ERRATA

p 46, line 21 should read: "5 minutes. The nuclei were collected by centrifugation."

p 47, lines 6 and 8. The pancreatic ribonuclease and pronase were dissolved in standard saline citrate at 2 mg/ml prior to adding to the DNA.

Fig. 3.2 legend should read: "A. ^{14}C-globin"
"B. ^{14}C-H5 plus ^{14}C-globin"
"C. ^{14}C-H5"

Fig. 6.5. The concentration of acrylamide in these gels is 4%.

CHICKEN HISTONE H5 mRNA

AND ITS GENES

A thesis submitted for

the Degree of

Doctor of Philosophy

in the

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by

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SUMMARY

1. The work described in this thesis forms part of an investigation of eukaryotic gene control. The system studied was the avian erythroid cell series since it is possible to isolate pure populations of the various cell types which have well-defined biochemical activities. These cells contain an unusual tissue-specific histone H5, which may be involved in the progressive repression of transcription observed as these cells differentiate. Although the gene controlling function of this histone must be at a very gross level, this represents a unique opportunity to investigate one facet of gene control. Probably the most sensitive technique is to assay for specific messenger RNA and gene sequences by hybridisation to an appropriate probe. The aim of this thesis was to prepare such a probe from H5 mRNA and to use it to calculate the reiteration frequency of the H5 gene in the chicken genome.

2. The cells employed were chicken reticulocytes since the only histone made in these cells is H5. Experiments were conducted which demonstrated that H5 mRNA is probably a minor species compared to globin mRNA in these cells. Furthermore, calculations indicate that the two mRNAs are probably of similar molecular weight which may complicate the isolation of H5 mRNA. As a result globin mRNA was first purified and characterised. Properties which may have proved useful in the separation of this mRNA from H5 mRNA are discussed. The globin mRNA was used to optimise techniques for the *in vitro* translation and identification of chicken mRNAs. This was considered necessary as mRNAs

from different sources vary in the conditions required for optimal translation and it was reasoned that mRNAs from the same cell would have similar optima.

3. Total polysomal RNA was fractionated on the basis of size and poly A content. Although large amounts of globin mRNA were present, H5 mRNA could only be detected in the non-poly A containing RNA. Even in this fraction however, there was still a large excess of globin mRNA which was difficult to remove due to the demonstrated similarity of their molecular weights.

4. Since it had proved impossible to isolate the H5 mRNA by conventional techniques, immunological methods of isolating the polysomes producing H5 were investigated. Using immunoadsorbents, mRNA was prepared in small amounts which programmed the synthesis *in vitro* of more than 70% H5. The yield and specificity were improved by modifying the procedure to indirect immunoprecipitation followed by oligo(dT)-cellulose chromatography. The resulting mRNA programmes the synthesis *in vitro* of more than 90% H5. The chemical purity of the mRNA is discussed.

5. The immunologically prepared H5 mRNA was not copied into cDNA by RNA-dependent DNA-polymerase. Since this was probably due to the lack of a 3' poly A tract on the mRNA, an enzyme was purified and characterised which would add such a tract. The enzymically modified mRNA could then be copied into cDNA of high specific activity.

6. The H5 cDNA was characterised in terms of size and fidelity of copying. By hybridisation analysis it was dem-

onstrated that the amount of contaminating rRNA and globin mRNA complementary sequences present in the cDNA was insignificant. The complexity of the cDNA was shown to be of the same size as the H5 mRNA and will back hybridise to this mRNA to greater than 75%. These results are discussed to demonstrate that the cDNA is a faithful copy of H5 mRNA. The possible uses of the resulting probe are also discussed.

7. The H5 cDNA was employed to quantify the number of H5 genes in the chicken genome. The significance of this result is discussed in terms of the known reiteration and organisation of histone genes in other species, and the possible role of H5 as a gene control agent.

STATEMENT

This thesis contains no material which has been accepted for the award of any other Degree or Diploma of any University, and to the best of my knowledge and belief contains no material previously published or written by any other person, except when due reference is made in the text.

A. SCOTT

December 1975

ACKNOWLEDGEMENTS

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NOMENCLATURE AND ABBREVIATIONS

1. Avian erythroid cells

The nomenclature used in this thesis is that of Sadgopal and Kabat (1968).

Cell type	Reference in this thesis	Alternative nomenclature (Lucas and Jamroz, 1961)
Dividing	Erythroblast	Erythroblast
Non-dividing but synthesising RNA and protein	Reticulocyte	Polychromatic erythrocyte
Inactive in macromolecular synthesis	Erythrocyte	Mature erythrocyte

2. Histones

Considerable confusion has arisen from the concurrent use of several systems of histone nomenclature. The system used in this thesis was taken from the CIBA Foundation Symposium on the Structure and Function of Chromatin (Bradbury, 1974). This nomenclature is logical and finding widespread acceptance.

(Histones)

Histone fraction	Ref.a	Ref.b	Ref.c	Ref.d (this thesis)
Lysine-rich	F1	Ia	KAP	1 (H1)
		Ib		
	F2c	V	KAS	5 (H5)
Slightly lysine-rich	F2a2	IIb1	ALK	2A(H2A)
	F2b	IIb2	KSA	2B(H2B)
Arginine-rich	F3	III	ARK	3 (H3)
	F2a1	IV	GRK	4 (H4)

a. Johns (1969, 1971)

b. Fambrough, Fujimura and Bonner (1968)

c. Gordon conference (1972)

d. Bradbury (1974)

3. Abbreviations

mRNA	messenger RNA
rRNA	ribosomal RNA
tRNA	transfer RNA
cDNA	complementary DNA
AMV	avian myeloblastosis virus
OD	optical density (subscript denoting wavelength at which measured)
poly A	polyadenylic acid
BSA	bovine serum albumin
Reverse transcriptase	RNA-dependent DNA-polymerase
Phosphocreatine Kinase	ATP Creatine:phosphotransferase (E.C.2.7.3.2)
Poly A polymerase	ATP Polynucleotidylexotransferase

CHAPTER 1

INTRODUCTION

AND

LITERATURE SURVEY

1.1 INTRODUCTORY REMARKS

This thesis is concerned with the mechanisms of differentiation which operate in the cells of eukaryotic organisms. In order to simplify the analysis, the model system used was the avian erythroid cell series. These cells undergo a progressive shutdown of macromolecular synthesis while still retaining their genomal material. It is therefore possible to isolate pure populations of cells having defined biochemical activities. Previous studies have concentrated on an examination of the changes in chromatin components, both RNA and protein, during the terminal differentiation of these cells, with the aim of identifying gene control agents. These studies have all demonstrated the need for an assay for specific genes and their transcription products, as a pre-requisite to the identification of specific gene control elements. This thesis is thus specifically concerned with the production of this assay system, which can be provided by complementary DNA prepared from purified messenger RNAs.

The cell series is unusual in containing a tissue-specific histone, H5, which has been suggested as the agent involved in the final shutdown of RNA transcription in these cells. The synthesis of this messenger RNA, and the organisation and control of the gene or genes coding for it, are thus of singular interest. Complementary DNA has been prepared from the purified messenger RNA for H5, and used to determine the gene reiteration frequency of this protein.

The remainder of this chapter consists of a literature review which is organised into the following topics:

(i) Eukaryotic differentiation and gene control

(ii) Chromatin structure and its relation to transcription

(iii) Histone messenger RNAs and genes

The review attempts to be comprehensive but by no means exhaustive; it contains the important findings relevant to the work presented but makes no attempt to list every reference. It is complete to October, 1975.

1.2 LITERATURE SURVEY

1.2.1 Gene Expression and Development

(1) Differentiation and its control

Cell differentiation is the process by which stable differences arise between the cells of an individual organism. Since the whole organism arises from a single cell, the fertilised ovum, this development must involve highly complex controls of the expression of the genetic information available to the cell. There are several lines of evidence which suggest that genetic information is not physically lost in the differentiated state, but is merely not expressed. For example, the expression of previously inactive genes has been demonstrated on stimulation of resting lymphocytes *in vitro* (Pogo, Allfrey and Mirsky, 1966), and after fusion on chicken erythrocyte nuclei with active HeLa cells (Harris, 1970). An even more convincing demonstration was provided by Gurdon and Woodland (1970)

who showed that nuclei derived from fully differentiated cells of *Xenopus laevis* contain all the information required to produce an entire, new adult.

If all cells have the same DNA content, then differentiation must occur by differential expression of the genes in different cells. In prokaryotes, where transcription and translation are coordinate, the control of gene expression seems to be largely transcriptional (Jacob and Monod, 1961). In eukaryotes however, the situation is complicated by the physical and temporal separation of these two processes. This has led to the postulation of four areas where controls may exist. These are:

(i) Control at the level of transcription of DNA into RNA (Gurdon, 1968; McCarthy and Duerksen, 1970). This will be dealt with in more detail later.

(ii) Control at the level of transfer of the RNA from the nucleus to the cytoplasm (Scherrer and Marcaud, 1968; Warocquier and Scherrer, 1969), since most of the RNA made in the nucleus is degraded there and never reaches the cytoplasm.

(iii) Control at the level of translation of the messenger RNA sequences into protein (Harris, 1970; Ilan and Ilan, 1971).

(iv) Control at the level of protein activity (Soll and Sonnenborn, 1970; Scherrer, K., 1973), by modulating the formation of active protein from inactive precursors, or by

changes in the rate of degradation of active protein.

Although examples of each have been advanced, transcriptional control is the best studied and is of most relevance to this thesis. It is therefore discussed in more detail in this survey.

(3) Transcriptional control

The evidence for the transcription of different RNA products in different cells is compelling. Firstly, different tissues display different families of RNA transcripts as measured by RNA-DNA hybridisation (McCarthy and Hoyer, 1964; Smith, Church and McCarthy, 1969), although the techniques employed only detect repeated sequences. Furthermore, the major species of cellular RNA (rRNA, tRNA, HnRNA) are synthesised at different times during embryonic development (Brown and Littna, 1964; Nemer and Infante, 1967). The most impressive evidence however is derived from studies using complementary DNAs to detect the presence of specific mRNAs. Such studies have demonstrated that the mRNAs for ovalbumin (Harris, Rosen, Means and O'Malley, 1974) and globin (Ramirez *et al.*, 1975) are produced in response to specific hormonal stimuli.

Thus there is ample evidence that differential gene transcription does occur but how this is controlled at the molecular level is still unclear, at least in eukaryotes. In general, however, selective gene transcription must depend on the recognition of specific initiation (and possibly termination) signals on the chromatin (Rutter, Goldberg and Pierrard, 1974). This

requires the existence of specificity-determining factors which could reside in either the chromosomal template or the DNA-dependent RNA-polymerase. Precedents for both of these exist in prokaryotes. For example:

(i) Template modifications affecting transcription in prokaryotes are well documented. Specific repressor proteins which regulate the transcription of the lac operon, and of phage λ have been isolated and extensively studied (Gilbert and Muller-Hill, 1966; Ptashne, 1967; Chadwick *et al.*, 1970). Both bind specifically to their repressor binding site in the absence of inducer (Riggs, Suzuki and Bourgeois, 1970; Adler *et al.*, 1972) and prevent transcription of the DNA distal to their point of attachment (Maizels, 1973). Such factors are involved in the control of a single gene or group of genes. Some proteins, however, such as the cyclic-AMP binding protein, affect the expression of large numbers of genes. This protein is a positive control element which affects the binding of DNA-dependent RNA-polymerase to the promoter site of genes associated with enzymes subject to catabolite repression (de Crombrugghe *et al.*, 1971; Eron *et al.*, 1971; Nissley *et al.*, 1971).

(ii) Bacterial RNA polymerases are composed of several subunits, some of which are interchangeable and seem to determine the specific-

ity of the enzyme. The RNA polymerase from *Escherichia coli* consists of a core of four subunits (Burgess, 1969) although the complete enzyme contains a fifth factor known as sigma (σ) (Burgess *et al.*, 1969). This factor is an anionic protein which is required for correct initiation at the promoter site (Bautz, Bautz and Dunn, 1969; Goff and Minkley, 1970; Sugiura, Okamoto and Takanami, 1970). New factors appear in *Escherichia coli* during infection by T4 (Bautz, 1972) and in *Bacillus subtilis* during sporulation (Losick and Sonensheim, 1970). Entirely new forms of the enzyme have also been described (Chamberlin, McGrath and Waskell, 1970) with quite different specificities (Dunn, Bautz and Bautz, 1971). Thus changes in the enzyme correlate with changes in the RNA transcribed.

The situation is less clear in eukaryotes since a number of the properties of eukaryotic chromatin make it difficult to investigate transcriptional controls. The major problems are:

(i) the much higher complexity of the DNA in eukaryotes compared to prokaryotes, which is estimated at several orders of magnitude (Mirsky and Ris, 1951). There are even large, unexplained differences in the DNA content of closely related eukaryote species (Strauss, 1971).

(ii) the high content (15-80%) of DNA sequences repeated at frequencies of up to 10^5 (Britten and Kohne, 1966). The function of these sequences is unclear but their presence causes problems in the analysis of RNA-DNA hybridisation studies.

(iii) the diploid nature of eukaryotes which complicates any genetic analysis. This problem coupled with the long generation time of eukaryotes also makes it difficult to isolate specific regulatory mutants.

(iv) the partition of the genome into a nucleus thereby severing the direct link between transcription and translation. This allows the introduction of regulatory steps involving the processing and transport of RNA to the cytoplasm.

Despite these problems, however, considerable data is available on how transcriptional control may operate in eukaryotes. Firstly, multiple forms of RNA polymerase are known to exist in the same cell (Roeder and Rutter, 1969) having different intracellular locations and synthesising different RNA molecules (Wobus, Panitz and Serfling, 1971; Chambon *et al.*, 1972). Thus, while it has never been proven, the concept of transcriptional control operating through changes in the RNA polymerase, is at least tenable here. The evidence for template modifications affecting transcription in eukaryotes is even less well defined. Arguably the best evidence is provided by studies show-

ing that specific mRNAs can only be transcribed from chromatin derived from cells either after hormonal stimulation (Harris *et al.*, 1974) or at certain stages of the cell cycle (Stein *et al.*, 1975). At all other times the mRNA is undetectable when the chromatin is transcribed *in vitro*. These studies can be criticised, however, on the grounds that bacterial RNA polymerase was used to transcribe the chromatin and this has an entirely different specificity from eukaryotic polymerase which initiates at different sites on the chromatin (Butterworth, Cox and Chesterton, 1971; Chambon *et al.*, 1972). The fact that such technically elegant studies as these are unable to provide unequivocal data on eukaryotic gene control merely emphasises the extreme difficulties inherent in these organisms. Nevertheless gene control elements must exist and a large amount of literature is devoted to their investigation. One of the major areas of interest is in the isolated components of chromatin.

(3) Chromatin components as a source of transcriptional control elements

In vivo, chromatin may be defined as the genomal material in the nucleus; the DNA and those other molecules physically and functionally associated with it. Chromatin prepared from a variety of tissues has the following approximate composition (by weight):- 40% DNA, 2% RNA, 38% protein (36% histone), (Bonner *et al.*, 1968). The exact content varies according to the method of preparation which makes it difficult to define chromatin *in vitro*. Isolated chromatin may bear only a lim-

ited relationship to *in vivo* chromatin due to the loss or acquisition of components during the isolation procedure. The best definition of chromatin *in vitro* is probably "the material isolated from the nucleus by chemically gentle means" (Appels, 1971). The functional significance of the components of isolated chromatin is difficult to determine.

Nevertheless, all of the chromatin components are potential sites for the modification of transcription.

(i) The DNA component. There are three ways in which changes in the DNA could modify transcription. These are:-

a. Specific loss or permanent turn-off of genes.

b. Specific markers or changes in DNA sequences to delineate transcriptional signals.

c. Amplification of specific genes.

The first of these seems untenable as a general mechanism because of the nuclear transplantation experiments and other evidence cited in section 1.2.1(1) of this survey. The second alternative is tenable since methylation of cytosine residues has been reported (Burdon, 1971), and this could change the template properties. As yet, however, no direct evidence has been reported. As regards the third possibility, eukaryotic DNA is characterised by a large content of repet-

itive (but not necessarily amplified) sequences which fall readily into two main classes (Britten and Kohne, 1968), the highly repetitive DNA (Walker, 1971), and the intermediate repetitive DNA. The major properties of the former are:

a. its existence as long contiguous simple sequences (Walker, 1971),
b. its association with constitutive heterochromatin (Yunis and Yasminch, 1971), and
c. its concentration close to centromeres (Jones, 1970; Pardue and Gall, 1970) or chromosome ends (Hennig and Walker, 1970).

It is apparently transcribed very slightly or not at all (Walker, 1971). The intermediate repetitive DNA by contrast, consists of short sequences interspersed more or less regularly among the non-repetitive DNA (Britten and Smith, 1970; Wu, Hurn and Bonner, 1972; Britten *et al.*, 1973) and is transcribed in the cell (Melli *et al.*, 1971; Greenberg and Perry, 1971). The genes coding for histones (Kedes and Birnstiel, 1971), tRNA and rRNA (Birnstiel *et al.*, 1969) are found in this DNA. Although the genes for the histones are not amplified (Weinberg *et al.*, 1972), the genes for tRNA and rRNA are amplified in a range of organisms (Attardi and Amaldi, 1970; Birnstiel *et al.*, 1969). Most mRNAs however, have been shown to be transcribed from the

non-repeated or unique DNA (Goldberg *et al.*, 1973; Birnie *et al.*, 1974). This has been demonstrated for several single genes which constitute major cellular products including silk fibroin (Suzuki, Gage and Brown, 1972), globin (Bishop, Pemberton and Baglioni, 1972; Harrison *et al.*, 1972), and ovalbumin (Harris *et al.*, 1973; Sullivan *et al.*, 1973). Hence amplification also does not seem to be a general mechanism for effecting transcriptional control.

(ii) The RNA component. RNA is always found associated with isolated chromatin but the amount present varies widely between preparations (Bonner *et al.*, 1968b). Huang and Bonner (1965) reported that about half of the RNA of pea-bud chromatin was a low molecular weight species which was covalently bound to histone. It was also purported to contain characteristically large amounts of dihydrouridine and dihydroribothymidine. The authors suggested that it may have been involved in gene control. More recent work however has disagreed with these findings and has been unable to reproduce these observations. It has been suggested that this cRNA is largely a degradation product of tRNA (Heyden and Zacchau, 1970) or more probably of rRNA (Artman and Roth, 1971; Tolstoshev and Wells, 1974). Thus there is no unequivocal evidence

to suggest that cRNA may be a gene control agent, indeed its very existence other than as an artefact of preparation is in serious doubt.

(iii) The protein component. By analogy with bacterial systems, one might expect to find control elements among the proteins associated with DNA. The analysis is complicated by the increased complexity of eukaryotic chromatin and the presence of large amounts of basic proteins known as histones. Specific gene control elements have not yet been identified.

Histones: These are, apart from the DNA, the best characterised of all the chromatin components. Their major properties are their low molecular weights, their high content of basic amino acids, and their strong affinity for DNA (Johns, 1971). One of their most striking features is their constancy in all well-defined eukaryotes in both amount and structure (Panyim and Chalkley, 1969; De Lange *et al.*, 1969). Furthermore, although they exhibit heterogeneity due to chemical modifications (Allfrey, 1971) and, in the case of lysine-rich histones, sequence differences specific to organs and tissues (Bustin and Cole, 1968, 1969), there are only five major histone species found in all cells irrespective of their source. At one time

or another, histones have been implicated in most of the functions of the eukaryotic chromosome, largely due to their association with DNA. However the view advanced by Stedman and Stedman (1950) that "the basic proteins of cell nuclei are gene repressors, each histone or protamine being capable of suppressing the activities of certain groups of genes" is now regarded as a gross over-simplification. The general properties of histones do suggest at least one of their roles. The highly conserved sequence of, say histone H4 (De Lange *et al.*, 1969) implies that each and every residue along the chain is essential for the function of the histone and that this function is identical in all eukaryotes. Usually proteins which have the same function between species show considerable variation in overall structure, presumably since slight differences in the metabolic environment permit some individuality. However, the physico-chemical properties of DNA are identical, irrespective of its source. When this is coupled with the fact that in any stretch of chromatin, there is an equal weight of DNA and histone (Bonner *et al.*, 1968a), there is compelling evidence for a major structural role of histones. It should be strongly emphasised, however, that this is not inconsistent with a gene inactiva-

tion role as well. In fact histones will inhibit the synthesis of RNA from DNA templates *in vitro* (Allfrey, Littua and Mirsky, 1963) but the constancy and relatively few species of histones found in a wide range of organisms does make the control of specific genes unlikely. Any histone-induced inactivation would probably be a very general effect.

Non-histones: Isolated chromatin also contains a variety of proteins with an acidic amino acid composition. Due to the wide variety of isolation methods used and the lack of any distinctive common properties they are, as a group, poorly characterised. Nevertheless, many authors have claimed that the group contains specific gene control elements, usually on the basis of circumstantial evidence. For example, the level of non-histone protein varies during the development of various organisms (Seale and Aaronson, 1973) and the amount is correlated with the RNA synthetic activity of the chromatin (Dingman and Sporn, 1964; Shelton and Neelin, 1971). Such claims cannot distinguish if the proteins are the cause or an effect of transcription, however. Several groups have reported tissue-specific non-histones (Loeb and Creuzet, 1969; Platz, Kish and Kleinsmith, 1970; Teng, Teng and

Allfrey, 1971) and changes in the species present during hormonal induction of specific genes (O'Malley, McGuire and Korenman, 1967; Spelsberg *et al.*, 1973). Once again, however, the origin (nuclear or cytoplasmic) and significance of these proteins cannot be determined. Indeed, all such studies which seek to identify putative gene control elements on a physical basis, such as polyacrylamide gels are of doubtful value since they may be present in only a few copies per genome (Kleinsmith, Heidema and Carroll, 1970; Elgin and Bonner, 1970), by analogy to the λ repressor (Gilbert and Muller-Hill, 1966), and would therefore be undetectable by these methods. More convincing results are obtained from *in vitro* chromatin reconstitution experiments using components from a range of sources. Such reconstituted chromatin can be transcribed with bacterial RNA polymerase and the RNA produced can be examined by hybridisation. Early studies concluded that the RNA species produced was determined by the source of the non-histone proteins used and not by the DNA or histones (Gilmour and Paul, 1970; Spelsberg, Hnilica and Ansevin, 1971). These experiments can be criticised however, since they could only detect the hybridisation of repeated sequences and they used bacterial RNA polymerase with its

attendant problems of specificity (see section 1.2.1(2) of this survey). More recently these experiments have been repeated using cDNA probes to detect the transcription of particular mRNAs (Gilmour and Paul, 1973, 1975; Stein *et al.*, 1975). These authors come to exactly the same conclusion, i.e. that it is the non-histones which determine the transcription of specific genes. Although they still use bacterial RNA polymerase, such experiments are a major step in the proof that non-histones contain specific gene controlling elements. The biggest problem may be isolating them from such a heterogeneous group as the non-histones.

Thus of the components of chromatin, cRNA seems unlikely to be a control element, histones are largely structural elements with a possibility of a broad transcriptional control, while non-histones seem to contain specific gene control elements (Stein, Spelsberg and Hnilica, 1974).

1.2.2 Relation of Chromatin Structure to Transcription

(1) Correlations of structure and function

In prokaryotes, genes are frequently controlled by the binding of a protein to a specific site on the DNA, thereby preventing the transcription distal to that site (section 1.2.1(2) of this survey). Although this may also be true for eukaryotes, the effect of DNA-protein interactions on the structure of the genetic

material is also important, since a variety of studies indicate that the physical state of the chromatin correlates closely with its biosynthetic activity. For example:-

(i) Characteristic "puffing" patterns are observed in the polytene chromosomes of Dipteran insects at particular stages of development (Beerman, 1959; Clever, 1968; Ashburner, 1970). These puffs are the sites of intensive RNA synthesis (Pelling, 1964; Ashburner, 1970) and are temporally and spatially correlated with changes in chromosomal proteins, particularly non-histones (Berendes, 1968; Holt, 1970; Helmsing and Berendes, 1971).

(ii) The segregation of active and inactive chromatin in Coccid insects shows a similar correlation between structure and function. In males of the species *Planococcus citri*, the paternal set of chromosomes is genetically inactive and occurs as heterochromatin where little or no RNA synthesis occurs (Berlowitz, 1965). The maternally derived chromosomes exist as euchromatin and carry out RNA synthesis.

(iii) The lampbrush chromosomes found in the oocytes of many animals and the spermatocytes of certain insects are characterised by the presence of DNA-containing lateral loops. These areas are the sites of

intensive RNA synthesis (Gall and Callan, 1962).

A variety of such observations in a range of eukaryotes has demonstrated a correlation between the fine structure of the chromatin and its transcriptional activity. Structural changes in the chromatin also accompany differentiation since as cells become more specialised and express fewer genes, heterochromatin replaces euchromatin (Hearst and Botcham, 1970). It has been reported that the genes present in heterochromatin are not expressed while those in euchromatin are expressed (Lyon, 1968). Although this may be only a matter of degree of expression, it is nevertheless clear that such structural changes could affect the binding of RNA polymerase to DNA and/or RNA elongation. The addition of histones to lampbrush chromosomes *in vitro* causes a retraction of the loops and a drastic inhibition of RNA synthesis (Allfrey and Mirsky, 1963). Although in all these cases it is impossible to determine if the changes in structure are a cause or effect of the change in transcription, such structural changes could constitute a coarse level of control affecting the expression of large blocks of genes. Some details of chromatin structure are therefore required to explain how such a control may operate.

(2) Chromatin structure

The length of DNA present in a eukaryotic nucleus is so great that extensive packing must exist in chromatin. Hydrodynamic, light scattering, and electro-

optical studies of chromatin (see Fredericq, 1971) indicate that the histones in the nucleohistone complex cause the DNA to be more compact than when it is in the form of free DNA. Characteristic X-ray diffraction patterns are exhibited by chromatin which are quite different from free DNA or isolated histones (Pardon, Wilkins and Richards, 1967). This was explained by the postulated existence of a higher order structure imposed upon the DNA by chromosomal proteins. Pardon and Wilkins (1972) suggested that this structure took the form of a continuous supercoiled fibre of diameter 100 Å and pitch 110 Å. Although the diffraction pattern is not fully explained by supercoil packing there is no doubt that histones are involved in whatever regular packing exists, since nucleohistone exhibiting the same diffraction pattern can be reconstituted *in vitro* from DNA and total purified histone (Bradbury *et al.*, 1974). Nevertheless, while it is obvious that one function of histones is the induction of the folded structure underlying the X-ray pattern, the extremely high sequence conservation of histones implies that each has a distinct role or there would be only one species, not five. The non-histones and the lysine-rich histones H1 and H5, are not required to produce the diffraction pattern (Murray *et al.*, 1970; Pardon and Richards, 1972; Bradbury *et al.*, 1974), while the other four histones are all required and in equimolar amounts (Bradbury *et al.*, 1974). The source of the DNA used in these experiments is irrelevant and thus there is no recognition of specific sequences

involved (Garrett, 1968; Baldwin *et al.*, 1975).

Models incorporating a continuous structure such as the supercoil have recently been seriously challenged and models consisting of a basic structure of subunits, which are themselves subject to further packing, have been proposed. The work of Hewish and Burgoyne (1973) demonstrated that an endogenous nuclease of rat liver would cleave the DNA in its chromatin into pieces which were multiples of a unit size (200 base pairs). Noll (1974) isolated such monomers by digesting Krebs ascites chromatin with micrococcal nuclease, and showed that these contained about 170 base pairs of DNA, a protein:DNA ratio of 1:1.3 and all 5 histone species. More than 90% of the chromatin could be shown to be in the form of such subunits. Numerous studies have since shown that such structures are found in a wide variety of tissues and organisms (Sahasrabuddhe and Van Holde, 1974; Lohr and Van Holde, 1975; Sollner-Webb and Felsenfeld, 1975; Axel, 1975; Oosterhof, Hozier and Rill, 1975; Oudet, Gross-Bellard and Chambon, 1975). Olins and Olins (1974) provided further evidence for a subunit structure by electron microscope visualizations of gently prepared chromatin. This had the appearance of "beads on a string" showing roughly spherical subunits with a diameter of about 70 Å and joined by a very short DNA string 15 Å wide.

This data suggests that the lowest level of chromatin structure is generated by the interaction of histones with DNA, but the details of the subunit components and their organisation have been gleaned from

studies of the histone-histone interactions. Aggregation has been a problem with isolated histones for some years; however Kornberg and Thomas (1974) suggested that this may be due to the denaturing conditions employed and extracted histones by milder procedures. Their histones were resolved into three groups; a complex of H3 and H4, another of H2A and H2B, and a third of uncomplexed histone H1. The use of a cross-linking agent identified the complexes as an $(H3\text{-}H4)_2$ tetramer, and poorly defined (H2A-H2B) oligomers. The mixing of both of these complexes with DNA is necessary and sufficient to generate the X-ray diffraction pattern seen by Pardon *et al.*, (1967). This led Kornberg (1974) to propose a model for the subunit composed of a core of an $(H3\text{-}H4)_2$ tetramer, 200 base pairs of DNA, and two (H2A-H2B) oligomers. This satisfied the stoichiometry of the chromatin (but see Wright and Olins, 1975), and by postulating the arginine-rich tetramer as the core of the repeat unit, it is possible to explain why these two histones are the last to be dissociated from DNA by mild extraction procedures (Ilyin *et al.*, 1971), and why they are the most highly conserved (De Lange *et al.*, 1969; Patthy, Smith and Johnson, 1973). The result would be a flexible structure capable of folding for compression within the chromosome. The existence of such complexes both in chromatin and free in solution has been confirmed (D'Anna and Isenberg, 1973, 1974a, b; Roark, Geoghegan and Keller, 1974; Burton, Hyde and Walker, 1975; Bonner and Pollard, 1975). Rubin and Moudrianikis

(1975) have also shown that H3 and H4 bind to DNA as a complex and, under mild conditions, H4 will not bind at all unless H3 is present, which also explains the cooperative binding of histones to DNA (Rubin and Moudrianikis, 1972). Recently, Thomas and Kornberg (1975) have demonstrated the existence of the predicted octameric histone complexes in chromatin.

The structure of the chromatin subunit has been examined both by physical and biochemical means. The neutron scatter data of Baldwin *et al.*, (1975) suggests that histones H2A, H2B, H3, and H4 comprise a multimeric protein unit occupying a separate region of space from the DNA. The data suggests that, as expected from the nuclease experiments, the DNA is external to the histone. This is supported by the nuclease and trypsin digestion studies of Weintraub (1975) and Weintraub *et al.*, (1975). Thus although the complete homogeneity of all subunits has not been proven, the structure of chromatin at this lowest level seems to consist of 1.5-2 turns of a coil of DNA (about 200 base pairs) wound on to the outside of a core of histones. This core probably contains two molecules each of H2A, H2B, H3 and H4, although there are other models which are tenable (Hyde and Walker, 1975; Li, 1975), but with only slight differences.

Whatever the exact details of chromatin subunit structure, there is general agreement that the histone interaction with DNA is totally lacking in specificity (Bradbury *et al.*, 1974, 1975; Griffith, 1975; Germond *et al.*, 1975; Oudet *et al.*, 1975). Any gross change in

transcription modulated by chromatin structure would require some change in this basic pattern of subunits. This could take the form of either open regions of DNA uncomplexed with histones or higher order folding of the basic unit. Uncomplexed regions of DNA do exist *in vitro* (Varshavsky, Ilyin and Georgiev, 1973, 1974; Oudet *et al.*, 1975), however this may well be due to artefacts in the preparation (Georgiev, 1974), and their *in vivo* significance is unknown. On the other hand, higher order packing of the subunits certainly does occur (Bram *et al.*, 1975; Oudet *et al.*, 1975) and is correlated with the extent of transcription from the chromatin (see section 1.2.2(1) of this survey). Thus anything which can change the structure of the chromatin can be potentially considered as a gene control element. Although some non-histones have been implicated in this process (Kleinsmith, 1975), the best example is the lysine-rich histones H1 and H5.

(3) Role of lysine-rich histones in chromatin structure and gene control

The lysine-rich histones H1 and H5 differ markedly from the other histones. They have by far the highest molecular weight and total lysine content of all the histones (Johns, 1971). Despite their very basic nature, however, they are the first fraction to be extracted from chromatin on raising the ionic strength (Ohlenbusch *et al.*, 1967). The greatest difference, however, lies in their innate variability. There are several distinct species of H1 with tissue-specific and species-specific differences in amino acid sequence

(Bustin and Cole, 1968; Kincade, 1969; Bustin, 1972) and in the proportion of the various species present (Kincade, 1969). H5 is also unusual in that it is polymorphic (Greenaway and Murray, 1971), and tissue-specific to nucleated red blood cells, i.e. birds, reptiles, and amphibians (Johns, 1971).

One of the major interests in the lysine-rich histones stems from their ability to act as chromatin cross-linking agents and thereby affect transcription. This cross-linking effect is well documented. For example, chromatin gels in water can be induced to shrink to about 10% of their original volume by dialysis against 0.15 M NaCl, but only in the presence of H1 (Bradbury, Carpenter and Rattle, 1973). Pure H1-DNA complexes react analogously (Bradbury *et al.*, 1974a), and NMR data suggests that H1 itself undergoes changes in its physical structure during this process (Bradbury *et al.*, 1975). These changes in H1 are probably responsible for the salt-induced contraction of chromatin (Bradbury *et al.*, 1974b). This effect can be visualised under the electron microscope where the presence of the lysine-rich histones correlates with a more compact, fibrillar structure formed by nucleosomes (Oudet *et al.*, 1975).

H1 has also been implicated in the contraction of chromatin at metaphase (Bradbury *et al.*, 1974c). This contraction is a series of precisely timed events and such a non-specific mechanism as the salt-induced contraction is unlikely to be involved. There is an accumulation of evidence, however, that specific mod-

ifications of the lysine-rich histones, particularly by phosphorylation, may be responsible for at least some aspects of this contraction. Although there are several phosphorylating events which make analysis complicated (Lake, 1974; Gurley, Walters and Tobey, 1974), studies with synchronous cells have demonstrated a specific phosphorylation event just prior to mitosis (Bradbury *et al.*, 1974c, d). There is an increase in the phosphorylating enzyme activity at this time (Gurley *et al.*, 1975), and addition of exogenous enzyme can advance mitosis (Bradbury *et al.*, 1974a). This event seems to phosphorylate specific sites in the histone (Langan, 1971; Adler *et al.*, 1971) and reduces the binding of the histone to DNA (Adler *et al.*, 1971; Adler, Langan and Fasman, 1973). This may increase histone-histone interactions and thereby bring about a contraction (Bradbury *et al.*, 1974b).

H5 also undergoes a conformational change on phosphorylation (Williams and Seligy, 1975) which affects the strength of binding to the DNA (Tobin and Seligy, 1975). An interesting point is that the only peptide so far sequenced which shows similarity between H1 and H5 contains one of the two specific phosphorylation sites (Sautiere *et al.*, 1975; Jones, Rall and Cole, 1974).

Thus these two histones are involved in cross-linking of chromatin and so could exercise a degree of gene control, possibly by limiting the availability of base sequences (Seligy and Lurquin, 1973; Seligy and Miyagi, 1974). However, although they can fulfil some

of the same functions, H1 and H5 must also have distinct properties, or, considering the high conservation of amino acid sequence in most histones, one would expect only one lysine-rich histone species. Johns (1971) has suggested that in species having nucleated erythrocytes, H5 has been evolved to finally repress all RNA, and thus protein synthesis: a biochemical equivalent of the enucleation process seen in mammals. As avian red blood cells mature they do lose their capacity for RNA synthesis (Attardi, Parnas and Attardi, 1970) and there is a concurrent change in the state of the chromatin from a diffuse to a condensed state (Brasch, Seligy and Setterfield, 1971). During this process H5 is the only histone made in the non-dividing reticulocyte (Appels and Wells, 1972). The mere presence of H5 does not prevent transcription (Seligy, Adams and Neelis, 1973) but decay of the mechanism for turnover of H5 may do so. Appels and Wells (1972) have demonstrated that H5 is in a state of dynamic equilibrium on to and off of the chromatin, and have proposed that this is necessary to maintain the chromatin in a derepressed state. This turnover may be dependent on the continued phosphorylation of the histone, as decreased phosphorylation leads to increased binding to the DNA (Tobin and Seligy, 1975). Thus the termination of transcription in erythroid cells may be due to an accumulation of H5 on the chromatin, resulting in a change in chromatin structure and restricting the accessibility of the DNA to RNA polymerase. Because of the large amounts present, the lysine-rich

histones, particularly H5, therefore represent a unique opportunity to study this gross type of gene control.

1.2.3 Histone Messenger RNAs and their Genes

(1) Synthesis of histones and their mRNAs

With the notable exception of H5, the synthesis of all histones is tightly coupled to that of DNA (Spalding, Kajiwara and Mueller, 1966; Borun, Scharff and Robins, 1967; Gallwitz and Mueller, 1969), as might be expected of proteins so intimately connected with DNA structure. Histone synthesis also stops rapidly if the synthesis of DNA is inhibited (Spalding *et al.*, 1966; Robbins and Borun, 1967; Gallwitz and Mueller, 1969). This is mirrored by the detection of translatable histone mRNA in polysomes only during S-phase of the cell cycle (Gallwitz and Breindl, 1972; Jacobs-Lorena, Baglioni and Borun, 1972). By the use of a cDNA probe complementary to HeLa cell histone mRNA, Stein *et al.*, (1975b) showed that mRNA sequences can be detected only in the RNA transcribed from S-phase chromatin *in vitro*, and not in the RNA transcribed from the chromatin of cells in other stages of the cell cycle. This suggests that a transcriptional control is operating, although the work can be criticised for the use of bacterial RNA polymerase. This is not the only control operating however, as there are major changes in the rate of degradation (or inactivation) of the mRNA during the cell cycle. Most histone mRNAs are synthesised during the first few hours of DNA replication (Borun *et al.*, 1967; Breindl and Gallwitz, 1974) in HeLa

cells, and are stable for approximately the duration of S-phase (Perry and Kelley, 1973) with a half-life of about 11 hours. However, if DNA synthesis is blocked, this mRNA selectively disappears from polysomes (Gallwitz and Mueller, 1969; Perry and Kelley, 1973; Breindl and Gallwitz, 1974) with a half-life of about 13 minutes (Gallwitz, 1975). This rapid disappearance is dependent on protein synthesis (Butler and Mueller, 1973; Breindl and Gallwitz, 1974) and hence translational control may be operating. The synthesis of all histones except H5, only during the S-phase of the cell cycle, is at least in part, a consequence of the rates of synthesis and turnover of their mRNAs. Histone H5 is of interest due to its postulated role in gene control and it is interesting that this mRNA is not subject to the same controls.

(2) Properties of histone mRNAs

The five HeLa histone mRNAs are from 150,000 to 220,000 daltons in molecular weight (Levy *et al.*, 1975b). All histone mRNAs examined are unusual in containing no polyadenylic acid tract (Adesnik *et al.*, 1972; Perry *et al.*, 1972). This poly A apparently has no function in protein synthesis (Williamson, Crossley and Humphries, 1974; Bard *et al.*, 1974) and it has been proposed that it has a role in processing and transport of mRNA (Edmonds, Vaughan and Nakazato, 197; Adesnik and Darnell, 1972; Adesnik *et al.*, 1972). Histone mRNA is certainly processed rapidly in the nucleus (Adesnik and Darnell, 1972; Schoechetman and Perry, 1972) but this may merely be due to the lack of requirement for

poly A addition, which takes a finite time. The demonstration of other naturally occurring mRNAs without poly A (Milcarek, Price and Penman, 1974; Nemer, Graham and Dubroff, 1974) which are also rapidly processed, supports this view. It has recently been proposed that poly A acts as a nuclease inhibitor (Levy *et al.*, 1975a) and could therefore affect the stability of the mRNA. This could explain the rapid turnover of histone mRNAs at the end of S-phase (Gallwitz, 1975) which is not observed with most mRNAs. If this hypothesis were correct, then one would predict that H5 mRNA may be unique among the histones in containing a poly A tract.

(3) Histone genes

The histone mRNAs can be isolated from sea urchin embryos in high yield and with a high specific radioactivity (Kedes and Birnstiel, 1971; Weinberg *et al.*, 1972). They can thus be used as a probe to detect their complementary sequences in the genome by hybridisation. This forms the basis of a purification of histone genes by fractionation of total cellular DNA, on the basis of G + C content, on caesium chloride gradients. By hybridisation of ^{3}H-mRNA to fractions from the gradient, it was demonstrated that the histone genes are of high G + C content (Kedes and Birnstiel, 1971; Birnstiel *et al.*, 1974). The histone mRNAs can be separated into four major species coding for different histones (Weinberg *et al.*, 1972; Grunstein *et al.*, 1973; Levy *et al.*, 1975b) and all four hybridise to this same DNA fraction. This was interpreted as being due to clustering of the genes for the various

histones; however the authors (Kedes and Birnstiel, 1971) were not certain that their labelled probe contained H1 mRNA, and so the presence of H1 genes in this DNA is uncertain. The clustering of the genes has since been unequivocally verified by cloning DNA fragments in plasmids (Kedes *et al.*, 1975). These results demonstrated that the genes for H2A, H2B, H3, H4 and *possibly* H1 are present on one piece of DNA as a cluster.

Hybridisation of labelled mRNA to total DNA shows that the genes for these histones are repeated some 400 to 1000-fold (species variation) in the sea urchin genome (Kedes and Birnstiel, 1971; Weinberg *et al.*, 1972), and thus the genes are probably present as a tandemly repeated block. Because of the high conservation of histone primary sequences, their mRNAs will hybridise to the histone genes of other species. In this way one can show that the histone genes are also repeated in *Drosophila* (Birnstiel, Weinberg and Pardue, 1973) and *Xenopus* genomes (Kedes and Birnstiel, 1971). However this may not be a general result for all organisms as the reiteration frequency in man and mouse has been shown to be only 10 to 20 (Wilson, Melli and Birnstiel, 1974).

The biological significance of the clustering and repetition of the histone genes is unknown. One possible explanation of the clustering is that it is connected with the coordinate control of histone synthesis although other proteins which are subject to coordinate control are known by genetic studies not to

be clustered in the DNA (Tan *et al.*, 1974; Ruddle, 1973). If the clustering is connected in any way with gene control, there is no reason, *per se*, why the H5 gene should also be clustered with the rest since it is subject to quite different control (Appels and Wells, 1972). The gene is only turned on in nucleated erythroid cells and mRNA synthesis is not linked to DNA synthesis. Hence an investigation of the H5 gene may help explain why the other histone genes are found as tandem repeats, at least in sea urchins. Such an investigation requires a probe for H5 genes and the major aim of this project is to synthesise such a probe.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

General chemicals were of A.R. grade. Ribonuclease A (pancreatic), ribonuclease T1, bovine serum albumin (Fraction V), phenylhydrazine (twice recrystallised), ATP (crystalline), GTP (type 1), creatine phosphate, and creatine phosphokinase (ATP Creatinephospho-transferase) were from Sigma. Oligo(dT)-cellulose (Type 1) and oligo(dT)$_{12-18}$ were from PL biochemicals. Proteinase K was from Merck, and all sera and media were from Commonwealth Serum Laboratories. Trichodermin was a gift of Dr. W. O. Godtfredsen of Leo Pharmaceutical Products Ltd., Ballerup, Denmark. Sarkosyl was a gift of CIBA-Geigy Ltd., Basle, Switzerland.

Isotopes were purchased from the following sources: - 5-^{3}H-dCTP (26.2 Ci/mmole) from New England Nuclear, ^{14}C(U)-Lysine (330 mCi/mmole), ^{14}C(U)-Leucine (311 mCi/mmole), ^{3}H-Lysine (6.6 Ci/mmole), ^{3}H-Leucine (13 Ci/mmole), and 5-^{3}H-dGTP (12 Ci/mmole) were from the Radiochemical Centre, Amersham. ^{14}C-potassium cyanate (23 mCi/mmole) was from ICN.

2.2 METHODS

Unless otherwise stated, all operations were performed at 4°C.

2.2.1 Avian Erythroid Cells

(1) Preparation of cells

The procedures followed were essentially those of Williams (1971). Mature erythrocytes were obtained from the blood of healthy White Leghorn - Australorp pullets (six months old). Reticulocytes were obtained from the blood of similar chickens which had been rendered anaemic by five daily injections of 2.5% phenylhydrazine in 47.5% ethanol (v/v) pH 7. All blood was obtained by heart puncture into NKM (0.14 M NaCl, 0.005 M KCl, 0.002 M $MgCl_2$) containing 1 mg/ml of heparin. The red blood cells were washed several times by centrifugation (1000 g, 5 minutes), and resuspension into NKM. The buffy, white-cell layer was removed during this operation by aspiration. When required, reticulocytes were further purified by centrifuging the cells through discontinuous isotonic BSA gradients (3800 g, 90 minutes) when they float at the interphase of BSA of densities 1.083 and 1.071 g/cm^3. This procedure removes any contaminating erythrocytes and white cells.

(2) In vitro incubation of cells

For the *in vitro* incorporation of ^{14}C-amino acids, washed reticulocytes were incubated in Eagle's medium modified for suspension culture (Eagle *et al.*, 1956), containing 10% dialysed chicken serum (freshly prepared), and buffered with bicarbonate/CO_2 to pH 7.0. Cells

were suspended at 10% (w/v) in the medium and equilibrated at 40°C for 10 minutes. The appropriate isotope was then added and the cells were slowly shaken in an atmosphere of 5% (v/v) CO_2. The incorporation was stopped by centrifuging the cells (1000 g, 3 minutes) and washing with ice-cold NKM, before isolation of the labelled proteins.

2.2.2 Histones

(1) Isolation of total histone

The method is based on the procedures of Dingman and Sporn (1964). Nuclei were released from washed packed cells by lysis in an equal volume of 0.5% (w/v) saponin in isotonic sucrose (0.294 M sucrose, 0.001 M $MgCl_2$, 0.001 M potassium phosphate, pH 6.8) for 10 minutes. The nuclei were collected by centrifugation (1800 g, 10 minutes) and washed with 10 ml of isotonic saline per 0.5 ml of packed nuclei. The nuclear pellet was similarly washed six times with 0.08 M NaCl, 0.02 M EDTA pH 6.4 (pH adjusted with 4 M NaOH), and twice with 0.147 M NaCl. The washed pellet was resuspended in 0.147 M NaCl, an equal volume of 4 M NaCl was added, and the suspension was gently agitated for 30 minutes. 10 M HCl was then added to a final concentration of 0.25 M and agitation continued for 30 minutes. The insoluble material was then removed by centrifugation (10000 g, 10 minutes) and the supernatant containing the histones was extensively dialysed against distilled water. The histone was then lyophilised and stored desiccated at -15°C.

(2) Purification of H5

Total chicken histone was dissolved in distilled water at 5 mg/ml and made 1.0 M in perchloric acid. This was stood on ice for 15 minutes to allow all histones except H1 and H5 to precipitate and the mixture was clarified by centrifugation (5000 g, 10 minutes). The supernatant containing only H1 and H5 was dialysed against distilled water and lyophilised. H5 was finally purified by either of two procedures.

(i) CM-cellulose chromatography (Johnson *et al.*, 1964). The lyophilised H1 + H5 was dissolved in 0.35 M NaCl, 0.1 M sodium formate pH 4.8 and loaded on to a solumn of Whatman CM52 carboxymethyl cellulose. The column was eluted with a linear salt gradient from 0.35 M NaCl, 0.1 M sodium formate pH 4.8, to 1.0 M NaCl, 0.1 M sodium formate pH 4.8. H1 was eluted at 0.46 M NaCl and H5 at 0.72 M NaCl. The purified histones were dialysed against distilled water and lyophilised.

(ii) Amberlite CG-50 chromatography (Greenaway and Murray, 1971). The lyophilised H1 + H5 was dissolved in 8% (w/v) guanidinium chloride, 0.1 M sodium phosphate pH 6.8, and loaded on to a column of Amberlite CG-50. This was eluted with a linear gradient of phosphate buffered guanidinium chloride from 8% to 20%. H1 was eluted to about 10% and H5 at 16% guanidinium chloride. The histone was then extensively dialysed and lyophilised.

Figure 2.1

Purification of chicken histone H5. Low pH-urea gels of

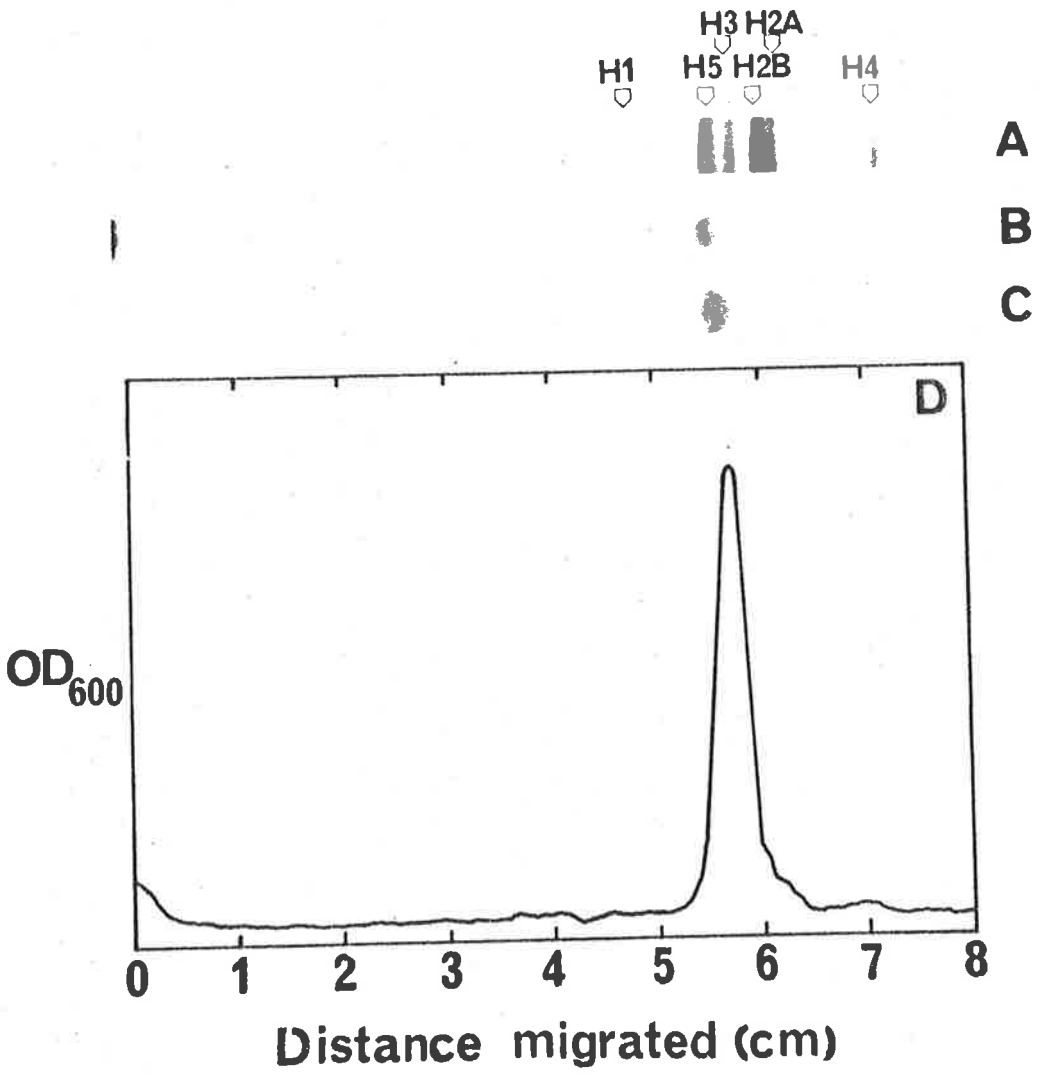
A. Acid-extractable nuclear proteins

B. Perchloric acid-soluble histones

C. H5 from CM-cellulose column

Electrophoresis was from left to right in all cases. The positions of the histones are marked.

D. Scan at 600 nm of C.


H3 H2A
H1
H5 H2B
H4
A
B
C
D
OD600
0
1
2
3
4
5
6
7
8
Distance migrated (cm)

(3) Analysis on polyacrylamide gels

Two different polyacrylamide gels systems, employing a different basis for separation, were used in the analysis of histones.

(i) Low pH-Urea (Paryim and Chalkley, 1969). The gels contained 15% acrylamide, 0.9 M acetic acid, and 2.5 M urea and were electrophoresed at 1.75 mA per gel for 4 hours. They were stained with 0.5% Amido black - 40% ethanol - 0.9 M acetic acid. After destaining in 40% ethanol, 0.9 M acetic acid, the gels were scanned with a Gilford spectrophotometer at 600 nm.

(ii) SDS-Urea gels (Swank and Munkres, 1971). The gels contained 10% acrylamide, 0.1% SDS, and 6 M Urea, and were electrophoresed at 1.5 mA per gel for 16 hours. They were stained in 0.25% Coomassie blue, 45% methanol, 1.6 M acetic acid, and destained in 25% methanol, 1.3 M acetic acid.

For the analysis of labelled proteins on either gel system, the gels were frozen with dry ice and sliced into 1 mm slices with a Mickle gel slicer (Mickle Laboratory Engineering Co., Surrey, England). The slices were incubated with 0.2 ml of 1% SDS overnight at 37°C. 2 ml of toluene-triton scintillation fluid (62.5% toluene, 0.2% PPO, 0.02% POPOP, 37.5% triton X-100) was added and radioactivity was determined in a Packard IV Tri-Carb scintillation spectrometer.

(4) In vitro labelling of histone

H5 was chemically labelled *in vitro* with ^{14}C using ^{14}C-potassium cyanate. To 9 mg of electrophoretically pure H5 in one ml of double distilled water was added 4 µCi of potassium ^{14}C-cyanate (23 mCi/mmole). This was incubated at 50°C for 2 hours to carbamylate any free amino groups, and then extensively dialysed against distilled water. The ^{14}C-H5 was then lyophilised. The specific activity of the protein was 700 d.p.m./µg.

(5) Preparation of total histone matrix

The matrix was synthesised by the glutaraldehyde cross-linking method of Avrameas and Ternynck (1969). Total chicken histone was dissolved in 0.1 M sodium phosphate buffer pH 7.0, to a final concentration of 150 mg/ml. To each millilitre of solution, 0.1 ml of 12.5% glutaraldehyde (freshly prepared) was added, dropwise. The mixture was left overnight to gel at room temperature. This matrix was then washed by homogenisation, three times in 0.2 M sodium phosphate buffer pH 7.3, three times in 0.01 M sodium phosphate buffer, 0.15 M NaCl, pH 7.3, and four times with sterile 0.025 M NaCl, 0.025 M Tris-Cl, pH 7.6, 0.005 M $MgCl_2$ containing 100 µg/ml of heparin at 4°C (Palacios *et al.*, 1973).

2.2.3 Antibodies

(1) Preparation of antisera

Anti-H5 serum was raised in rabbits by injection of 2 mg of electrophoretically pure H5 in complete

Freund's adjuvant, every 2 weeks. When required, rabbits were bled from an ear vein and the blood allowed to clot at 37°C for 1 hour. The clot was stored overnight to contract at 0°C, and the serum clarified by centrifugation (5000 g, 15 minutes).

Anti-rabbit gamma globulin serum was raised in goats by injection of 2 mg of ammonium sulphate purified (Palacios *et al.*, 1973) rabbit gamma globulin in complete Freund's adjuvant every 2 weeks. The goats were bled from the carotid artery, and serum prepared as above.

(2) Detection of antibodies

Antisera were tested against a variety of antigens on Ouchterlony diffusion plates. To eliminate precipitation of the antigens in the agar, the gel had the following composition:- 0.8% agarose, 0.15 M NaCl, 0.01 M sodium phosphate buffer pH 7, 0.01% merthiolate. Antigens were diluted in saline and placed in peripheral wells with antiserum in a central well. These were incubated at room temperature in a humid cabinet until precipitin lines formed. This method may fail to detect antibodies present in low titre.

(3) Purification of antibodies

Antibodies were rendered specific and ribonuclease free by the procedures of Shapiro *et al.*, (1974).

(i) Ammonium sulphate fractionation. Antisera were made up to 40% saturation with ammonium sulphate and left on ice for 30 minutes. The precipitate of gamma globulin was collected by centrifugation (5000 g, 10 minutes), and the

precipitation was repeated. The gamma globulin was then resuspended in a small volume of 0.015 M NaCl, 0.01 M sodium phosphate buffer, pH 7.2.

(ii) Affinity chromatography. Three affinity columns were prepared by the methods of Shapiro *et al.*, (1974), containing H5, chicken globin, and rabbit gamma globulin. The purified proteins were bound to cyanogen bromide-activated Sepharose 4B (Cuatrecasas, 1970) and packed into columns. The gamma-globulin fractions were purified by chromatography first on the globin-Sepharose (to remove anti-globin activity) and then on the H5-Sepharose (anti-H5) or gamma-globulin-Sepharose (goat anti-rabbit serum). The antibody formed a white band at the top of the second column which, after washing, was eluted with 0.1 M glycine pH 2.8. This was then dialysed against 0.015 M NaCl, 0.01 M sodium phosphate pH 7.2, and rendered ribonuclease-free by passage through a column containing CM-cellulose (5 x 10 cm) over 5 cm of DEAE-cellulose.

(4) Quantitation of antibodies

The amount of gamma-globulin present in purified antibody preparations was determined from the $O.D._{280}^{1\ cm.}$; a value of 1.4 for a 1 mg/ml solution was used (Schechter, 1974).

To quantitate the amount of antibody present in a preparation, precipitin reactions were carried out

(Shapiro *et al.*, 1974). The reactions were performed in Corex tubes and any resulting precipitate was collected by centrifugation (5000 g, 15 minutes). The pellets were washed three times by resuspension in 0.015 M NaCl, 0.01 M sodium phosphate, pH 7.2, and centrifugation, and dissolved in 1 M NaOH. The $O.D._{280}^{1\ cm.}$ was then used to calculate the amount of protein precipitated. This method can be used to calculate the amount of antibody required to precipitate a fixed amount of antigen.

2.2.4 Isolation and Analysis of RNA

All procedures involving RNA employed sterile glassware and solutions and were carried out as rapidly as possible at 0°C unless otherwise stated.

(1) Polysome preparation

Washed reticulocytes were lysed by the addition of 2 volumes of ice-cold 2 mM $MgCl_2$ and vortex mixing for 2 minutes. An equal volume of TKM (0.01 M Tris-Cl pH 7.0, 0.2 M KCl, 0.002 M $MgCl_2$) was then added to restore tonicity, and the nuclei were removed by centrifugation (5000 g, 10 minutes). The supernatant was again centrifuged to remove any remaining nuclei, and the polysomes were collected by centrifuging (210,000 g, 60 minutes) through a pad of 50% (w/v) sucrose in TKM, in a Beckman Ti50 rotor. The polysome pellet was then rinsed twice in ice-cold TK (10 mM Tris-Cl pH 7.0, 15 mM KCl) before suspension in the required buffer.

(2) Preparation of globin mRNA (Pemberton et al., 1972)

Polysomes were suspended in TK buffer by aspiration with a pipette and $^1/_{10}$ volume of 300 mM EDTA pH 7.0 was

added. This dissociated the polysomes into ribonucleoprotein (RNP) particles which were fractionated by centrifugation through linear 10-40% (w/v) sucrose gradients in TK (160,000 g, 16 hours), in the Beckman SW41 rotor. The 20S RNPs were collected and ethanol precipitated by the addition of $^{1}/_{10}$ volume of 1 M NaCl, two volumes of RNAase-free ethanol, and storage at -15°C for a minimum of 4 hours. The RNPs were collected by centrifugation (20,000 g, 20 minutes) and dried *in vacuo*. They were then dissolved in 10 mM Tris-Cl pH 7.0, 0.1% SDS containing 250 µg/ml of Protease K, incubated at 37°C for 3 minutes to degrade any protein, and fractionated once more on sucrose gradients in NET (0.1 M NaCl, 0.01 M Tris-Cl, 0.001 M EDTA, pH 7.2) buffer. The 10S RNA was collected and ethanol precipitated.

(3) Preparation of H5 mRNA by immunoadsorption (Scott and Wells, 1975)

Polysomes were dissolved in polysome buffer (0.025 M NaCl, 0.005 M $MgCl_2$, 0.025 M Tris-Cl, pH 7.6) containing 100 µg/ml of heparin to a concentration of 25 A_{260} per ml. 25 µg/ml of purified anti-H5 was then added and mixed for 60 minutes to allow it to bind to the nascent peptides. Precipitation of these complexes was then induced by the addition of 20 mg of histone matrix per 10 A_{260} units of polysomes, for a further 60 minutes before it was collected by centrifugation (5000 g, 10 minutes). This matrix was washed four times with 0.15 M NaCl, 1% Triton X-100, 1% sodium deoxycholate in polysome buffer, once with polysome buffer, and resuspended in

Tris-SDS pH 5.3 (0.1 M Tris-Cl pH 5.3, 1% SDS). The RNA was purified by two extractions with phenol saturated with Tris-SDS pH 5.3. The RNA was collected by two ethanol precipitations, and resuspended in a small volume of deionised formamide. This was warmed briefly to disaggregate the RNA, diluted by the addition of four volumes of NET and fractionated on sucrose gradients in NET as above.

(4) Preparation of H5 mRNA by immunoprecipitation (Shapiro et al., 1974)

Polysomes were dissolved in immunoprecipitation buffer (0.025 M Tris-Cl pH 7.6, 0.15 M NaCl, 0.005 M $MgCl_2$, 0.5 mg/ml heparin) to 25 A_{260} units per millilitre, and 40 µg/ml of purified anti-H5 was added. This mixture was stirred at 0°C for 60 minutes before the antibody-polysome complexes were precipitated by the addition of purified goat anti-rabbit gamma globulin serum (1 mg/30 µg anti-H5). After 2 hours at 0°C (Shapiro and Schimke, 1975) the solution was layered over discontinuous sucrose gradients containing 3 ml of 15% sucrose over 6 ml of 30% sucrose (both in immunoprecipitation buffer plus 1% Triton X-100, and 1% sodium deoxycholate) in Beckman SW41 rotor tubes. The precipitate was sedimented by centrifugation (20,000 g, 20 minutes) in the Beckman SW41 rotor, rinsed with immunoprecipitation buffer, and sedimented through a second similar gradient. The RNA was extracted from the final precipitate and fractionated as described previously (section 2.2.4.(3)).

(5) Sizing of nucleic acids on formamide gels (Pinder, Staynov and Gratzer, 1974)

Nucleic acids were electrophoresed on polyacrylamide gels containing formamide to estimate their size. The gels were 4% or 10% acrylamide, 0.02 M diethylbarbituric acid pH 9.0 (adjusted with 1 M NaOH) in formamide, 9 cm in length. These were overlaid with formamide buffered to pH 9.0 with 0.02 M diethylbarbituric acid, and the sample layered under this in buffered formamide containing 5% sucrose and a trace of bromophenol blue. The electrode buffer was 0.02 M NaCl which was recirculated to maintain the pH. Gels were electrophoresed at a constant current of 5 mA per gel and then scanned at 280 nm, or stained in toluidine blue (0.05% in 50 mM sodium acetate pH 5.5) and destained in water. For the size determination of labelled nucleic acids, the gels were sliced into 1 mm slices and dissolved in NCS scintillant (Ward, Wilson and Gillian, 1970). Radioactivity was determined in a Packard IV scintillation spectrometer.

(6) In vitro translation of RNA

RNA was translated in the wheat embryo cell-free system of Shih and Kaesberg (1973) as modified (Scott and Wells, 1975). 50 µl reaction mixes contained 0.025 M Tris-acetate pH 8.0, 85 mM KCl, 3.8 mM magnesium acetate, 2.3 mM dithiothreitol, 1 mM ATP, 0.25 mM GTP, 8 mM creatine phosphate, 4 µg of creatine phosphokinase, 1 µCi of ^{3}H-leucine or ^{3}H-lysine, mRNA as required, and 25 µl of wheat embryo extract. These were incubated at 30°C for the required time and the tRNA

was then deacylated by adjusting the mixture to 0.1 M in NaOH followed by incubation at 37°C for 15 minutes. For determination of radioactivity incorporated, the mixtures were precipitated by the addition of 1 ml of 20% TCA, 0.1% leucine or lysine, and storage at 0°C for 15 minutes. The precipitates were collected on to GF/A filters and then washed with TCA, ethanol, and ether (Boime, Aviv and Leder, 1971). Radioactivity was determined after drying and addition of a toluene scintillant, in a Packard IV scintillation spectrometer.

For analysis of the products produced in the system, the TCA precipitate was collected by centrifugation (5000 g, 10 minutes) and washed three times with 5 ml of 20% TCA. This was finally washed in ethanol to remove the TCA (which quenches strongly) and once in ether. The proteins were dissolved in the appropriate loading buffer and analysed on polyacrylamide gels as described (section 2.2.2(3)).

(7) Oligo (dT)-cellulose chromatography

Oligo (dT)-cellulose was equilibrated in 0.3 M NaCl, 0.01 M Tris pH 7.0, 0.001 M EDTA and packed into a small column. This was flushed with 0.1 M NaOH to render it ribonuclease-free and re-equilibrated with the first high-salt buffer. RNA was loaded in high-salt buffer at 50 OD_{200} units/ml and flushed through at 0.5 ml/minute, monitoring the eluant OD_{260} on a Uvicord I. The unbound RNA was reapplied to the column, re-eluted and stored. The column was then washed with 0.1 M NaCl, 0.01 M Tris-Cl pH 7.0, 0.001 M

EDTA, and the bound RNA eluted with 10 mM Tris-Cl pH 7.0, 0.001 M EDTA. If required, the column was re-equilibrated with high-salt buffer and the bound RNA was re-chromatographed to remove any contaminating non-polyadenylated RNA. All fractions were then ethanol precipitated.

(8) Sizing of poly A tracts

RNA was dissolved in degradation buffer (0.4 M NaCl, 0.02 M EDTA, 0.02 M Tris-Cl pH 7.2) containing 2 µg/ml of RNAase A and 10 Units/ml of RNAase T1. This was then incubated at 37°C for 60 minutes during which time all RNA eccept poly A would be degraded (Adesnik and Darnell, 1972). The remaining RNA was ethanol precipitated, dried *in vacuo* and analysed on 10% polyacrylamide gels in formamide as described (section 2.2.4(5)). 4S, 5S RNA and oligo $(dT)_{15}$ were included as standards since under these conditions RNA electrophoreses according to the log of its molecular weight (Morrison, Merkel and Lingrel, 1973; Pinder, Staynov and Gratzer, 1974).

(9) Enzymic addition of poly A

1 µg of RNA was incubated in a covered Corex tube at 30°C for 3 hours, in a 30 µl incubation mixture containing:- 70 mM Tris-acetate pH 8.6, 10 mM dithiothreitol, 1 mM ATP, 1 mM $MnCl_2$, and 50 µg/ml of poly A polymerase (Mans and Huff, 1975). The modified RNA was then ethanol precipitated and dried *in vacuo*.

2.2.5 Nucleic Acid Hybridisation

(1) Production and sizing of cDNA (Kemp, 1975)

cDNA was synthesised in a 50 µl mixture containing 50 mM Tris-Cl pH 8.3, 8 mM dithiothreitol, 8 mM $MgCl_2$, 0.66 mM dATP, dTTP, 0.1 mM ^{3}H-dCTP, dGTP, 100 µg/ml Actinomycin D, up to 1 µg mRNA, 0.1 µg oligo $(dT)_{12-18}$ and AMV reverse transcriptase. The mixture was incubated at 37°C for 1 hour, treated with 0.3 M NaOH for 15 minutes at 37°C, and purified by chromatography on a 1 x 25 cm column of Sephadex G-50 in 0.1 M NH_4HCO_3 or 0.2 M NaCl (Verma *et al.*, 1972). The high molecular weight fraction was collected and either lyophilised (0.1 M NH_4HCO_3) or ethanol precipitated (0.2 M NaCl). The highest molecular weight cDNA was obtained after fractionation on 10-40% sucrose gradients in NET. The DNA was sized on 10% polyacrylamide gels in formamide as described (section 2.2.4(5)).

(2) Preparation of total chicken DNA

20 ml of washed erythrocytes were lysed by the addition of 50 ml of 2 mM $MgCl_2$ and vortex mixing for 5 minutes. The nuclei were removed by centrifugation (10,000 g, 10 minutes) and washed once more with 50 ml of 2 mM $MgCl_2$. The nuclei were suspended in 50 ml of NKM, and made up to 200 ml with 1% sarkosyl (sodium lauryl sarcosinate) by small additions over a period of 1-2 hours with continuous mixing. A further 50 ml of 4 M NaCl was then added and the suspension extracted with an equal volume (250 ml) of water saturated phenol at room temperature for 15 minutes. The aqueous phase was separated by centrifugation (5000 g, 15 minutes)

and the DNA precipitated by the addition of two volumes of redistilled ethanol at -15°C. The DNA was spooled out on a glass rod, drained and dissolved in 200 ml of standard saline citrate (0.15 M NaCl, 0.015 M sodium citrate pH 7.4) by stirring overnight at 37°C. Pancreatic ribonuclease, preincubated at 80°C for 20 minutes to inactivate DNAase, was added to 50 µg/ml and incubated at 37°C for 4 hours. Pronase, predigested at 37°C for 2 hours, was then added to 25 µg/ml and incubated at 37°C overnight. The DNA was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (50:50:1) and once more spooled out after the addition of two volumes of absolute ethanol. This was lyophilised to remove any traces of phenol and sonicated prior to use to a size of about 500 nucleotides in length.

(3) Calculation of activity of S1 nuclease

S1 nuclease was prepared to step four by the procedure of Vogt (1973) and stored frozen at -80°C. The assay for S1 was carried out in a corex tube containing 0.2 ml of calf thymus DNA (2 mg/ml, denatured, sonicated), 0.1 ml of 0.003 M sodium acetate, 0.05 M NaCl, 0.001 M $ZnSO_4$, 5% glycerol pH 4.6, and 20 µl of enzyme. The tube was incubated at 45°C for 10 minutes with a blank containing no enzyme, and the reaction stopped by the addition of 250 µl of 25% perchloric acid, 0.75% uranyl acetate. These were stood at 0°C for 15 minutes and the precipitate removed by centrifugation (3000 g, 10 minutes). 0.5 ml of the supernatant was diluted to 2.5 ml with distilled water

and the OD_{260} read against the blank tube. The activity is defined as:-

$$\text{Activity} = (OD_{260} \times 3) \text{ units/ml.}$$

(4) RNA-DNA hybridisations

Hybridisations were carried out in 100 µl mixtures containing 5000 cpm of globin cDNA or 2000 cpm of H5 cDNA, and varying amounts of mRNA in hybridisation buffer (0.18 M, NaCl, 0.01 M Tris-Cl, 0.001 M EDTA, 0.05% SDS pH 7.0). The samples were overlaid with paraffin oil, heated briefly at 100°C and incubated for 4 hours. The reaction was stopped by chilling and assayed using S1 nuclease. For the assay, 80 µl of the mixture was diluted in 250 µl of 0.03 M sodium acetate, 0.05 M NaCl, 0.001 M $ZnSO_4$, 5% glycerol pH 4.6 containing 12 µg of sonicated, denatured, calf thymus DNA. Two 150 µl samples were incubated with or without 4 units of S1 nuclease for 45 minutes at 30°C. 75 µg of carrier BSA was then added followed by 1 ml of 10% TCA to precipitate the undergraded DNA. After 15 minutes at 0°C, the precipitate was collected on to GF/A filters, washed with TCA, ethanol, and ether, and dried. Radioactivity was determined in a scintillation spectrometer.

(5) DNA-DNA hybridisations

Hybridisations were carried out in 10 ml screw-topped tubes containing 10 mg/ml sonicated chicken DNA and about 50,000 cpm/ml of cDNA or total ^{3}H-labelled DNA, in hybridisation buffer. The DNA was denatured by boiling for 5 minutes and incubated at 60°C to the Cot value desired. To assay the reaction, 50 µl

aliquots were diluted into 2 ml of 0.3 M NaCl, 30 mM sodium acetate, 0.001 M $ZnSO_4$, 5% glycerol pH 4.6 and frozen until assayed. When all Cot values were sampled, 1 ml aliquots were incubated with or without 150 units of S1 nuclease for 30 minutes at 37°C. All samples were made up to the same DNA concentration to standardise the effects of quenching, and TCA precipitated as above.

2.2.6 Other Analytical Procedures

(1) Protein estimation

Unless otherwise stated, protein was estimated by the method of Lowry *et al.*, (1951). BSA dissolved in water was used as a standard for all estimations, assuming $A^{1\%}_{1cm}$ = 6.61 at 278.5 nm (Peters and Blumenstock, 1967).

(2) Preparation of total globin (Moss and Thompson, 1969)

Washed erythrocytes were lysed by vortex mixing with two volumes of 2 mM $MgCl_2$ for 5 minutes and the nuclei removed by centrifugation (10,000 g, 10 minutes). The pH of the supernatant was adjusted to 5.15 with 0.5 M acetic acid and non-haemoglobin proteins removed as a precipitate by centrifugation (10,000 g, 10 minutes). To 20 ml of the supernatant was added 30 ml of Drabkins solution (0.012 M $NaHCO_3$, 0.8 mM KCN, 0.6 mM $K_3Fe(CN)_6$) 20 ml of 1.0 M Tris-Cl pH 7.4, and 30 ml of distilled water. The resulting cyanomethaemoglobin was fractionally precipitated between 40% and 80% ammonium sulphate saturation at 0°C. The precipitate was dissolved in distilled water, clarified by

centrifugation (10,000 g, 10 minutes) and precipitated by the addition of ten volumes of acid-acetone (acetone containing 1% HCl). The globin was collected by centrifugation (10,000 g, 10 minutes), washed twice with acetone and dried *in vacuo*.

(3) Preparation of α and β globins

Haemoglobin prepared by ammonium sulphate fractionation as above was fractionated on a column of Bio-Rex 70 by a convex gradient from 2 M to 9 M Urea pH 1.9. After removal of urea on a Sephadex G-50 column equilibrated with 10% formic acid, the globins were lyophilised.

CHAPTER 3

ISOLATION AND CHARACTERISATION OF CHICKEN GLOBIN mRNA

3.1 INTRODUCTION

Appels (1971) has shown that in the developing avian erythroid cell series, the early dividing erythroblast synthesises all of the histones, the mature erythrocyte none, and the non-dividing, immature reticulocyte, only H5. Since most histone mRNAs are turned over rapidly in the absence of DNA synthesis (Gallwitz, 1975), probably the only histone mRNA which the reticulocyte contains is that for H5. This cell therefore represents a unique opportunity to isolate the mRNA for H5 uncomplicated by the presence of the other histone mRNAs. The major protein product of this cell however, is globin which constitutes over 90% of all protein synthesised (Sadgopal and Kabat, 1969). This infers that the major mRNA species in these cells are those coding for the various chicken globins. In those few cases where histone mRNAs have been isolated and sized (Grunstein *et al.*, 1973; Levy *et al.*, 1975) it has been found that the molecular weight of the mRNA is approximately proportional to the molecular weight of the histone for which it codes. On this basis, one can calculate that the molecular weight of H5 mRNA will be from 200,000-230,000 daltons. However, all globin mRNAs so far isolated are about 200,000-220,000 daltons in molecular weight (Bishop *et al.*, 1972; Harrison *et al.*, 1972). Thus, due to the large amount of globin mRNA and the similar molecular weights, any procedure which attempts to physically separate H5 mRNA on the basis of its size will probably result in contamination with globin mRNA. It is there-

fore essential to isolate and characterise globin mRNA since any difference in the properties of the mRNAs (e.g. poly A content) may provide a basis for their separation.

There are two other compelling reasons for isolating this mRNA. Firstly, by hybridisation of the mRNA to any complementary DNA, it is possible to estimate the percentage of the cDNA which is complementary to globin mRNA. The quantitation of this contamination is essential to be able to draw any conclusions from hybridisation experiments using H5 cDNA. One must be quite sure that the results are not due to such contaminating sequences. This is dealt with in more detail in chapter 6. Secondly, the mRNA is required for optimisation of the *in vitro* translation system, which will be used to identify H5 mRNA. Although such systems could be tested by using, for example, rabbit globin mRNA, the salt concentration for optimum translation varies from one mRNA to another (Schmeckpeper, Cory and Adams, 1974; Efron and Marcus, 1973). The rationale is that a mRNA from the same cell will have a similar salt optimum, and hence the need for *chicken* globin mRNA for optimisation.

The results in this chapter demonstrate that chicken globin mRNA can be isolated from reticulocytes as a 10S RNA species on aqueous sucrose gradients. This corresponds to a length of 650 nucleotides (210,000 daltons) on polyacrylamide gels containing formamide, of which 50 nucleotides are present as a poly A tract. This tract is presumably responsible for

its binding to oligo (dT)-cellulose. The mRNA can be efficiently translated in a wheat embryo cell-free translation system and has been shown to programme the synthesis of both α and β globins. Globin is the only major product.

3.2 IDENTIFICATION OF GLOBIN mRNA

3.2.1 Assay System for *in vitro* Translation Products

Putative mRNA was identified by its *in vitro* translation in the presence of radioactive amino acids and the subsequent examination of the radioactive proteins produced. It was therefore necessary to have methods of identifying the products of translation. This requires:-

(i) the production of standards, i.e. purified samples of the expected products which are themselves usually radioactively labelled with a different radio-isotope

(ii) a means of identifying the products and comparing them with the standards

In vitro translation products are frequently identified by extracting them from the assay system specifically, by such means as antibodies (Kemp, Partington and Rogers, 1974) or solvents in which they are preferentially soluble (Gallwitz and Breindl, 1972). Such methods demonstrate that specified products are made but cannot comment upon what other products, i.e. contaminants, may also be made. Since it is necessary to prepare pure mRNAs it is essential to examine the total *in vitro* translation products and this is best done on polyacrylamide gels.

(1) Preparation of ^{14}C-labelled standards

The products of particular interest in this thesis are the histones, particularly H5, and the globins. To ensure products which were the same as those produced *in vivo* reticulocytes or erythroblasts were incubated in the presence of ^{14}C-labelled amino acids and the histones and globins extracted from these as in chapter 2. The methods described for preparing purified H5, while giving an electrophoretically pure product (see figure 2.1), also exhibited very low yields. As a result, unlabelled H5 was chemically labelled with ^{14}C-cyanate(K^+) and used in most of the experiments described in this thesis.

(2) Separation of globin and H5 on polyacrylamide gels

The usual method of analysing histones is the low pH-urea polyacrylamide gel electrophoresis system of Panyim and Chalkley (1969). Figure 3.1 shows the distribution of radioactivity when ^{14}C-H5 and ^{14}C-globin standards are electrophoresed on these gels. While there is obviously a good separation of H5 and globin on these gels, globin and H1 electrophorese coincidentally. In order to differentiate between these two proteins, several SDS gel systems were tested. These have the advantage that proteins migrate according to their size, in general, and not according to their charge, and hence give an independent means of identifying proteins. However, as figure 3.2 demonstrates, ^{14}C-histone will not enter Weber and Osborn (1969) SDS gels. This may be due to the absence of urea which is necessary to prevent

Figure 3.1

Separation of H5 and globin by electrophoresis on low pH-urea polyacrylamide gels. Electrophoresis was from left to right in all cases. The photograph is of a parallel gel containing total chicken erythrocyte histones.

A. ^{14}C-H5

B. ^{14}C-H5 plus ^{14}C-globin

C. ^{14}C-globin

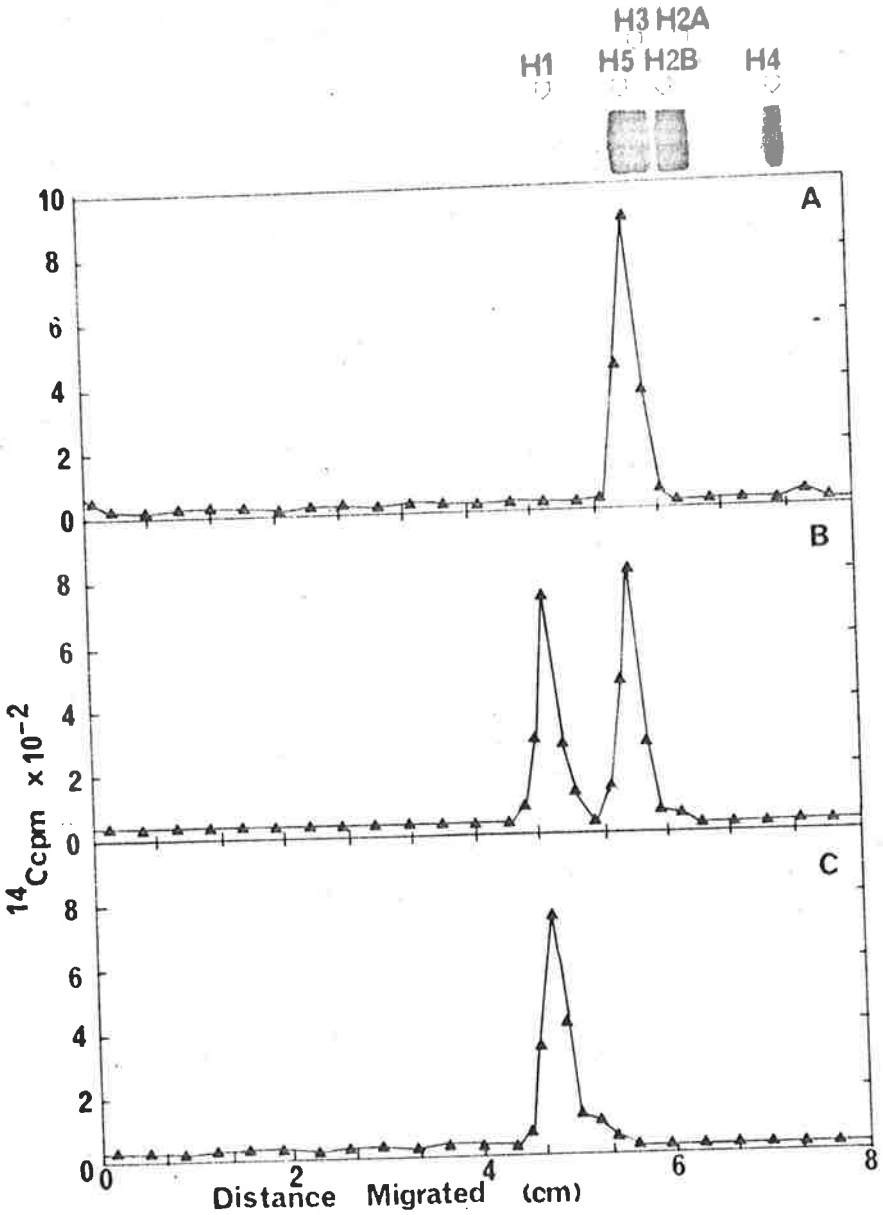

H3 H2A
H1
H5 H2B
H4
A
B
C
10
8
6
4
2
0
^{14}Ccpm x10^{-2}
0
2
4
6
8
Distance Migrated (cm)

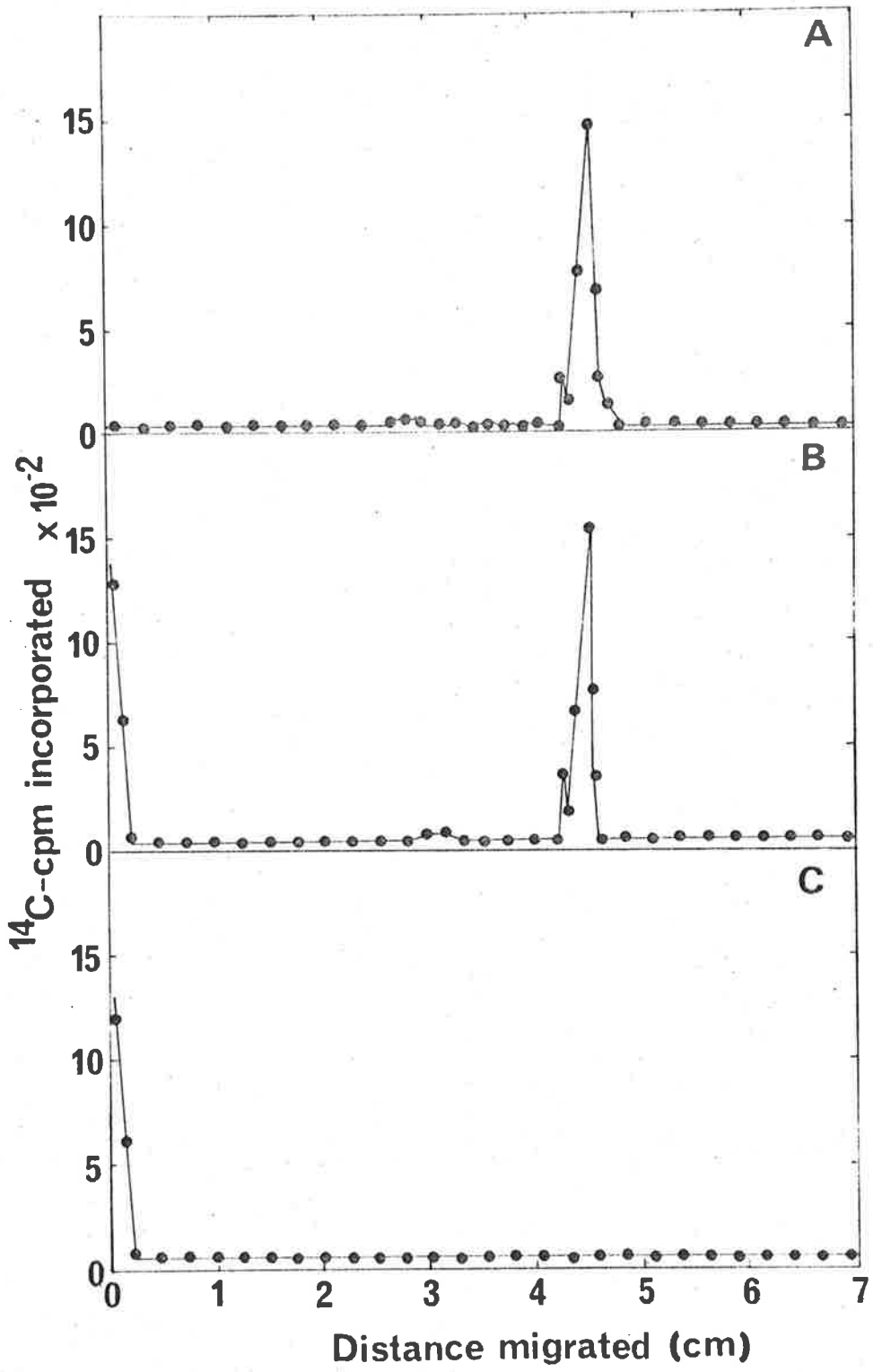
Figure 3.2

Attempted separation of H5 and globin on polyacrylamide gels containing SDS (Weber and Osborn, 1969). Electrophoresis was from left to right in all cases.

A. ^{14}C-H5

B. ^{14}C-H5 plus ^{14}C-globin

C. ^{14}C-globin


A
B
C
15
10
5
0
^{14}C-cpm incorporated x 10^{-2}
0
1
2
3
4
5
6
7
Distance migrated (cm)

aggregation of histones, particularly following acid extraction (Thomas and Kornberg, 1974). To test this hypothesis, the standards were electrophoresed on SDS gels containing urea (Swank and Munkres, 1971). As shown in figure 3.3, H5, Hl and globin are all separated on this gel system. Since the two gel systems separate on different principles, it was assumed that if a translation product electrophoresed with a standard on both gel systems, then it was identical to that standard.

Figure 3.4 demonstrates that the *in vivo* ^{3}H-labelled, and chemically ^{14}C-labelled H5 co-electrophorese on both gel systems. The use of chemically ^{14}C-labelled H5 as a standard on all further gels was thus justified.

3.2.2 *In vitro* Translation System

The properties required of an *in vitro* translation system are that it should exhibit a high sensitivity to added mRNA and have a low background due to endogenous mRNA. Although several cell-free systems were tested, only the wheat embryo cell-free system of Shih and Kaesburg (1971) exhibited these properties. Since the optimum salt concentration for translation in this system varied, the effect of salt concentration on translation was examined. As shown in figure 3.5, the optimum conditions for translation of chicken globin mRNA were 85 mM K^{+} and 3.8 mM Mg^{++} compared with 65 mM K^{+} and 3.6 mM Mg^{++} for rabbit globin mRNA. Figure 3.6 shows the response of the system to added chicken globin mRNA at these salt conditions. This demon-

Figure 3.3

Separation of H5 and globin by electrophoresis on SDS-urea polyacrylamide gels. Electrophoresis was from left to right in all cases. The photograph is of a parallel gel containing total chicken erythrocyte histone.

A. ^{14}C-H5

B. ^{14}C-H5 plus ^{14}C-globin

C. ^{14}C-globin

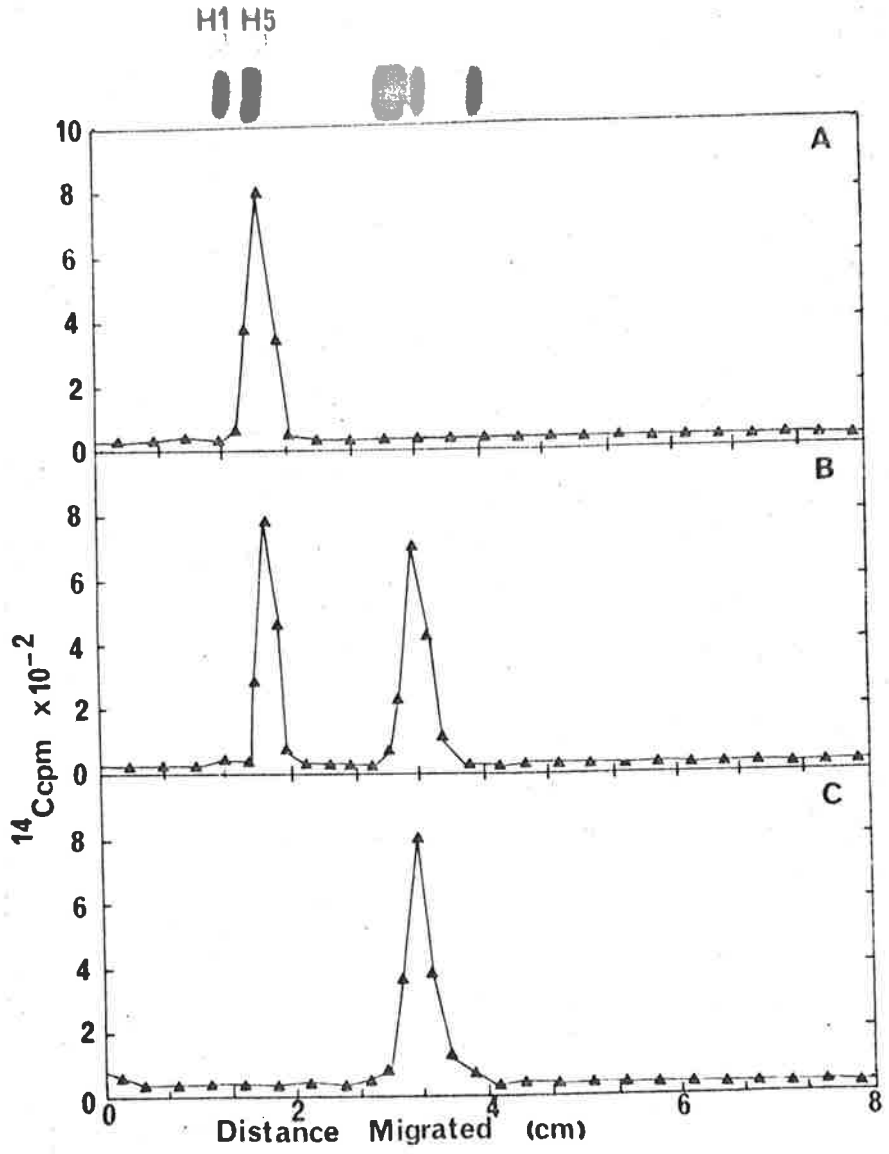

H1 H5
A
B
C
10
8
6
4
2
0
^{14}Ccpm x10^{-2}
0
2
4
6
8
Distance Migrated (cm)

Figure 3.4

Co-electrophoresis of H5 labelled with ^{3}H-leucine *in vitro*, and chemically labelled with ^{14}C-cyanate (K^{+}). The ^{14}C accounted for 10% spillover into the ^{3}H channel which has been subtracted.

A. Low pH-urea polyacrylamide gel

B. SDS-urea polyacrylamide gel

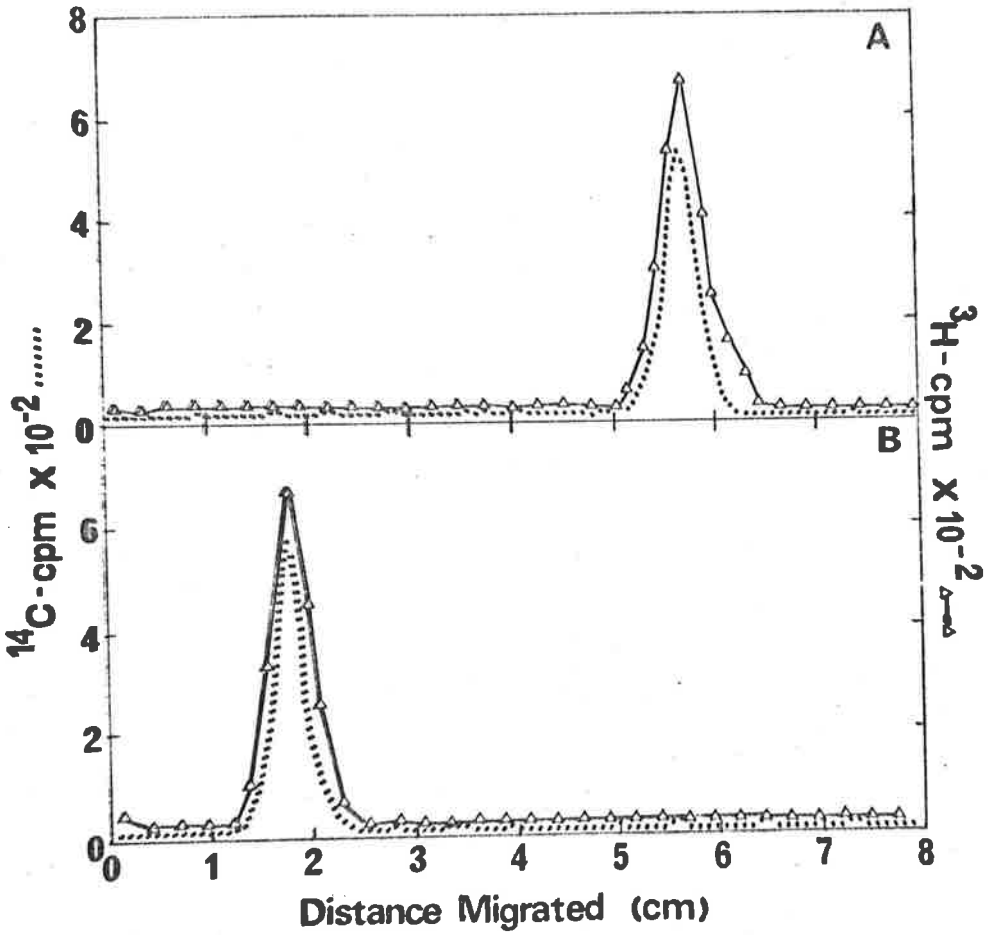
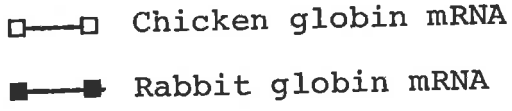

A
B
^{14}C-cpm X 10^{-2}
^{3}H-cpm X 10^{-2}
Distance Migrated (cm)
0
1
2
3
4
5
6
7
8

Figure 3.5

Effect of varying KCl and Mg^{++} concentrations on the efficiency of translation of rabbit and chicken globin mRNAs. The mRNAs were translated as described in chapter 2 but at varying magnesium concentrations (at 85 mM KCl), or KCl concentrations (at 3.6 mM Mg^{++}).



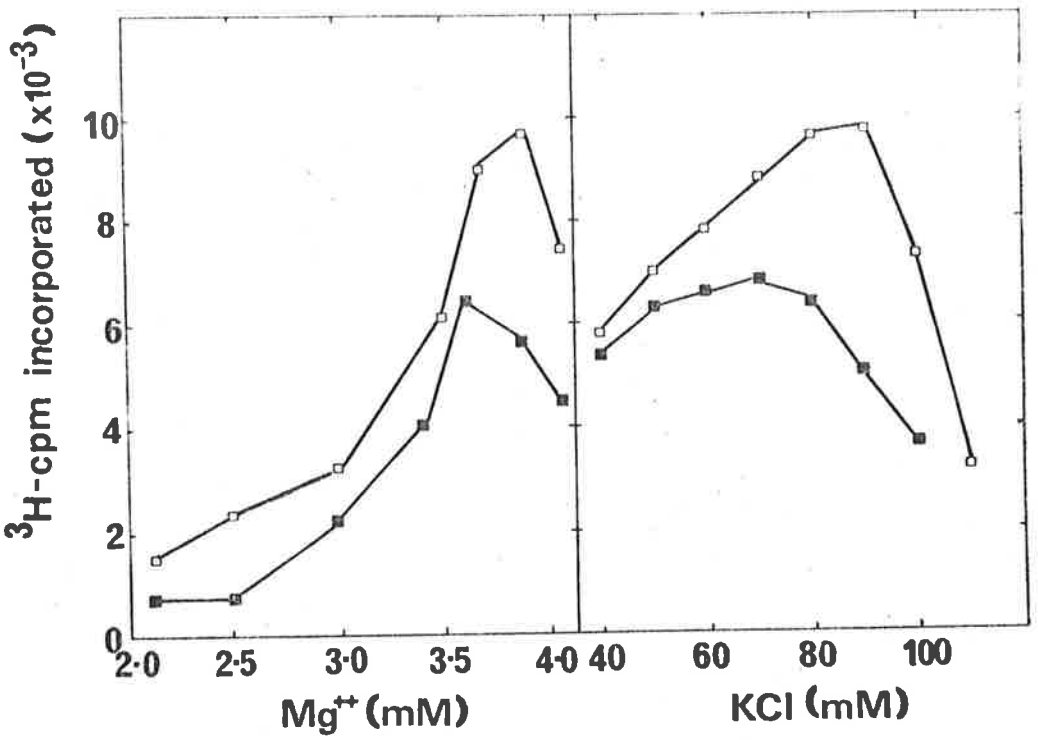
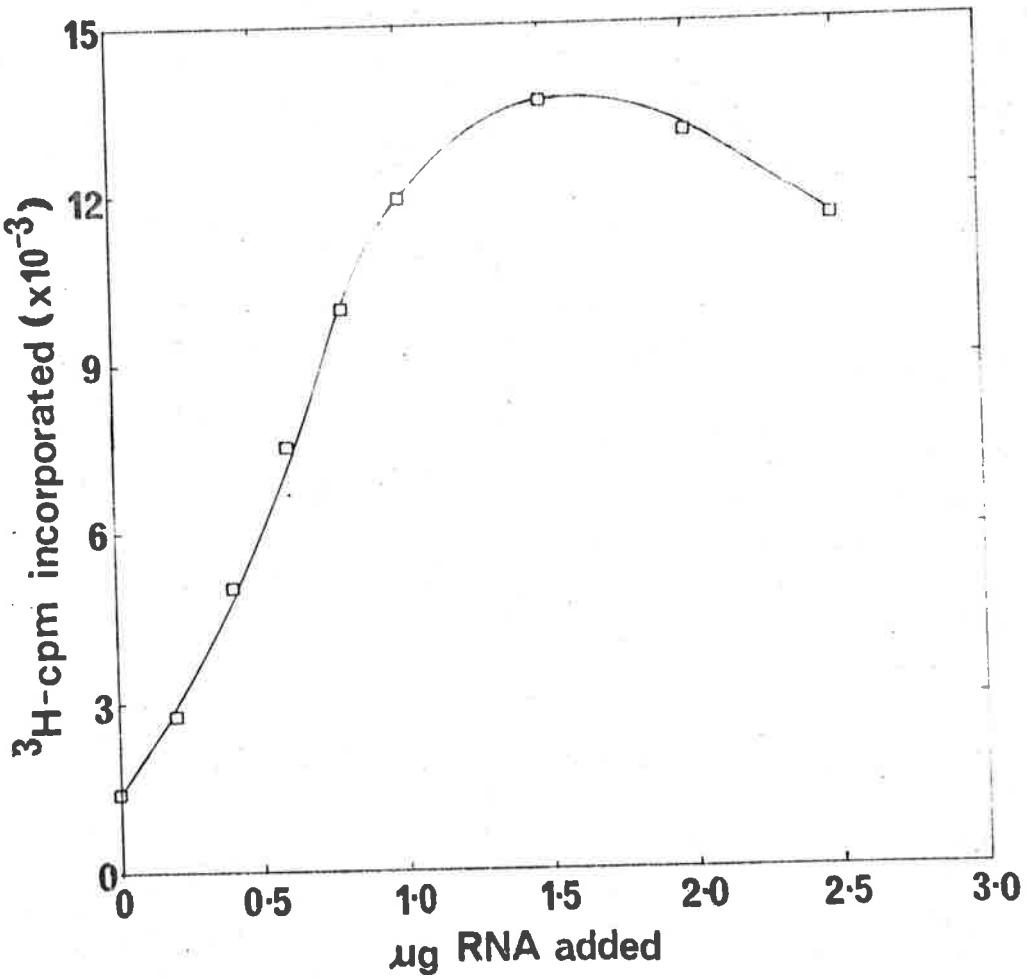

^{3}H-cpm incorporated (x10^{-3})
0
2
4
6
8
10
2·0
2·5
3·0
3·5
4·0
Mg^{++} (mM)
40
60
80
100
KCl (mM)

Figure 3.6

Effect of increasing concentrations of mRNA on translation in the wheat embryo system. Standard 50 µl incubation mixes contained the indicated amounts of chicken globin mRNA and were assayed as described in chapter 2.


15
12
9
6
3
0
^{3}H-cpm incorporated (x10^{-3})
0
0·5
1·0
1·5
2·0
2·5
3·0
µg RNA added

strates the high degree of sensitivity of the system which can easily detect as little as 0.1 µg of mRNA. As shown in figure 3.7, the reaction was linear for over 45 minutes at the optimal salt conditions and with non-saturating amounts of mRNA present.

These results characterised the cell-free system for a number of important parameters; however, the measurement used was the incorporation into TCA-insoluble material. It was essential to prove firstly that this material was globin and secondly that the globin chains were complete and were not being prematurely terminated as has been described (Schmeckpeper *et al.*, 1974). Figure 3.8 shows the analysis of the *in vitro* translation products of chicken globin mRNA on gels. This demonstrated that the only product detectable was globin and it was the same size as normal globin produced *in vivo*.

3.2.3 Isolation of Globin mRNA

Three basic methods were used to prepare globin mRNA using polysomes as a starting material. These were:-

(1) Size-dependent separation

As noted in the introduction, all globin mRNAs prepared are of 200,000 to 220,000 daltons in molecular weight. Polysomes were therefore extracted with phenol at pH 9.0 to yield polysomal RNA with a maximum of RNA containing poly A (Brawerman, 1972). This was then fractionated on a sucrose gradient (figure 3.9a) and the 8-12S RNA was collected. When re-centrifuged

Figure 3.7

Effect of time of incubation on incorporation of ^{3}H-leucine into protein, in the wheat embryo translation system. 50 µl incubation mixes containing 0.6 µg of chicken globin mRNA were incubated for the indicated times before assaying for translation.

□—□ mRNA stimulated incorporation

■—■ endogenous incorporation

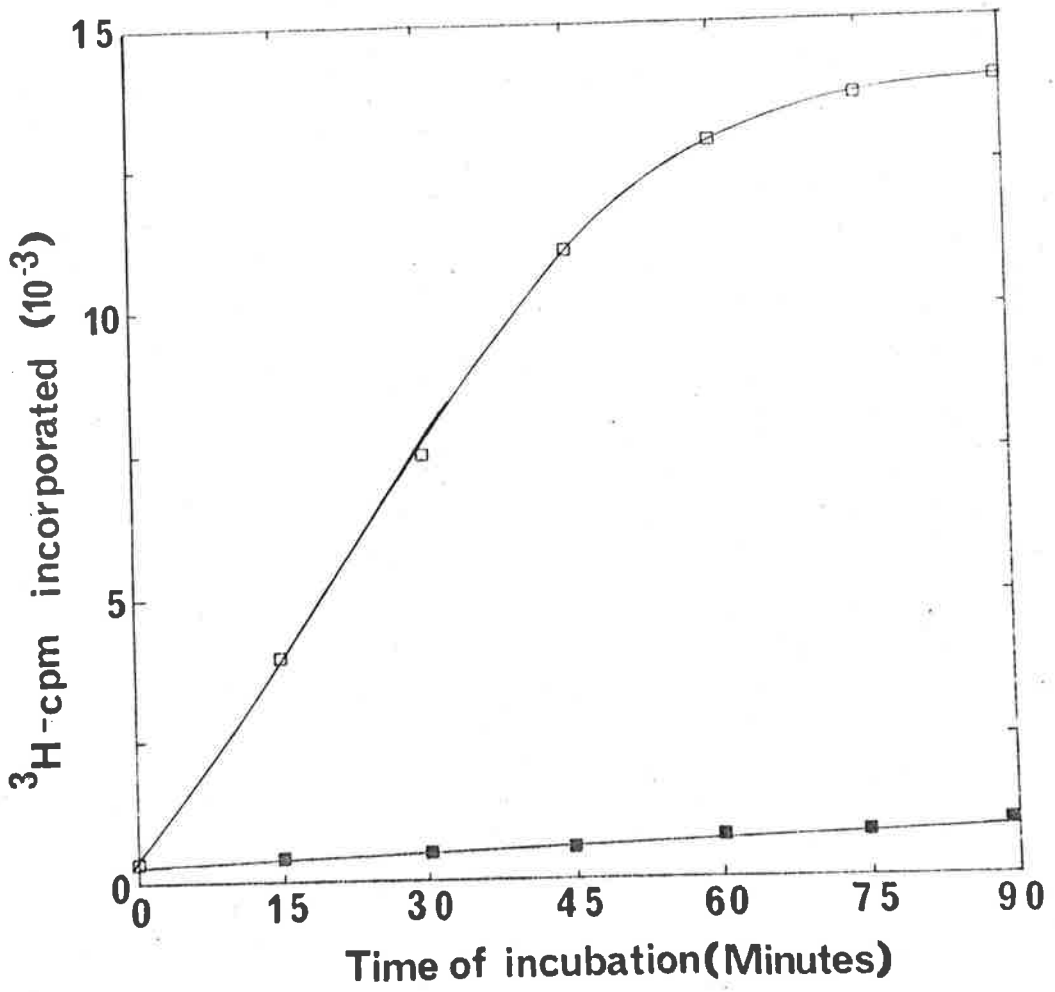

15
10
5
0
0
15
30
45
60
75
90
^{3}H-cpm incorporated (10^{-3})
Time of incubation(Minutes)

Figure 3.8

Analysis of the *in vitro* translation products of chicken globin mRNA. 0.6 µg of chicken globin mRNA was translated in the wheat embryo translation system, and the products examined by electrophoresis on

A. SDS-urea polyacrylamide gels

B. Low pH-urea polyacrylamide gels

The position of ^{14}C-labelled standards is indicated. Electrophoresis was from left to right in both cases.

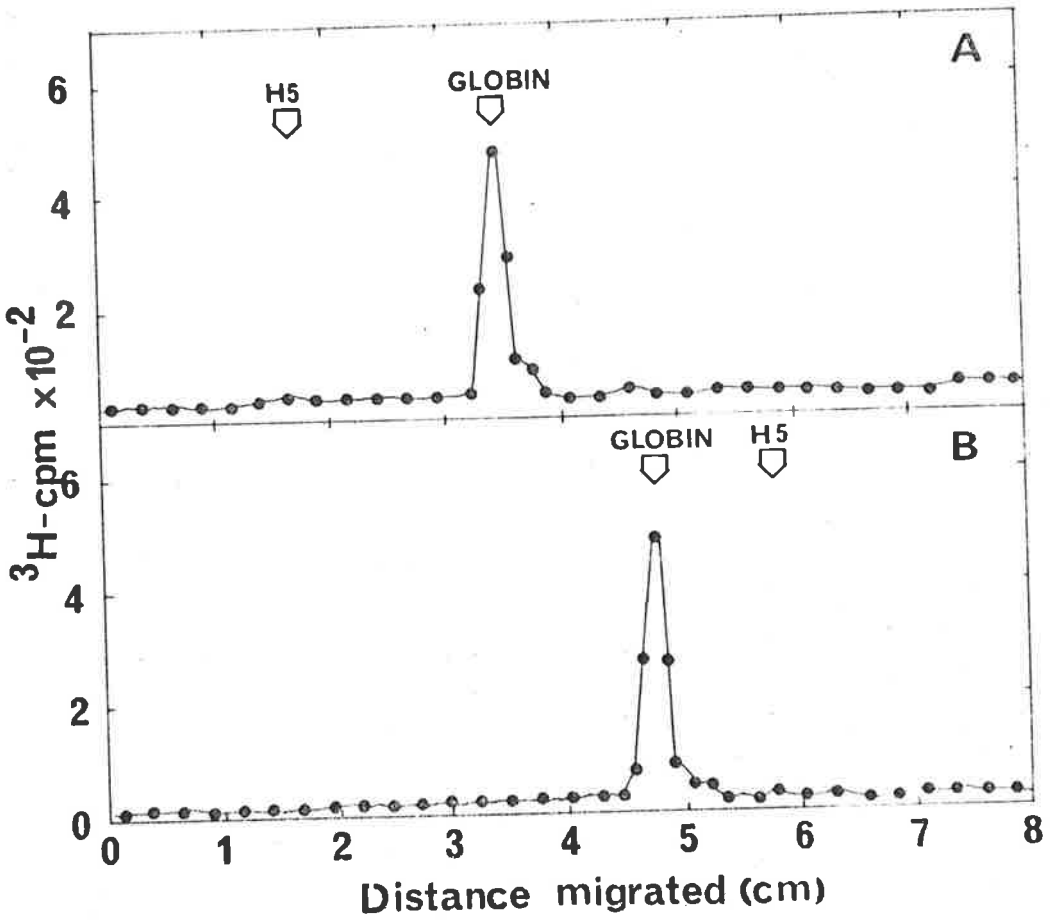
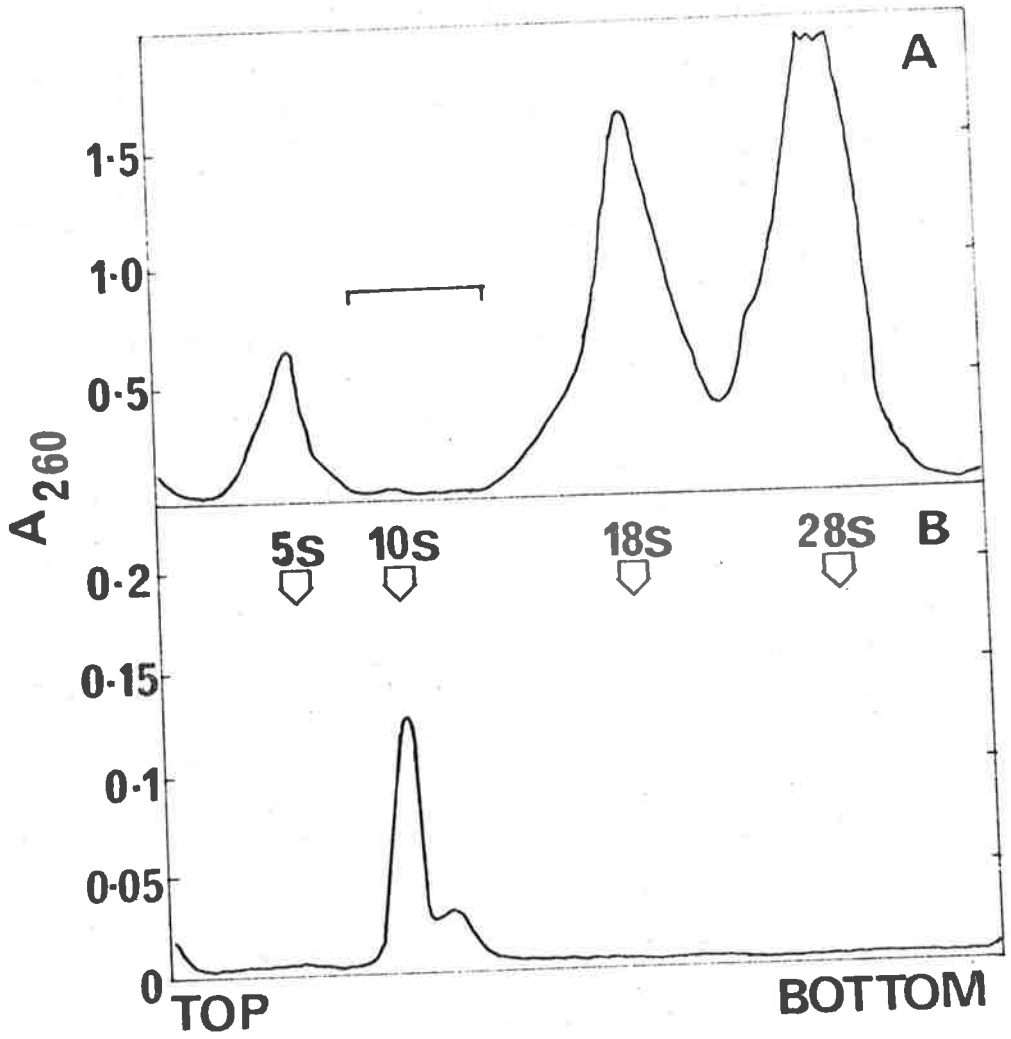

A
H5
GLOBIN
B
GLOBIN
H5
^{3}H-cpm x10^{-2}
Distance migrated (cm)
0
2
4
6
0
1
2
3
4
5
6
7
8

Figure 3.9

Purification of globin mRNA by phenol extraction of reticulocyte polysomes.

A. Fractionation of polysomal RNA on sucrose gradients

B. Re-purification of the 10S RNA from above.


A
B
1·5
1·0
0·5
A_{260}
5S
10S
18S
28S
0·2
0·15
0·1
0·05
0
TOP
BOTTOM

this yielded a 10S peak (figure 3.9b) which was assayed, by translation.

(2) Oligo (dT)-cellulose chromatography

Oligo (dT)-cellulose has the ability to bind poly A-containing RNA (Aviv and Leder, 1972) and most mRNAs have such a poly A tract (Adesnik and Darnell, 1972). Polysomal RNA prepared as above was therefore fractionated on oligo (dT)-cellulose (figure 3.10a) and the poly A-containing RNA fraction was further fractionated on a sucrose gradient (figure 3.10b). The 10S RNA produced was also assayed, by translation.

(3) Ribonucleoprotein particles

A recurrent problem of mRNA isolation is ribonuclease degradation, but this can be minimised by only partially dissociating polysomes to ribonucleoprotein particles (Pemberton *et al.*, 1972). Figure 3.11a shows the profile at 260 nm of such RNP particles fractionated on a sucrose gradient. By analogy to Pemberton *et al.*, (1972), the globin mRNA should be in a 20S RNP which was accordingly dissociated with SDS and re-centrifuged. As shown in figure 3.11b, this did yield a 10S RNA which was assayed for mRNA, by translation.

Figure 3.12 shows the stimulation of translation caused by the addition of equal, non-saturating amounts of the three RNA preparations listed above, to the wheat embryo cell-free system. It was routinely observed that the mRNA derived from RNPs was the most active and this was used as globin mRNA. The RNA prepared as in *(2)* will not stimulate at all unless first purified on a sucrose gradient.

Figure 3.10

Preparation of globin mRNA by fractionation on oligo (dT)-cellulose.

A. Elution of RNA from oligo (dT)-cellulose column

B. Fractionation of 'bound' RNA on sucrose gradients.

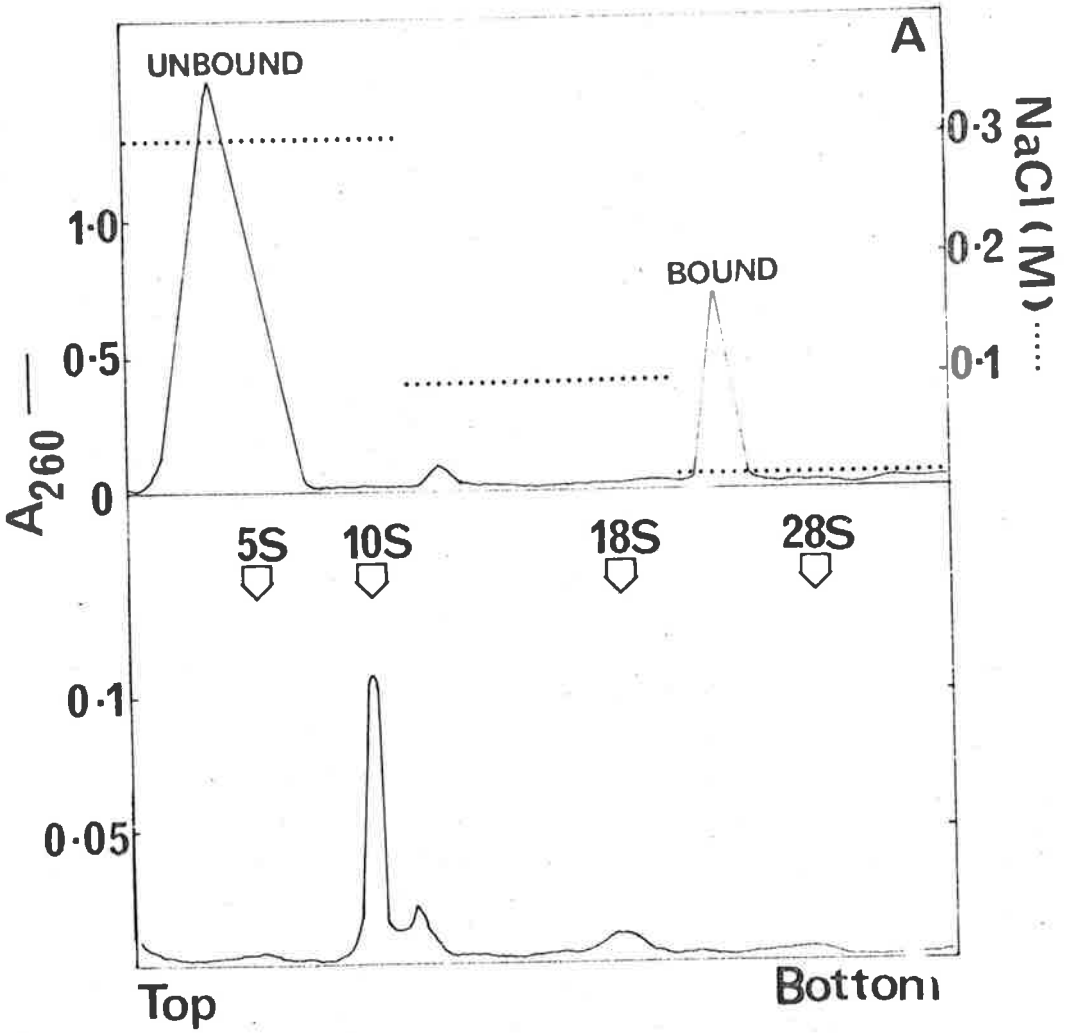

A
UNBOUND
BOUND
1·0
0·5
0
A_{260}
0·3
0·2
0·1
NaCl (M)
5S
10S
18S
28S
0·1
0·05
Top
Bottom

Figure 3.11

Preparation of globin mRNA from ribonucleo-protein particles.

A. Fractionation of RNP-particles on sucrose gradients

B. Fractionation of 20S RNPs on SDS-sucrose gradients.

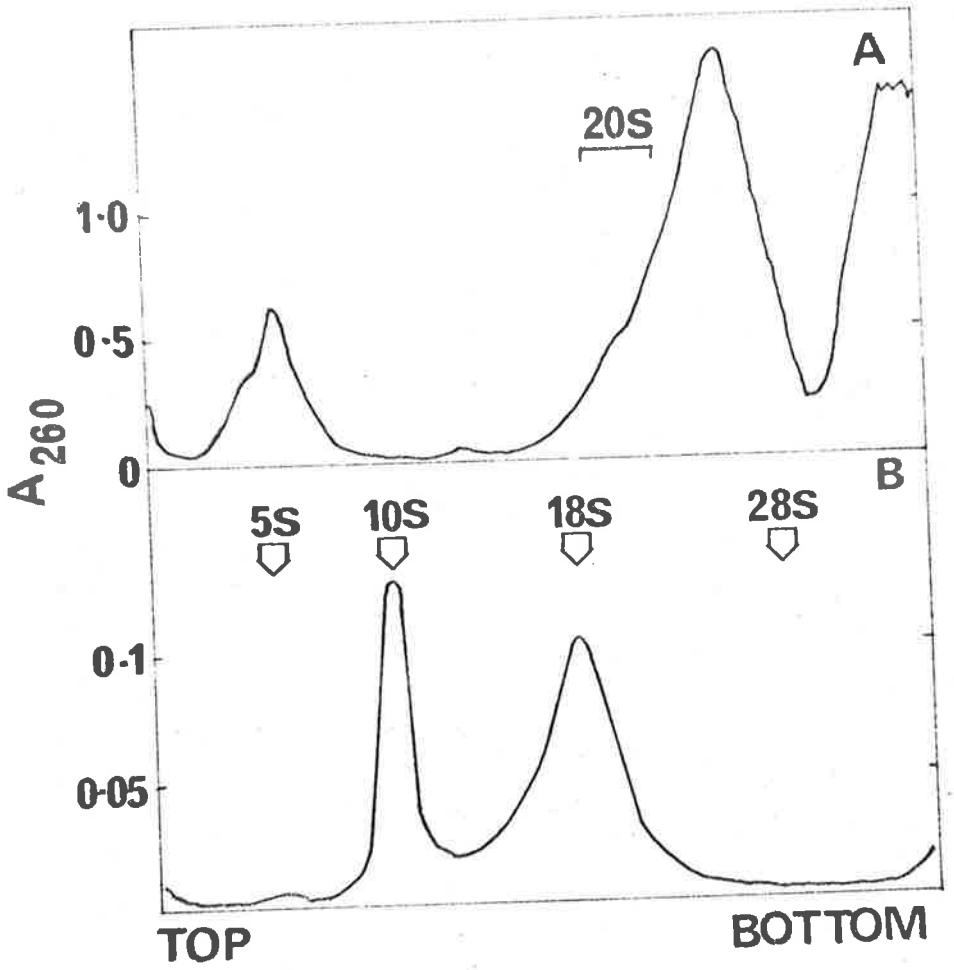

A
20S
1·0
0·5
A_{260}
0
B
5S
10S
18S
28S
0·1
0·05
TOP
BOTTOM

Figure 3.12

Translation of globin mRNAs prepared by different procedures. 0.6 µg of chicken globin mRNA prepared

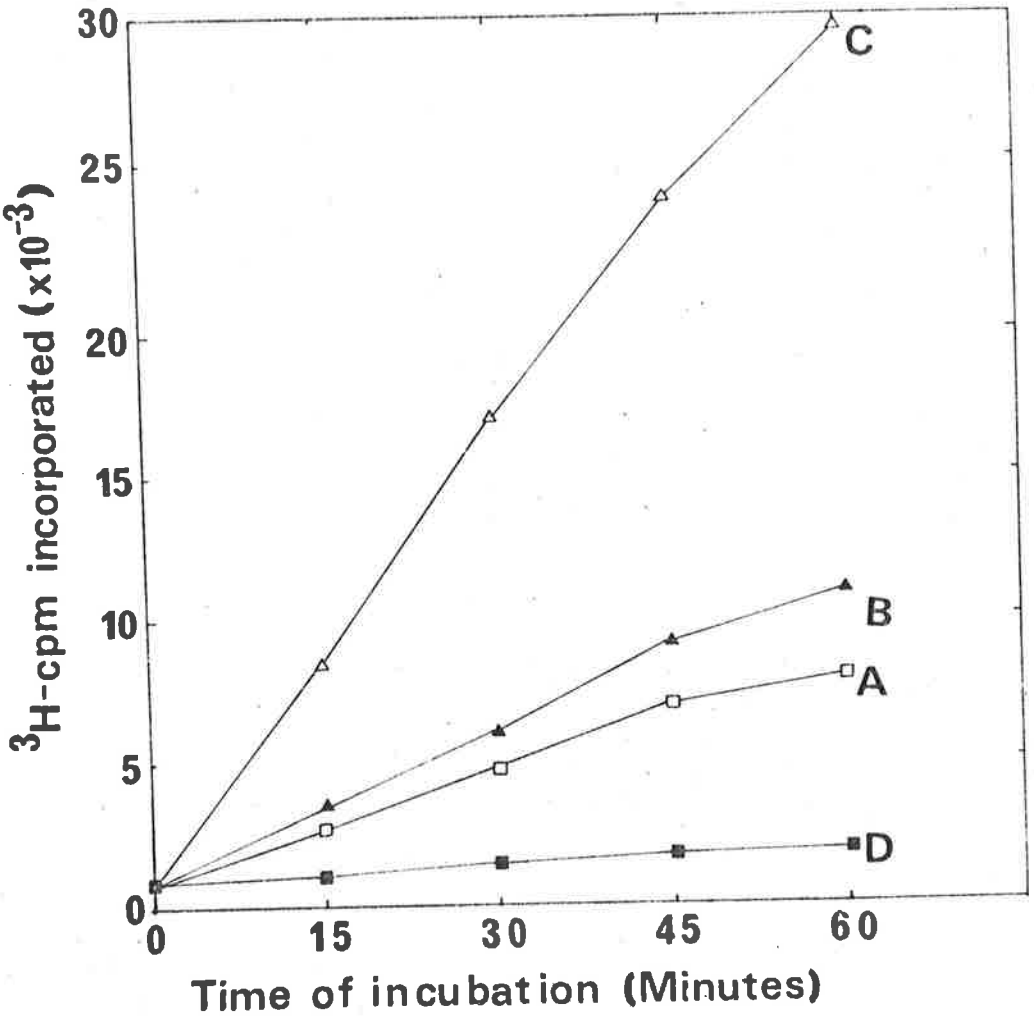
A. From phenol-extracted polysomes (figure 3.9B)

B. By binding to oligo (dT)-cellulose (figure 3.10B)

C. From RNP particles (figure 3.11B)

were translated in separate 50 µl assay mixtures and the incorporation into protein was measured.

D. Endogenous incorporation (No added mRNA).


30
25
20
15
10
5
0
^{3}H-cpm incorporated (x10^{-3})
C
B
A
D
0
15
30
45
60
Time of incubation (Minutes)

It was noted that treatment of the RNPs with Protease K gave the mRNA a longer storage life, possibly due to small amounts of ribonuclease which were present unless so treated. The only product detected on translation of the globin mRNA was globin (figure 3.8).

3.3 CHARACTERISATION OF GLOBIN mRNA

3.3.1 Purity of mRNA

Figure 3.8 has already demonstrated that the only major translation product of chicken globin mRNA was globin. Figure 3.13 shows that this mRNA gave two closely separated bands on electrophoresis on polyacrylamide gels containing formamide. By analogy to rabbit globin mRNA (Hamlyn and Gould, 1975) these are probably the mRNAs for α and β globin, and not due to contaminants. No other RNA species were visible on such gels.

The translation products were also analysed on Bio-gel columns when two labelled products were resolved, as shown in figure 3.14. By comparison with labelled standards, these corresponded to α and β globin, and could be employed to estimate the proportions of each globin made. Since the α and β globins contain different amounts of leucine (Matsuda *et al.*, 1971, 1973), the radioactive amino acid incorporated, this corresponded to an α:β ratio of 58:42 respectively. Hence the mRNA contains both α and β globin mRNAs.

A further indication of the purity of the mRNA can be obtained by copying with AMV reverse transcrip-

Figure 3.13

Electrophoresis of chicken globin mRNA on polyacrylamide gels in formamide. The position of known standards is indicated and used to calculate the molecular weight of the mRNA.

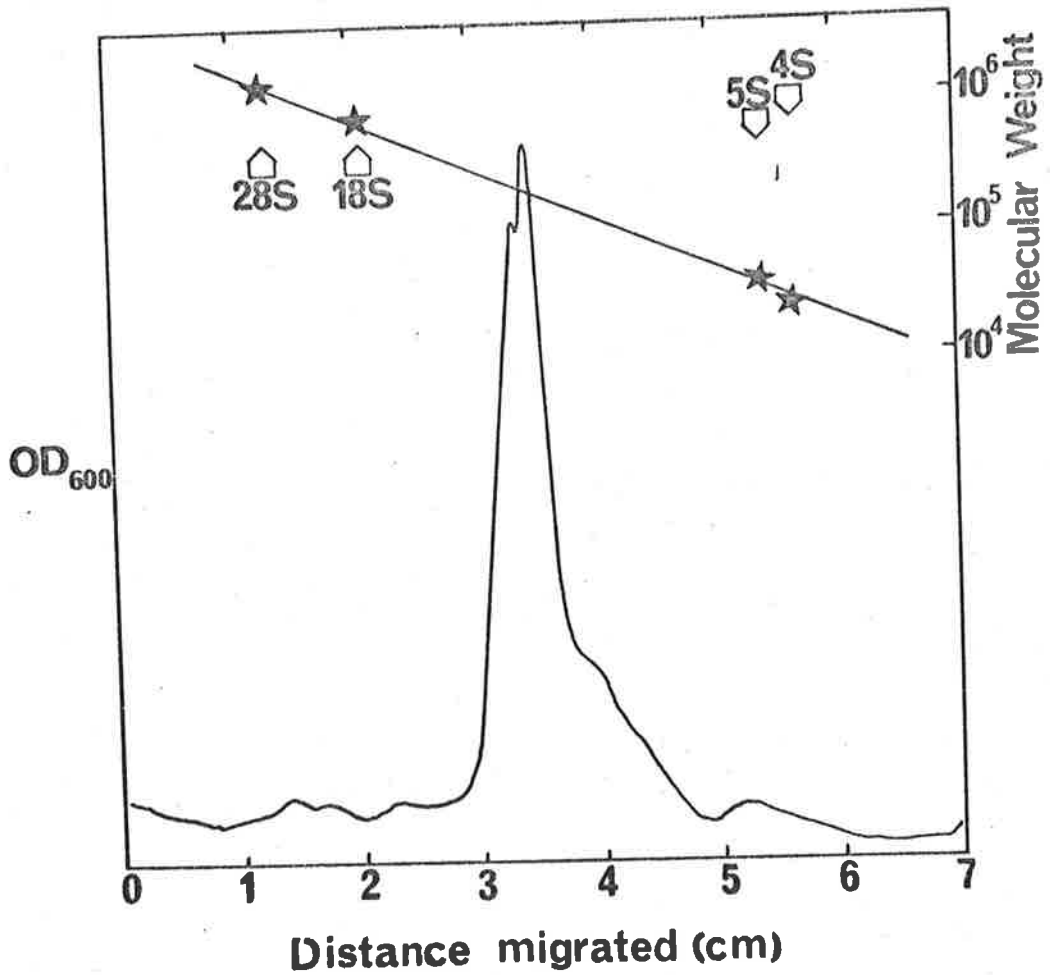
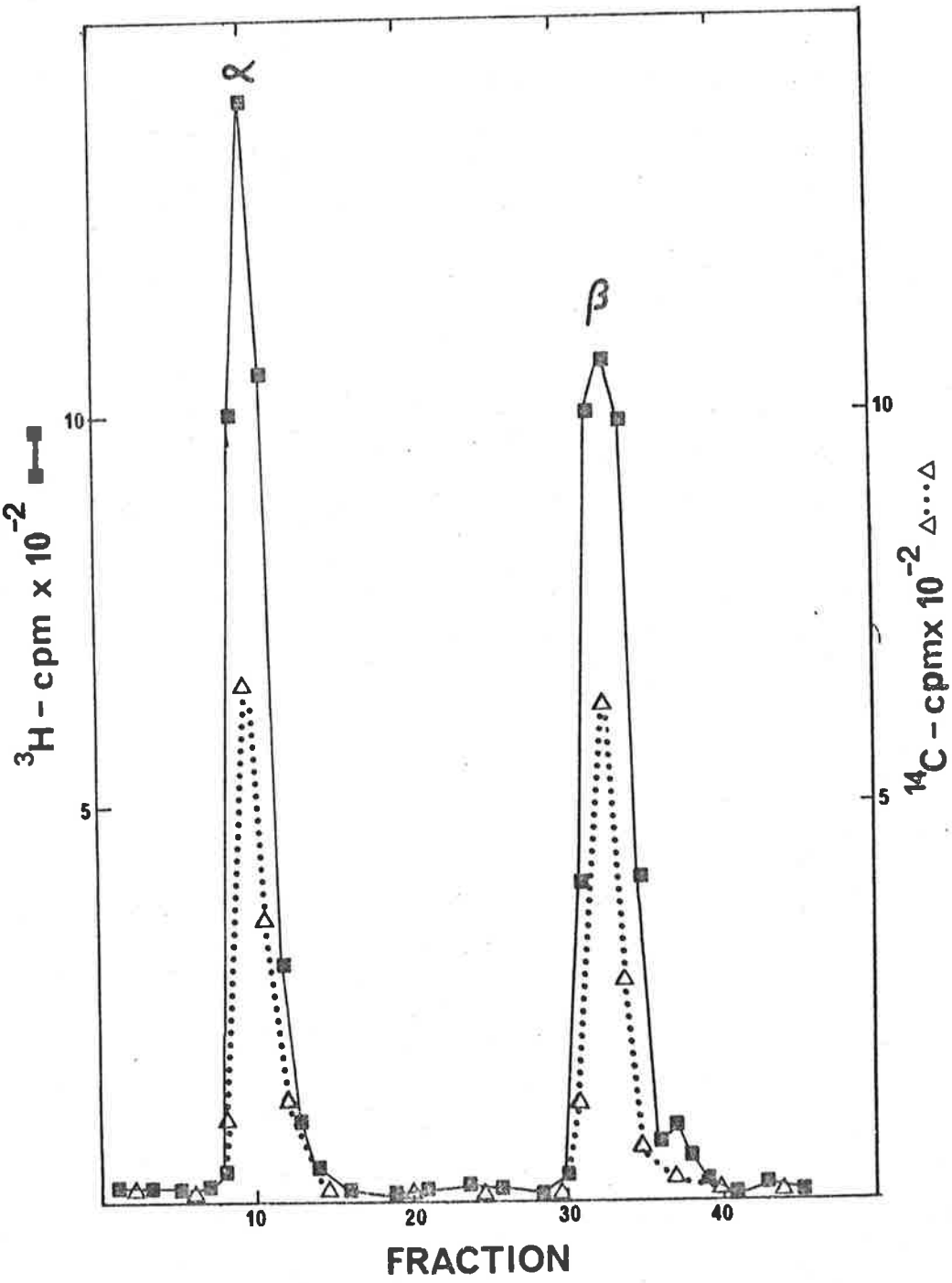

28S
18S
5S
4S
10^6
10^5
10^4
Molecular Weight
OD_{600}
0
1
2
3
4
5
6
7
Distance migrated (cm)

Figure 3.14

Analysis of the products of *in vitro* translation of chicken globin mRNA, on Bio-Rex 70 columns. The position of ^{14}C-labelled globin standards is indicated.


α
β
10
5
10
5
10
20
30
40
FRACTION
^{3}H - cpm x 10^{-2}
^{14}C - cpmx 10^{-2}

tase. This enzyme copies poly A-containing RNAs, once per molecule. The mRNA was copied to over 80% into full-length cDNA copies (R. Crawford, personal communication). Thus at least 80% of the RNA molecules contained a poly A tract and were not simply 10S-sized, non-mRNA contaminants.

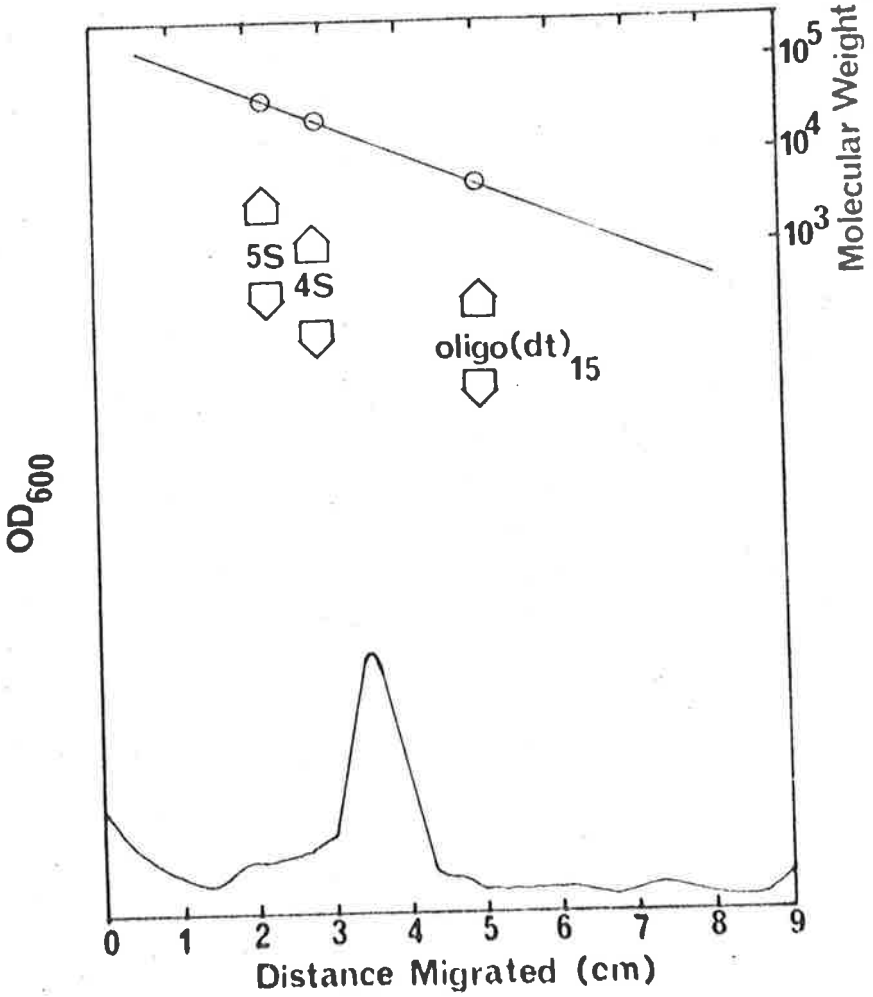
3.3.2 Size and Poly A Content

Figure 3.13 shows that chicken globin mRNA electrophoresed as two closely separated bands on 4% acrylamide gels in formamide. Since such gels remove secondary structure, they can be used to accurately measure molecular weight (Pinder *et al.*, 1974). Figure 3.13 also plots the mobility of the chicken globin mRNA against RNA species of known molecular weight. From this data, it was calculated that the mRNA had a molecular weight of 210,000 which corresponds to 650 nucleotides.

α globin contains 141 amino acid residues and β globin 146 (Matsuda *et al.*, 1971, 1973), and so require approximately 435 nucleotides to code for them. Obviously a large amount of the mRNA was not coding for completed globin. It was likely that at least some of the extra nucleotides were present as a poly A tract since the mRNA bound to oligo (dT)-cellulose (figure 3.10). The size of this tract was determined by degradation of the mRNA by T1 and pancreatic ribonuclease under conditions where all except poly A was degraded (see chapter 2). The undergraded poly A was then sized on polyacrylamide gels in formamide since it has been shown that poly A runs anomalously on

Figure 3.15

Estimation of the size of the polyadenylic acid tract from globin mRNA. 65 µg of globin mRNA was degraded leaving only the poly A tract. This was electrophoresed on a 10% polyacrylamide gel in formamide using oligo $(dT)_{15}$, 4S and 5S RNAs as standards. The positions of the RNAs were determined by scanning the gel at 280 nm before staining or at 600 nm after staining with toluidine blue.


10^5
10^4
10^3
Molecular Weight
5S
4S
oligo(dt)$_{15}$
OD_{600}
0 1 2 3 4 5 6 7 8 9
Distance Migrated (cm)

aqueous gels (Morrison *et al.*, 1973). Figure 3.15 shows that the poly A fragment prepared in this way from chicken globin mRNA had a molecular weight of 15,800 which corresponds to 46 adenylic acid residues.

3.4 CONCLUSIONS

Although globin mRNAs have been isolated from a variety of organisms, it was important to isolate and characterise this mRNA from chickens in order to use it as a control in the proposed isolation of H5 mRNA.

The major findings in this study were that, like other globin mRNAs, chicken globin mRNA is of 210,000 daltons in molecular weight, and consists of two similar RNA species, probably coding for α and β globin. The RNA has a poly A tract of about 50 residues in length. Earlier estimates of the length of such poly A tracts were much higher partly due to the use of non-denaturing gels where poly A runs anomalously (Morrison *et al.*, 1973). Another discrepancy arises from the method used to label the RNA before isolation and sizing of the poly A, since the length of the poly A tract is shortened with time (Sheiness and Darnell, 1973). Thus RNA labelled by a short pulse will be newly synthesised and with a longer tract than steady-state, long-term labelled RNA (Pemberton and Baglioni, 1973). The RNA used in this study was not labelled and so represents an average. The estimate agrees well with recent estimates for rabbit (Burr and Lingrel, 1971; Hunt, 1973) and mouse globin mRNAs (Morrison *et al.*, 1973).

The mRNA was readily prepared from ribonucleoprotein particles in a high degree of purity and could be accurately and efficiently translated in the wheat embryo cell-free system. The messenger coded for the synthesis of both α and β globin in similar amounts, and these were the only major products synthesised. The salt concentration for optimal translation of chicken globin mRNA was higher than that for rabbit globin mRNA, and full length chicken globin chains were made at this concentration. This is important since it has been shown that at the concentration giving optimal incorporation of labelled amino acids, full length proteins may not be made (Schmeckpeper *et al.*, 1974). Since the translation products were to be identified on polyacrylamide gels, this was essential.

CHAPTER 4

STUDIES ON THE PHYSICAL ISOLATION OF H5 mRNA

4.1 INTRODUCTION

Those mRNAs which have been purified are usually the major mRNA species present in the cell, e.g. globin mRNA in erythroid cells, immunoglobulin mRNA in the lymphocyte or myeloma (Milstein *et al.*, 1972) ovalbumin mRNA in the oviduct (Rhoads, McKnight and Schimke, 1971), silk fibroin mRNA from the silk gland of *Bombyx mori* (Suzuki and Brown, 1972), keratin mRNA from the feather (Partington, Kemp and Rogers, 1973). They are also characterised by an unusual size or some other feature which distinguishes them from other cellular RNAs, such as poly A content. These properties together form the basis of most published mRNA isolation procedures. The mRNA for H5 however, is atypical of this group as it is probably only a minor species (over 90% of the protein synthesised is globin (Sadgopal and Kabat, 1968)). This implies that large amounts of material must be processed and therefore large-scale preparative methods must be examined. Since the predicted size of H5 mRNA is 200,000-230,000 daltons, and the chicken globin mRNA is 210,000 molecular weight, this fractionation on the basis of size may be impractical, particularly in view of the much larger amount of globin mRNA present. However, chicken globin mRNA binds to oligo (dT)-cellulose (figure 3.10) while all other histone mRNAs isolated do not bind (Thrall *et al.*, 1974). Thus separation on this basis may be feasible.

The results in this chapter demonstrate that H5 constitutes only 4% of the protein made by the chicken

reticulocyte. The mRNA is undetectable in polysomal RNA fractionated on sucrose gradients but is detectable in the RNA which does not bind to oligo (dT)-cellulose. It is of approximately 10-12S in size and probably has no poly A tract. However, even under optimal fractionation conditions, the H5 mRNA is still not the major species detected, but globin mRNA. The procedures employed are therefore insufficiently selective to constitute an effective separation of pure H5 mRNA.

4.2 QUANTITATION OF H5 mRNA IN RETICULOCYTES

Appels and Wells (1972) have demonstrated that H5 is synthesised in chicken reticulocytes, but the percentage of total protein synthesis which is H5 has not been investigated. This could be a useful indication of the proportion of mRNA in the cell which codes for H5 although it assumes that mRNA is rate-limiting and that all mRNAs are translated at equivalent rates.

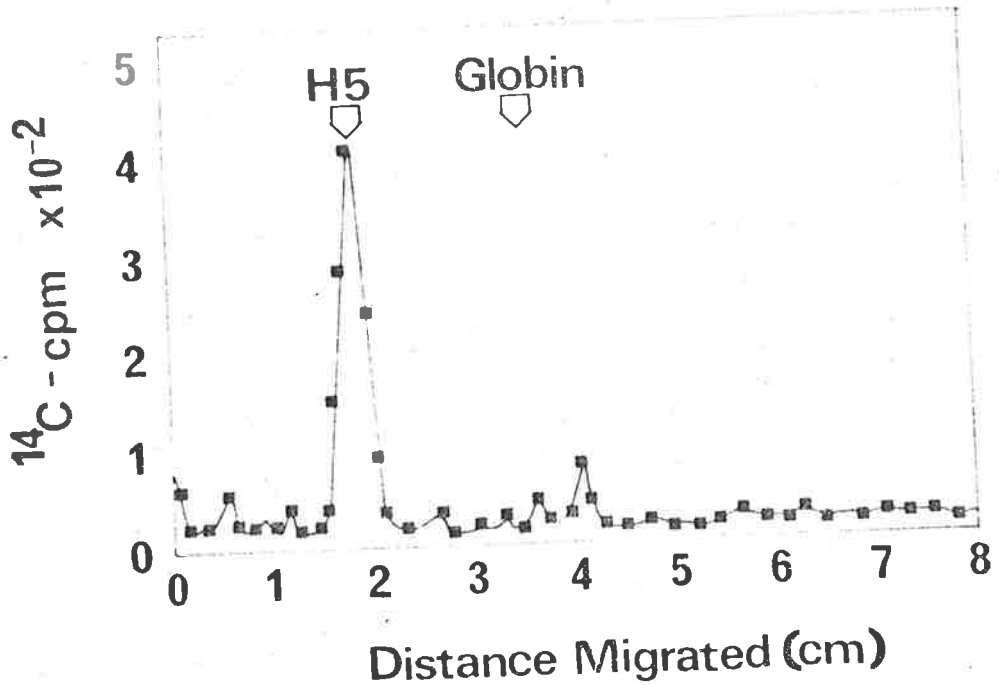
The problem was investigated by labelling cells *in vitro* with ^{14}C-leucine and then estimating the amount of label in H5, globin, and total protein. The amount of label incorporated into total protein was estimated by lysing cells with 20% TCA, and precipitating the protein on to glass fibre filters. By counting the filters one can estimate the counts per minute (but not the amount of ^{14}C-leucine) incorporated. Similarly, one can prepare clean nuclei and estimate the number of counts per minute incorporated

into nuclear proteins. In order to calculate the percentage of these counts which were incorporated into H5, proteins were extracted from the nuclei and fractionated on Swank and Munkres (1971) gels as described in chapter 2. As shown in figure 4.1, approximately 70% of the nuclear counts were in H5. The difficulty however was to convert the figures for counts per minute incorporated into moles of ^{14}C-leucine incorporated, which required an estimate of the counting efficiency. The efficiency was determined using ^{14}C-globin as a standard. The ^{14}C-globin was counted as an aqueous solution using a toluene-triton scintillation fluid and by adding ^{14}C-toluene as an internal standard, the specific activity of the protein was determined. Having standardised this protein, it could then be added to the TCA precipitates in order to calculate the counting efficiency under these conditions. The figures together were used to calculate the percentage of total protein synthesis which was H5, as 3.8%.

These calculations are subject to several errors, since they assume that there are no effects due to preferential loss or extraction. For this reason, the final figure of 3.8% can only be used as an indication. Using the same approach, however, the percentage of protein synthesis which is globin was calculated to be 92%, which agrees well with previous estimates.

Figure 4.1

Analysis of the radioactive proteins in the nuclei of reticulocytes labelled with ^{14}C-leucine, on SDS-urea polyacrylamide gels.


H5
Globin
^{14}C - cpm x10^{-2}
5
4
3
2
1
0
0
1
2
3
4
5
6
7
8
Distance Migrated (cm)

4.3 SIZE-DEPENDENT SEPARATION OF H5 mRNA

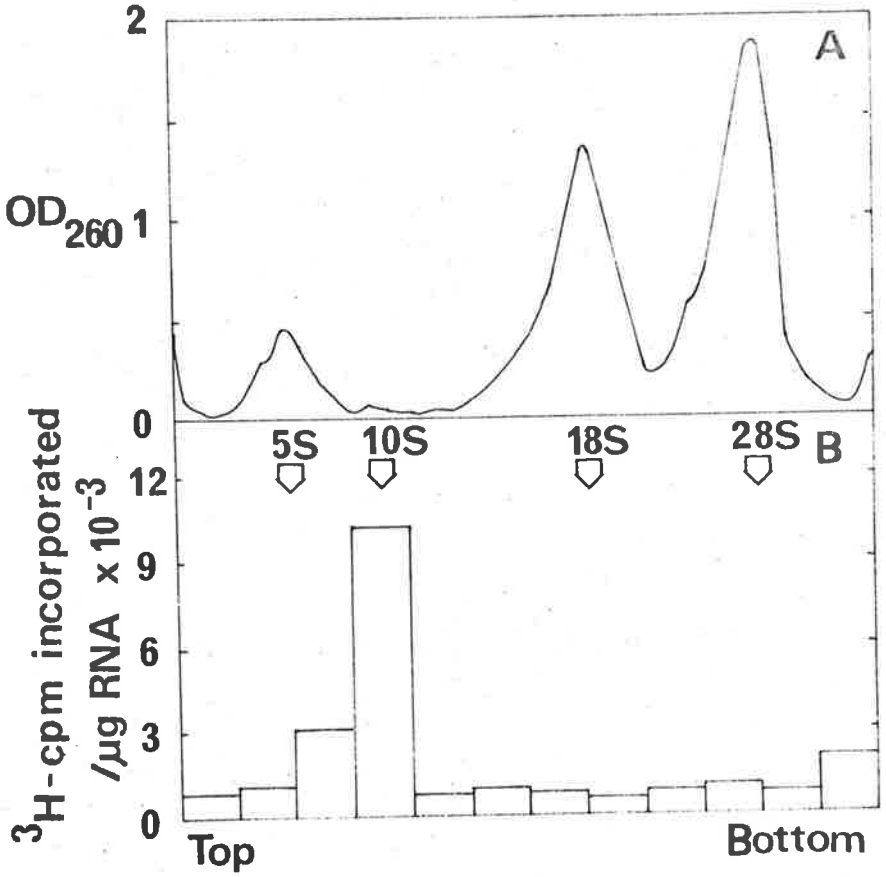
Fractionations based on the size of RNA have the advantage that they are simple to perform and large amounts of RNA can be handled. Since H5 mRNA is probably present as a minor species in chicken reticulocytes, the ability to handle bulk amounts is important and thus size-dependent fractionations were investigated as, at least, a partial separation procedure.

The RNA to be fractionated was prepared by extraction of reticulocyte polysomes with phenol to yield total polysomal RNA. Brawerman, Mendecki and Lee (1972) have shown that the pH of the extracting medium has a marked effect on the partition of poly A-containing RNA between aqueous and phenol phases. Thus far more poly A-containing RNA is extracted into the aqueous phase at pH 9.0 than at pH 7.0, and this can be used to partially fractionate RNA on the basis of poly A content. Since the presence or absence of a poly A tract in H5 mRNA was unknown, RNA was extracted at pH 9.0, 7.0, and 5.4 (Thrall *et al.*, 1974). When this RNA was fractionated on sucrose gradients (figure 4.2a), the OD_{260} profiles were indistinguishable. This was predictable however, as the only major species present were the ribosomal RNAs. Fractions from this gradient were translated in the wheat embryo translation system to identify mRNA. As indicated in figure 4.2b, only the region around 10S significantly stimulated translation *in vitro*, even at high RNA concentrations. When the products of these translations were electrophoresed on SDS-urea gels the only major

Figure 4.2

A. Fractionation of RNA extracted from reticulocyte polysomes on sucrose gradients.

B. Efficiency of translation of polysomal RNA fractions in the wheat embryo cell-free system.


2
A
OD260 1
0
5S
10S
18S
28S
B
12
9
6
3
0
3H-cpm incorporated
/µg RNA x 10-3
Top
Bottom

product detected was globin, however, at all pH values (figure 4.3). This should not be taken to indicate that the RNA contains no H5 mRNA as there would still be a large excess of globin mRNA present. In fact, Knochel (1975) has recently demonstrated that such RNA preparations do contain H5 mRNA but that in order to detect their translation it was necessary to preferentially extract them from the cell-free system. For the purpose of this study, it is sufficient to note that globin and H5 mRNAs are probably not separable on sucrose gradients, although this cannot be stated with certainty as H5 mRNA was not detected.

4.4. FRACTIONATION OF H5 mRNA ON OLIGO (dT)-CELLULOSE

A variety of methods are available for the separation of RNA according to its content of polyadenylic acid. These include phenol extraction at different pHs (Brawerman *et al.*, 1972) and the preferential binding of poly A-containing RNAs to such materials as millepore filters (Lee, Mendecki and Brawerman, 1971), unmodified cellulose (Schutz, Beato and Feigelson, 1972), poly U bound to filters (Kates, 1973), cellulose (Kates, 1970), and sepharose (Lindberg and Persson, 1972), or oligo (dT) bound to cellulose (Aviv and Leder, 1972). The most widely used of these is oligo (dT)-cellulose since it can be rendered ribonuclease-free by washing with alkali, has a high capacity, and is simple to prepare and use. It was used in this study to attempt fractionation of H5 mRNA on the basis of its possible lack of a poly A tract (see chapter 3).

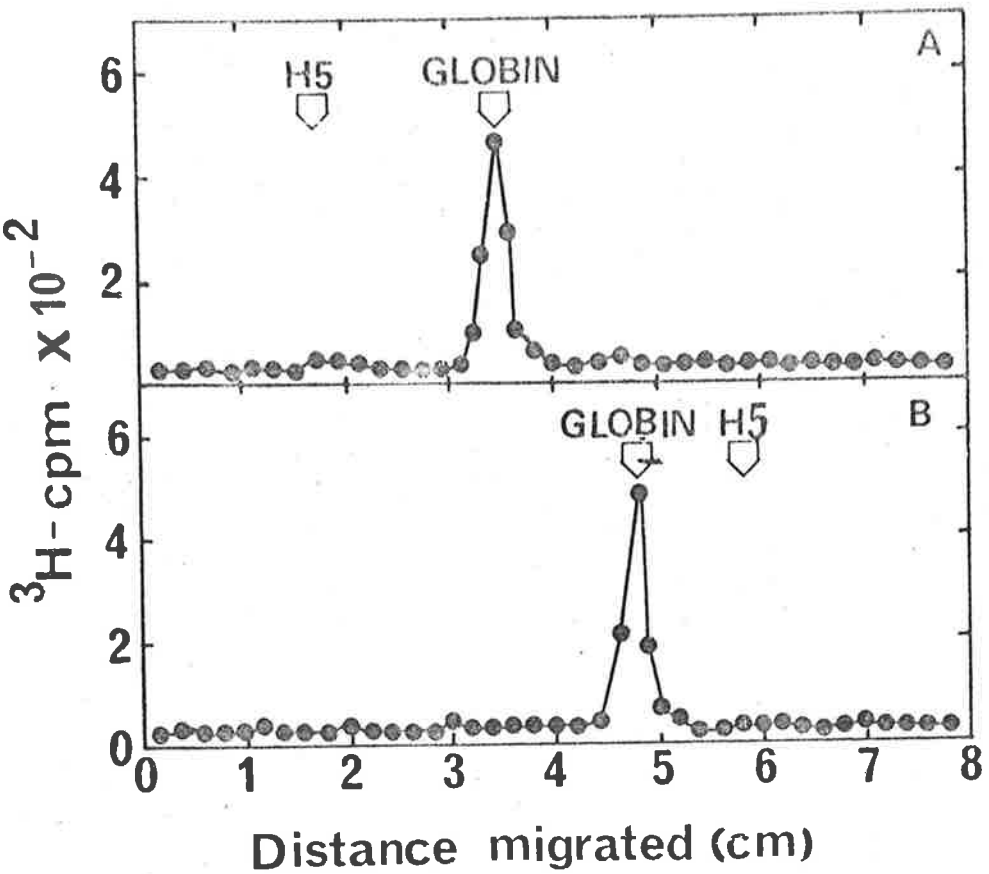
Figure 4.3

Analysis of the products produced by translating 10S polysomal RNA *in vitro*. The position of ^{14}C-labelled standards is indicated.

A. SDS-urea gels

B. Low pH-urea gels

Electrophoresis was from left to right in both cases.


A
H5
GLOBIN
B
GLOBIN
H5
^{3}H-cpm X 10^{-2}
0
2
4
6
0
1
2
3
4
5
6
7
8
Distance migrated (cm)

Total polysomal RNA was extracted as in section 4.3 and fractionated on oligo (dT)-cellulose as described in chapter 2. The bound (figure 4.4) and unbound (figure 4.5) RNAs were then fractionated on sucrose gradients and the fractions were translated *in vitro*. These results (figures 4.4 for bound, 4.5 for unbound) again indicated that only the 10S region would stimulate translation, in both cases. However, when the translation products were identified on gels, some H5 mRNA was detected in the unbound RNA (figure 4.6) but not in the bound RNA (figure 4.7). In both cases, however, the major product was still globin.

These results indicated that globin mRNA preferentially bound to oligo (dT)-cellulose but that a significant amount did not bind. The presence of subfractions of mRNA without poly A (Milcarek *et al.*, 1974; Nemer *et al.*, 1974) means that this result is not unique but is unfortunate in that though H5 mRNA does not bind, there was still too much globin mRNA present in the unbound RNA to make this procedure useful as a preparative method on its own.

4.5 CONCLUSIONS

The results in this chapter clearly indicate that active H5 mRNA can be isolated from polysomes and translated to produce full-length H5 *in vitro*. However, as predicted, the size of this mRNA is sufficiently close to globin mRNA to make separation of these two impossible on sucrose gradients. It is encouraging in another sense, however, as it should be possible to

Figure 4.4

A. Fractionation of the polysomal RNA which binds to oligo (dT)-cellulose on sucrose gradients.

B. Efficiency of translation of 'bound' RNA fractions in the wheat embryo cell-free system.

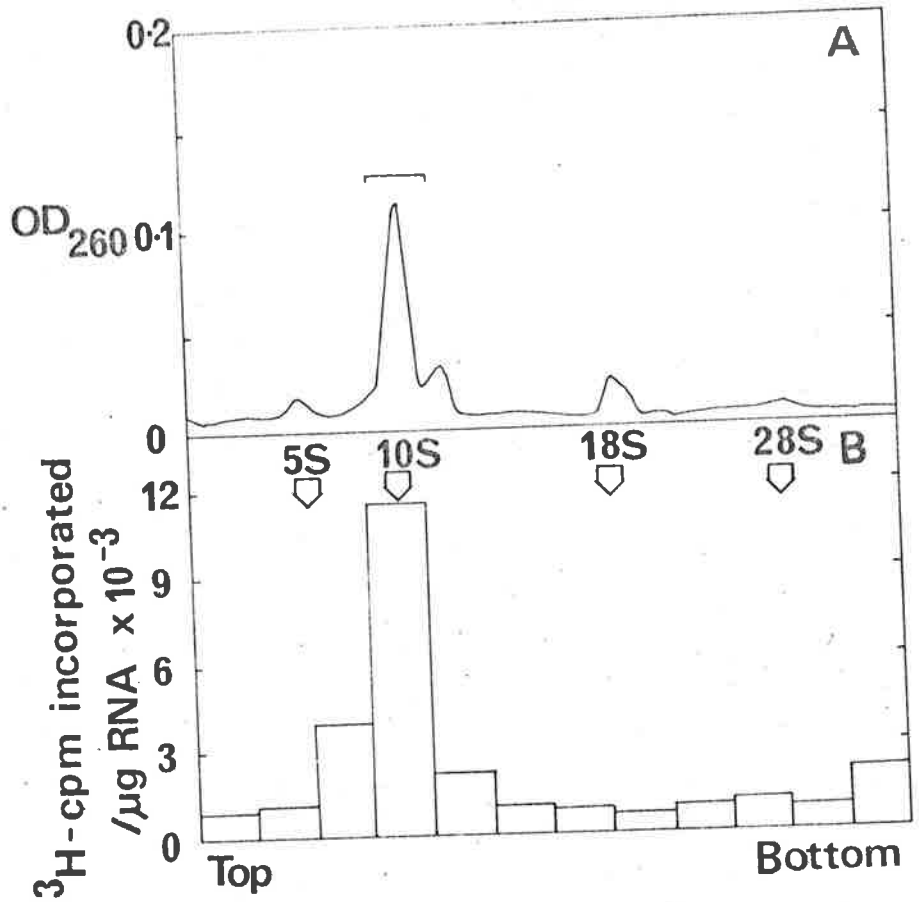

0·2
A
OD260 0·1
0
5S
10S
18S
28S
B
12
9
6
3
0
3H-cpm incorporated
/µg RNA x 10-3
Top
Bottom

Figure 4.5

A. Fractionation of the polysomal RNA which does not bind to oligo (dT)-cellulose on sucrose gradients.

B. Efficiency of translation of 'unbound' RNA fractions in the wheat embryo cell-free system.

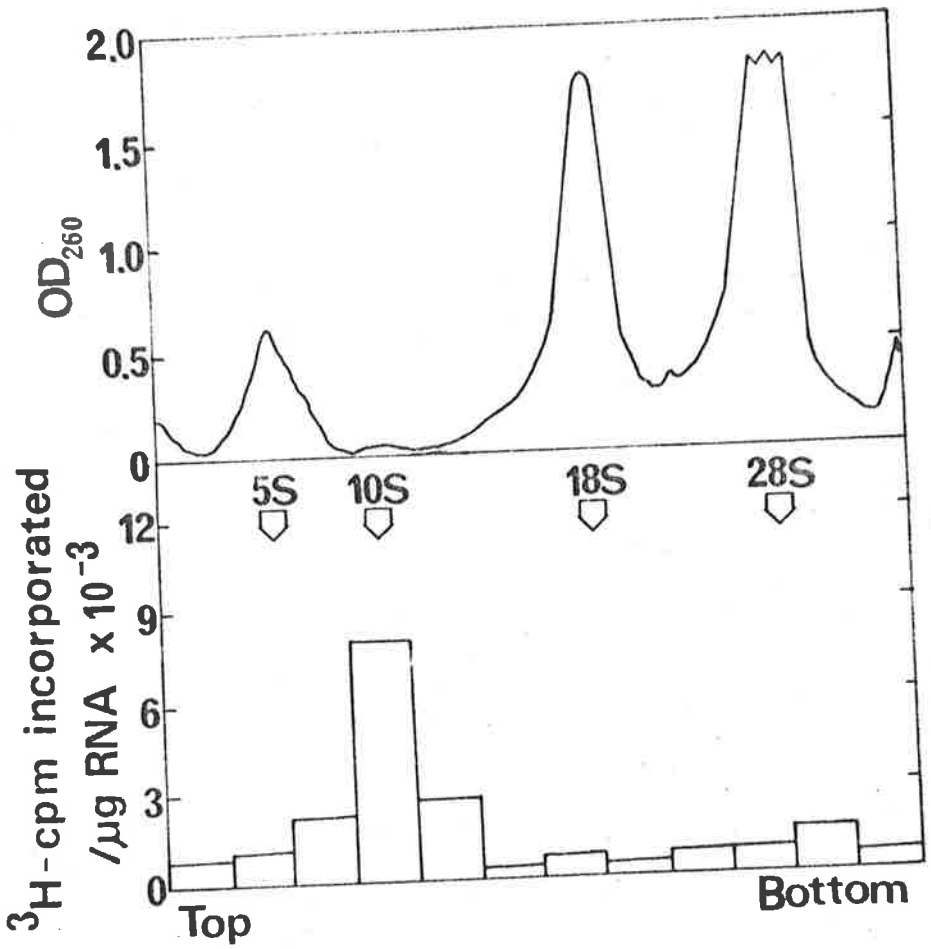

2.0
1.5
1.0
0.5
0
OD260
5S
10S
18S
28S
12
9
6
3
0
3H-cpm incorporated
/µg RNA x 10-3
Top
Bottom

Figure 4.6

Identification of the products of *in vitro* translation of 10S RNA which does not bind to oligo (dT)-cellulose.

A. SDS-urea gels

B. Low pH-urea gels

Electrophoresis was from left to right in both cases. The position of ^{14}C-labelled standards is indicated.

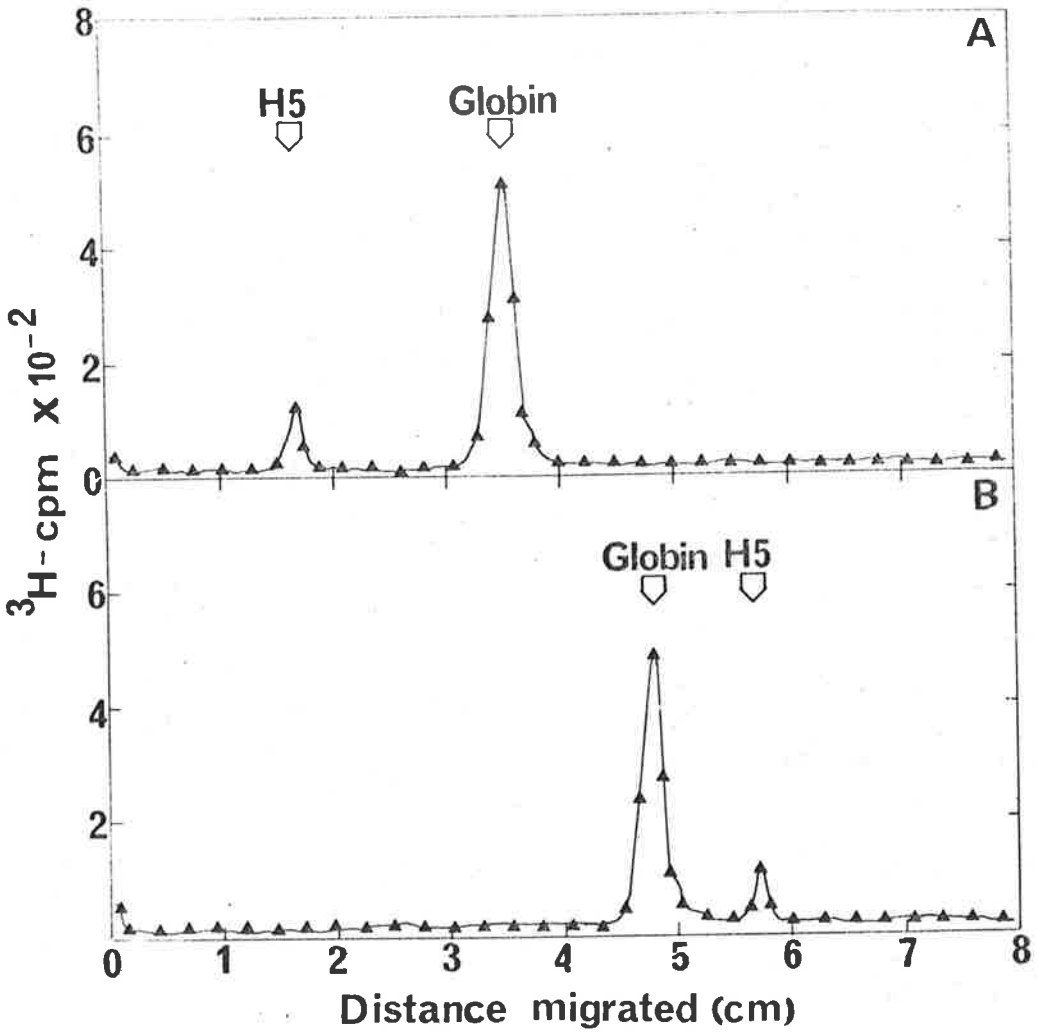

A
H5
Globin
B
Globin H5
^{3}H-cpm X 10^{-2}
Distance migrated (cm)
0
1
2
3
4
5
6
7
8

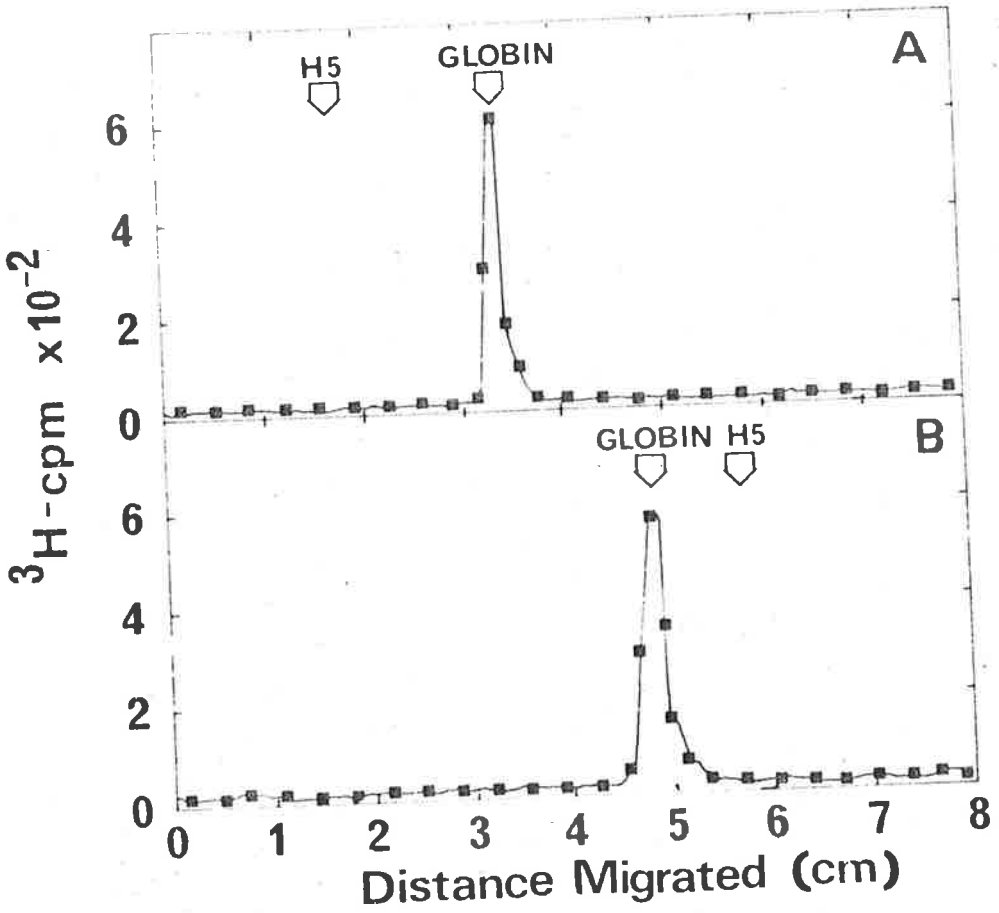
Figure 4.7

Identification of the products of *in vitro* translation of 10S RNA which binds to oligo (dT)-cellulose.

A. SDS-urea gels

B. Low pH-urea gels

Electrophoresis was from left to right in both cases. The position of ^{14}C-labelled standards is indicated.


H5
GLOBIN
A
GLOBIN H5
B
^{3}H-cpm x10^{-2}
0
2
4
6
0 1 2 3 4 5 6 7 8
Distance Migrated (cm)

separate H5 mRNA from the ribosomal RNAs simply on sucrose gradients. Although the fractionation on oligo (dT)-cellulose was insufficient to yield pure H5 mRNA, the result is nevertheless encouraging in that it provides a method for removing *most* of the globin mRNA and may prove useful in combination with other techniques.

Since such closely similar mRNAs as those coding for α and β globin (Hamlyn and Gould, 1975) and the histones (Levy *et al.*, 1975) can be separated on high resolution polyacrylamide gels, such a technique may be useful for separation of globin and H5 mRNAs. However, such fractionations rarely produce pure mRNA and in this case there is an excess of the globin mRNAs which would complicate the process. The basic problem is that there are insufficient clear-cut differences between the mRNAs to allow a decisive fractionation. Recently immunological procedures have been used to isolate the polysomes synthesising specific proteins (Shapiro *et al.*, 1974; Palacios *et al.*, 1973; Schechter *et al.*, 1974; Boyer *et al.*, 1974). Since these rely on differences in the proteins which are readily differentiated by antibodies, such procedures were investigated as a possible alternative to separation on physical properties of the mRNA.

CHAPTER 5

IMMUNOLOGICAL ISOLATION OF H5 mRNA

5.1 IMMUNOADSORPTION

5.1.1 Introduction

mRNA molecules are polymers of 4 basic units and in terms of physical properties are quite similar. It is therefore difficult to separate them unless there are gross differences in size, poly A content, or buoyant density, and they are present in fairly large amounts. This restricts mRNA isolation to a few specific systems which possess these properties. H5 mRNA is a good example since it is difficult to purify it away from the much larger amounts of globin mRNA. Proteins, on the other hand, are polymerised from over twenty basic units and take up stable, specific conformations. They can therefore be recognised by antibodies which can differentiate quite readily between very closely related proteins (Boyer *et al.*, 1974). Hence the resolution based on differences in proteins is potentially far higher than that which can be attained by employing differences in the mRNAs coding for them. This can be used to isolate mRNAs by precipitating the nascent peptides on polysomes making a particular protein. The mRNA can then be isolated from these polysomes. In practical terms, however, there are inherent problems in such an approach. For example, though antibodies were used to isolate the polysomes making α-glucosidase and thus prepare the mRNA, the mRNA was not pure and was partially degraded (Hartlief and Koningsberger, 1968). The specificity of the reaction was reported to be low due to the binding of immunoglobulin to polysomes

(Holme *et al.*, 1971). This binding was apparently via the F_c portion of the immunoglobulin (Kern, Helmreich and Eisen, 1961; Swanson *et al.*, 1969) which led to the use of $F(ab')_2$ fragments to improve the specificity (Holme, Boyd and Sehon, 1971; Delovitch *et al.*, 1972). All of these procedures relied upon direct precipitation of the nascent peptides by the added antibody which necessitated a high concentration of polysomes and antibody to form a precipitate. These conditions were probably the cause of the lack of specificity (Palacios *et al.*, 1973) and they can easily be overcome by indirectly precipitating the antibody-polysome complexes. The first method of doing this was by binding small amounts of antibody to diluted polysomes and precipitating these with an immunoadsorbent consisting of either insolubilised antigen (Palacios *et al.*, 1973) or antigen bound to an insoluble support such as aminocellulose (Sidorova, Trudolyubova and Lerman, 1974).

Since H5 mRNA proved difficult to separate on a physical basis these procedures were investigated as a possible alternative.

5.1.2 Preparation of H5 mRNA

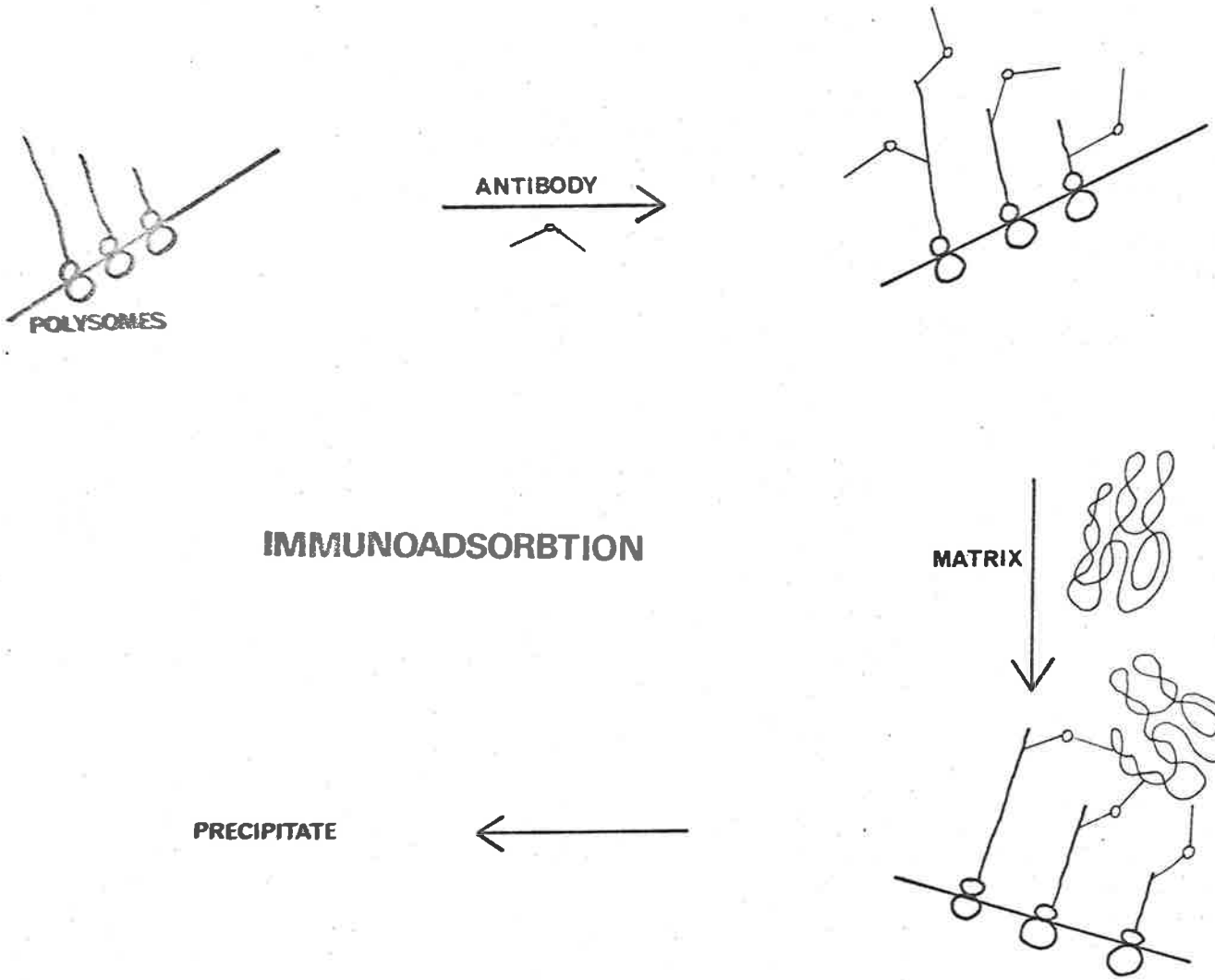
(1) General considerations

The overall rationale of the immunoadsorption reaction is illustrated in figure 5.1. The specificity of the reactions is affected by three factors:-

(i) The inoic strength. Non-specific adsorption of antibodies to polysomes is much

Figure 5.1

Rationale of the immunoadsorption reaction.


ANTIBODY
POLYSOMES
IMMUNOADSORBTION
MATRIX
PRECIPITATE

greater at low ionic strength (Sidorova *et al.*, 1974). The specificity of the reaction was maximised by carrying it out in the presence of 0.14 M KCl.

(ii) The specificity of the antibody. Any contaminating antibodies, particularly if reactive against chicken globin, may precipitate unwanted polysomes. It is also desirable to add as little protein as possible to reduce the chances of ribonuclease contamination. Antibody preparations must therefore be as highly purified and specific only to H5, as possible.

(iii) The size of the precipitate. Polysomes can easily be trapped within the antibody-antigen matrix and are difficult to remove by conventional washing procedures (Palacios *et al.*, 1973). It is therefore essential to use the minimum amount of antibody and antigen matrix which will precipitate the polysomes, although the reaction must also occur in a reasonable time and at low temperatures to minimise ribonuclease attack. Thus it is essential to optimise yield while minimising contamination.

These factors are dealt with below.

(2) Specificity of the reaction

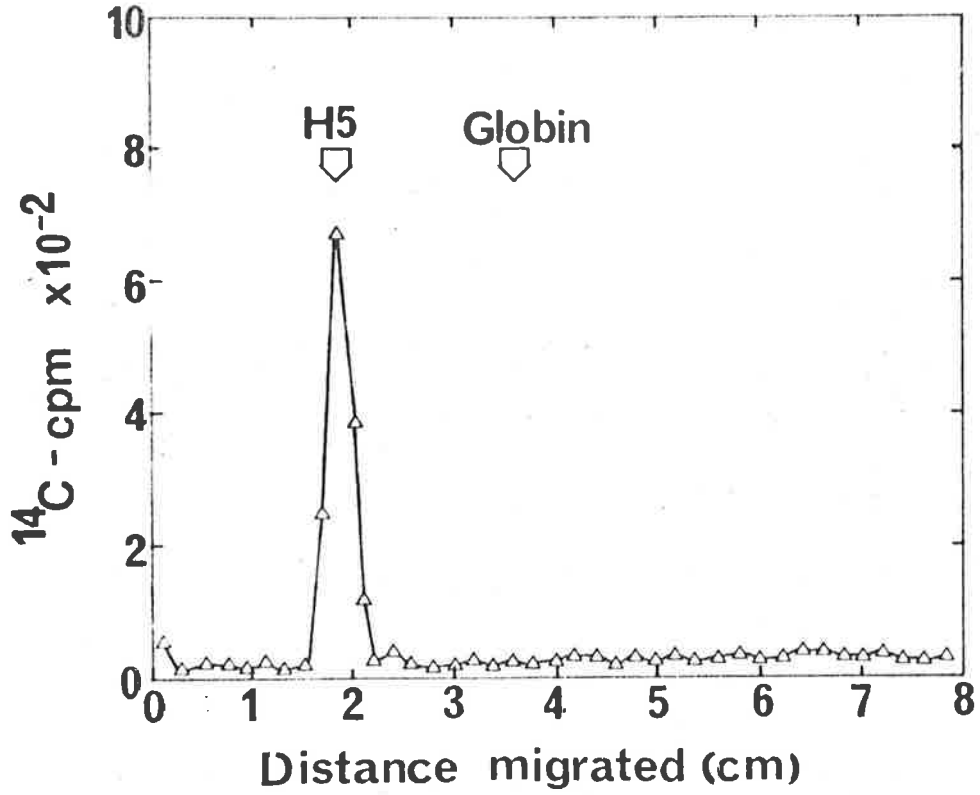
This was ensured by extensive purification of the reagents used. Hence the anti-H5 was induced by injection of electrophoretically pure H5 into rabbits.

The purified gamma globulin (chapter 2) was then chromatographed on a globin-Sepharose affinity column which removed any anti-globin activity (since this is the major protein being made and therefore the most likely contaminant) as well as any antibodies which bind non-specifically. The unbound antibody was then chromatographed on a column of H5-Sepharose to which anti-H5 binds. Thus most of the other gamma globulin was removed and the specificity of the antibody assured. Although the resulting antibody would only react with H5, and not with chicken globin, chicken H1 or calf thymus histone on an Ouchterlony diffusion plate, this assay is not sensitive enough to detect small amounts of other antibodies. The contaminating protein which is most critical is globin, and thus the specificity of the antibody was tested by adding a twenty-fold excess of ^{14}C-globin to ^{14}C-H5 and precipitating this with purified anti-H5. The precipitate was washed and dissociated by electrophoresis on SDS-Urea gels. The labelled proteins precipitated by the anti-H5 were then identified by slicing and counting the gel. As shown in figure 5.2, only ^{14}C-H5 was precipitated by the anti-H5 even in the presence of a large excess of ^{14}C-globin.

The final purification of the antibody by chromatography on a combined CM-cellulose/DEAE-cellulose column was designed to remove ribonuclease. The efficiency of this process was gauged by incubation of purified 18S and 28S rRNA with the antibody at various stages of purification. The RNA was then

Figure 5.2

Analysis of the proteins precipitated by purified anti-H5 from a mixture of ^{14}C-H5 and ^{14}-C-globin, on an SDS-urea gel.


H5
Globin
^{14}C - cpm x10^{-2}
Distance migrated (cm)
0
2
4
6
8
10
0
1
2
3
4
5
6
7
8

treated with formamide to expose any hidden breaks by removing secondary structure, and the RNA was examined on sucrose gradients. As shown in figure 5.3, the final purified antibody has little if any ribonuclease activity.

The immunoadsorbent used in this study was a matrix of protein cross-linked with glutardehyde (Palacios *et al.*, 1973) to render it insoluble. In order to maintain the minimum sized pellets, pure H5 should theoretically be used to prepare this matrix. However this required large amounts of H5 which was difficult to prepare, and hence total histone was employed as the matrix material. This contains about 25% H5 (Appels, 1971) and is readily prepared in large amounts. Total histone can be used since H5 is the only histone being made in the reticulocyte, and the specificity of the reaction is ensured by the antibody used. The only problem may be in the increase in non-specific entrapment caused by the larger pellet. This is dealt with in the next section.

(3) Optimisation of the reaction

There are three variables which must be optimised to obtain the highest degree of specificity. These are:-

(i) the concentration of polysomes which must not be too high (Palacios *et al.*, 1973),

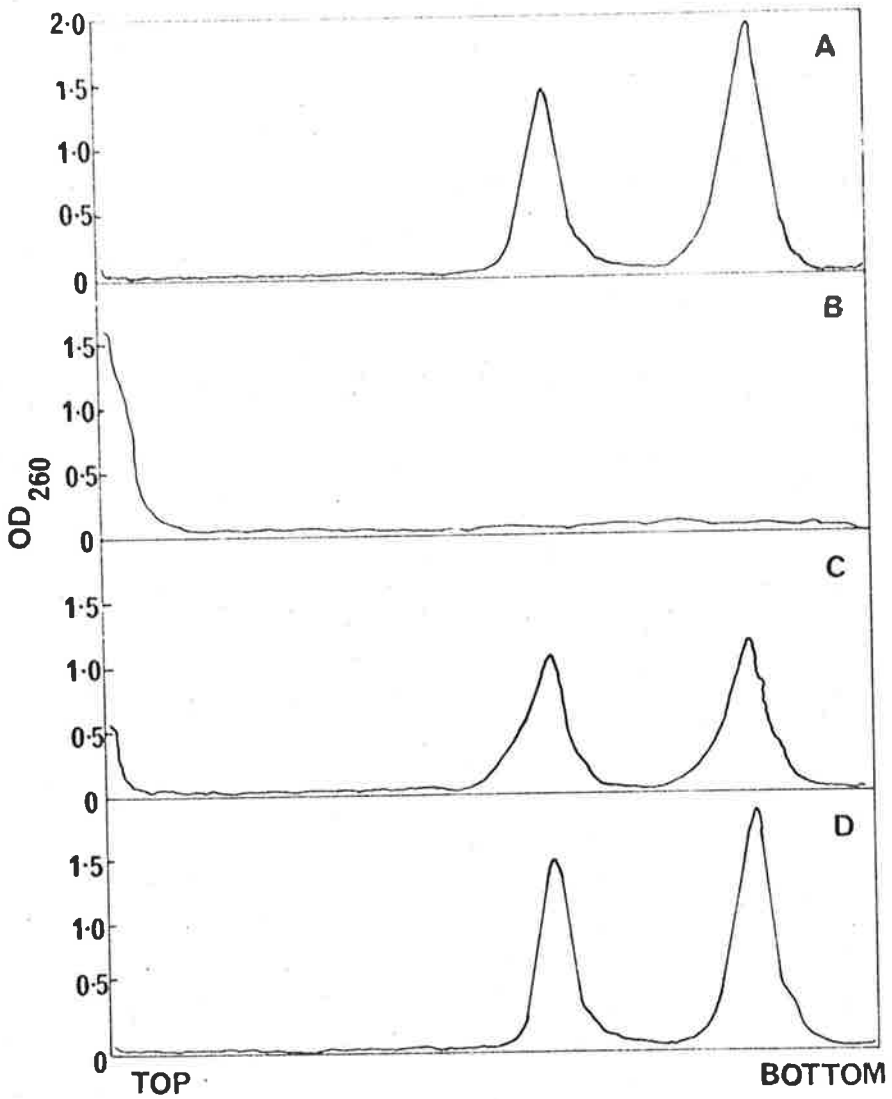
(ii) the amount of antibody and antigen matrix added, which must give maximum yield without compromising specificity,

(iii) the time of incubation of the reagents,

Figure 5.3

Analysis of ribonuclease activity in anti-H5. Sucrose gradient fractionation of 18S and 28S RNA after incubation with anti-H5 at various stages of purification.

A. Unincubated 18S and 28S RNA
B. Incubated with clarified serum
C. Incubated with affinity-column purified antibody
D. Incubated with final antibody preparation.


2·0
1·5
1·0
0·5
0
A
B
C
D
OD 260
TOP
BOTTOM

which must allow a reasonable yield with minimum ribonuclease breakdown.

Figure 5.4a shows a titration curve for the precipitation of anti-H5 by H5, which demonstrated that 1 mg of anti-H5 was precipitated by 35 µg of H5. As shown in figure 5.4b, however, it required 285 µg of total histone matrix to precipitate 1 mg of anti-H5. Thus if 25% of the matrix was H5, this means that not all the H5 was available for reaction with antibody. This figure was used to calculate the amount of matrix required to precipitate a given quantity of anti-H5, and it only remained to calculate the amount of anti-H5 to add. Before this could be done, however, it was important to find the minimum time required for the reaction at 0°C, the temperature required for minimum ribonuclease activity. As shown in figure 5.5, the reaction of anti-H5 with histone matrix (as measured by removal of antibody from the supernatant) was essentially complete within 60 minutes of addition.

Having calculated these two parameters, it was then possible to optimise the concentration of polysomes to use by carrying out the reaction with constant amounts of antibody and varying the concentration of polysomes. The yield was measured by extracting the RNA from the precipitate with phenol and quantitating this. As shown in figure 5.6, above 20 A_{260} of polysomes per millilitre, there was little increase in the yield of RNA. There could be several reasons for this, such as:-

(i) insufficient antibody to precipitate more

Figure 5.4

Precipitin curve for the precipitation of

A. Pure H5

B. Total histone matrix

by 1 mg of purified anti-H5.

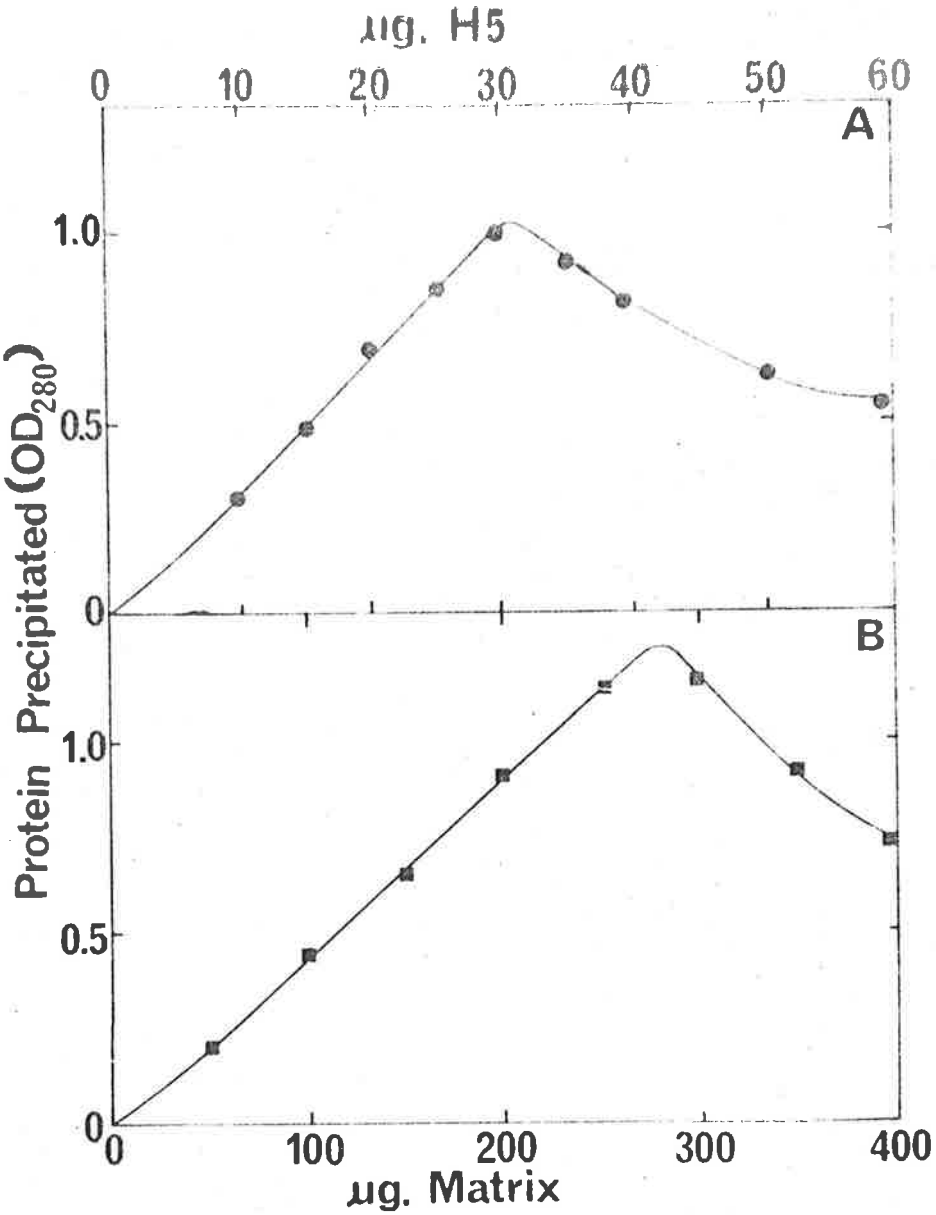

µg. H5
0
10
20
30
40
50
60
A
1.0
0.5
0
B
1.0
0.5
0
0
100
200
300
400
µg. Matrix
Protein Precipitated (OD280)

Figure 5.5

Time course of the precipitation of H5 by anti-H5 at 0°C.

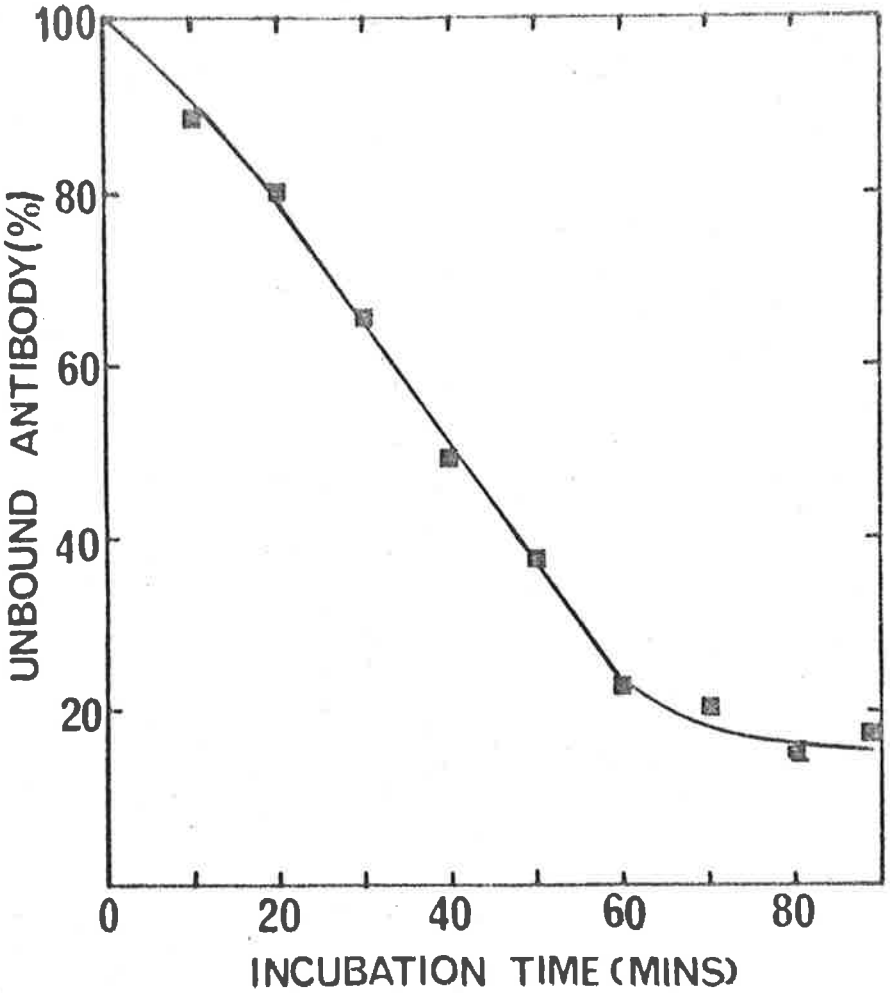
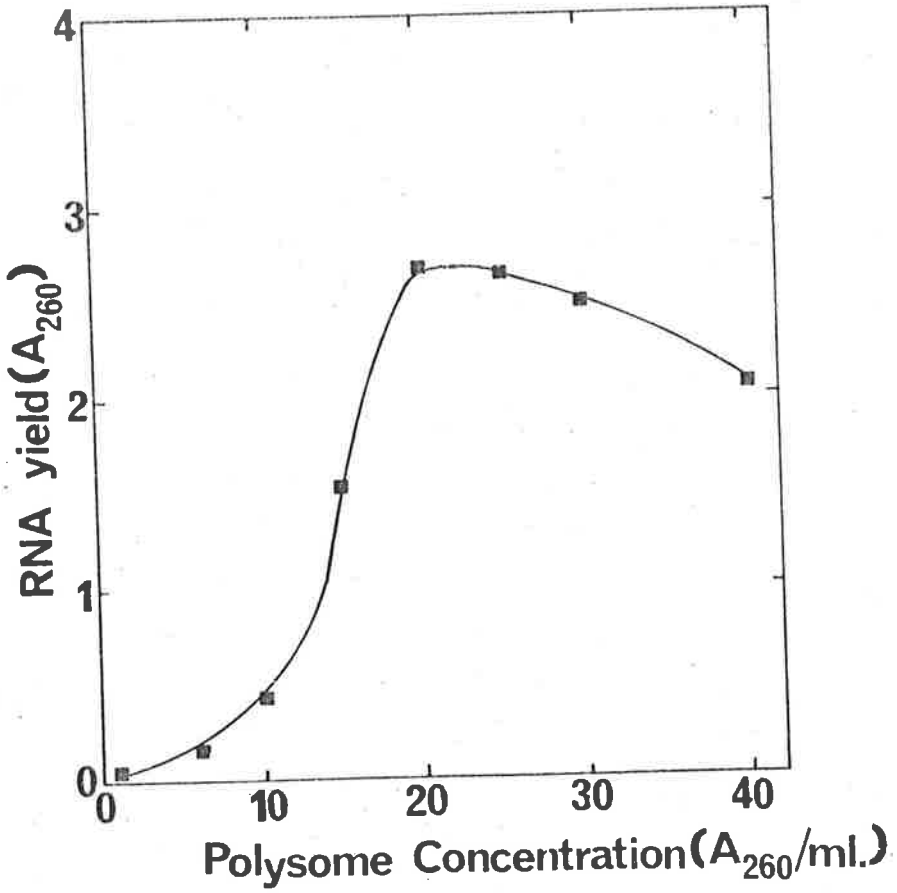

100
80
60
40
20
UNBOUND ANTIBODY(%)
0
20
40
60
80
INCUBATION TIME (MINS)

Figure 5.6

Effect of polysome concentration on the precipitation of polysomes by the immunoadsorption reaction.


4
3
2
1
0
0
10
20
30
40
RNA yield(A_{260})
Polysome Concentration(A_{260}/ml.)

polysomes,

(ii) inhibition of the reaction above this figure. The first of these is unlikely as the same conclusion was reached at several different antibody concentrations. Thus the second possibility seems to be the explanation although the reason why this occurred was not examined. In a similar way, the amount of antibody to add was investigated by adding increasing amounts of antibody (plus the appropriate amount of matrix) at different polysome concentrations. As shown in figure 5.7, the yield of RNA did not increase much at over 1 mg of antibody per 400 A_{260} of polysomes. Thus the amount of antibody to add and the concentration of polysomes required for optimal yield were calculated. However, it was essential that the mRNA was pure and the yield was secondary to this. Hence it was essential to calculate the purity of the mRNA at various values of these parameters. This was carried out by extracting the RNA from the washed matrix, fractionating on a sucrose gradient and testing the RNA for mRNA activity. As shown in figure 5.8, mRNA activity was detected only in the 10S RNA fractions. When these translation products were examined on SDS-Urea gels, however, it was found that the maximum yield did not coincide with the maximum purity of the mRNA. As shown in figure 5.9, the 10S RNA extracted at increasing polysome concentrations contained mostly H5 mRNA; however above 15 A_{260} per ml, the amount of globin present increased substantially. Similarly, the translation products programmed by the

Figure 5.7

Effect of antibody concentration on the precipitation of polysomes by the immunoadsorption reaction.

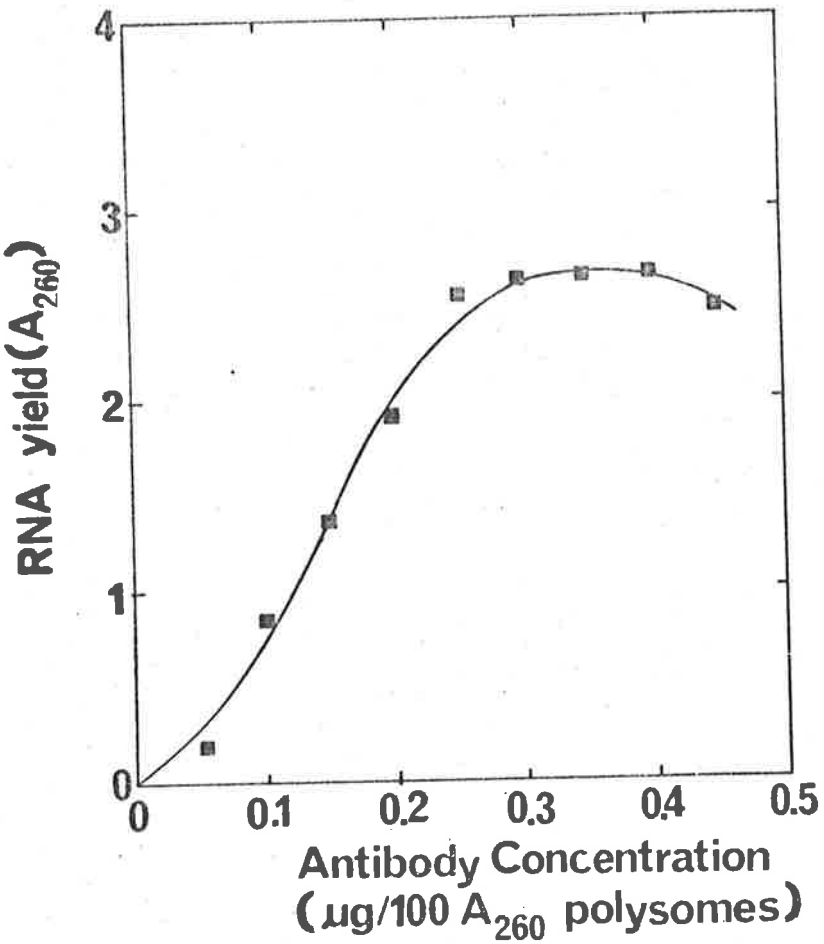

RNA yield (A_{260})
0
1
2
3
4
0
0.1
0.2
0.3
0.4
0.5
Antibody Concentration
(µg/100 A_{260} polysomes)

Figure 5.8

A. Fractionation of RNA prepared by the immunoadsorption reaction on sucrose gradients

B. Efficiency of translation of the RNA fractions from above, in the wheat embryo cell-free system.

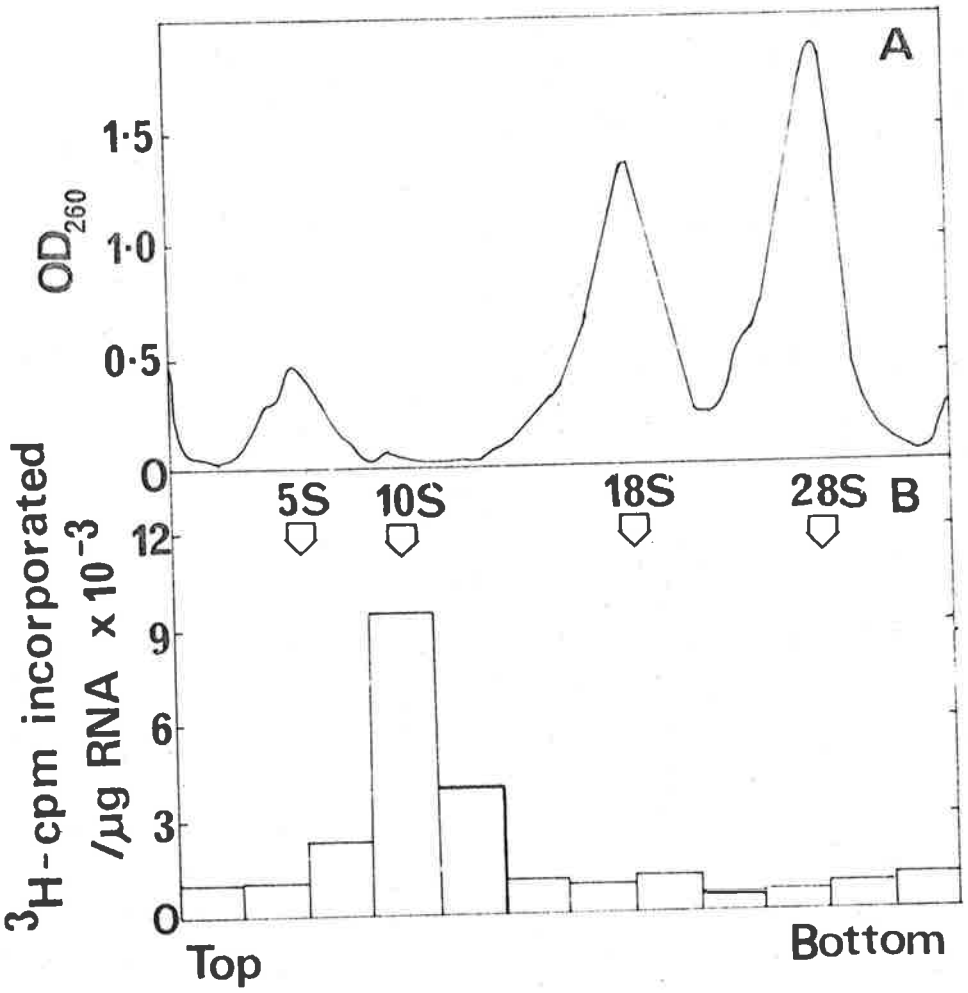

A
1·5
1·0
0·5
0
OD260
5S
10S
18S
28S
B
12
9
6
3
0
3H-cpm incorporated
/µg RNA x10-3
Top
Bottom

Figure 5.9

Analysis of the *in vitro* translation products of the 10S RNA prepared by the immunoadsorption reaction on SDS-urea polyacrylamide gels. The concentration of polysomes used in the immuno-adsorption reaction was

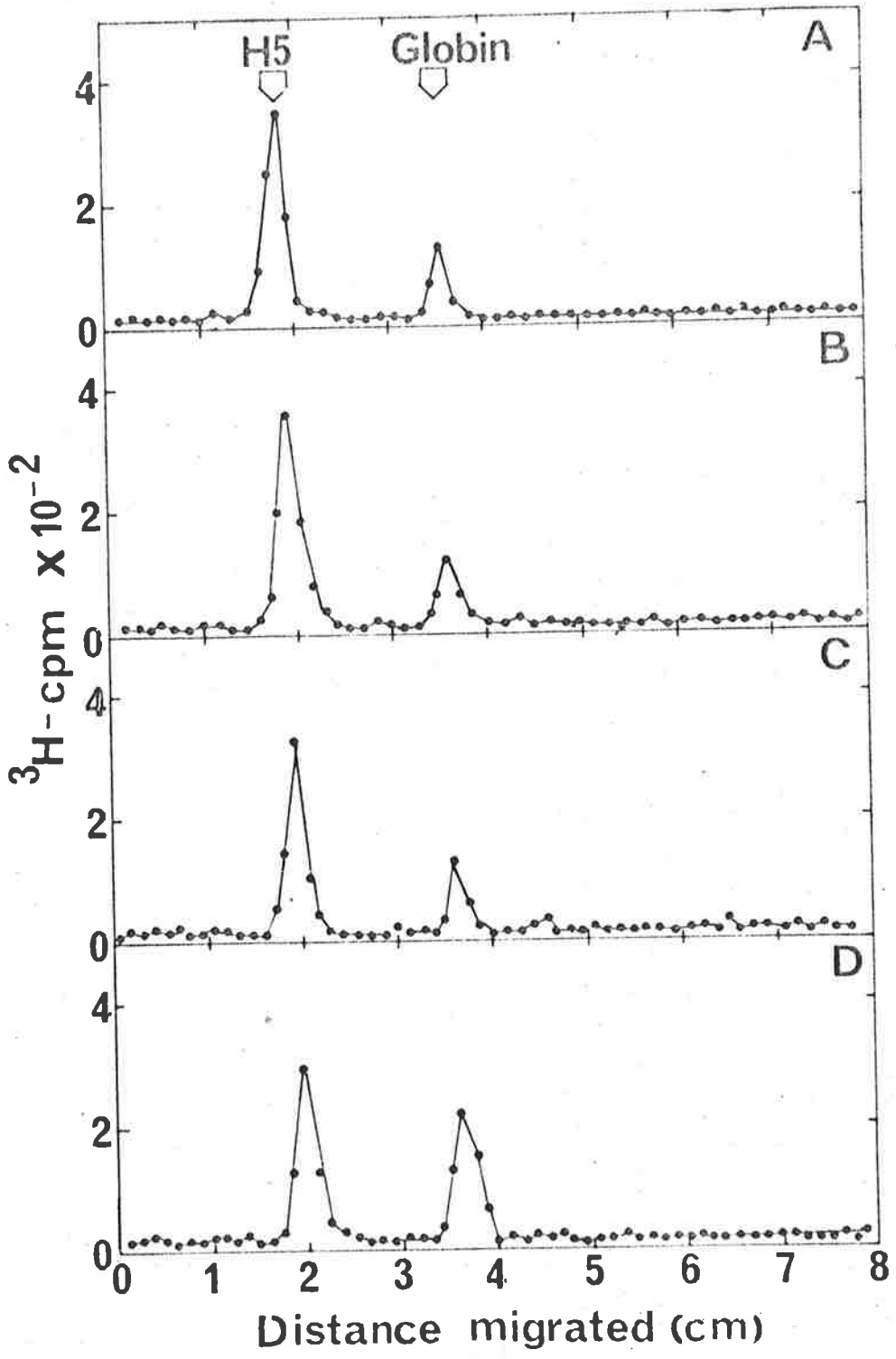
A. 10 A_{260}/ml

B. 15 A_{260}/ml

C. 20 A_{260}/ml

D. 25 A_{260}/ml

Electrophoresis was from left to right in all cases. The position of ^{14}C-labelled standards is indicated.


H5
Globin
A
B
C
D
^{3}H-cpm X 10^{-2}
0
2
4
0 1 2 3 4 5 6 7 8
Distance migrated (cm)

mRNA isolated at increasing antibody concentrations were largely H5 but the amount of globin present increased above 1 mg per 400 A_{260} of polysomes. Since this was close to the conditions for maximum yield, the result was encouraging. In the case of the polysome concentration, however, it was necessary to sacrifice some RNA yield (not necessarily yield of H5 mRNA) to attain optimal purity of the mRNA. Obviously the effect of changing one parameter may be to change the optimum for another parameter; however to investigate all possible combinations was impractical and probably unproductive as the final reaction conditions for the best compromise of yield with specificity agree well with those found by Palacios *et al.*, 1973).

Nevertheless, the yield of mRNA produced in this way was extremely low. Due to the low concentration of polysomes required, larger reaction volumes were impossible to handle and thus methods of improving the efficiency of the reaction were investigated. One problem could be due to the rapid run-off of ribosomes during the preparation of polysomes from chicken reticulocytes (G. Partington, personal communication). This could result in fewer nascent peptides being available for antibody binding per mRNA with a consequent reduction in efficiency. One way of improving this was to use a drug which prevents termination of peptide synthesis and thereby gives the maximum number of nascent peptides for binding. Such a drug is Trichodermin which, at low concentrations, specifically inhibits the termination of protein synthesis (Wei

TABLE 5.10

Level of Trichodermin (µg/ml)	A_{260} units of polysomes eluted from matrix	Total mRNA activity in 5S-18S RNA (total cpm)
0	2.4	9700
0.2	3.4	12400
0.5	4.7	15500
1.0	6.8	19100

Effect of various levels of trichodermin on the yield of immunoadsorbed polysomes and messenger RNA. 640 A_{260} units of polysomes were used to prepare H5-synthesising polysomes by immunoadsorption as described (see chapter 2). The 5S-18S RNA from these polysomes was translated at non-saturating concentrations in the wheat embryo system and the incorporation of ^{3}H-leucine into protein was measured.

et al., 1974). As shown in table 5.10, the addition of Trichodermin at a level of 1 µg/ml to all solutions resulted in a substantial increase in the yield of H5 mRNA. The purity of the mRNA was essentially unaffected by this addition, as determined by examination of the translation products.

5.1.3 Conclusions Concerning Immunoadsorption

Several important conclusions can be drawn from the results presented in this section. Firstly, it is quite possible to isolate undergraded H5-synthesising polysomes from reticulocytes. The overall specificity of this immunoadsorption reaction can be gauged by estimating the contamination with non-specifically bound polysomes, using some of the available data. Thus, the reticulocyte synthesises approximately 92% globin and 3.8% H5 (section 4.2). If we assume that this corresponds to the proportion of mRNAs in the cell (as do Palacios *et al.*, (1973)), then of the 640 A_{260} units of polysomes used to prepare H5 mRNA in table 5.10, 588.8 A_{260} were synthesising globin (i.e. 92% of 640). From figure 5.9a, the purified mRNA programmes the synthesis of 25% globin, and if we equate this with the percentage of globin mRNA present, then 1.7 A_{260} of the 6.8 A_{260} eluted from the matrix (25% of 6.8) were bound non-specifically. Thus the contamination with non-specifically bound polysomes

$$= \frac{\text{Amount of globin-synthesising polysomes bound}}{\text{Total globin-synthesising polysomes originally present}}$$

$$= \frac{1.7}{589} \times 100\%$$

$$= 0.3\%$$

This figure implies certain assumptions, for example, that globin and H5-synthesising polysomes are of the same size and therefore may be a minimum estimate; however it compares favourably with the 2% contamination reported by Palacios *et al.*, (1973) using this same technique. There are two probable reasons for this improvement. Firstly, the protein in this case constituted a much smaller percentage of total synthesis resulting in a much smaller precipitate, despite the use of total histone in preparing the matrix. Secondly, the antibody purification procedure employed was more rigorous resulting in higher specificity and reduced contamination.

The mRNA produced in this fashion was 10S in size on aqueous sucrose gradients and reasonably pure. The yield is still very poor, however. Since it would be necessary to remove the globin mRNA before the H5 mRNA could be used to prepare cDNA, the yields are too low to be useful, even with the improvement wrought by addition of Trichodermin. Whatever the reason for the low yield, methods of overcoming it are required. The technique of indirect immunoprecipitation was therefore investigated as it has been reported as being more efficient (Shapiro *et al.*, 1974).

5.2 INDIRECT IMMUNOPRECIPITATION

5.2.1 Introduction

While in many respects similar to immunoadsorption, indirect immunoprecipitation differs mainly in that the antibody-polysome complexes are precipitated

by the use of antibodies directed against the first antibody. Thus in this case an anti-(anti-H5) was used. The rationale of this procedure is shown in figure 5.11. The advantage of this technique besides its higher yield (Shapiro *et al.*, 1974) lies in the added specificity which can be achieved by the use of a second highly purified antibody. Far less total protein is added with a consequent reduction in possible ribonuclease contamination and in the size of the precipitate formed. This small precipitate causes less non-specific entrapment of polysomes and is easily washed to remove any contaminants which do bind. The method is a logical extension of the immunoadsorption technique, requiring only the addition of a second highly purified antibody to the reagents already in use.

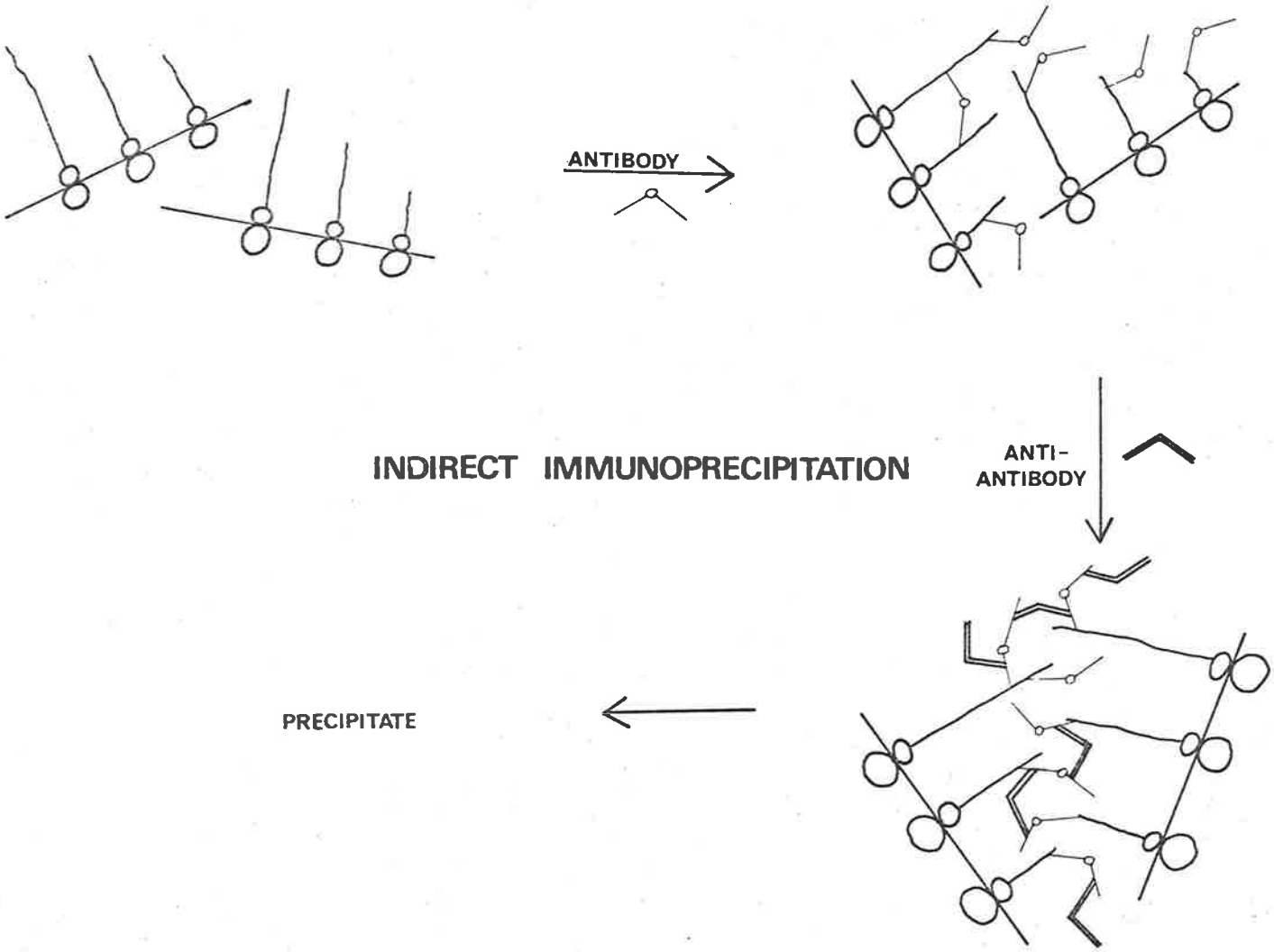
5.2.2 Preparation of H5 mRNA

(1) Reagents

The only extra requirement for this procedure was a purified antibody capable of precipitating anti-H5. This was raised in goats and purified analogously to anti-H5. The purified antibody only reacted with rabbit gamma globulin on an Ouchterlony immunodiffusion plate and not with chicken H5, chicken globin or total chicken histone. As a more sensitive test for low levels of anti-globin activity, anti-(anti-H5) was used to precipitate anti-H5 in the presence of ^{14}C-globin. After washing, the precipitate was dissolved and the radioactivity determined. As there was no radioactivity above background (data not shown), there was no anti-globin activity detectable even by this sensitive

Figure 5.11

Rationale of the indirect immunoprecipitation reaction.


ANTIBODY
INDIRECT IMMUNOPRECIPITATION
ANTI-
ANTIBODY
PRECIPITATE

test.

(2) Optimisation of reaction

As in the immunoadsorption reaction, the importance of optimising the reaction cannot be overstated. The minimum amount of both antibodies conducive to good yield, must be added to retain maximum specificity. It was therefore necessary to calculate the minimum amount of anti-(anti-H5) required to precipitate a fixed amount of anti-H5. Figure 5.12 shows the precipitin curve for this reaction (at 37°C), and demonstrates that 31 mg of anti-(anti-H5) was required to precipitate 1 mg of anti-H5. This data was used to determine the amount of each antibody required for optimal precipitation. The second variable which was examined was the time course of the reaction at 0°C since at this temperature the effects of ribonuclease are minimised. As shown in figure 5.13, the reaction of anti-(anti-H5) with the amount of anti-H5 required for optimal precipitation as determined above, was essentially complete within 75 minutes, under the conditions employed.

Having this data it was then possible to optimise the reaction in terms of amount of antibody required. Hence a fixed amount of polysomes were incubated with increasing amounts of anti-H5 at 0°C for 60 minutes (from figure 5.6), followed by incubation with the appropriate amount of anti-(anti-H5) (from figure 5.12) at 0°C for 75 minutes (from figure 5.13). The precipitate was collected, washed, and the RNA extracted with phenol to determine the yield. As shown in figure 5.14

Figure 5.12

Precipitin curve for the precipitation of anti-H5 by 1 mg of anti-(anti-H5).

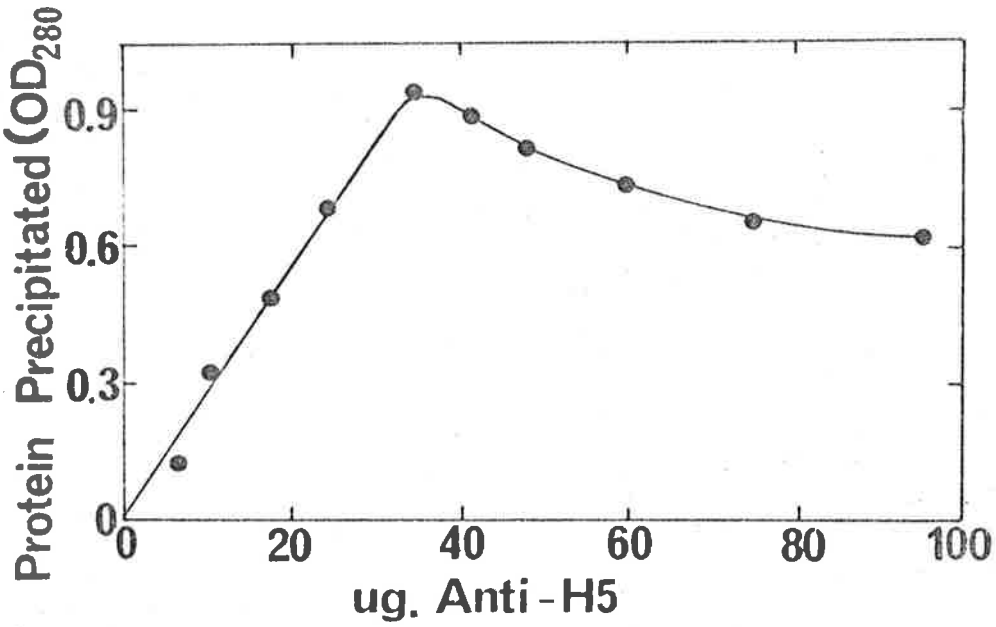

Protein Precipitated (OD280
0.9
0.6
0.3
0
0
20
40
60
80
100
ug. Anti-H5

Figure 5.13

Time course of the precipitation of anti-H5 by anti-(anti-H5) at 0°C.

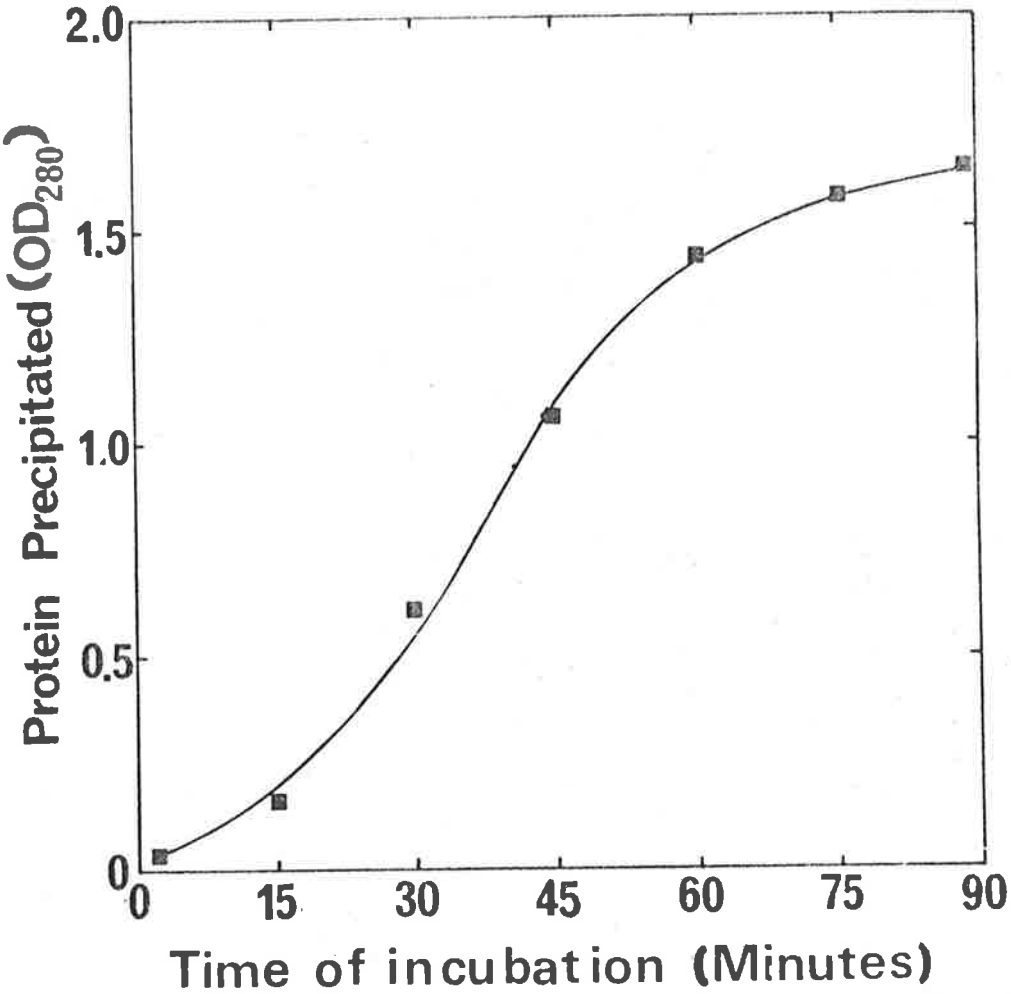
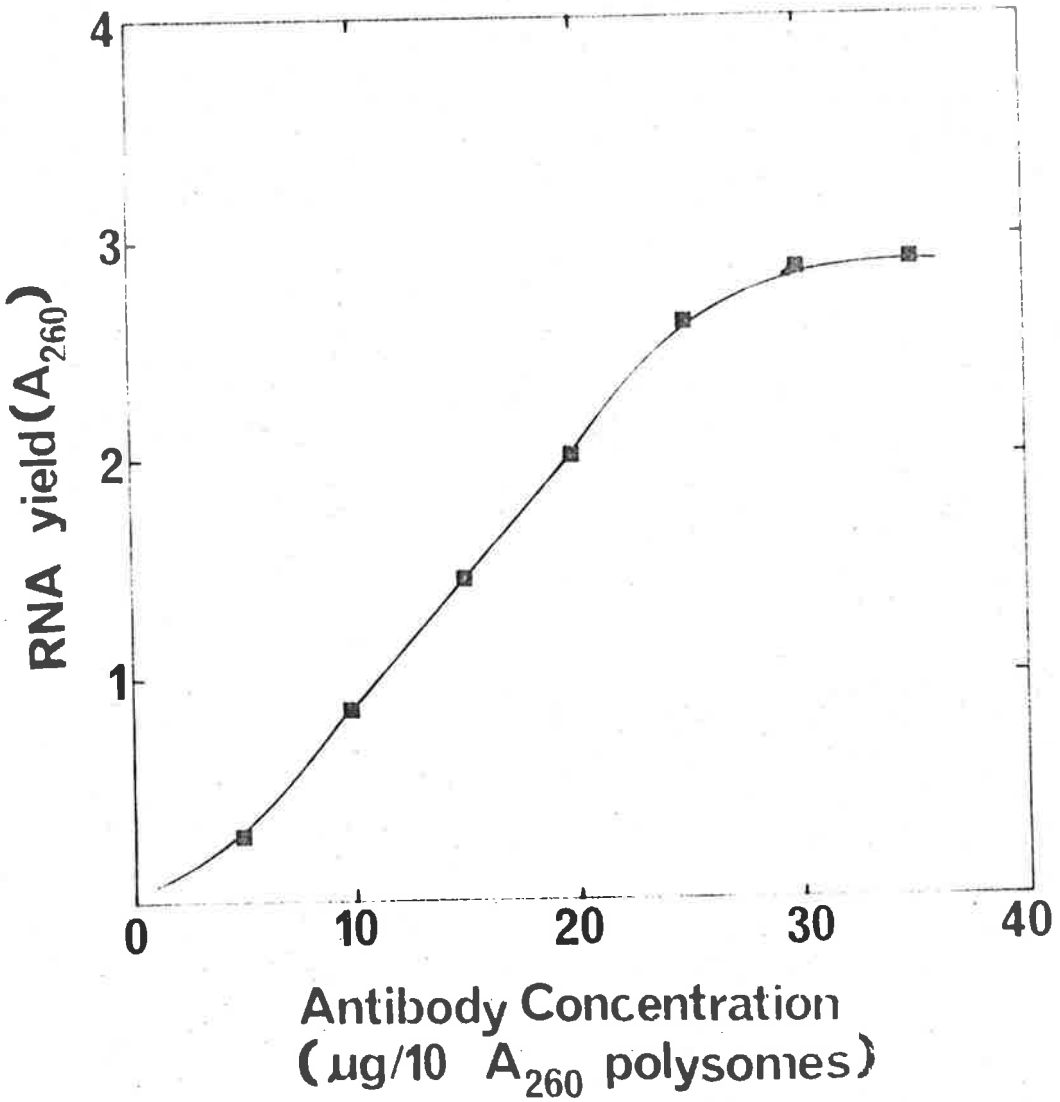

Protein Precipitated (OD_{280})
2.0
1.5
1.0
0.5
0
0
15
30
45
60
75
90
Time of incubation (Minutes)

Figure 5.14

Effect of anti-H5 concentration on the precipitation of polysomes by the indirect immunoprecipitation reaction.


RNA yield(A_{260})
4
3
2
1
0
10
20
30
40
Antibody Concentration
(μg/10 A_{260} polysomes)

adding more than 25 µg of anti-H5 per 10 A_{260} units of polysomes did not increase the yield of RNA extracted. One other variable, the optimum concentration of polysomes remained to be examined. When the yield of RNA was determined as described above using varying concentrations of polysomes, it was found that concentrations over 15 A_{260} per ml decreased the yield of RNA slightly, as shown in figure 5.15. However, when the incubation time with anti-(anti-H5) was increased to 120 minutes, the concentration could be increased to 25 A_{260} per ml and gave larger yields of RNA, as is also shown in figure 5.15. This must mean that the binding of anti-H5 to polysomes affects its reaction with anti-(anti-H5).

Thus the reaction was optimised in terms of yield of RNA; however, the yield is less important than the purity and integrity of the mRNA. As shown in figure 5.16, the RNA prepared in this manner was not visibly degraded and all mRNA activity, as measured by translation, was in the 10S fraction, as before. Increasing the amount of antibody used up to the optimum also had no effect on the purity of the mRNA. The translation products are shown in figure 5.17.

5.2.3 Conclusions on Indirect Immunoprecipitation

Using the optimal conditions described here, it was possible to prepare microgram amounts of H5 mRNA which, when translated, coded for the synthesis of approximately 90% H5 and 10% globin, as shown in figure 5.17. Hence both the yield and specificity of

Figure 5.15

Effect of polysome concentration on the precipitation of polysomes by the indirect immunoprecipitation reaction. Anti-H5 was incubated with the polysomes for 75 minutes before the addition of anti-(anti-H5). This was then incubated for

A. 75 minutes

B. 120 minutes

before collection and washing of the precipitate, and extraction of the RNA contained therein (chapter 2).

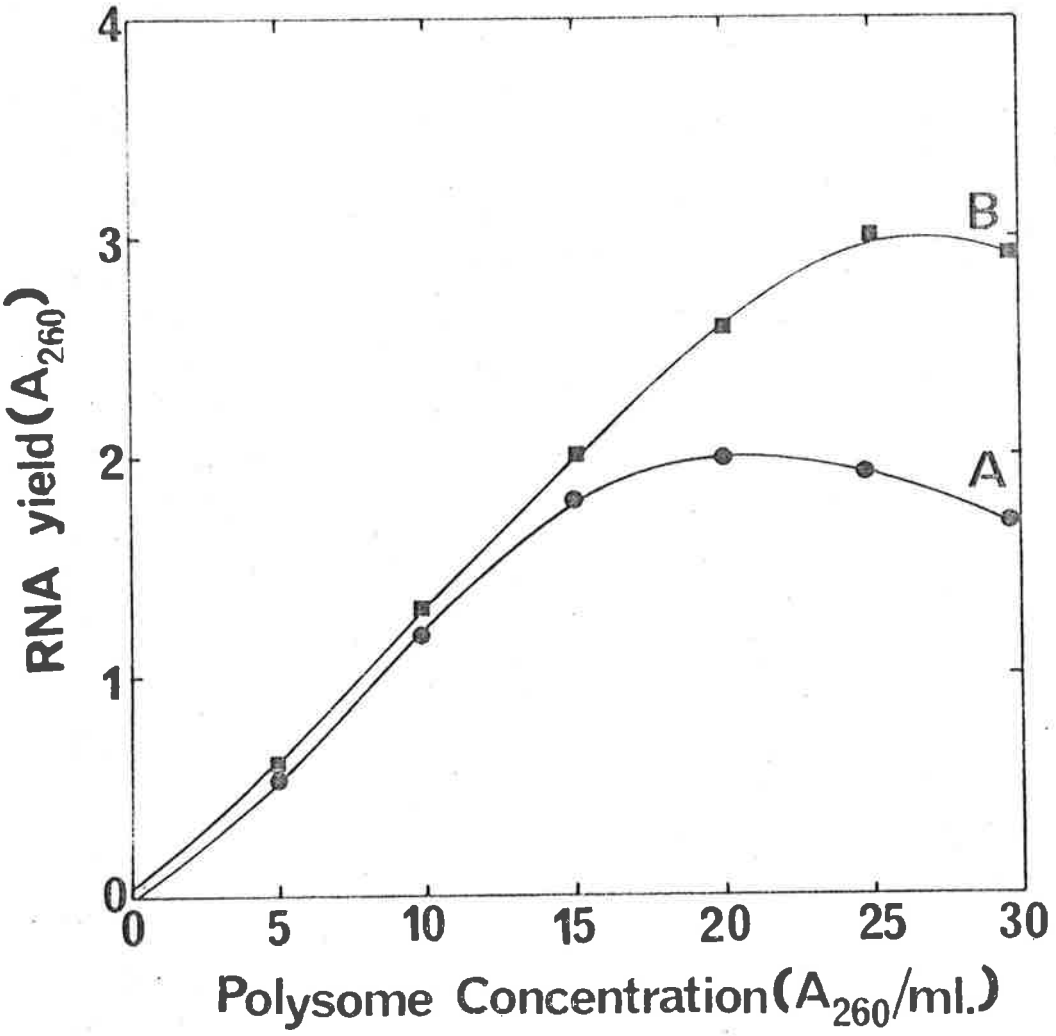

RNA yield(A_{260})
Polysome Concentration(A_{260}/ml.)
B
A
0
1
2
3
4
0
5
10
15
20
25
30

Figure 5.16

A. Analysis of the RNA prepared by the indirect immunoprecipitation reaction on sucrose gradients

B. Efficiency of translation of the RNA fractions from above in the wheat embryo cell-free system.

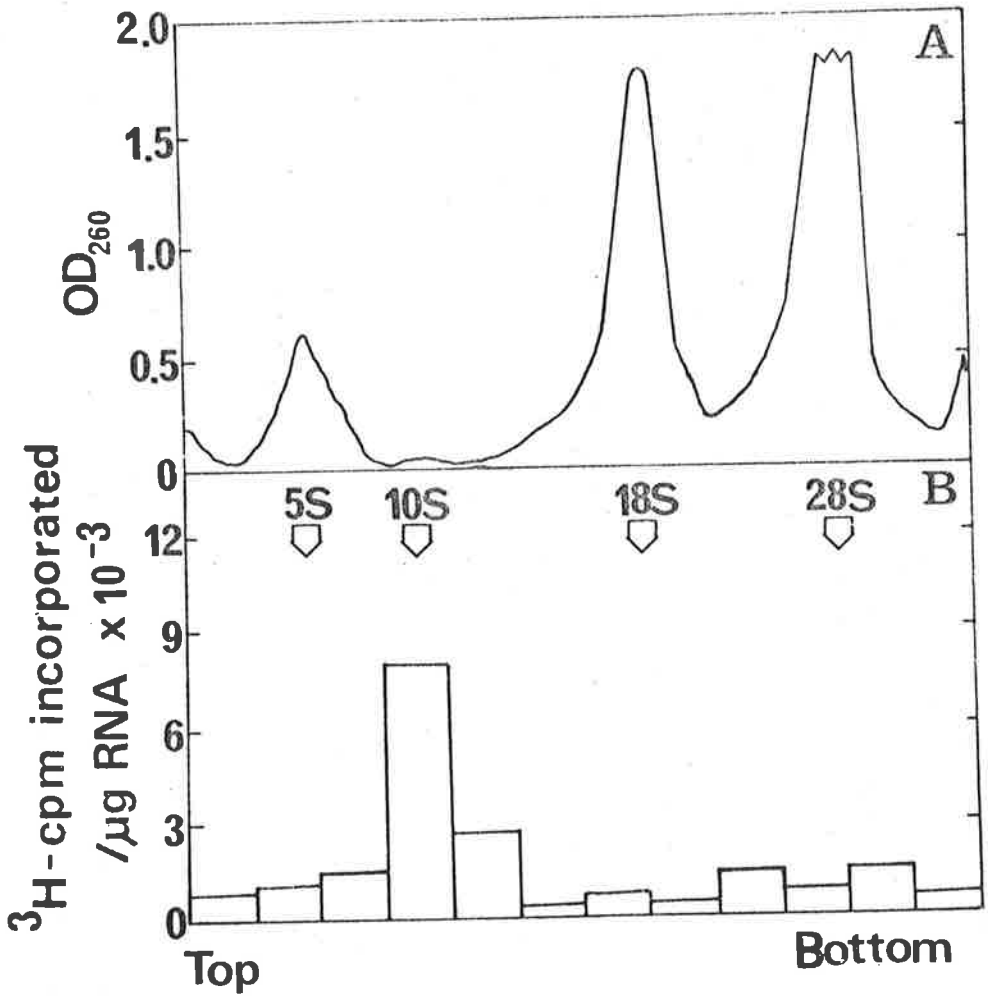

2.0
1.5
1.0
0.5
0
OD_{260}
A
5S
10S
18S
28S
B
12
9
6
3
0
^3H-cpm incorporated
/µg RNA x 10^{-3}
Top
Bottom

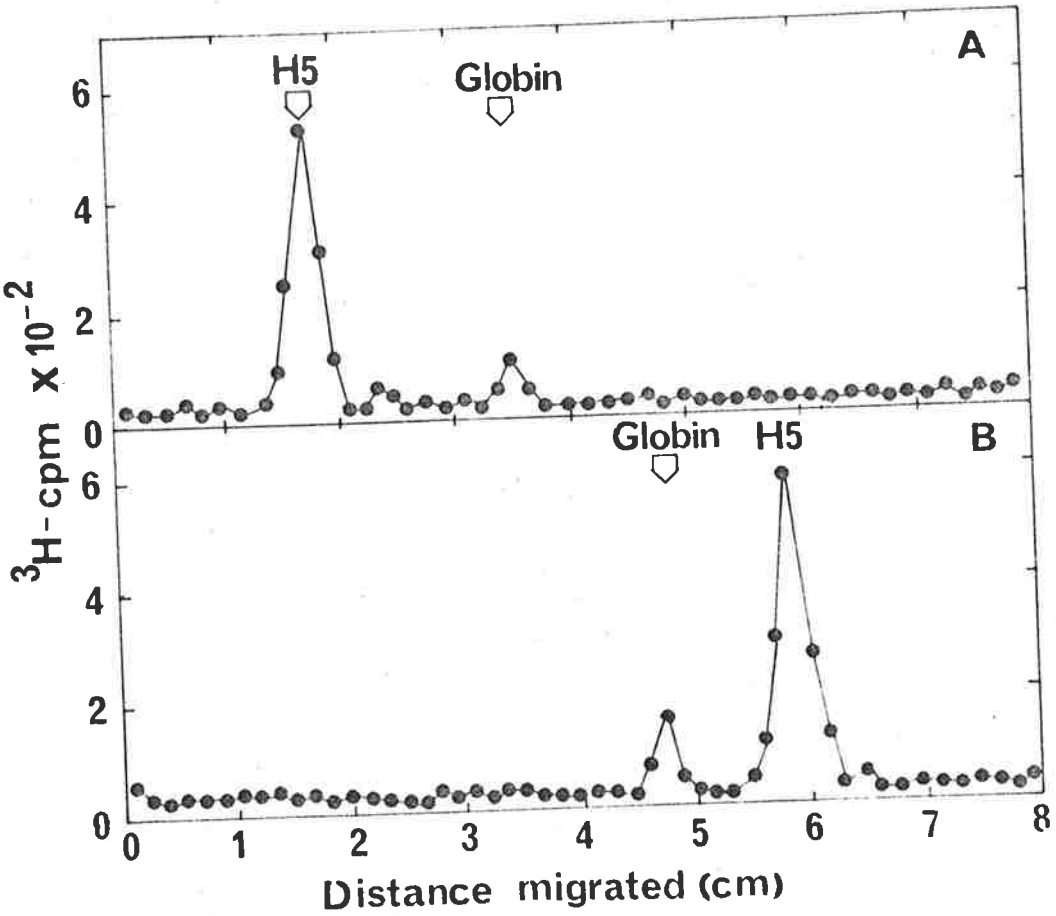
Figure 5.17

Analysis of the *in vitro* translation products of 10S RNA prepared by indirect immunoprecipitation on

A. SDS-urea gels

B. Low pH-urea gels

Electrophoresis was from left to right in both cases. The position of ^{14}C-labelled standards is indicated.


A
H5
Globin
B
Globin
H5
^{3}H-cpm X 10^{-2}
0
2
4
6
0
1
2
3
4
5
6
7
8
Distance migrated (cm)

this reaction were better than those of the immuno-adsorption reaction. The amounts produced meant that further purification of the H5 mRNA in an effort to remove the residual globin mRNA were feasible.

5.3 PURITY AND PROPERTIES OF H5 mRNA

When the H5 mRNA produced by indirect immuno-precipitation was translated it coded for the synthesis of 10% globin which meant that some globin mRNA must still have been present. In an effort to remove this, the mRNA was chromatographed twice on oligo (dT)-cellulse and the mRNA re-purified by sucrose gradient centrifugation. When this mRNA was translated, there was insufficient globin contaminant produced to accurately quantitate it; however it was certainly less than 5% as shown in figure 5.18.

This final H5 mRNA preparation was therefore 'pure' as defined by translation. The mRNA ran as 10S on aqueous sucrose gradients after disaggregation with formamide or by heating (McKnight and Schimke, 1974), as predicted from the size of H5 histone. Insufficient mRNA was available to determine its exact size on formamide gels however, and thus the purity of the preparations could not be tested in this way. This is dealt with further in the next chapter. The mRNA was copied to less than 1% with reverse transcriptase under conditions where globin mRNA is copied to over 70% (R. Crawford, personal communication). This property, together with its lack of binding to oligo (dT)-cellulose, means that H5 mRNA probably contains no poly

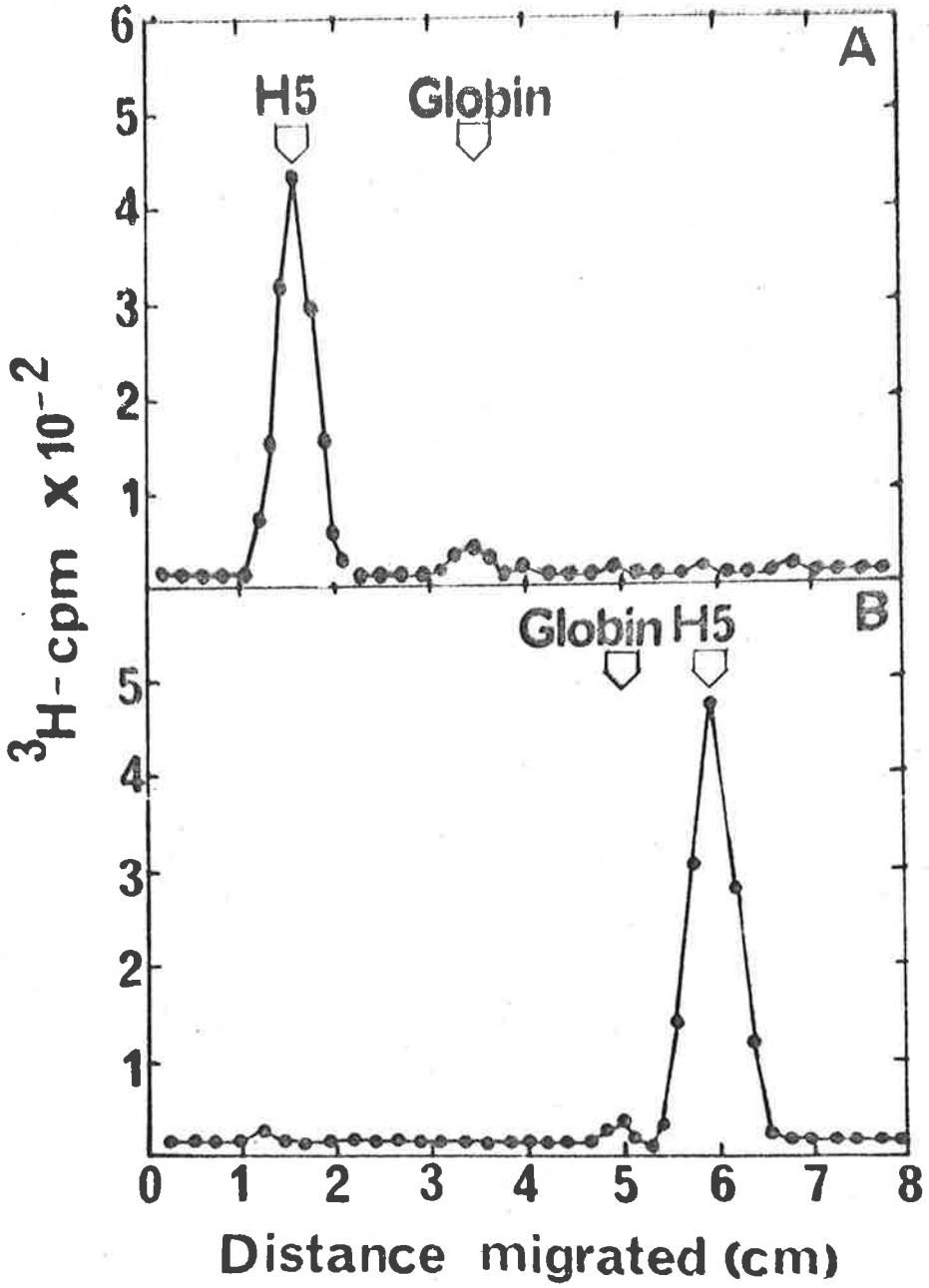
Figure 5.18

Analysis of the *in vitro* translation products of H5 mRNA. The 10S RNA prepared by indirect immuno-precipitation was purified by 2 cycles of oligo (dT)-cellulose chromatography (see chapter 2). 0.5 µg of the unbound RNA was then translated in the wheat embryo cell-free system and the products analysed on

A. SDS-urea gels

B. Low pH-urea gels

Electrophoresis was from left to right in both cases. The position of ^{14}C-labelled standards is indicated.


A
H5
Globin
B
Globin
H5
^{3}H-cpm x10^{-2}
Distance migrated (cm)
0
1
2
3
4
5
6
7
8

A tract, and is thus like other histone mRNAs in this respect.

The major drawback with this procedure was that the yield of mRNA was still extremely low. Considering the purification procedure, however, this is not surprising, and it is doubtful if it could be greatly increased without affecting the purity of the mRNA. Sufficient mRNA could still be produced to prepare cDNA however, and to use this in an investigation of the purity of the H5 mRNA and its gene reiteration frequency.

CHAPTER 6

PRODUCTION AND CHARACTERISATION OF H5 cDNA

6.1 INTRODUCTION

The major aim of the work described in this thesis was to prepare a probe which could be used to detect H5 gene sequences or H5 mRNA by hybridising to them. Two forms of probe are generally used, highly labelled mRNA or a cDNA copy prepared from mRNA by copying it with an RNA-dependent DNA polymerase. The RNA probes suffer from several disadvantages. Firstly, they are often impossible to label to high specific activities which makes DNA-excess reactions difficult to perform due to the large amount of mRNA which must be added (Melli *et al.*, 1971). Secondly, the mRNA obviously cannot be used to quantitate the amount of mRNA present in a sample as it will only hybridise to mRNA-complementary sequences. Thirdly, RNA probes suffer from stability problems and may be degraded on prolonged incubation which makes it difficult to obtain high Cot values and to interpret the results at these Cot values. The cDNA probe on the other hand suffers from none of these disadvantages. Since it is made *in vitro*, several highly-labelled deoxynucleotides can be used in its synthesis giving extremely high specific activities. The cDNA is thus only required in trace amounts and will not interfere with reassociation reactions (Melli *et al.*, 1971). The cDNA is also produced from mRNA and so is complementary to both mRNA and one strand of the DNA in the gene. Hence it can be used to quantitate both mRNA and genes. Thirdly, the cDNA is extremely stable and very high

Cot values can be readily obtained.

The probe of choice is therefore cDNA produced from purified mRNA by an RNA-dependent DNA-polymerase. The previous chapter showed that H5 mRNA could be purified; however it also demonstrated that it will not act as a template probably due to the lack of a poly A tract. This tract normally hybridises to oligo (dT) which then acts as a primer for the enzyme. The first requirement therefore is to modify the H5 mRNA by addition of a 3' polyadenylic acid tract before a cDNA probe can be made from it. The results described in this chapter show that H5 mRNA can be enzymically modified by addition of such a tract and copied into cDNA. This cDNA is shown to be a faithful copy of H5 mRNA, with only minor amounts of contaminating sequences complementary to globin mRNA and rRNA.

6.2 ADDITION OF A 3' POLYADENYLIC ACID TRACT TO H5 mRNA

Any method of adding polyadenylic acid must cause a minimum of damage to the mRNA, it must add to the 3' end, and must add sufficient adenylic acid residues to allow the modified mRNA to be efficiently copied into cDNA. The most promising way of doing this was offered by a poly(A)-polymerase enzyme which has been isolated from corn by Mans and Huff (1975). The analogous enzyme, prepared from waxy maize by J. R. E. Wells, was characterised to assess its suitability.

As the addition reaction was carried out at 30°C, ribonuclease degradation could have been a major problem and it was thus essential to add only a minimum

amount of enzyme, for the minimum time, compatible with effective poly A addition. Since the aim was to copy the H5 mRNA into cDNA, the poly A addition reaction was monitored by conversion of *E.coli* tRNA into a template for Reverse Transcriptase. It was also imperative to obviate any losses as only very small amounts of mRNA were available. This was achieved by carrying out the poly A-polymerase reaction in a centrifuge tube, ethanol precipitating the RNA, and carrying out the cDNA preparation in the same tube. It was feasible that substrates from the first reaction might precipitate and so interfere with the copying. This was tested by using globin mRNA and demonstrating that the efficiency of copying was unaffected after carrying out this regime, as shown in table 6.1.

Figure 6.2a shows the effect of increasing poly A-polymerase concentration on the conversion of tRNA into a template for Reverse Transcriptase. This gave the optimal concentration of enzyme, and the time course of the reaction at this concentration is shown in figure 6.2b. In both cases, the amount of cDNA produced from the modified tRNA decreased at higher concentration of enzyme or longer incubation time. This could be due to ribonuclease activity degrading the tRNA, and as the size of the cDNA affects the rate of hybridisation (Britten and Kohne, 1968), the size of cDNA produced from RNA templates must be checked. Figure 6.3 shows the size of the cDNA produced from chicken globin mRNA and *E.coli* tRNA after treatment with poly A-polymerase. Since these cDNAs were the same

TABLE 6.1

RNA	Polyadenylated ?	% copy
Chicken globin mRNA	No	72.1
Chicken globin mRNA	Yes	70.0
E.coli tRNA	No	0.15
E.coli tRNA	Yes	3.3

Effect of polyadenylation of RNA on its subsequent copying by AMV reverse transcriptase. RNA samples were polyadenylated and then ethanol precipitated, as described (see chapter 2). The RNA was then transcribed by reverse transcriptase using ^{3}H-dGTP and ^{3}H-dCTP. The percentage copy was calculated by assuming that the RNA and cDNA contained 25% dGMP, and 25% dCMP.

Figure 6.2

A. Conversion of *E.coli* tRNA into a template for AMV reverse transcriptase by increasing amounts of poly A-polymerase.

B. Time course of the conversion of tRNA into a template for reverse transcriptase by poly A-polymerase.

In both cases, the quantity measured was the incorporation of ^{3}H-dGTP into cDNA.

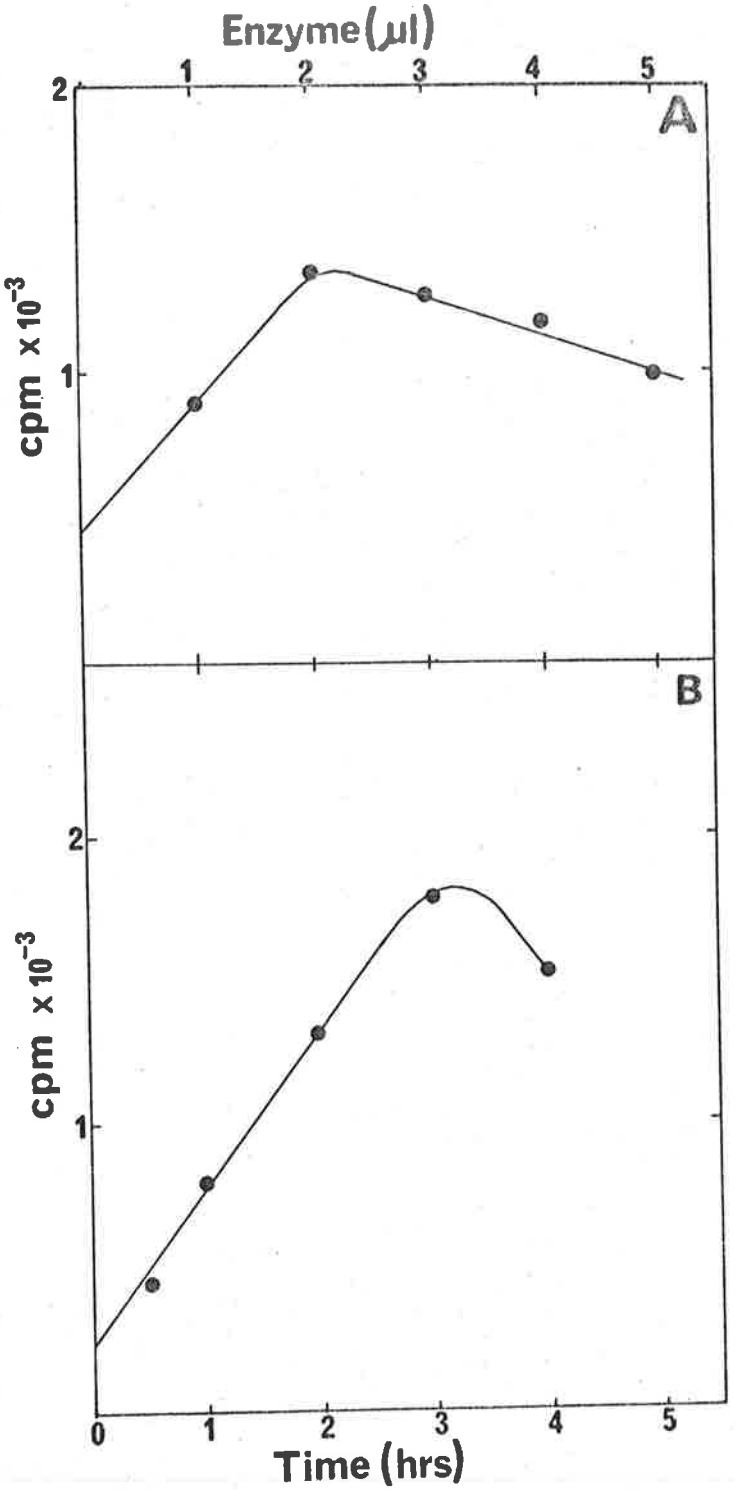

Enzyme (µl)
1
2
3
4
5
A
cpm x10^-3
2
1
B
cpm x10^-3
2
1
0
1
2
3
4
5
Time (hrs)

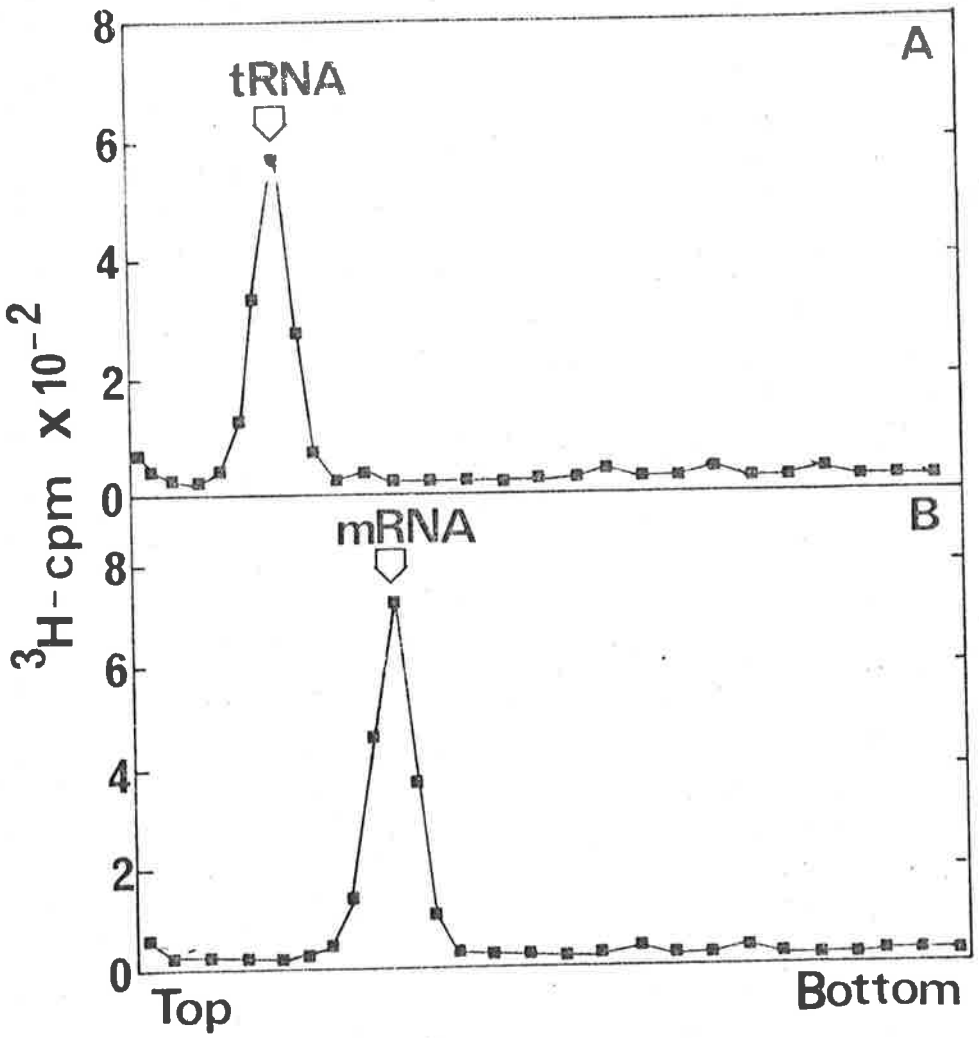
Figure 6.3

Estimation of the size of cDNA produced from tRNA and chicken globin mRNA after addition of a poly A tract, on sucrose gradients.

A. *E.coli* tRNA

B. Chicken globin mRNA.

The positions of the RNA templates are indicated.


A
tRNA
B
mRNA
^{3}H-cpm X 10^{-2}
8
6
4
2
0
Top
Bottom

length as the template from which they were copied, ribonuclease was not a problem under these conditions.

Thus poly A-polymerase had the ability to modify RNAs such that they could be copied by Reverse Transcriptase into cDNAs of reasonable size. One disturbing factor, however, was the much higher efficiency of copying globin mRNA after enzyme treatment than similarly treated tRNA. This may have been due to the higher degree of secondary structure in tRNA which may reduce its efficiency as a template, or natural poly A-containing RNAs may be an inherently better template. Whatever the reason, it is clear that a minor contaminating RNA species may be more efficiently transcribed and so constitute an inordinately large percentage of the cDNA. Although the H5 mRNA had been purified by oligo (dT)-cellulose chromatography to remove poly A-containing RNA, this possibility must still be considered. Hence the cDNA produced must be characterised.

6.3 CHARACTERISATION OF H5 cDNA

The H5 cDNA prepared from H5 mRNA as described above was characterised in terms of:-

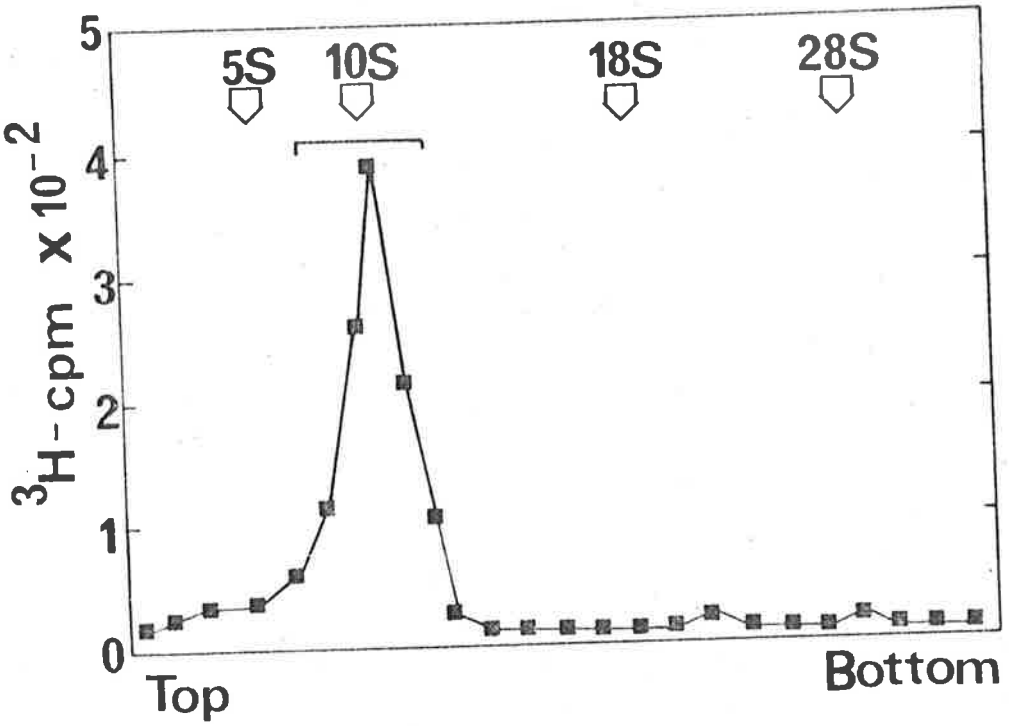
(i) its size (since this affects the rate of hybridisation; Britten and Kohne, 1968), and

(ii) its content of non-H5 sequences, since it was essential to show that any hybridisation was due to H5 cDNA and not due to contaminating sequences.

Figure 6.4 shows the size of the purified cDNA

Figure 6.4

Estimation of the size of the cDNA produced from polyadenylated H5 mRNA on sucrose gradients. The fraction indicated by the bracket was collected and used as H5 cDNA.

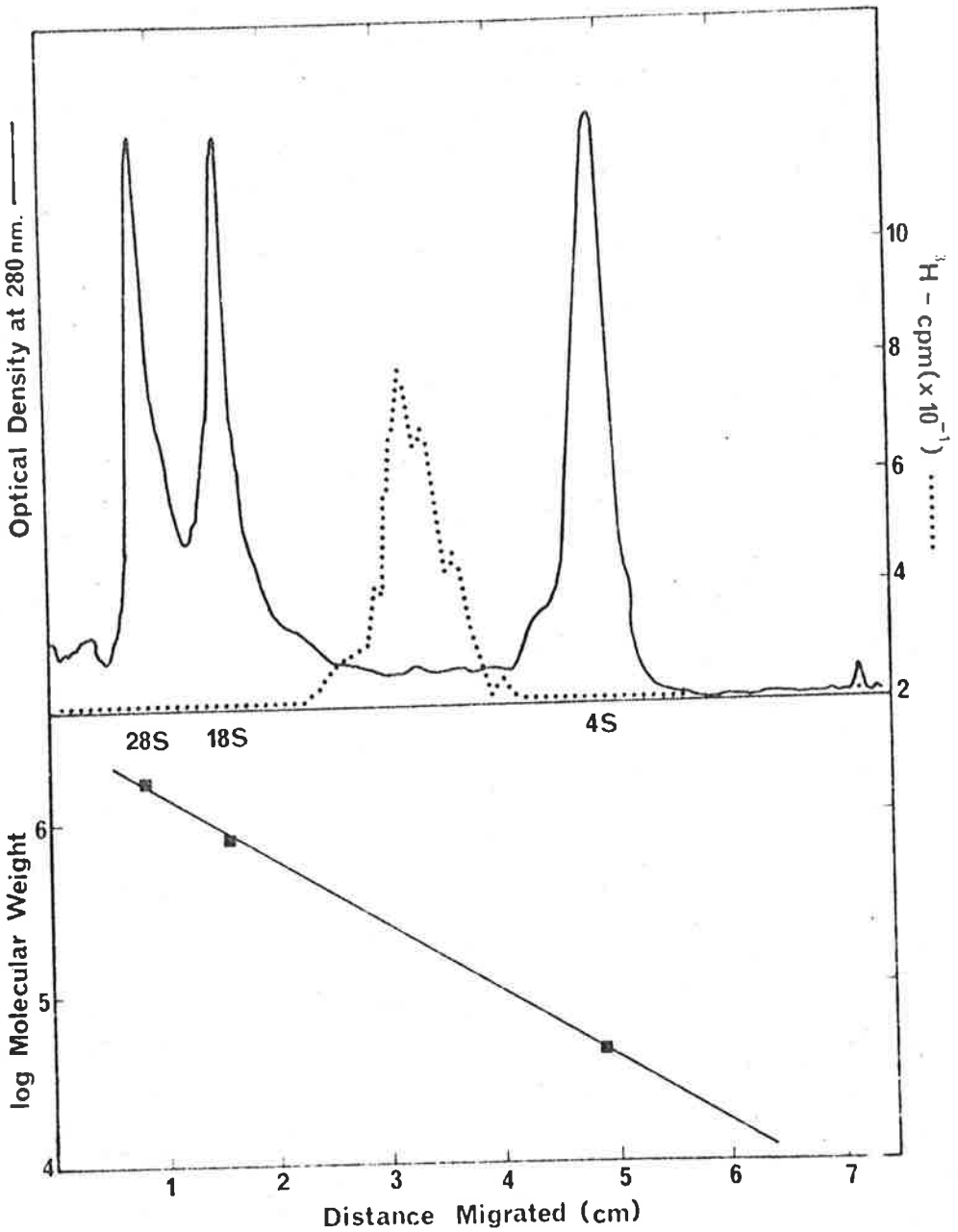

5S
10S
18S
28S
^{3}H-cpm X 10^{-2}
5
4
3
2
1
0
Top
Bottom

after fractionation on a sucrose gradient. As some small molecular weight material was present, the larger molecular weight cDNA was collected as indicated, and its average size determined by electrophoresis on polyacrylamide gels containing formamide, as shown in figure 6.5. The average size of this cDNA, by reference to known RNA standards, was 450 nucleotides in length.

There were two likely contaminants of the H5 mRNA which may have been copied into cDNA; these were globin mRNA and ribosomal RNA. Although globin mRNA was a minor species as assayed by *in vitro* translation, it was possible that it might have been preferentially transcribed into cDNA as described in section 6.2. The rRNA was also a possibility as 10S sized degradation products have been described (Bishop *et al.*, 1972) and their representation in the H5 mRNA was unknown as they would not be translated. The percentage of these two species represented in the cDNA was calculated by hybridising the cDNA to a large excess of each RNA and assaying for hybrids using S1 nuclease. As shown in table 6.5, only 3.2% of the cDNA was protected from degradation by S1, in the presence of rRNA. Hence only 3.2% of the sequences in the cDNA were complementary to rRNA. With globin mRNA, however, the situation was more complicated. Using globin mRNA prepared from RNP particles, over 85% of the cDNA formed hybrids at a Rot of 5×10^{-1}. However, if this mRNA was purified by oligo (dT)-cellulose chromatography, only 3% of the cDNA was complementary to it,

Figure 6.5

Estimation of the size of H5 cDNA on polyacrylamide gels containing formamide. The position of known standards is indicated and used to calculate the average size of the cDNA.


Optical Density at 280 nm.
^{3}H - cpm(x 10^{-1})
10
8
6
4
2
28S
18S
4S
log Molecular Weight
6
5
4
1
2
3
4
5
6
7
Distance Migrated (cm)

as shown in table 6.6. The explanation was that the globin mRNA contained trace amounts of H5 mRNA which could hybridise to the cDNA at the high Rot value used. When this contaminating H5 mRNA was removed by oligo (dT)-cellulose chromatography, however, only the 3% of the cDNA complementary to globin mRNA was protected from degradation. Although these experiments showed that chicken globin mRNA and rRNA were not major contaminating sequences in the cDNA, they could not prove that the cDNA was complementary to H5 mRNA. This was indicated however by back-hybridising the cDNA to H5 mRNA. This reaction proceeded to more than 75% completion as shown in figure 6.7. The kinetics of such a reaction are dependent on the sequence complexity of the reacting species and this can be calculated by comparison with a kinetic standard (Wetmur and Davidson, 1968; Kemp, 1975). The standard used was the hybridisation of rabbit globin mRNA to its cDNA. These hybridised in a single sharp transition with a mid-point ($Rot_{\frac{1}{2}}$) of 5×10^{-4} as shown in figure 6.7. This corresponds to a complexity of 1300 nucleotides for α plus β globin mRNA, since it is known that cDNA probes to these two mRNAs do not cross-hybridise to any appreciable extent (Kacian *et al.*, 1973). By contrast, H5 mRNA hybridised to its cDNA with a similar sharp transition but with a $Rot_{\frac{1}{2}}$ of about 2.8×10^{-4}, as shown in figure 6.7. This corresponds to a complexity of some 730 nucleotides which was the approximate size of the H5 mRNA. This indicated that H5 mRNA was coded for by a single species of mRNA. The low $Rot_{\frac{1}{2}}$

TABLE 6.6

RNA added	Rot (mole.sec.litre^{-1})	% Hybridisation
18S rRNA	6.8×10^{-1}	2.2
28S rRNA	7.2×10^{-1}	1.0
'Crude' globin mRNA	5.0×10^{-1}	85.4
'Purified' globin mRNA	5.5×10^{-1}	3.0
H5 mRNA	3.0×10^{-1}	89.0

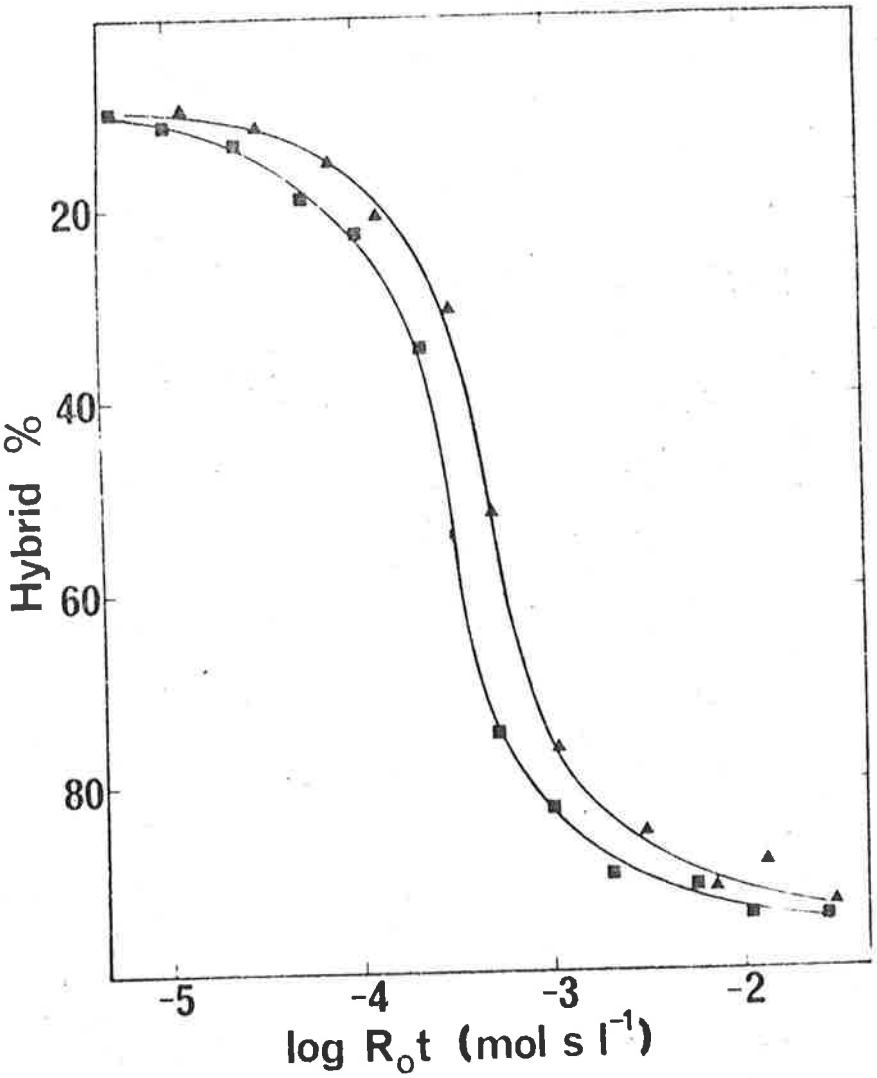
Extent of cross-hybridisation of H5 cDNA. H5 cDNA was hybridised to several electrophoretically pure RNA samples to the indicated Rot values. The percentage of cDNA hybridised was then calculated by S1 nuclease assays (see chapter 2). The percentage has been corrected by subtraction of the background due to S1-insensitive counts (about 4%). The 'crude' and 'purified' chicken globin mRNA refer to the same sample before and after purification by oligo (dT)-cellulose chromatography.

Figure 6.7

Rot curve for the hybridisation of rabbit globin mRNA and H5 mRNA to their respective cDNAs.

■—■ H5 mRNA-cDNA

▲—▲ chicken globin mRNA-cDNA


Hybrid %
20
40
60
80
-5
-4
-3
-2
log $R_o t$ (mol s l^{-1})

value and the sharpness of the transition also indicated that the H5 cDNA was a faithful copy of H5 mRNA.

6.4 CONCLUSION

The results in this chapter demonstrate that it is possible to modify an RNA by the enzymic addition of a poly A tract, and thus produce a cDNA from it using AMV Reverse Transcriptase. Using these methods it is theoretically possible to prepare cDNA to small amounts of any RNA. The technique is therefore of far wider applicability than this particular case.

A cDNA was prepared from H5 mRNA using these procedures and was shown to be a faithful copy of the mRNA by hybridisation analysis. The cDNA probe can be used to quantitate the amount of complementary sequences present in any sample of nucleic acid. One example of this was provided by the hybridisation of the H5 cDNA to globin mRNA prepared from RNPs, which showed that this mRNA contained contaminating H5 mRNA. This was therefore an independent confirmation of the work of Knochel (1975) who reached the same conclusions. The fact that the contaminant was removed by oligo (dT)-cellulose chromatography was also predictable from the results in chapter 4 which showed that H5 mRNA does not bind to this material.

The cDNA could also be used to quantitate the amount of mRNA present in a cell or the number of gene sequences present in the genome. This latter possibility was of special interest since most histone genes seem to be reiterated to varying extents.

CHAPTER 7

REITERATION FREQUENCY OF THE GENES CODING FOR H5 IN THE CHICKEN GENOME

7.1 INTRODUCTION

Complementary DNA hybridises only to its complementary sequence, and with a very high degree of specificity. When this is coupled with the inherent stability and high specific radioactivity characteristic of cDNA, the sensitivity of the probe can be gauged. The rate at which the probe hybridises to its complement is dependent on the concentration of the complement (Melli *et al.*, 1971). The amount of hybrid formed, however, is determined by the product of rate and time, hence the concept of Cot (Co x t). By plotting percentage of hybrid against Cot (or log Cot for convenience), one generates a reassociation curve which is characteristic of the nucleic acid being studied (Britten and Kohne, 1968). By comparison of such a curve with the appropriate standard, the concentration of the sequences complementary to cDNA in any nucleic acid can be calculated. For example, all other factors being equal, the rate at which two cDNAs hybridise to the DNA of an organism is proportional to the reiteration frequencies of these genes in the genome of that organism. This is used to calculate the reiteration frequencies of genes by comparison with a known standard such as the globin gene.

Histones are one of the few genes which are reiterated. This varies from 400 - 1000-fold in sea urchins (Kedes and Birnstiel, 1971) to 10 - 20-fold for man and mouse (Wilson *et al.*, 1974). Avian species are between these two extremes in terms of evolution and so the reiteration frequency of chicken histone genes is

interesting. The reason for the reiteration is unknown. In sea urchins the histone genes are present as tandemly repeated blocks (Birnstiel *et al.*, 1974) and this may be involved with the co-ordinate control of histone synthesis, or in the linkage of histone mRNA synthesis to DNA synthesis. Histone H5 is subject to neither of these controls, and indeed is only made in red blood cells. Thus the reiteration frequency of this particular histone is of special interest.

The work in this chapter shows that the genes for histone H5 are reiterated some 10-fold in the chicken genome.

7.2 CALCULATION OF REITERATION FREQUENCY OF H5 GENES

The reiteration frequency of the H5 gene can be estimated by annealing the cDNA from H5 mRNA with a vast excess of total chicken genome DNA (Melli *et al.*, 1971). Before this can be done, however, the kinetics of hybridisation of unique gene sequences must be known. Two independent estimates of this standard were made, one with labelled total genomic DNA and the other with cDNA to chicken globin genes.

Total genomic DNA labelled with ^{3}H-dGTP was sheared to 500 nucleotides average length and hybridised with a vast excess of unlabelled DNA giving the reassociation curve shown in figure 7.1. The major portion of the DNA, the unique sequences (Sullivan *et al.*, 1973), hybridised with a $Cot_{\frac{1}{2}}$ of 1.2×10^{3}. There was also some 20% of repeated sequences which hybridised in a broad transition at lower Cot values.

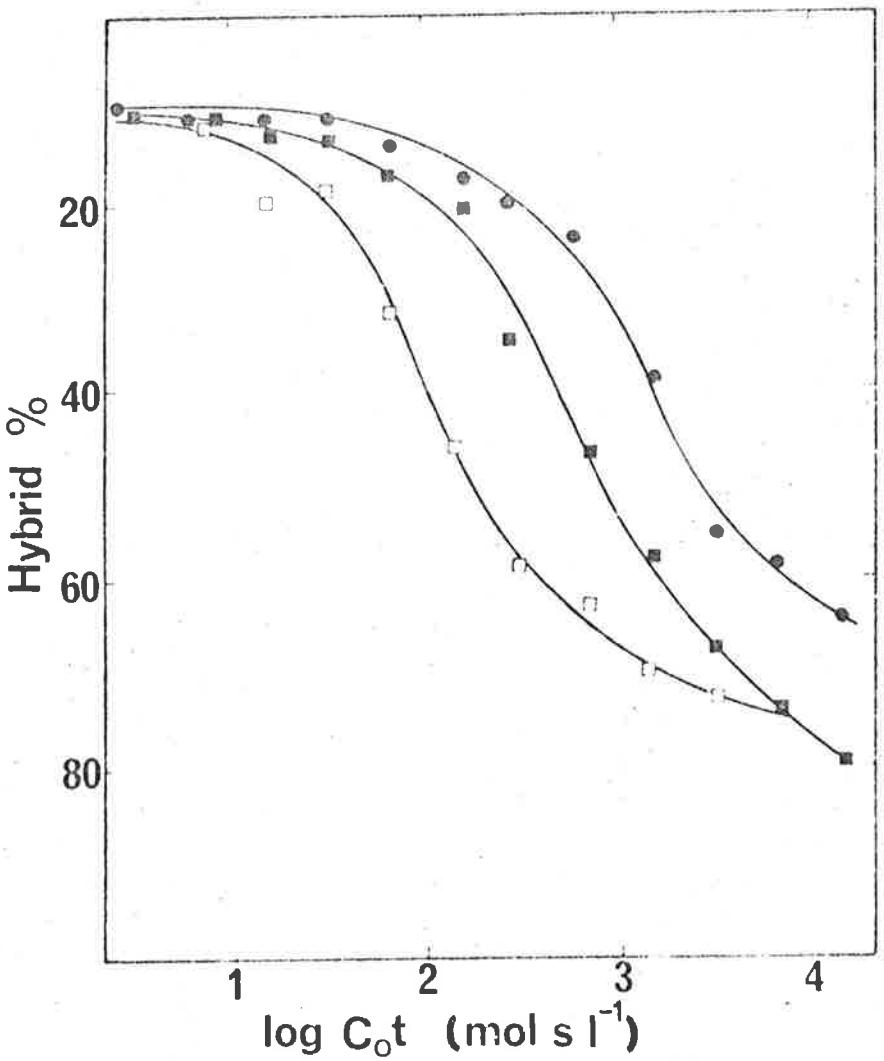
Figure 7.1

Reassociation of H5 cDNA, chicken globin cDNA and labelled chicken DNA, to an excess of unlabelled chicken nuclear DNA. The percentage of hybrid was estimated using S1 nuclease, as described (chapter 2).

●—● ^{3}H-chicken DNA-DNA

□—□ H5 cDNA-DNA

■—■ chicken globin cDNA-DNA


Hybrid %
20
40
60
80
1
2
3
4
log $C_o t$ (mol s l^{-1})

Chicken globin cDNA hybridised to total chicken DNA in a single sharp transition with a $Cot_{\frac{1}{2}}$ of 1.2×10^{3}. This is indicative of the fact that there are only one or two copies of the globin genes in the chicken genome. The hybridisation of H5 cDNA to chromosomal DNA, on the other hand, while still a single sharp transition had a $Cot_{\frac{1}{2}}$ of 1.2×10^{-2}. This means that the rate of the reaction was an order of magnitude faster than the unique rate. Since the globin and H5 cDNAs were of similar size and their G + C content, which also affects hybridisation rate (Britten and Kohne, 1968), was probably very similar (on the basis of the amino acid analysis of the proteins), then this difference in rate must reflect a reiteration of the H5 gene in the chicken genome. The extent of reiteration is directly proportional to the $Cot_{\frac{1}{2}}$ value compared to the kinetic standard (Melli *et al.*, 1971), and so the H5 is reiterated some 10 times in the chicken genome. Furthermore, as the reaction went to over 75% of completion, the minor contaminating species in the H5 cDNA were not responsible for the hybridisation.

7.3 CONCLUSION

The most significant conclusion from the results presented in this chapter is that the gene coding for H5 in the chicken genome is reiterated about 10-fold. This is similar to the histone genes in man and mouse (Wilson *et al.*, 1974), but far less than the reiteration frequency in sea urchins (Kedes and Birnstiel, 1971). The globin genes in chickens, on the other

hand, are not reiterated at all, and are thus similar to the genes coding for globin in rabbit, man, and duck (Bishop *et al.*, 1972; Harrison *et al.*, 1972; Kan *et al.*, 1975).

CHAPTER 8

FINAL DISCUSSION

8.1 INTRODUCTION

The aim of this chapter is to draw together the main results from this thesis and assess their importance and significance. The major point which is made is that while these results are interesting in their own right their major significance lies in their ability to be further exploited. These techniques and results can form a basis for an investigation of H5 genes and their relation to other histone genes. This in turn may lead to some understanding of the control of expression of H5 genes and of histone genes in general. To this end, the further studies which could be carried out are discussed in some detail.

8.2 HISTONE H5 mRNA

The results presented in this thesis indicate that the messenger RNA coding for H5 can be isolated from chicken reticulocytes by appropriate techniques. H5-synthesising polysomes were isolated by indirect immunoprecipitation and their mRNA extracted. This mRNA was of about 10S in size on aqueous sucrose gradients, undegraded, and programmed the synthesis of more than 90% H5 in the wheat embryo cell-free system. The success of this method depends heavily upon the antibodies used. These must exhibit a high degree of specificity and react rapidly with their antigen at low temperatures. As a result, many antibody preparations were found to be unsuitable for this technique. A further disadvantage was the low yield of H5 mRNA produced by this procedure, which made it impossible

to determine the exact size of the mRNA by electrophoresis on polyacrylamide gels containing formamide. Although hybridisation to H5 cDNA indicated that the H5 mRNA was of about 700 nucleotides in length (figure 6.7), and of high purity, it is still important to check this on gels. It is therefore *essential* to produce larger amounts of H5 mRNA before the work with this mRNA can continue. The information available in this thesis indicates how this may be achieved. For example, the 5-18S polysomal RNA which does not bind to oligo (dT)-cellulose, could be fractionated on high resolution polyacrylamide gels. The H5 mRNA could then be located by hybridisation of H5 cDNA (prepared as described in this thesis) to the RNA fractions.

Probably the most important problem which must be investigated is whether H5 mRNA as well as H5 protein is still being made in reticulocytes. If the H5 mRNA were simply more stable than the mRNAs for the other histones, then the synthesis of H5 and any gene controlling functions which it exhibits could be subject to some form of translational control.

8.3 HISTONE H5 GENES

The results presented in this thesis indicate that cDNA can be produced from small amounts of non-polyadenylated RNA. Using the techniques described in chapter 2, H5 cDNA was produced and shown to be a representative copy of its RNA template, *provided* that poly A-containing RNA was removed first. This implies either that the polyadenylation process was not as efficient as it might be, or that natural poly A-

containing RNA was inherently more efficiently copied. Whatever the reason for this poor copying, the amount of cDNA must be increased substantially before further work can be carried out. If the poly A-polymerase reaction can be improved and thereby improve the copying efficiency, then this will result in larger amounts of cDNA. However, if this is not feasible, then more H5 mRNA must be produced for copying into cDNA.

Using the available cDNA it has been demonstrated that the gene coding for H5 is reiterated 10-fold in the chicken genome. While this result is interesting in itself when compared to histone gene reiteration frequencies in other organisms, it is impossible to draw any conclusions regarding its significance without further data. Since H5 is expressed in a different fashion from the other histones, then an investigation of the relationship of H5 genes to the other histone genes may indicate how the expression of histone genes is controlled and why these genes are clustered, at least in sea urchins. The two major questions to be answered are:-

(i) Is the reiteration frequency of H5 genes different from that of the other histone genes in chickens? and

(ii) Are the H5 genes clustered with the other histone genes in chickens?

If the H5 genes were clustered with the other histone genes as tandem repeats, then this clustering probably would not be responsible for the co-ordinate expression of histone genes as H5 is made quite independently

of the other histones.

8.4 FURTHER STUDIES

Assuming that the amounts of H5 mRNA and cDNA available can be increased as outlined in sections 8.2 and 8.3, the major lines of investigation to be followed are:-

(i) the synthesis of H5 mRNA in reticulocytes: It is important to establish if H5 mRNA is not being synthesised in reticulocytes as this would introduce the possibility of translational control of H5 synthesis.

(ii) the reiteration frequency of the other histone genes in chickens: Since histone H5 is different from the other histones in many of its properties (see chapter 1), it is of some interest to establish whether the genes coding for this histone are reiterated to the same extent as those coding for the other histones. The mRNAs for the other histones could be isolated from any dividing, non-erythroid chicken tissue, e.g. fibroblasts, by established procedures (Jacobs-Lorena *et al.*, 1972; A. Scott and P. Krieg, unpublished results) and cDNA prepared from this as described in chapter 2. This cDNA could then be employed in estimating the reitration frequency of the genes coding for the other histones, as has been done for H5 genes.

(iii) the possible clustering of the genes for H5 with the genes for the other histones: This

could be investigated by hybridisation of cDNA to H5 and to all other histones, to metaphase chromosomes *in situ*, or to fractionated DNA (Kedes and Birnstiel, 1971). While such studies are suggestive of clustering, an unequivocal answer could be obtained by showing that the genes are all present on a single piece of DNA, by subculture cloning (Kedes *et al.*, 1975).

Such studies as these may give some indication of the way in which histone genes, in particular H5 genes, are controlled. The work described in this thesis was designed to make these investigations possible.

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