposed that *oop* RNA can be extended to

cover the immunity region, and therefore acts as a primer for the immunity transcript. Phage proteins *c*II and *c*III are positive regulators of *c*I transcription and it has been suggested that the products of these genes might act as antiterminators to enable the extension of *oop* RNA, rather than acting at p_{re} the promoter which was invoked for *c*I expression. The second function suggested for *oop* RNA was a primer for DNA synthesis and some evidence has been presented to suggest that *oop* RNA may be covalently linked to DNA and that *oop* RNA may stimulate *in vitro* DNA synthesis from a λ DNA template (Hayes and Szybalski, 1975). This dual role for *oop* RNA may play a part in the 'decision' between lysis and lysogeny.

A second phenomenon, that of transcriptional activation of λ replication has also been described. A repressed λ chromosome will not replicate even when O and P proteins are supplied by a helper phage (Thomas and Bertani, 1964). The epistatic block of the repressor was overcome by mutations (ri^c) that allowed constitutive transcription around the *cro*-OP region (Dove *et al.*, 1971) and, conversely, if transcription of this region was prevented by mutation then the mutant DNA could not be replicated by a helper phage. It has been suggested that transcriptional activation may:

(1) change the structure of the DNA in the *c*II-O region to allow its recognition by the replication complex,

(2) cause a translocation of this region to the site where replication takes place.

Replication of 186 DNA differs from λ replication in several facets; like P2 and φX174, 186 is dependent on host *rep* function and in addition requires the host initiation functions *dna*A and *dna*C, none of which are used by λ phage (Hooper, 1979). Replication of 186 and P2 proceeds rightwards (Chattoraj and Inman, 1973; Bertani and Bertani, 1971) but λ replication is bidirectional (Schnös and Inman, 1970). If the transcript of the *ori* region in 186 serves as a primer of DNA replication then it must be transcribed rightwards and therefore could be an extension of the A transcript *per se* since this must surely be rightward originating from the promoter associated with the operator located between 76.3 and 81.0%. However, A transcription is not sufficient for the transcription of the *ori* region because this region was not active when the A gene was defective or when *dna*C protein was inactivated.

Like *oop* RNA, transcripts of pJF16 were found in the prophage but their function is unknown (Section VI.D.2).

When DNA synthesis was inhibited by a *dna*C*ts* mutation in the host, the transition to late gene expression began between 15 and 25 minutes after infection. This implies either that DNA synthesis was not needed for the activation of late genes or that inhibition of replication was not complete and late genes were transcribed from replicating DNA.

Even during wild type lytic development the fragment cloned in pJF16 was relatively inactive when compared with the fragment in pJF15 which must be completely transcribed since the A gene spans the *Pst*I fragment in pJF15. pJF16 encodes two alleles of gene A and in view of the inactivity

of this fragment it seems unlikely that the A gene extends beyond these alleles (near 87.0%) to overlap *ori* at 92.9 ± 1.8%. Thus 186 gene A probably differs from the replication gene of ϕX174 which overlaps the origin of replication (Francke and Ray, 1972; Weisbeek and van Arkel, 1976; Eisenberg *et al.*, 1977). It is also unlikely that any other gene needed during the lytic cycle is encoded on this fragment.

Although the exact function of transcription around *ori* cannot be determined from the information available its presence does correlate closely with 186 DNA replication and therefore it may activate replication or provide a primer for DNA synthesis.

7. Transcription of the cohesive ends

The transcriptional activity of the cohesive ends of phage 186 is of particular interest because it may provide information about the mechanism of late gene activation.

P2 late gene expression is dependent on at least two essential genes, A and *ogr*. Gene A is required for DNA synthesis as well as *ogr* expression (Sunshine and Sauer, 1975). Ogr^+ mediates the transition to late transcription probably by a modification of the host machinery (Sunshine and Sauer, 1975) while the requirement for A protein may be at the level of DNA replication or the transcription of *ogr per se*. Late gene transcription proceeds in both directions, although the majority of genes are read rightward (Lindahl, 1971) and thus *ogr*, which is located to the right of the late genes between D and *att*, must be separated from its site(s) of action. The left-most polarity group of P2,

genes Q and P, is transcribed leftwards (Lindahl, 1971) and therefore it is unlikely that late gene transcription would be initiated at a single site near the righthand end of the chromosome and proceed rightward across the cohered ends and through the late genes.

Late gene transcription of phage λ is activated by the product of gene Q and does not require DNA synthesis. The evidence suggests that Q protein acts at a single site between genes Q and S and that transcription proceeds across the reannealed ends and then genes A through J, all of which are transcribed rightward (Herskowitz, 1973). The form of λ late mRNA is not certain.

Therefore a λ mode of late gene expression is characterized by the action of a phage protein at a single site adjacent to the gene encoding this activator followed by transcription across the reannealed ends and the late genes. In contrast to this P2 late gene expression requires two gene products which regulate transcription at a site(s) separated from the control genes and is probably not associated with transcription across the cohered ends of the vegetative phage.

Evidence of transcription across the cohered ends of 186 would support a 'λ like' mode of late gene activation.

The cohesive ends of 186 were transcribed during the late phase of 186 development, however, this was not invariably associated with late gene expression. The appearance of transcripts of the reannealed ends was coincident with late gene expression after the induction of a

186*cIts* prophage. If the prophage was defective in gene A a low level of RNA from the cohered ends and the late genes was found and if gene B was defective or protein synthesis inhibited then transcripts of the cohered ends were present but late gene transcription did not ensue. When host DNA synthesis was prevented by a *dnaCts* mutation both late genes and the cohesive ends were transcribed. The transcript of the cohesive ends may therefore act as a leader for late gene transcription and the B protein may act to elongate this transcript. If this occurs then 186 combines features of both P2 and λ late gene activation. λ late genesare included on a transcript that crosses the cohesive ends but the late gene activator (Q protein) acts at a site adjacent to the Q gene. In contrast to this P2 *ogr* acts at sites distant from the *ogr* gene but probably does not cross the cohered ends.

If 186 late genes are transcribed from a promoter at the right end of the 186 chromosome, then the activation of the P2 late genes by 186 gene B in the hybrid phage would not require gene B to interact with the heterologous P2 promoters. However, it has been suggested that P2 genes P and Q are transcribed leftward (Lindahl, 1971; Geisselsoder *et al.*, 1973) which would be incompatible with rightward transcription across the cohesive ends in a hybrid phage.

Therefore although 186 late gene transcription resembles P2 late expression in many ways, transcription across the cohesive ends cannot be excluded as the mode for 186 late gene activation.

8. Control of B gene transcription

The data presented in Sections VI.D.4 and 7 suggests that 186 A protein was required for the efficient transcription of gene B. The poor complementation between A and B mutant phage implies that A protein acts in *cis* to stimulate B transcription (Hocking, 1977). Furthermore, B transcription can be *trans*-activated from a prophage (either 186*cIts* or 186*cItsAam*5) and from a cloned restriction fragment 65.5 to 76.3% (Section V.E.4) by a superinfecting phage. The observation of *trans*-activation conflicts with the poor complementation suggesting *cis*-action of A protein. The order of genes on the cloned restriction fragment is identical to the vegetative map rather than the prophage map and therefore one cannot reconcile this contradiction by proposing a different mode of control during the lytic and prophage states. One simple explanation might lie in the difference in sensitivity of the two tests. Complementation was conducted in liquid and phage production was assayed 90 minutes after coinfection. In contrast plate tests were used to measure recovery of functions from the prophage. Nevertheless, the striking difference in marker rescue of B from the prophage when compared to the recovery of all other late functions (at least some of which would be enzymatic rather than structural) strongly suggests that the B gene was *trans*-activated by the superinfecting phage. One thing is certain, B protein is not under direct repressor control, but since it was not transcribed constitutively by the prophage it must be indirectly controlled. The control of B gene expression remains a paradox.

The *ogr* gene of P2 is not under direct immunity control nor is its operon split in the prophage (Sunshine and Sauer, 1975); *cis*-acting A gene is required for the full expression of *ogr* (Sunshine and Sauer, 1975) but it is not known whether the dependence on A function is at the transcriptional level. Therefore, the control of P2 *ogr* and 186 gene B appears to be very similar.

A model for control of 186 transcription incorporating the results presented in Sections III, IV and VI will be described in Section VII and compared with the transcriptional controls of phage P2 and λ.

SECTION VII

GENERAL DISCUSSION

SECTION VII

GENERAL DISCUSSION

A discussion of the results presented has been included in each section of this thesis and so the aim of this general discussion is to present an overall model for the control of 186 transcription and then to compare the features of this with the controls operating in the related phage P2 and also for λ.

Further experiments essential to an increased understanding of 186 transcription will be suggested.

1. Controls of 186 transcription

(a) Transcription of the prophage

Two segments of 186 DNA were transcribed by an uninduced lysogen (Fig. 7.1) and therefore the genes encoded by these transcripts are probably required for the maintenance of a stable prophage. The immunity transcript would code for at least *c*I, the phage repressor which directly represses genes needed for lytic development. In addition *int* may also be transcribed by the prophage and the repressor protein may exert an epistatic block on *int* catalysed excision. The function of the second region (87.0 to 93.8%) is not known, but like P2, 186 may require an additional function for stability of the prophage.

(b) Heat induction

The transcripts made soon after heat induction of a 186*cIts* prophage are illustrated in Fig. 7.1.

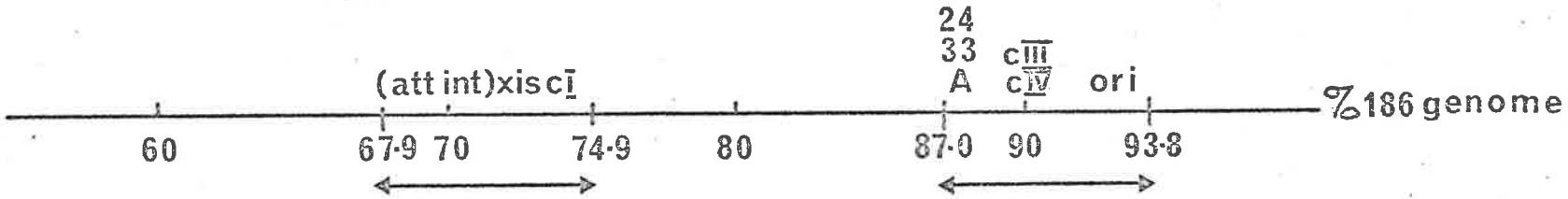
FIGURE 7.1. Regions of the 186 Genome Transcribed in the Prophage State

Transcripts made by an uninduced prophage originate in the two regions depicted in the figure, but the extent and direction of transcription is unknown.

The genes *c*III and *c*IV are required for lysogeny (Huddleston, 1970) and it has been proposed that they are located on the *Pst*I fragment, 87.0 to 93.8%.

There is no evidence for a 186 *xis* function and it has been suggested that if such a function exists it might be located in the immunity region.

These same fragments are transcribed five to seven minutes after heat induction of the prophage but the extent of transcripts may differ from the prophage transcripts.


24
33
A
cIII
cIV
ori
(att int)xis cI
% 186 genome
60
67·9
70
74·9
80
87·0
90
93·8

The same two regions of 186 DNA transcribed by the prophage were expressed five minutes after heat induction of the prophage. This may be due to the persistance of transcripts made by the prophage or alternatively although the transcripts originate from the same fragments that are active in the prophage the extent of transcription may have increased to include different genes. The immunity region of 186, as defined by the deletion 67.9 to 74.9%, has a coding potential for about 700 amino acids. *Int* and *c*I proteins of λ have been purified and their molecular weights found to be 36,000 to 44,000 daltons (330 to 400 amino acids) and 27,000 daltons (245 amino acids) respectively (Ptashne, 1971; Nash, 1977). Thus the 186 immunity region has sufficient DNA to include *int* and *c*I genes of similar size to their λ counterparts as well as *att* (λ *att* is at most 100 base pairs and has only a 15 base homology with the host site (Davis and Parkinson, 1971; Hradecna and Szybalski, 1969; Landy and Ross, 1977)). Although there is no evidence for a 186 *xis* function, λ *xis* is small (about 80 amino acids, Nash, 1977) and so a 186 *xis* could also be encoded by this region. P2, closely related to 186, does have a gene, *cox*, that is required exclusively for excision and which has been mapped to the right of P2 C gene (Lindahl and Sunshine, 1972).

The A/*ori* transcript made at the time of excision has an unknown function. During the lytic cycle its appearance correlated closely with phage replication and a role as a primer for rightward DNA synthesis or in transcriptional activation of replication has been proposed. A transcript from this region was also made constitutively by the prophage leading to the suggestion that a second phage

protein (analogous to P2 Z gene) was needed for stable lysogeny, and that either *c*III and/or *c*IV may be encoded on this tract of DNA.

A second alternative is that this RNA is analogous to λ *oop* for which a dual function has been proposed (Honigman *et al.*, 1976). 186 RNA from this region could only function in the two capacities if two transcripts, a leftward leader for *c*I transcription and a rightward primer for DNA synthesis, were made.

It seems more likely that transcription five minutes after induction was a carry over, from the prophage state, of transcripts required by the prophage and that during lytic development a different transcript associated with phage replication originates from this fragment.

(c) Lytic development of 186

A schematic representation of the proposed model for transcription during 186 lytic development is shown in Fig. 7.2.

The order of early transcripts was deduced from the results obtained by induction and infection with 186 in the presence of chloramphenicol. The early phase of 186 development can be divided into two distinct stages:

(1) early transcription which proceeds without the need for any phage coded products (Fig. 7.2). Genes included on the fragment transcribed were X and probably *dho*. As discussed in Appendix I(c) the *dho* gene is transcribed from a repressor controlled

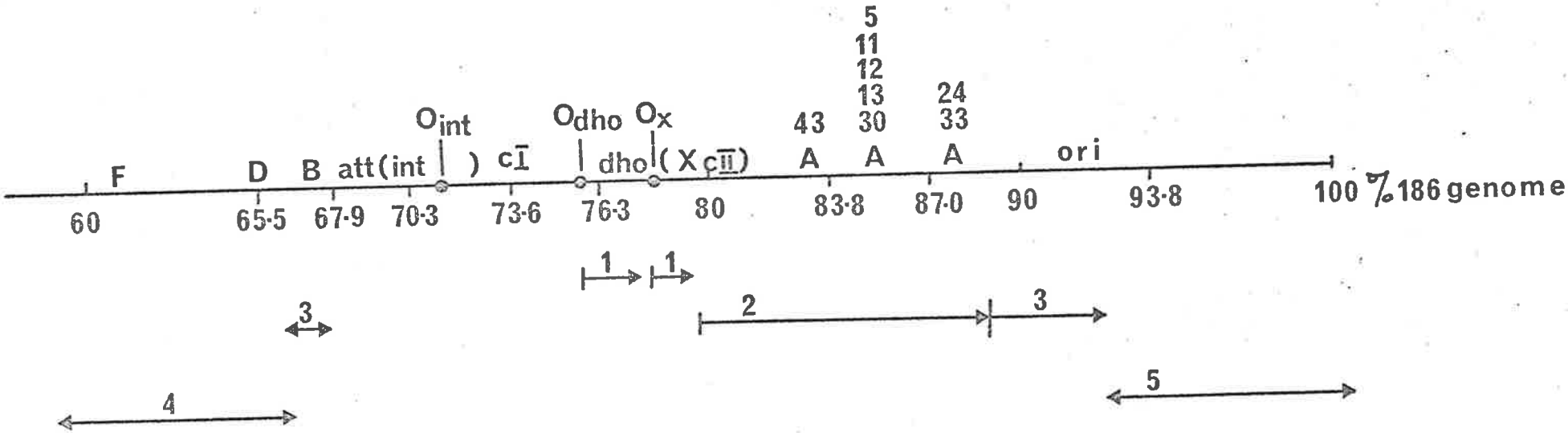
FIGURE 7.2. The Proposed Model for Transcription During 186 Lytic Development

This figure shows the genes and sites encoded by the right 40% of the 186 genome. The relative location of *int* with respect to o_{int} and X with respect to *c*II are unknown and this is indicated by brackets.

The operator sites under *c*I control are represented by closed circles on the map.

The proposed transcripts are indicated below the map. Although the direction of transcription has not been determined by hybridization to separated DNA strands, the direction of some transcripts has been inferred from the relative locations of the operator sites and the genes under their control. Where the direction is unknown the transcripts have an arrow at either end.

Early transcripts (1) are proposed to cover the genes *dho* and X. X is the positive effector for the delayed early transcripts (2). The transcripts denoted (3) are dependent on the A gene. The B gene regulates the appearance of the late gene transcripts (4) that extend from approximately 2.3 to 65%. The transcript arising from the cohesive ends (5) is not made until late in the lytic cycle but no gene controlling the appearance of this transcript has been identified.


5
11
12
13
24
33
Oint
Odho
Ox
43
30
F
D
B
att(int
)
cI
dho
(XcII)
A
A
A
ori
60
65·5
67·9
70·3
73·6
76·3
80
83·8
87·0
90
93·8
100 % 186 genome
1
1
2
3
3
4
5

promoter that is to the left of the 76.3% *Pst*I restriction site but it is not known whether transcription from this promoter is dependent on the proposed positive regulator, X. It has been suggested that X may permit the elongation of the early transcript from the essential promoter to cover gene A and probably *c*II and therefore the simplest model demands that the promoter for *dho* is independent of X function. The identity of early transcripts could be verified by either cloning these genes on to separate fragments or by translation of early mRNA, with the subsequent identification of the products.

(2) delayed-early transcription during which three additional regions of DNA are transcribed; these transcripts cover the immunity region, gene B, the A gene and probably *c*II although the relative locations of X and *c*II are unknown. It has been postulated that *c*II is the positive regulator needed to establish *c*I and *int* expression, and that the A protein is needed for efficient transcription of gene B; the existence of a new gene, X, has been proposed to account for the absence of transcripts from part of pJF17.2 and pJF15 in the presence of chloramphenicol. The delay in gene A expression may be beneficial to ensure that host replication is inhibited by *dho* and thus that phage replication is optimal since 186 utilizes many host functions for its DNA synthesis (Hooper, 1979).

The A gene is essential for DNA replication and the next stage in phage development was DNA synthesis

which was associated with transcription of the A/*ori* fragment in pJF16.

The final phase of 186 development was the transcription of the late genes which requires both A and B functions. The requirement for A is unclear, it is needed for efficient transcription of the B gene and in addition A*am* phage show poor complementation with phage mutant in any structural gene implying that *cis*-action of the A product is essential for late transcription. Late genes may only be transcribed from replicating DNA and thus the requirement for A protein may be indirect. Experiments with a *dnaCts* host at non-permissive temperatures have failed to clarify this point. If it can be shown by DNA/DNA hybridization that 186 does not replicate in a *dnaCts* host at 41.5°C then one must conclude that DNA replication is not essential for late transcription and that the A protein acts directly with B to activate late genes.

If the need for A protein is direct, one could postulate that A acts in *cis*, together with B protein (in *cis* or *trans*) to stimulate late transcription from a promoter at the right end of the vegetative chromosome. The A protein would then be required to function in three capacities, as the DNA replication protein, as the positive effector of B transcription, and as the positive effector of late transcription in concert with B protein. Therefore, it is more likely that A is needed at the level of DNA replication to provide either a replicating template for late transcription or to increase the number of DNA copies to provide sufficient late gene transcripts for a productive

infection.

The B protein may alter the phage DNA so that the host RNA polymerase will recognise late promoter(s) or the polymerase itself may be the target of B action.

(d) Genes under repressor control

186 has at least two sites of repressor action and the genes X and *dho* are under direct repressor control. The major or essential operator has been located between 76.3 and 81.0% by restriction mapping of 186*del*2, a virulent phage with a 0.4 kb insertion that inactivates this operator (Saint, 1979; Finnegan, unpublished observations). A better estimate of its position could be obtained by an EM heteroduplex analysis. It has been proposed that X is under the direct control of this operator and therefore A and *c*II are only indirectly controlled.

The harmful effect of *dho* inserted into pJF32, a plasmid clone from 186*del*2, can be overcome by growth in a 186 immune cell indicating not only that *dho* is under repressor control but also that its operator is distinct from the essential operator controlling X. Moreover, the evidence presented in Appendix I(c) suggests that the promoter and operator for *dho* are to the left of 76.3% or span the *Pst*I restriction site at 76.3%.

The *c*II gene is not expressed constitutively by a prophage and therefore must also be under *c*I control. The relative positions of *c*II with respect to X and the major phage operator have not been determined but the simplest model suggests that *c*II would lie in the same

operon as X.

The late genes of 186 cannot be *trans*-activated from the prophage by a superinfecting virulent phage suggesting that they too may be under direct repressor control. However, it is more likely that the primary requirement for the *cis*-acting A gene prevents their expression from the prophage.

Some evidence was presented in the previous section (VI.E.5) to suggest that *int* and *c*I may be co-ordinately controlled. As seen in phage λ, *int* may be transcribed from a promoter which is distinct from the *c*I promoter and which is under repressor control. Fig. 7.2 illustrates the probable locations of the phage operator sites.

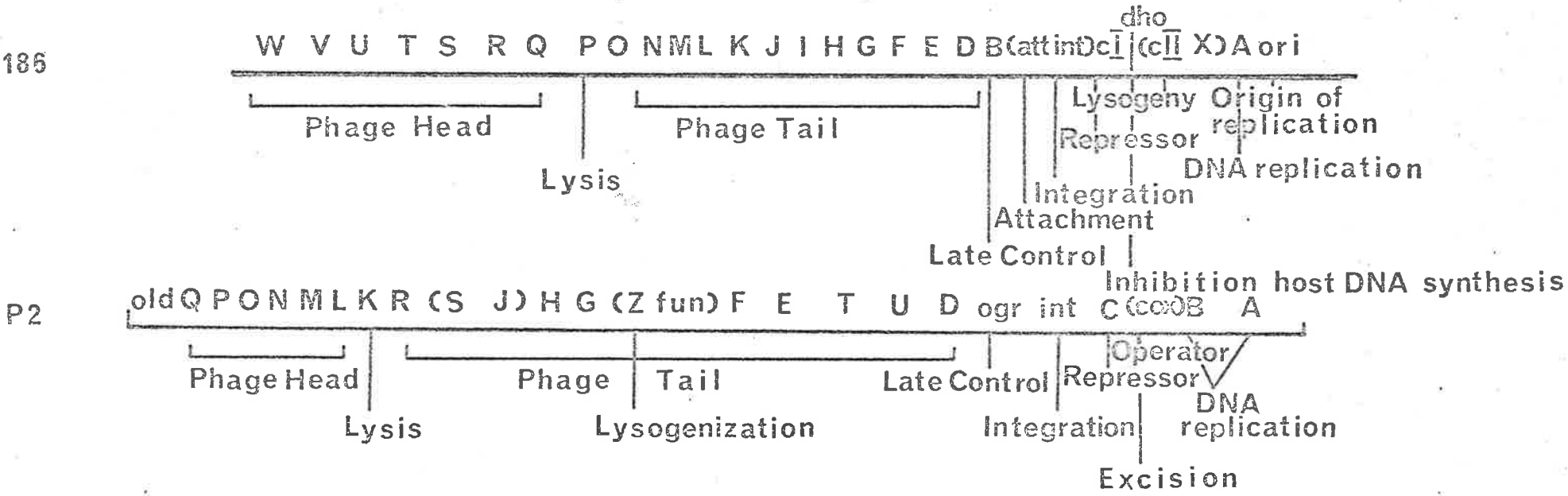
In contrast to the other early genes discussed, gene B is not under repressor control because it can be *trans*-activated from a repressed prophage. The control of the B gene is unclear but it required gene A product for efficient transcription and so the A protein is probably the positive effector of B transcription. This in itself is a paradox since A is apparently required in *cis* for B expression (implied by the poor complementation of A*am* and B*am* phage) and yet B can be *trans*-activated from the prophage by a superinfecting phage.

2. A comparison between 186 and P2 transcription

A few studies of P2 transcription have been reported and the essential control genes have been described. The genetic maps of P2 and 186 are presented in Fig. 7.3 for

FIGURE 7.3. The Genetic Maps of 186 and P2

The relative location of the known genes of 186 and P2 are shown in the figure. The gene functions are listed below the map. Where the relative location of a pair of genes is unknown the genes are included within brackets, for example, 186 genes *c*II and X or P2 genes S and J.


186
W V U T S R Q P O N M L K J I H G F E D B (attint) dho cI cII X) A ori
Phage Head
Phage Tail
Lysis
Late Control
Attachment
Integration
Repressor
Lysogeny
Origin of replication
DNA replication
Inhibition host DNA synthesis
P2
old Q P O N M L K R (S J) H G (Z fun) F E T U D ogr int C (cox) B A
Phage Head
Lysis
Phage
Tail
Lysogenization
Late Control
Integration
Operator
Repressor
DNA replication
Excision

easy comparison of gene function and map location.

Like 186, early P2 transcripts originate exclusively from the right half of the genome while the majority of late genes are encoded by the left half of the chromosome; 22 to 24 minutes after infection two-thirds of the transcripts arose from the left half (Geisselsoder *et al.*, 1973). Transcription throughout the lytic cycle was asymmetric with about 95 to 98% of RNA being made from the H strand; L strand transcripts were specific and so could not be attributed to H strand contamination of the L strand preparation (Lindqvist and Bøvre, 1972).

It is clear that P2 development can be divided into at least two phases, early and late, and several requirements for the transition to late transcription have been identified. These are described and compared to their 186 counterparts below:

(1) P2A*am* phage have approximately normal early transcription, both in the degree of asymmetry and amount, but show no transition to late transcription (Lindqvist and Bøvre, 1972; Geisselsoder *et al.*, 1973). The A gene of P2 is analogous to 186 gene A; both have properties of *cis*-acting proteins, are essential for DNA replication, and it appears that both genes are needed for late transcription.

(2) P2 has a second DNA replication gene, B, which acts well in *trans* and is probably involved in a step subsequent to DNA nicking catalysed by P2 A gene (Geisselsoder, 1976). B therefore depends on gene A for its activity although in the model proposed by Lindahl (1971) it is

transcribed before gene A. The B gene has been mapped to the left of A and is under the control of an operator, which is mutated in virulent phage, and which is located between genes C and B.

Transcription of a P2B*am* phage was similar to wild type early after infection but showed only a slight increase in P2 directed transcription later in the lytic cycle (Lindqvist and Bøvre, 1972; Geisselsoder *et al.*, 1973). The increase was inhibited by infection of a str^r host to reduce the level of amber suppression (Geisselsoder *et al.*, 1973). Although P2 B and the proposed 186 gene X are located in the same relative position on each genome the proposed function of X and the known function of B are in no way analogous. No 186 equivalent to P2 gene B has been described.

(3) infection of a rep^- host cell with P2 resulted in normal early transcription but once again there was only a slight increase of transcription late in the lytic cycle. Therefore, P2 DNA replication must be essential to provide the appropriate template for late transcription or to increase the gene dosage. This finding is in keeping with the observation that the two genes needed for replication, A and B, were also required for late transcription. The effect of blocking 186 DNA replication using a *dnaCts* host at a non-permissive temperature was investigated and the transition to late transcription was observed. Phage production was inhibited under these conditions but no check was made to ascertain whether DNA replication was completely prevented and thus late genes may have been transcribed

from replicating templates. Until further experiments have been carried out to clarify this point it is not possible to conclude whether 186 late expression is dependent on DNA replication or the A gene product *per se*.

(4) Sunshine and Sauer (1975) described another gene, *ogr*, that is essential for P2 late expression. A mutation in the α subunit of RNA polymerase (Sunshine and Sauer, 1975; Fujiki *et al.*, 1976), termed gro_{109}, inhibited the transcription of P2 late genes even though early transcription and P2 replication were unaffected. A P2 mutant, *ogr*, able to overcome the effects of gro_{109} was isolated and it has been proposed that the expression of P2 late genes was mediated by an interaction between a phage product (*ogr*) and a bacterial protein (*gro*). The gro_{109} mutation was specific for P2; the related phage P2*hydis*, 299 (Sunshine and Sauer, 1975), and 186 (Egan, pers. commun.) were unaffected by the mutation.

The *ogr* gene of P2 is very similar to 186 gene B both in map position (Fig. 7.3) and in function. However, the mode of action of these two genes cannot be identical because gro_{109} does not inhibit 186 development. An equivalent host mutation, blocking 186 late transcription, should exist if 186 B gene interacts with a host protein as proposed for P2 *ogr*.

The control of *ogr* resembles that of 186 B gene; it is not under direct immunity control nor is it a part of an operon split by integration into the bacterial chromosome (Sunshine and Sauer, 1975). The *cis*-acting A gene is required for *ogr* expression, but it is not known

whether A is needed for *ogr* transcription or if it is only needed to provide a replicating template for late transcription. The A gene of 186 is also essential for B gene expression, both at the level of B transcription and perhaps to provide a suitable template for late transcription.

The recovery of *ogr* from a P2 lysogen has not been reported and so it is not known whether *ogr*, like 186 B, can be *trans*-activated from the prophage.

As discussed in Section VI.E.8, when 186 control genes are located in *cis* with P2 structural genes (that is in hybrid phage) the phage are viable and thus 186 must activate P2 structural genes. Therefore, even though 186 B and P2 *ogr* are not identical the mode of B action must be compatible with the activation of heterologous late genes. Geisselsoder *et al.* (1973) considered it unlikely that P2 late transcription was initiated at a promoter near the right end of the chromosome with transcription across the cohered ends and then rightward from genes Q through D. The two left-most genes are transcribed leftward and thus late transcription from a promoter at the right end of the chromosome would interfere with the expression of these genes. Activation of P2 late genes in the hybrid phage could occur by

(i) alteration of the host RNA polymerase (probably α subunit) by 186 gene B to allow recognition of P2 late promoters.

(ii) activation of a 186 late promoter on the right end of the vegetative chromosome

with transcription across the cohesive ends and then rightward through the late genes.

Although the second alternative would interfere with the proposed leftward transcription of P2 genes P and Q, it by-passes the need for 186 B protein to act on heterologous promoters. 186 is not affected by the *gro* mutation in *E. coli* suggesting that even if B interacts with the RNA polymerase the site of action must differ from the *ogr* site. Transcription of 186 reannealed ends was detected late in development and was observed in B*am* phage and in the presence of chloramphenicol suggesting that if late transcription proceeds from a rightward promoter then B protein may act to elongate this transcript.

It would be interesting to discover if P2*ogr* phage could borrow 186 B protein for the activation of P2 structural genes and similarly if 186B*am* could use P2 *ogr* function. It is possible that the failure to isolate reverse hybrids with P2 control genes associated with 186 structural proteins (Hocking, 1977) was due to the incompatibility of P2 *ogr* and 186 late promoters.

A satellite phage, P4, which is dependent on the presence of P2 or an antigenically related phage for productive infection has been isolated (see Barrett *et al.*, 1973, for a review). P4 head size and DNA content are one-third the size of the P2 helper, and although there is no homology between the two genomes P4 structural components are identical to P2 capsid proteins. P4 is able to

replicate and integrate in the absence of a helper phage but is dependent on the helper for its structural components. P4 is able to *trans*-activate P2 late gene expression from a P2 prophage or in a *rep*$^-$ cell in which P2 late transcription does not normally occur; however, although it can borrow 186 structural proteins during a mixed infection it cannot *trans*-activate 186 genes from the prophage (J.B. Egan, pers. commun.).

A P4 gene, δ, that is required for *trans*-activation of P2 genes has been identified and it has been shown that P4δ6 needs a replicating P2 phage as a helper (Souza *et al.*, 1977). Even though P2 gene B is essential for DNA replication a P2B*am* phage can act as a helper for P4δ6, however, it appears that P4 may enable a P2B*am* phage to replicate its DNA (Six and Lindqvist, cited by Souza *et al.*, 1977). It has been proposed that P4 δ may function as an antiterminator because P4 can suppress the effect of polar mutations in P2 (Sunshine *et al.*, 1976). Furthermore, it has been suggested that δ may correspond to P2 *ogr* because neither P4δ$^+$ nor P4δ6 can *trans*-activate a P2 helper in a gro_{109} host. A mutation of P4, *oγρ*, which overcomes *gro* has been isolated but it has not yet been ascertained whether δ6 and *oγρ* lie within the same gene (Souza *et al.*, 1977).

The inability of P4 to *trans*-activate 186 late genes from a prophage emphasizes the difference between the late promoters of P2 and 186. In addition P4 does not *trans*-activate P2 late genes from a P2.186 hybrid prophage (E. Six, pers. commun.) suggesting that activation requires

a function or a site encoded by the right end of the P2 chromosome.

The major phage operator in P2 has been mapped between the genes C and B by the location of *vir* mutations (Lindahl, 1971). It is not known whether there is more than one site of repressor control in P2, but the location of the essential operator is similar for 186 and P2. P2 does not interfere with the host DNA replication and therefore probably does not encode a *dho* function (Hooper, 1979) but in contrast to 186 it does inhibit host RNA synthesis.

In the P2 prophage at least four genes, C, *fun*, *old* and Z are known to be active (Lindahl, 1974; Bertani, 1976). The phage repressor, C, and a second gene Z, which is adjacent to *fun*, are required for the stability of the prophage. P2 *old* inhibits the growth of phage λ in a P2 lysogen and the *fun* gene product converts *E. coli* C to sensitivity to 5-fluoro-deoxyuridine. A 186 prophage does not interfere with λ and so does not encode an *old* function. It is not known if 186 has a *fun* gene because the sensitivity of the host to 5-fluoro-deoxyuridine has not been tested (Hocking, 1977). The 186 repressor *c*I, and perhaps 186 *int*, was active in the prophage, and a second region 87.0 to 93.8% was also transcribed. This may encode a function similar to P2 Z, although the map position is unrelated. Transcripts between 10.0% and about 61.0% would not have been observed because no restriction fragments from this interval were used in hybridization experiments. Therefore additional function(s) analogous to P2 Z or *fun* in map location would have remained undetected.

In conclusion, P2 and 186 do show many similarities in the overall pattern of transcription and some features of its control. However, this study has revealed a number of basic differences:

(1) there is no evidence for a P2 equivalent to X

(2) 186 *dho* has no counterpart in P2

(3) 186, in contrast to P2, does not inhibit host RNA synthesis

(4) although 186 is able to activate P2 late genes on the same chromosome, 186 B and P2 *ogr* clearly differ in some aspects of their mode of action.

The control and direction of *int* transcription which are basic to any comparison of induction properties of 186 and P2 have not yet been investigated.

3. Comparison of control elements in 186 and λ transcription

The genes controlling the sequential appearance of λ proteins and the transcripts made are shown in Fig. 7.4 (Echols and Murialdo, 1978).

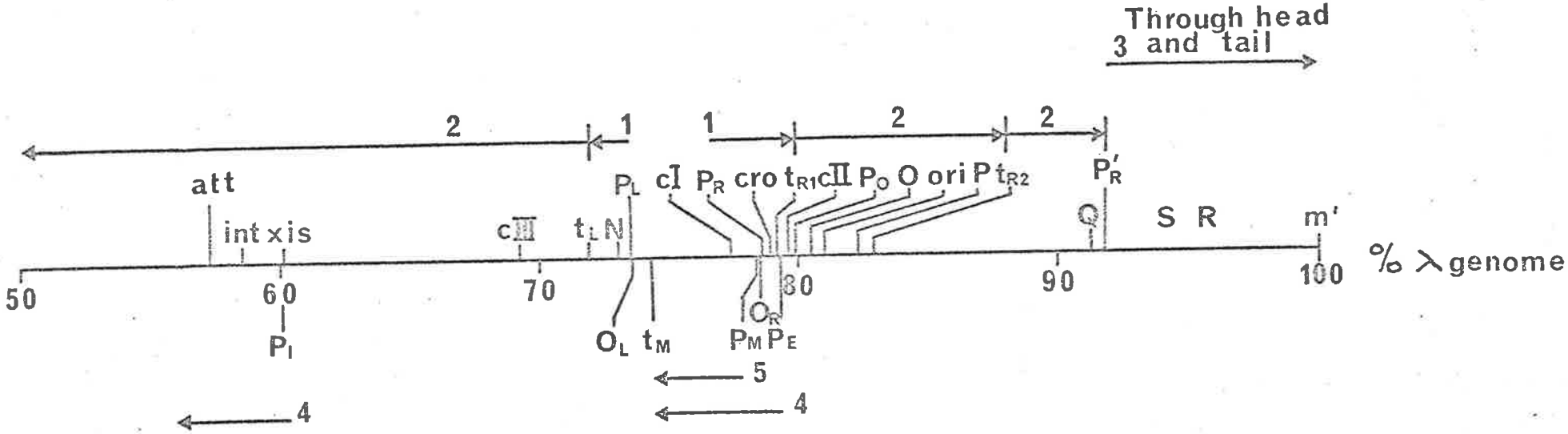
Early transcripts of phage λ arise from two promoters, p_L and p_R, located on either side of the *c*I gene. Transcription of genes N and *cro*, the products of which are essential to λ development, does not require a phage coded protein. N protein is needed for the transcription of delayed early genes and it has been demonstrated that the three sites of N control are located between *c*III and N,

FIGURE 7.4. The Transcription Map of Phage λ

The transcripts essential to lytic development are depicted above the map which shows the control genes and the sites at which they act.

Early transcripts (1) of λ cover the genes N and *cro* and terminate at t_L and t_{RI} respectively. The N protein allows elongation of both leftward and rightward early transcripts (2) to cover *c*III, the recombination and integration genes to the left of N, and *c*II, the replication genes and Q which are transcribed rightward. The late gene transcripts (3) are activated by Q at a promoter between genes Q and S.

*C*II and *c*III act together to stimulate transcripts required to establish lysogeny (4) which are depicted below the map. The major transcript made in the prophage state (5) covers the *c*I gene and is maintained by the *c*I protein itself.


Through head
3 and tail
2
1
1
2
2
att
int
xis
cIII
tL
N
PL
cI
PR
cro
tR1
cII
PO
O
ori
P
tR2
P'R
Q
S
R
m'
% λ genome
50
60
70
80
90
100
PI
OL
tM
OR
PM
PE
5
4
4

cro and *c*II, and P and Q. N protein acts as an antiterminator (Lozeron *et al.*, 1976) to allow the elongation of early transcripts beyond t_L, t_R and t_{R2} to include *c*III and the non-essential genes coding for phage recombination and integration functions to the left of N, the *c*II and DNA replication proteins O and P, as well as gene Q which activates late gene expression. It has been proposed that phage coded sites located in or near p_L and p_R are required for N action (Salstrom and Szybalski, 1978).

Gene Q is thought to act preferentially in *cis* (Echols *et al.*, 1976) on a promoter between genes Q and S to initiate expression of S and R then A through to J, all of which are transcribed rightward. Late gene expression can be stimulated from a repressed prophage by the presence of Q protein and therefore is not under direct repressor control, nor is it directly controlled by N protein. A low level of Q independent expression of late genes occurs, probably by the extension of rightward early transcripts.

Superficially λ and 186 transcription follows a similar pattern of sequential gene expression with early genes activating delayed early transcription which in turn controls DNA replication and late gene expression. Within this framework the details of control mechanisms differ. It is probable that 186 has only one repressor controlled promoter for essential genes, with the operator mapping to the right of *c*I; in comparison λ has two essential promoters with the associated operators on either side of the *c*I gene. λ gene N which is essential for the expression of all other genes acts in *trans* at three distinct sites. Although the

mode of action of X is not known, it has been proposed that it acts to elongate the early transcript and thus is similar to λ gene N.

Late transcription of 186 depends on two early genes, B and A; gene A is needed for B transcription and may also be required for phage replication to provide a replicating template. The mode of B action is not understood but it may combine features of both P2 and λ late control. It appears that the B protein may act at a site distant from the B gene (like P2 *ogr*) to activate transcription across the cohesive ends (like λ) perhaps from a replicating template (like P2).

In addition, λ codes for another early control gene which is not present in 186. The λ *cro* gene binds to the same DNA binding sites as the λ repressor, at least in o_R (Johnson *et al.*, 1978), and ten minutes into the lytic cycle the early leftward and rightward transcripts were repressed by *cro* binding near p_L and p_R. Phage defective in *cro* were not viable and it is probable that *cro* turns off N transcription and so prevents the premature death of the host. There was some evidence for the repression of early 186 transcripts late in the lytic cycle which did not occur in a B*am* phage suggesting the B protein or another gene under B control may partially shut down early promoters.

Cro is also important in the temperate response in phage λ because it controls the level of *c*I protein in the cell both indirectly by its action on *c*II and *c*III and by repression of *c*I transcription *per se*. The transcription of *c*I initiated by *c*II and *c*III proceeds along the antisense

strand of DNA encoding *cro* and there is evidence to suggest that the overlapping transcription of this region interferes with transcription in both directions.

It has been suggested that the balance between *cro* and *c*I is important in the 'decision' between the temperate and lytic responses of phage λ (Herskowitz, 1973; Oppenheim *et al.*, 1977b).

Two λ proteins, in addition to *c*I and *int*, are required to establish the prophage state. *C*I transcription is initiated by the action of *c*II and *c*III at p_{re}. Once repression is established *c*I protein turns off the transcription of *c*II and *c*III and regulates its own transcription from a second promoter p_{rm} (Reichardt and Kaiser, 1971). *C*II and *c*III also exert a positive regulatory effect on integrative recombination by the stimulation of *int* transcription from a site distinct from that involved in the establishment of repression (Katzir *et al.*, 1976; Chung and Echols, 1977). Thus the two events needed to establish the prophage state are subject to co-ordinate positive regulation.

186 *c*I transcription is also dependent on the synthesis of at least one other phage protein, *c*II. Like λ *c*II and *c*III, 186 *c*II cannot be required for the maintenance of the prophage state because the DNA encoding this gene was not active in the prophage. It was proposed that 186 *int* transcription was dependent on *c*II but it is not clear if a single promoter is involved or if like λ, the two genes share a positive regulator but are transcribed from separate promoters.

The λ prophage is maintained by the action of the repressor and the majority (80%) of transcripts made by an uninduced prophage come from the immunity region. Of the remainder 2% arose from the *ori* segment (*oop* RNA) and the rest were evenly distributed across the *int* to *b2* region (Hayes and Szybalski, 1973). A 186 prophage transcribed two different segments of DNA, the immunity region and the fragment 87.0 to 93.8% which probably codes for a second function required for prophage stability.

Therefore it can be seen that 186 conforms to the overall pattern of phage development established for the lambdoid group of phage. The three phases of transcription are sequentially controlled by phage coded proteins. Until the molecular mechanisms involved in 186 transcription have been elucidated one cannot decide whether λ and 186 control show similarities at the molecular level. The information available suggests that 186 X and λ N may share a common mechanism and that the action of 186 gene B may share features in common with both P2 and λ control elements.

4. *Int* gene and the split operon model

One basic difference between P2 and 186 lies in the excisability of the 186 prophage after the lifting of repression. A derepressed P2 prophage cannot excise from the host chromosome and it has been proposed (Bertani, 1970) that the P2 *int* gene is part of a constitutive operon that is physically split by phage integration. A direct prediction from this model is that the phage *att* site must lie between *int* and its promoter and thus *int* must be

transcribed rightward (Fig. 7.3). The *int* operon of 186 probably differsfrom that of P2 and so the location of the *int* promoter and direction of *int* transcription are of great interest in a comparison of the two phage.

The frequency of *int* catalysed recombination at *att* site greatly exceeds normal phage recombination and thus the relative positions of *int* and *att* can be determined by recombination experiments between 186*int*$^{-}$*D*$^{+}$ and 186*int*$^{+}$*Dam* phage.

The direction of *int* transcription can be determined by a two step hybridization experiment. In the first step mRNA which is most likely to contain *int* transcripts (for example, RNA labelled five minutes after induction) is hybridized to strands of 186 DNA which can be separated by poly rUG/CsCl density gradient centrifugation (Finnegan, 1974). The hybridized RNA is then eluted and rehybridized with filters containing λppJF18 or pJF35 DNA to determine which strand was complementary to immunity RNA. In order to determine the actual direction of transcription the separated DNA strands must be oriented with respect to the 186 genetic map.

The fragment cloned in λppJF18 codes for both *c*I and *int* and thus *int* mRNA cannot be separated from *c*I transcripts in this way. Two insertion mutants of 186 have been isolated, one, 186*ins*3 with an insertion at 70.3% (in the *int* gene) and the other, 186*ins*2, at 73.6% (in *c*I); in each case the insertion carries two additional *Hin*dIII restriction sites which are located within about 900 base pairs. Although the insertions destroy *int* and *c*I

activity respectively the location of the insertion with respect to the gene end points is not known.

The coding potential of 67.9 to 70.3%, that is the region between the left end of the immunity segment and the insertion into the *int* gene, is about 240 amino acids and thus could include almost the entire *int* gene. Hybridization of mRNA to a *Hin*dIII fragment from 186*ins*3 (61.3 to about 70.3%) should exclude *c*I RNA and select for *int* RNA. If this clone is used in place of λppJF18 for the second round of hybridization it should be possible to determine the direction of *int per se* rather than immunity region transcripts.

5. The future

A number of experiments still remain to increase the understanding of the control of 186 transcription.

The direction of transcription of the genes on each restriction fragment can be determined by the two step hybridization procedure described in the preceding paragraphs. This knowledge will verify the relative positions of genes and their promoter/operator regions proposed in the model presented in Section VII.1. The direction of transcription of the late genes may provide some insight into their control.

The RNA polymerase binding sites of phage λ have been located by a number of different techniques including binding to DNA fragments (Jones *et al.*, 1977), protection from endonucleolytic cleavage (Allet and Solem, 1974) and

the direct visualization of RNA polymerase bound to linear λ DNA (Vollenweider and Szybalski, 1978). The binding of RNA polymerase to restriction endonuclease generated fragments of λ DNA was assayed by the retention of DNA/polymerase complex on nitrocellulose filters. The location of the fragments on the λ map allowed the placement of phage promoters; it was found that the efficiency of retention (determined by the required polymerase to DNA ratio) varied for different fragments (Jones *et al.*, 1977). The cloned restriction fragments of 186 could be purified from the vector DNA by sucrose gradient centrifugation and used in a study of this nature to locate RNA polymerase binding sites. Late promoters may not be detected in such a study if the binding of the polymerase depends on an interaction with the B protein and/or a replicating template.

A second study in which the polymerase binding sites were visualized by electron microscopy using a glutaraldehyde/benzyldimethylalkyl-C_{12}, C_{14}-ammonium chloride technique gave good agreement with the fragment binding study (Vollenweider and Szybalski, 1978). The binding under these conditions was judged to be 84% specific and the sites corresponded to the major λ promoters, minor *in vivo* promoters, potential promoters and initiation sites of *in vitro* transcription, and in addition a few sites corresponding to known regions of termination were seen. Thus such a study would indicate a maximum rather than a minimum number of RNA polymerase binding sites.

Purification of the 186 *c*I protein will allow the location of repressor controlled operator sites by the

retention of restriction fragments binding the repressor on nitrocellulose filters (Riggs *et al.*, 1968).

Combining the information derived from these three studies, that is the direction of transcription, location of phage promoters and the position of phage operators, should allow a more detailed description of the 186 operons. This analysis can be extended to include the late 186 genes by using the cloned 186 *Bam*HI restriction fragments to determine the direction of late transcripts.

In vitro transcripts can be correlated with *in vivo* transcripts and so perhaps increase the understanding of the protein interactions required for faithful transcription.

The biological study of the expression of genes from cloned restriction fragments has yielded information about the requirements of certain genes for their expression, for example, *c*I and late genes. In the future the cloned immunity region will be valuable for sequencing studies and this and other fragments are a potential source of 186 proteins.

The use of cloned restriction fragments in the study of viral transcripts and protein interactions is a general method that can be employed if suitable deletion phage are unavailable or if the size range of restriction fragments renders them unsuitable in Southern transfer hybridization. Cloning of restriction fragments has the added advantage of allowing the genetic content of individual fragments to be determined. Overlapping fragments suitable for the isolation of specific mRNA sequences by two step hybridization

procedures can be generated by using different restriction endonucleases. The wide range of enzymes and cloning vehicles available should ensure that any viral genome can be restricted to provide fragments suitable for this type of analysis.

APPENDIX I

EXPRESSION OF NON-ESSENTIAL FUNCTIONS FROM CLONED 186 DNA

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EXPRESSION OF NON-ESSENTIAL FUNCTIONS FROM CLONED 186 DNA

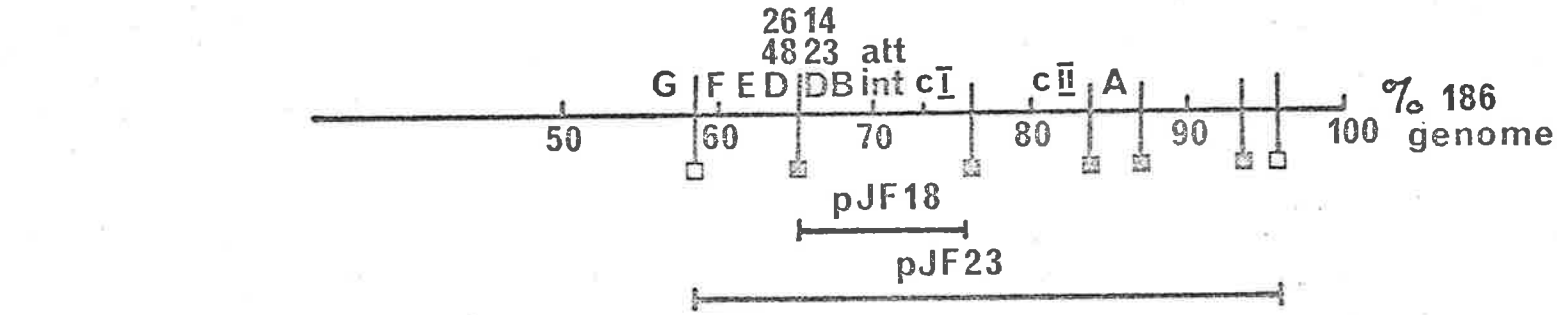
(a) Expression of *c*I

A plasmid clone which expresses the 186 *c*I gene should confer immunity to infection by 186 in the same way as a 186 prophage will prevent superinfection of the host. Immunity of a *sup*$^+$ host carrying either pJF18 or pJF23 (Fig. A1.1) to 186*c*I infection was tested and the efficiency of plating (*eop*) compared to *eop* on *sup*$^+$(pBR322). The *c*I gene on the fragments cloned in both pJF18 and pJF23 carries a temperature sensitive mutation and so the immunity tests were done at 30°C which is permissive for the 186 *c*I*ts* protein. The results of this comparison are presented in Table A1.1. The *eop* of 186*c*I was reduced to 73% by the presence of the plasmid pJF18 in the host cell and to 42% by pJF23. The presence of pJF23 in the host cell also reduced the plaque size dramatically while pJF18 had no obvious effect on plaque morphology. It is apparent that the 186 *c*I gene was not effectively expressed by the DNA cloned into pJF18 and pJF23, when compared to a 186*c*I*ts* prophage which confers complete immunity to superinfection by 186*c*I.

Expression of *c*I may be dependent on another gene as exemplified by the need for *c*II and *c*III gene products in the transcription of λ *c*I gene from p_{re}. Once established *c*I maintains its own expression from a second promoter p_{rm} (Reichardt and Kaiser, 1971). A second 186 gene required for lysogeny, *c*II, was isolated by Huddleston (1970) but its exact function is unknown. The *c*II gene has been mapped

FIGURE AI.1. A Cleavage Map of the Region 50 to 100% of the 186 Genome Showing the Location of the *Bam*HI and the *Pst*I Restriction Sites

The *Pst*I sites to the left of 65.5% have not been mapped and therefore have not been included in the figure. The fragments cloned to give the plasmids pJF18 and pJF23 are indicated below the map. The genes encoded by each fragment are indicated above the map.



BamHI Pst I

Cleavage sites

TABLE AI.1. Plating Efficiency of 186*c*I *sup*$^+$(pBR322), *sup*$^+$(pJF18) and *sup*$^+$(pJF23)

Host	Plaque forming units/ml			Average
	a	b	c	*eop*
sup$^+$(pBR322)	6.3×10^9	3.8×10^9	2.7×10^9	1.0
sup$^+$(pJF18)	5.8×10^9	2.9×10^9	1.4×10^9	.73
sup$^+$(pJF23)	2.9×10^9	1.5×10^9	1.1×10^9	.42

The results of triplicate experiments a, b and c are presented in the table.

to the right of *att* (Hocking, 1977) but its position relative to the *Pst*I site at 76.3% is unknown. The coding potential of the region 74.9 to 76.3% (that is, the rightward limit of the deletion in 186*del*2 to *Pst*I site) is approximately 140 amino acids. It has been proposed that the promoter, operator region for *dho* is encoded by this region (Appendix I(c)) and furthermore, that the *c*II gene is included in either this operon or the essential X operon to the right of 76.3% (Section VII.I(c)). Therefore it is likely that *c*II does not map entirely (if at all) on pJF18. The expression of *c*I from pJF18 may be reduced or absent unless *c*II function is provided by an infecting phage and this may account for the limited immunity (27%) to an infecting 186*c*I phage.

In contrast the large *Bam*HI fragment (11.2 kb) cloned in pJF23 should code for all known control functions as it extends from 58.7 to 96.0%. The immunity of cells carrying this plasmid was also found to be incomplete, with *eop* of an infecting 186*c*I phage reduced to 42%. The plaques that were formed were much smaller than those on the sup^+(pBR322) or sup^+(pJF18) bacterial lawns. One must conclude therefore that the expression of 186*c*I from cloned restriction fragments was insufficient to confer complete immunity to 186 infection of the host.

A plaque morphology mutation of 186 producing a 'super-clear' phenotype has been located on the left half of the genome but little is known about its function or accurate map location (S.M. Hocking, pers. commun.). It is possible that this function is equivalent to P2 Z gene which is

required for lysogeny of some strains although Z function appears to be needed for maintenance of the prophage rather than initiation of C gene expression (Bertani, 1976). The need for another function to give complete immunity and which maps in position analogous to P2 Z could be tested by co-cloning the *Bam*HI restriction fragment (37.0 to 58.7%) into the tetracycline resistance gene in pBR325 (C. Bolivar, unpub. obs.) and then the *Hin*dIII/*Eco*RI fragment (61.3 to 92.0%) into the chloramphenicol resistance gene of the same vector.

Could the instability of pJF18 and pJF23 observed during plasmid DNA preparation lead to the loss of immunity to 186 infection? Twenty single colonies of *sup*$^+$(pJF18) were selected at random and tested for the presence of the *Pst*I fragment cloned in pJF18 by marker rescue of D*am*23 and B*am*57. All colonies gave rescue of wild type function indicating that the plasmid was intact. Therefore it seems unlikely that the breakdown of pJF18 could be contributing to the low level of *c*I expression.

(b) Expression of *int*

The instability of both pJF18 and pJF23 during DNA preparation has been alluded to in previous discussions. The following observations were made during attempts to purify pJF18 DNA at 37°C (that is, non-permissive conditions for the *cIts* gene on the plasmid):

(1) chloramphenicol amplification resulted in the isolation of a plasmid slightly larger than pBR322, that contained one *Pst*I restriction site (a single band was observed on an agarose gel after *Pst*I digestion). No pJF18

DNA was detected although had it been present as a minor component it may not have been seen on the gel.

(2) preparation of pJF18 DNA from 100 ml cultures in which the host was lysogenic for 186D*am*48 gave pure pJF18 DNA. Chloramphenicol was not added during the logarithmic phase of growth to amplify the plasmid DNA, but instead, cells were harvested in stationary phase. Litre cultures prepared in this way did not always give pure pJF18 and a breakdown plasmid was sometimes present.

(3) a second isolate of pJF18, derived from 'shot-gun' cloning of *Pst*I digested Hy5 DNA also proved to be unstable. A single 100 ml preparation grown in a non-lysogenic host, but without chloramphenicol amplification yielded pJF18 DNA and in addtion two extra bands, one slightly smaller than pBR322 and the second of about 2.4 kb.

Preparation of pJF23 DNA posed a similar problem. The products of a single preparation grown in a 186 lysogenic host and amplified with chloramphenicol are shown in Fig. 5.4. pJF23 DNA represented by the bands at 4.4 kb and 11.2 kb, the pBR322 and *Bam*HI fragments respectively, was a minor component of the preparation. The majority of DNA was from 3.6 kb to 4.1 kb approximately, but because there was so much DNA in this region of the gel it was impossible to ascertain whether the band comprised a single species or whether there was a heterogenous mixture of DNA in this size range.

The overnight culture used to inoculate the broth for plasmid DNA preparation was derived from a single colony

and it is clear that although the breakdown of pJF18 and pJF23 does not yield the same product on each occasion, the process must be relatively specific because the plasmid formed can be visualized after restriction as one or sometimes two bands on a gel. An explanation of this phenomenon is that the *int* gene is being expressed from the cloned DNA and that *int* catalysed recombination between the phage *att* site and a second site within the plasmid DNA results in plasmid breakdown. One can predict, from the Campbell model of phage integration and excision (Campbell, 1962) that two circular DNA molecules would be formed. Only one of these would retain the plasmid coded functions necessary for autonomous replication, and thus this plasmid would survive while the other would be lost.

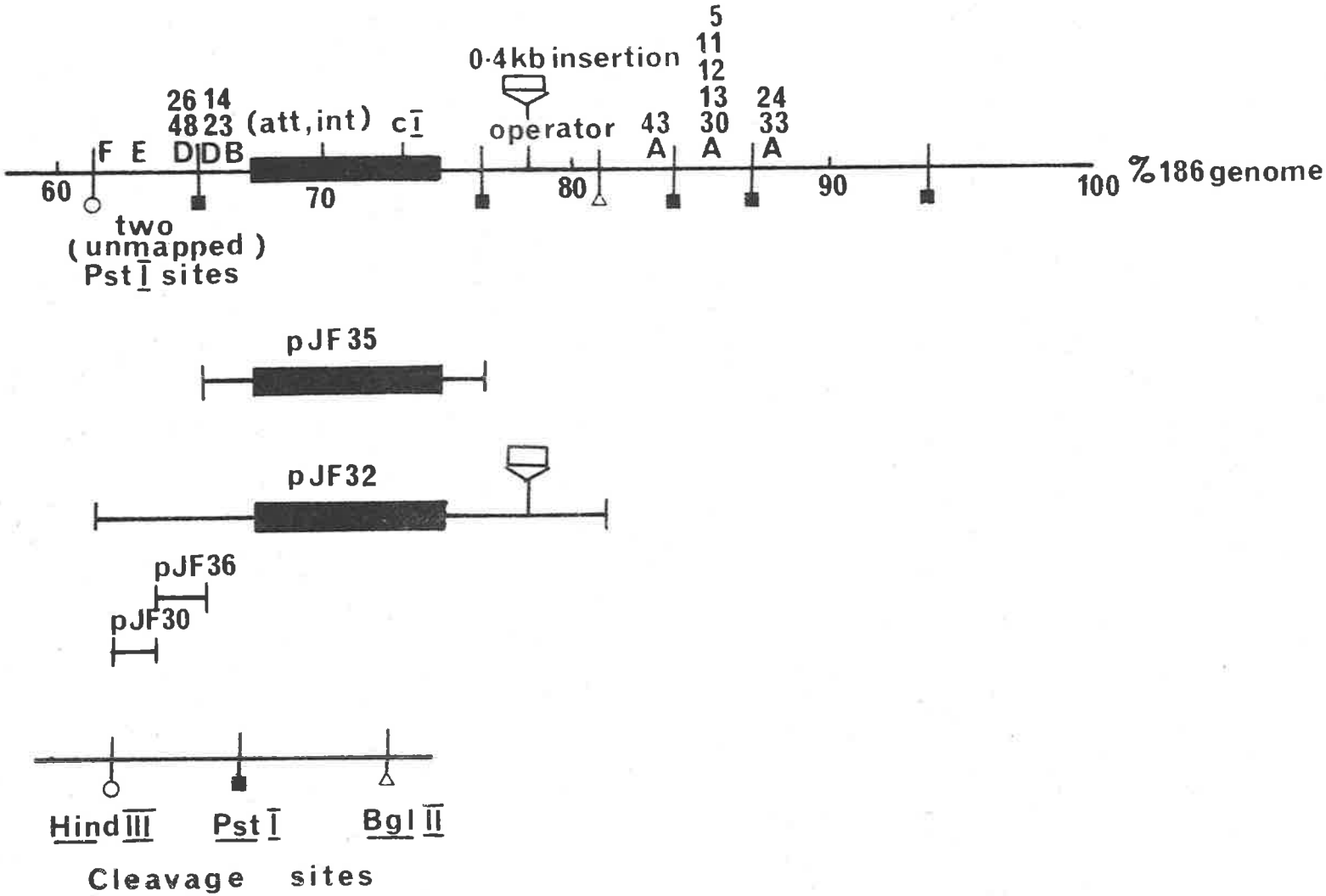
Two observations that support this proposal have been made. Plasmid stability, at least for pJF18, was enhanced by growth in a cell lysogenic for 186. The repressor provided by the prophage would inhibit *int* expression from the cloned DNA and thus prevent *int* mediated recombination. If the repressor was titrated then plasmid breakdown would follow. The greater instability of pJF23 even in a lysogenic host can be attributed to the fact that the 186 DNA of this plasmid contains a second operator site mapping to the right of 76.3% which is absent from pJF18. Therefore fewer copies of pJF23 would be needed to titrate the repressor and thus the plasmid would be less stable.

The *PstI* fragment (65.5 to 76.3%) from 186*del*2 was cloned in pJF35. This fragment is equivalent to the fragment cloned in pJF18 but it has a deletion from 67.9 to 74.9% (Fig. A1.2) which covers *int* and probably *att*. This

FIGURE A1.2. A Map of the Region 60 to 100% of the 186*del*2 Genome

The position of the 2.1 kb deletion has been determined by an EM heteroduplex analysis (R.M. O'Connor, pers. commun.) and the 0.4 kb insertion has been placed between the *Pst*I restriction site at 76.3% and the *Bgl*II site at 81.0% (Saint, 1979; E.J. Finnegan, unpublished observations).

The fragments cloned to give the plasmids pJF32, pJF18 and pJF35 are indicated below the map.


5
11
12
13
24
33
0·4kb insertion
26 14
48 23
(att, int)
cI
operator
43
30
F E D D B
A
A
A
60
70
80
90
100
% 186 genome
two
(unmapped)
Pst I sites
pJF35
pJF32
pJF36
pJF30
Hind III
Pst I
Bgl II
Cleavage sites

plasmid is stable in a non-lysogenic host as one would predict if *int* catalysed recombination was responsible for the breakdown of pJF18 and pJF23.

The problem of plasmid instability could be overcome by cloning these same fragments from a 186*int*⁻ phage.

(c) Expression of *dho*

Collins (1977) predicted that the cloning of some DNA sequences into plasmid vectors might be precluded by the expression of the functions encoded by that DNA, for example:

(1) DNA coding for an enzyme which breaks down an essential metabolite, interferes with host macromolecular synthesis or breaks down host macromolecules.

(2) DNA coding for functions, such as histones, that interact directly with host macromolecules.

In addition DNA containing tandom repeats has been found to be unstable when the hybrid plasmid is present in large numbers (S.N. Cohen, cited by Collins (1977)).

The *dho* function of 186, which is postulated to inhibit host DNA synthesis, would interfere with cell growth and thus be difficult to clone if it was expressed by the recombinant plasmid. *Dho* is expressed early after induction and must be under repressor control in the prophage state, therefore it was proposed that it may be included in the A transcripton. *Dho* has not been genetically mapped but it was proposed that it may be located between *c*I and gene A, that is, on the *Pst*I fragment cloned in pJF17.

pJF17 was initially cloned by purification of DNA from low melting agarose and the recombinant plasmid was selected in a host lysogenic for 186. DNA was prepared and used in the transformation of *sup*$^+$ non-lysogenic host; the transformed cells appeared to be healthy and the presence of the recombinant plasmid was confirmed by marker rescue of A*am*43. However, DNA preparation of pJF17 from either a lysogenic or a non-lysogenic host gave poor yields.

A second isolate, pJF17.2, from a 'shot-gun' cloning of *Pst*I digested Hy5 DNA, gave normal yields of plasmid from a non-lysogenic host. This isolate of *Pst*I fragment 76.3 to 83.8% was used for all hybridization studies.

One explanation of these observations is that this *Pst*I fragment does in fact code for a *dho* function, but that part or all of the promoter for this gene is on the adjacent fragment (65.5 to 76.3%) cloned into pJF18. When the pJF17 fragment was cloned in one orientation into pBR322 the *dho* gene was expressed by read-through transcription initiated from a plasmid promoter, but expression was at a low level and only partially inhibited host and plasmid replication resulting in low plasmid yields. In the opposite orientation (pJF17.2) the *dho* gene was not expressed and hence the yield of plasmid DNA was unaffected.

The orientation of the *Pst*I fragment in pJF17 and pJF17.2 can be compared by a heteroduplex analysis of the two plasmids, linearised by digestion with *Eco*RI. If the orientation of the fragment differs in the two isolates then heteroduplexes will contain a substitution bubble; alternatively, if the *Pst*I/*Eco*RI region of the plasmid is

too short to form a stable duplex then the heteroduplex will have a region of double stranded DNA with two equal single stranded tails.

This theory leads one to the prediction that a plasmid in which the *Pst*I fragments of pJF18 and pJF17 are cloned adjacent and in the normal orientation would inhibit cellular DNA synthesis in the absence of phage repressor. The *Hin*dIII/*Bgl*II restriction fragment (61.3 to 81.0%) of 186*del*2 was cloned into pBR322 and the recombinant was selected by transformation of a host lysogenic for 186. DNA from the plasmid (pJF32, Fig. A1.2) was purified and was used in the transformation of a non-lysogenic sup^+ host. Four separate attempts were made to transform the non-lysogen but only one colony carrying this plasmid was isolated. The competence of the cells was verified at each attempt by transformation with pBR322 and pJF32 DNA was used at the normal level of 0.01 µg and 0.1 µg per assay as well as 1.0 µg per assay. pJF32 DNA transformation of the lysogenic host proceeded at normal frequency. The single isolate of sup^+ (pJF32) grew poorly both on agar plates and in liquid culture which contained many dead cells. The identity of the plasmid was verified by marker rescue of alleles from genes F, E, D and B and by the absence of gene A. The plasmid in this isolate is a potential source of a dho^- mutation.

The isolation of a plasmid with the properties predicted for a clone having a *dho* gene provides the strongest evidence for the 186*dho* function. In earlier discussion of the *dho* function it was suggested that *dho* was included in the gene

A transcript (Section V.E), however the successful isolation of pJF32 in a lysogenic cell renders this unlikely because the *Hin*dIII/*Bgl*II fragment in pJF32 was derived from 186*del*2. This phage carries, in addition to the 2.1 kb deletion from 67.9 to 74.9%, an insertion to the right of 76.3% which destroys the phage operator site making the phage virulent. If *dho* was under the control of this operator, and assuming that the insertion was stable, then pJF32 should inhibit cellular DNA synthesis in the lysogenic as well as the non-lysogenic host. 186 may thus have two sites of repressor action, with mutation in the operator for gene A being sufficient for virulence. 186 utilizes many of the host functions for its DNA synthesis (Hooper, 1979); *dho*, a non-essential gene, may have evolved to maximise 186 replication by the inhibition of bacterial synthesis. In agreement with this is the proposal based on the properties of pJF17 and pJF17.2 that *dho* is transcribed from a promoter, operator region that is located to the left of 76.3% on the adjacent *Pst*I fragment included in pJF18 rather than the essential promoter. Fig. A1.3 illustrates the proposed map for this region. Further work that is needed to support this idea is:

(1) location of the A gene operator by physical location of the addition in 186*del*2 which destroys this site.

(2) identification of promoter sites by sequencing data or the visualization of RNA polymerase binding sites under the electron microscope (Vollenweider and Szybalski, 1978).

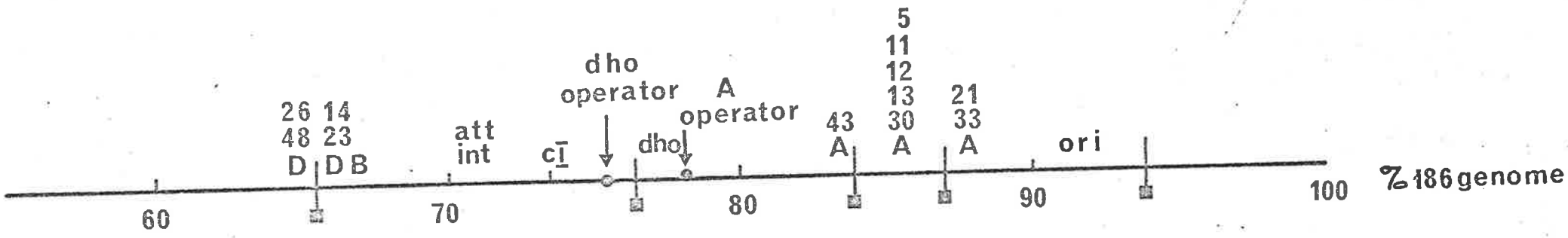
(3) mapping of the A operator with respect to *dho*,

FIGURE AI.3. The Proposed Map of the Control Region of the 186 Genome

The proposed location of two operators, the *dho* operator and A operator are indicated on the map.

The *int* gene must also be under *c*I control, but it has been suggested that this control may not be at the level of transcription (Section VI.E.5 and Section VII.1(c)) and so no *int* operator has been included on this map.

The relative locations of *dho* and *c*II or *c*II and the A operator are unknown and for this reason *c*II has not been included on this map.


26 14
48 23
D DB
att
int
cI
dho
operator
A
operator
dho
5
11
12
13
30
43
A
A
21
33
A
ori
60
70
80
90
100
λ 186 genome

this will only be possible once *dho* has been identified by the isolation of a mutation or a deletion covering this region.

Like pJF32, plasmids in which λ gene N have been cloned are not recovered by transformation unless the host cell can provide a functional λ repressor to inhibit expression of the N gene.

Bernardi and Bernardi (1976) reported the cloning of the λ *Eco*RI fragments into the vector pSC101. 'Shot-gun' cloning gave all fragments with the exception of the fragment that codes for the immunity region of λ (65.6 to 81.0%). Attempts to isolate clones of this fragment following the purification of the DNA from agarose also failed until the transformants were grown at 30°C; this represents the permissive temperature for the repressor gene of the immunity fragment, which carried a temperature sensitive mutation. In the absence of repressor, λ N protein, which acts as an antiterminator, would be made by the cloned immunity fragment. Uncontrolled expression of N protein may interfere with normal termination of cellular transcripts and so select against plasmids carrying the immunity fragment.

A second example of the harmful effect of the N function of lambdoid phage was described by Hershfield *et al.* (1974). When cloning the *E. coli trp* operon from φ80ptl90 transducing phage they observed that the φ80 immunity fragment, which was located on the restriction fragment adjacent to the *trp* operon, was always co-cloned in trp^+ recombinants. They concluded that for viability of the recombinant plasmid the φ80 repressor protein was essential to inhibit expression

of the ϕ80 N gene which was located on the same fragment as the *trp* operon.

The evidence presented in this section points to the expression of 186 genes from cloned fragments of the early or control region of the phage which is in contrast to the absence of late gene expression.

Although the immunity region cloned in plasmids pJF18 and pJF23 does not confer complete immunity to superinfection the efficiency of plating 186*c*I is decreased in cells hosting either plasmid.

The instability of pJF18 and pJF23 could be attributed to *int* catalysed recombination between the phage *att* site and another site within the plasmid. This idea is supported by the stability of pJF35, in which *int* has been deleted.

There are two 186 genes which would be potentially lethal when expressed from a cloned restriction fragment. The first, *dho*, inhibits bacterial DNA synthesis soon after induction, and was successfully cloned by selection of the recombinant plasmid in a host lysogenic for 186. Transformation of a non-lysogen resulted in a single colony carrying this plasmid suggesting that *dho* was actively expressed by the recombinant plasmid.

The second gene that would be lethal if it was expressed by a hybrid plasmid is P, the gene for cell lysis. All known alleles of this gene were cloned in pJF10 but the fact that this plasmid was viable in a non-lysogenic host provides further evidence for the lack of expression of 186 late genes from cloned fragments.

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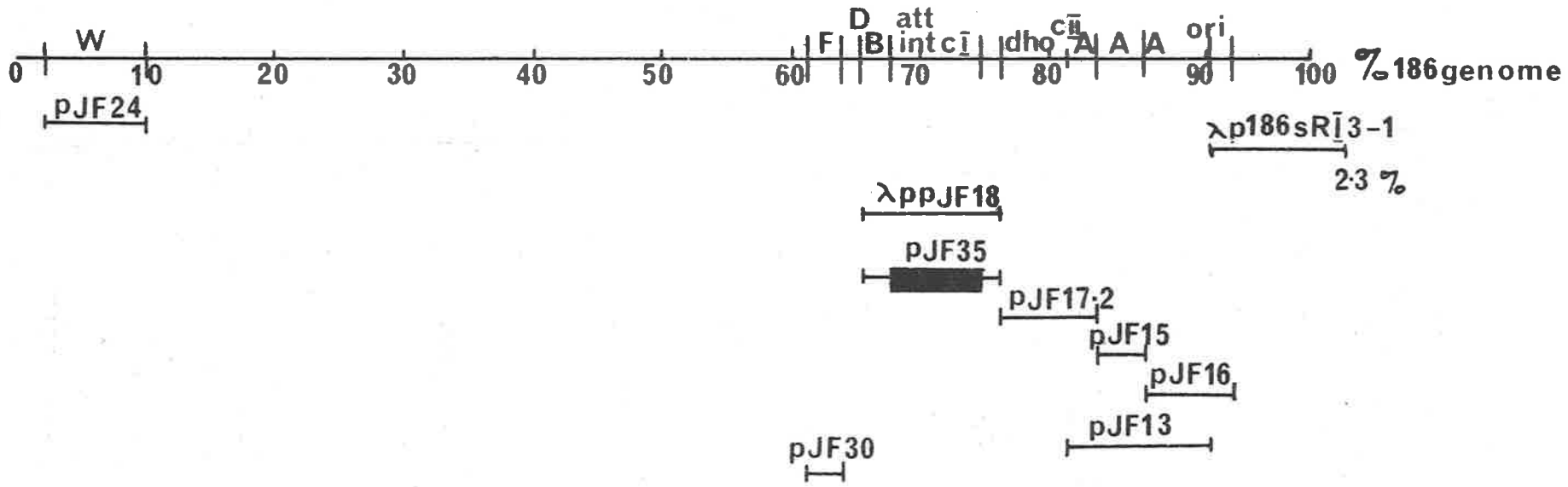
FIGURE 6.1.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