Complementary DNA To Sea Urchin Histone Messenger RNA.

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Parechinus angulosus

CERTIFICATE OF SUPERVISOR

In terms of paragraph eight of 'General regulations for the degree of Ph.D.' I, as supervisor of the candidate, D.E. Woods, certify that I approve of the incorporation into this thesis of material that has already been published or submitted for publication.

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To

My Parents

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SUMMARY

A preparation of 9S RNA was isolated from early blastula sea urchin embryo polyribosomes by phenol extraction followed by repeated sucrose density gradient centrifugation. When incubated in an ascites cell free protein synthesizing system this preparation of RNA was shown to support the synthesis of sea urchin histones confirming that this fraction contained histone mRNA.

Prior to reverse transcription of the histone mRNA it was necessary, due to the lack of a 3'OH poly(A) tail, to polyadenylate the histone mRNA *in vitro*. This was achieved using either a poly(A) polymerase which was isolated from E.coli or a polynucleotidylexotransferase which was isolated from maize seedlings. This *in vitro* polyadenylation rendered the histone mRNA suitable as a template for reverse transcriptase and yields of cDNA as high as 15-20% of the input mRNA have been achieved. When these cDNA preparations were hybridized back to histone mRNA the kinetics of the hybridization reaction indicated that the cDNA contained complementary sequences to RNA molecules other than histone mRNA. Hybridization of the cDNA to purified ribosomal RNA showed that the cDNA was extensively contaminated (85% of hybridizable cDNA) with sequences complementary to rRNA derived from the large ribosomal subunit; the histone mRNA preparation was therefore contaminated with rRNA which became polyadenylated and reverse transcribed *in vitro* with the histone mRNA. Purification of a histone mRNA specific cDNA fraction was achieved by hybridization of purified rRNA to the cDNA followed by fractionation on hydroxylapatite and isolation of single stranded cDNA. After further purification to remove nonhybridizable cDNA, the histone mRNA specific cDNA constituted 84% of the hybridizable DNA (compared to 15% in the original cDNA).

This histone mRNA specific cDNA was used in hybridization experiments to investigate the cytoplasmic localization of maternal histone mRNA in the unfertilized sea urchin egg and first cleavage stage embryos. It was shown that in the unfertilized egg the maternal histone mRNA was localized exclusively in ribonucleoprotein particles with no histone mRNA in the polyribosomes. After fertilization the cytoplasmic localization of the histone mRNA was shown to change and in particular, coupled with the first cleavage telophase (period of DNA synthesis), there was a transfer of histone mRNA from the ribonucleoprotein fraction to the polyribosomes.

PART 1

INTRODUCTION

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PART 1

INTRODUCTION

1.1 DEVELOPMENTAL BIOLOGY OF THE SEA URCHIN EMBRYO DURING EARLY DEVELOPMENT

The cells of the cleaving sea urchin embryo differ in a fundamental respect from other dividing procaryotic or eucaryotic cells studied : there is no growth, no net accumulation of RNA or protein, and no change in dry weight during early embryonic development (for review see Giudice, 1973).

1.1.1 DNA SYNTHESIS

In the unfertilized sea urchin egg there is a larger amount of DNA per cell than would be expected for an echinoid haploid nucleus (0.8 - 1.0 pg DNA/nucleus). This was shown by Piko et al. (1968) to be due to the presence of large amounts of mitochondrial DNA which comprised about 65% of the total egg DNA. Embryonic development leads to an increase in the number of nuclei per embryo without a parallel increase in the number of mitochondria and by the gastrula stage the nuclear DNA content overwhelms that of the mitochondria.

Although there is no net increase in mass of the embryo up to the prism stage there is a marked increase in the DNA content per embryo (Giudice, 1973). When does this DNA synthesis start ? A number of results using different species of echinoderms and different methodology indicate that some DNA synthesis takes place even before the pronuclei of the fertilized egg have fused (Nemer, 1962; Brookbank, 1970). The DNA synthesis during the first two cleavages after fertilization of Strongylocentrotus purpuratus was accurately studied by Hinegardner et al. (1964) and it was shown that the bulk of the DNA synthesis starts during telophase and lasts about 13 minutes until the beginning of the interphase. These results are in agreement with those of Nemer (1962) with Paracentrotus lividus and have more recently been confirmed by Fansler and Loeb (1969) and Hobart et al. (1977).

1.1.2 RNA SYNTHESIS

In the mature sea urchin egg little of no RNA synthesis is detectable (Nemer and Infante, 1967a; Levner, 1974) and there is no detectable accumulation of RNA prior to the feeding larvae stage (Wu and Wilt, 1974). The fact that RNA synthesis starts soon after fertilization was first shown by Wilt (1963, 1964) and Nemer (1963). Immediately after fertilization labelled precursors were found incorporated into 4S RNA; this has been shown to be due to turnover of the terminal CCA of tRNA (Glisin and Glisin, 1964). The results of Renaldi and Monroy (1969) indicated that RNA synthesis, in the form of newly synthesized high molecular weight RNA, may start as early as the first cleavage stage but RNA synthesis in the first cleavage stage has not been conclusively demonstrated. Slater and Spiegelman (1970) however have shown that a complex family of RNA was synthesized at, or prior to, the 4 cell stage and subsequent development was shown to be accompanied by an increase in both the number of distinct classes of RNA and the average molecular weight of the RNA. A marked increase in the rate of RNA synthesis has been shown to take place at the 8-16 cell stage (Wilt, 1970).

The RNA synthesized during early cleavage stages is DNA-like, metabolically labile RNA (Nemer, 1963; Wilt, 1964) and the results of kinetic, autoradiographic and cell fractionation experiments have shown that in the pregastrula embryo over 90% of this newly synthesized DNA-like RNA turns over in the nuclei (Aronson and Wilt, 1969; Emerson and Humphreys, 1970; Hogan and Gross, 1972; Aronson et al., 1972). Analysis under denaturing conditions indicates that this RNA is about 8000-9000 nucleotides in length (Dubroff and Nemer, 1975).

This DNA-like RNA is the only detectable class of RNA synthesized during cleavage stages (Glisin and Glisin, 1964; Gross et al., 1964), whereas rRNA is synthesized at the late gastrula and pluteus stages (Nemer and Infante, 1967). Ribosomal RNA does not constitute a significant percentage of the RNA synthesized at any stage of development, but was shown by labelling experiments over extended periods to accumulate in the embryo relative to DNA-like RNA (see Giudice, 1973). This preferential accumulation of rRNA is much greater at later than early developmental stages. Accumulation of newly synthesized rRNA was detected by Emerson and Humphreys (1970) in embryos after the first half of the blastula stage but other investigators (Giudice

and Mutolo, 1967; Slater and Spiegelman, 1970; Sconzo and Giudice, 1971) indicated that rRNA is not produced until the late blastula or early gastrula stage. The synthesis of 5S rRNA however starts very much earlier than 18S and 26S RNA in the development of the sea urchin embryo (Emerson and Humphreys, 1970; O'Melia and Vallee, 1972).

As described above a large percentage of the newly synthesized RNA turns over in the nucleus. The fraction of this newly synthesized RNA which leaves the nucleus, during cleavage stages, becomes divided equally between the polyribosome and a free ribonucleoprotein (RNP) fraction sedimenting from 10-74S (Infante and Nemer, 1968; Dworkin and Infante, 1976) but the percentage of the newly synthesized RNA in the RNP fraction decreases with development. The RNAs present in the RNP and the polyribosomal fraction are of two distinct types but both have mRNA properties indicating that the fractionation of the RNA between the polyribosomes and RNP is not random (Dworkin and Infante, 1976). The polyribosome bound fraction of the newly synthesized RNA is found associated with light polyribosomes rather than heavy ones (Rinaldi and Monroy, 1969; Kedes and Gross, 1969a,b). Kedes and Gross (1969a) initially suggested that the mRNA bound to the light polyribosomes may represent the mRNA specific for histone synthesis.

In the sea urchin three classes of newly synthesized cytoplasmic mRNA have been distinguished : a) poly(A) containing mRNA, b) non-histone poly(A)$^-$ mRNA and c) the histone mRNA, the most prominent class during the mid-blastula phase (Nemer et al., 1974, 1975; Fromson and Duchastel, 1975; Fromson and Verma, 1976). These cytoplasmic mRNA molecules have a broad size range of average length about 2000 nucleotides of which up to 200 nucleotides could be poly(A) and the average half life of mRNAs has been shown, by two different methods, to be about 5.5 hours (Nemer et al., 1974, 1975; Nemer, 1975; Galau et al., 1977).

In the sea urchin embryo transcription of mitochondrial DNA begins shortly after fertilization and it has been estimated that 50-80% of the newly synthesized RNA during early cleavage consists of mitochondrial transcripts of a distinct size class between 11-17S (Chamberlain and Metz, 1972).

1.1.3 PROTEIN SYNTHESIS

The mechanism by which protein synthesis can be regulated is of great interest in molecular biology. The sea urchin egg offers an ideal system of study due to the very rapid "switching on" of protein synthesis which takes place at fertilization thus providing a suitable system for the comparison of the protein synthetic machinery in the unfertilized (inactive) and fertilized egg (active) (for review see Giudice, 1973; Davidson, 1976). As mentioned earlier, the total amount of protein per embryo does not change noticeably throughout development up to the prism stage (Fry and Gross, 1969).

Proteins are actively synthesized during oogenesis but the level of protein synthesis is low in the resting unfertilized egg (Epel, 1967; Humphreys, 1969). One of the most striking phenomena which accompanies fertilization is the rapid increase in the rate of protein synthesis (Epel, 1967; Gross, 1967). The results of Epel et al. (1967) showed an apparent lag of 5-10 minutes between the cortical events of fertilization and the increased protein synthetic activity. The results of Timourian and Watchmaker (1970) however have shown that the protein synthetic rate increases 1-2 min after fertilization and showed that the apparent lag reported by Epel (1967) was due to the release of ^{14}C-valine from the egg. The rate of increase of protein synthesis following fertilization was shown to be approximately 15 fold within 20 min of fertilization (Epel, 1967; Humphreys, 1969); the incorporation rate rising rapidly to a plateau in cleavage stages and then showing another significant increase before gastrulation (Gross and Cousineau, 1964; Berg, 1968).

The stimulation of protein synthesis after fertilization has been investigated at the subcellular level using cell free systems prepared from unfertilized and fertilized sea urchin eggs, and it has been shown that a greater number of ribosomes are in the form of polyribosomes after fertilization showing that the stimulation of protein synthesis involves the formation of polyribosomes and not merely the activation of polyribosomes present in the egg (Stavy and Gross, 1969; Humphreys, 1969; Rinaldi and Monroy, 1969; Fromson and Nemer, 1970).

A more detailed discussion on the protein synthetic activity will be given below where the significance of maternal mRNA is discussed.

1.1.4 MATERNAL mRNA

The increased rate of protein synthesis after fertilization has been shown to take place even in the absence of new RNA synthesis, which was eliminated either by enucleation (Denny and Tyler, 1964) or by inhibition of transcription with actinomycin D (Gross, 1967). This phenomenon indicates that the RNA of the protein synthetic machinery (rRNA, tRNA and mRNA) necessary for increased protein synthesis is present in the egg before fertilization i.e. maternal (Gross et al., 1964; Gross, 1967). In the absence of RNA synthesis this maternal RNA can permit a near normal rate of protein synthesis and support apparently normal development up to blastulation (Gross and Cousineau, 1963).

The most direct proof for the presence of maternal mRNA in sea urchin eggs came from its extraction and characterization by translation in cell free systems (see below). Slater and Spiegelman (1966) isolated RNA from unfertilized sea urchin eggs and estimated from its translational capacity (compared with the activity of viral mRNA) that it contained about 4% mRNA. Similar experiments were carried out with Xenopus laevis oocytes and 2-3% of the RNA was assumed to be mRNA (Davidson et al., 1966). Similar estimates as to the amounts of maternal mRNA in unfertilized eggs have come from the analysis of the amount of poly(A) containing RNA (Davidson, 1976). From these estimates Davidson (1976) has calculated that there are 0.05 - 0.1 ng of maternal mRNA present per sea urchin egg.

Maternal mRNA constitutes more than 85-90% of the polyribosomal mRNA during the first two hours of development (Humphreys, 1971), the remainder being newly synthesized mRNA. The maternal mRNA in the unfertilized egg has recently been shown to contain a methylated "capped" 5' terminus (Hickey et al., 1976; Pirrone et al., 1977). Methylation of the 5' terminal cap on the maternal mRNA was put forward as a possible mechanism for the activation of protein synthesis at fertilization but the results of Pirrone et al. (1977) strongly oppose this hypothesis.

In the unfertilized egg the maternal mRNA is localised in cytoplasmic ribonucleoprotein (RNP) particles (Gross et al., 1973b). These RNP particles are similar to the informosomes described by Spirin (1966). Gross et al. (1973) showed that mRNAs isolated from different postribosomal fractions stimulated the synthesis of different proteins and the RNA from one

fraction in particular was shown to support the synthesis of histones. Skoultchi and Gross (1973) have shown sequence homology between maternal and embryonic histone mRNAs by competition hybridization experiments. Similarly, unfertilized sea urchin egg RNA (total) was shown to compete in competition hybridization experiments with both total (Farquhar and McCarthy, 1973) and fractionated labelled individual histone mRNA preparations (Lifton and Kedes, 1976).

The identification of a particular class of maternal mRNA, such as the histone mRNA fraction, offers the opportunity to study particular species of RNA coding for specific proteins. An investigation into the stimulation of synthesis of a defined class of well characterized proteins is therefore possible and may give an insight into the mechanism by which maternal histone mRNA becomes available for protein synthesis. The high rate of histone synthesis during early sea urchin embryonic development (Moav and Nemer, 1971; Seale and Aronson, 1973; Ruderman and Gross, 1974) should aid such an investigation.

Histone synthesis is tightly coupled to the rate of DNA synthesis and also to the rate of cell division (Kedes et al., 1969b; Ruderman and Gross, 1974). These factors together with the fact that histones have not been isolated from the cytoplasm of unfertilized eggs (although this has been attempted, Benttinen and Comb, 1971) argue against the presence of a stockpile of histones in the unfertilized egg. Histone synthesis has been shown to be present at the 32 cell stage and represents about 5% of the total protein synthesis whereas at the mid-blastula state it represents 25-40% of the total protein synthesis (Seale and Aronson, 1973). Ruderman and Gross (1974) have shown that histone synthesis starts as early as the first cleavage stage.

The identification of histone mRNA in the maternal RNA and the fact that it is a major fraction of the newly synthesized mRNA raises the question as to the function of the maternal histone mRNA. Is the rate of histone mRNA synthesis insufficient to produce the required amounts of mRNA? The amount of the histone mRNA in the embryo which is maternal compared with newly synthesized has been investigated by protein labelling experiments in the presence and absence of actinomycin D, which blocks incorporation of label into the 9S histone mRNA fraction (Kedes et al., 1969a;

Ruderman and Gross, 1974). During cleavage stages about 60% of the histone synthesis is actinomycin D resistant i.e. maternal mRNA dependent. The percentage actinomycin D resistant synthesis drops during development which may be due to degradation or turnover of the maternal mRNA templates. From these actinomycin D studies Davidson (1976) has estimated the amount of maternal histone mRNA per egg as about 4-8% of the total maternal mRNA.

The results described above have relied on indirect methods (mRNA translatability) for the analysis and quantitation of maternal mRNA. The use of mRNA specific complementary DNA probes would represent a more direct and more accurate method to carry out these investigations.

1.2 THE USE OF COMPLEMENTARY DNA PROBES FOR THE INVESTIGATION OF RNA METABOLISM

Complementary DNA (cDNA) probes have been used extensively in hybridization analyses for the elucidation of gene frequency, determination of sequence relationship between nucleic acids, for detecting and quantitating mRNA in very low concentration and determining the sequence complexity of RNA populations (see below).

The first workers to report the successful synthesis of a cDNA from a specific mRNA (globin mRNA) *in vitro* using viral reverse transcriptase were Ross et al. (1972) and Verma et al. (1972). The specificity of the probe in hybridization reactions, i.e. their ability to discriminate among very similar sequences during hybridization, was investigated by comparing the hybridization of rabbit globin cDNA to rabbit, mouse and duck globin mRNA (Leder et al., 1974). Despite the similarities in amino acid sequences of the globins of these three species, variations were found in the rates and extent of hybridization showing that very similar sequences could be distinguished by this type of assay. These results together with those of Harrison et al. (1974) demonstrated that the cDNA to globin mRNA were faithful but partial transcripts and indicated that a maximum of 2% base errors occurred during transcription with reverse transcriptase.

cDNA preparations can be labelled to very high specific activities and for this reason they have replaced labelled globin mRNA and other mRNAs, which cannot be easily labelled, for hybridization analyses of gene number and structure (e.g. Harrison et al., 1972). Globin cDNA has been used in

hybridization analyses to determine the reiteration frequency and organization of globin genes; for example Harrison et al. (1972) found that the bulk of mouse globin cDNA hybridized to unique mouse DNA indicating that mouse globin mRNA is transcribed from unique sequences. Similar results have been obtained with duck globin cDNA hybridized to duck DNA (Packman et al., 1972; Bishop and Rosbash, 1973). Harrison et al. (1974), using various size fragments of mouse DNA, have reported that these unique sequences were interspersed with repeated DNA sequences. The preparation and purification of cDNA to the mRNA of specific human globins has permitted the estimation of the number of genes coding for individual human globins (α, β, γ and δ) (Ottolenghi et al., 1974, 1975; Kan et al., 1975 and Old et al., 1976).

Complementary DNAs have been extensively used to investigate the presence, complexity and subcellular localization of mRNA. Imaizumi et al. (1973), using a cDNA to duck globin mRNA, showed the presence of globin mRNA sequences in the heterogeneous nuclear RNA (HnRNA) suggesting that the HnRNA contains high molecular weight precursors to the globin mRNA. Comparison of $Cot_{\frac{1}{2}}$ values of the cDNA:globin mRNA and cDNA:HnRNA reactions led to the estimate of 0.3 - 1.5% as the percentage of globin mRNA in the HnRNA. Similar investigations to determine the primary product of transcription of the ovalbumin genes demonstrated that the initial product of transcription was the same size as the polysomal ovalbumin mRNA (McKnight and Schimke, 1974).

Young et al. (1974) reported a titration technique for determining the amount of a particular mRNA in an RNA preparation by hybridization with cDNA. This gave more accurate estimates of, for example, the percentage globin mRNA in a particular RNA preparation than could be obtained from the comparison of $Cot_{\frac{1}{2}}$ values of cDNA:RNA reactions. These titration reactions are carried out with a fixed amount of cDNA incubated with increasing amounts of RNA under hybridizing conditions. Reaction mixtures are incubated to a Cot value (determined from the cDNA concentration) of 10-20 times the $Cot_{\frac{1}{2}}$ of the particular cDNA:mRNA reaction. These conditions allow completion of even the slowest reaction ensuring that any sequences complementary to the cDNA, even in very low concentration, will form hybrids with the cDNA (Young et al., 1974). Titration plots (% cDNA hybridized v/s RNA:cDNA ratio) show an almost linear relationship at low levels of hybridization. The curvature of the titration plots was shown to be due to size heterogeneity

in the cDNA preparation (Young et al., 1974). From titration curves it is possible to determine the concentration of mRNA in an RNA population by comparing the initial slope of the titration with that of the cDNA:mRNA titration or the inflection points of the two curves (for example see Gilmour et al., 1974; Humphries et al., 1976). By this titration method it is possible to detect extremely small amounts of mRNA and Humphries et al. (1976) have detected as little as 5×10^{-5}% globin mRNA in the cytoplasmic RNA of lymphoma cells. Using such information Humphries et al. (1976) have been able to calculate the number of globin mRNA molecules per cell in erythroid and non-erythroid tissue. Similarly the number of ovalbumin mRNA molecules per tubular gland cell of the chick oviduct before and after stimulation with oestrogen have been determined (Harris et al., 1975). These results indicated that cDNAs to specific messenger RNAs could be used as extremely powerful tools for the investigation of transcriptional and translational control mechanisms.

1.3 SEA URCHIN HISTONE mRNA

Sea urchin histone mRNA, unlike other mRNAs studied (e.g. globin and ovalbumin mRNAs discussed above), can be labelled *in vivo* to high specific activities due to high levels of histone mRNA synthesis in the absence of rRNA synthesis during early cleavage stages. For this reason histone mRNAs, and not histone cDNAs, have been used extensively in hybridization analyses for the elucidation of the structural organization of the histone genes (for review see Kedes et al., 1976).

The high G + C content of histone mRNA, and therefore histone genes (51-58%, Grunstein and Schedl, 1976), allowed the purification of histone DNA from the bulk of the DNA by CsCl (Kedes and Birnstiel, 1971) and CsCl-actinomycin D (Birnstiel et al., 1974) centrifugation. The development of plasmid technology has also allowed the purification of histone DNA from unfractionated EcoRl digested total sea urchin DNA by sub-culture cloning in E.coli (Kedes et al., 1975). By digestion of "purified" histone DNA with specific restriction endonucleases the histone coding sequences have been shown to alternate with spacer DNA (Schaffner et al., 1976; Portman et al., 1976). The coding sequences have been shown to be on the same DNA strand and arranged with 5' - 3' polarity in the sequence H1 $\longrightarrow$H2A $\longrightarrow$ H3 $\longrightarrow$ H2B $\longrightarrow$ H4 (Gross et al., 1976b).

The availability of a probe for sea urchin histone mRNA would allow, by the techniques described above (1.2), an investigation into histone mRNA metabolism in sea urchin eggs and embryos. The questions which could possibly be answered are :

a) When does maternal histone mRNA first become utilized in sea urchin embryos ?

b) Is the amount (number of molecules) of histone mRNA increased drastically at any stage of development ?

c) If there is a constant amount of histone mRNA per cell or embryo does this indicate preferential degradation of maternal histone mRNA with the onset of new histone mRNA synthesis ?

d) At the 16 cell stage, where the first morphological differentiation occurs (four macromeres, eight mesomeres and four micromeres) is there an equal segregation of cytoplasmic constituents containing histone mRNA ?

e) Is all the maternal histone mRNA, which is stored in RNP particles in the unfertilized egg, utilized during early development (there is no accumulation of newly synthesized histone RNA in these RNP particles during development; Dworkin and Infante, 1976) ?

Answers to these questions would contribute substantially to an understanding of RNA metabolism during early embryonic development.

The preparation of specific probes for histone mRNA could possibly be approached as follows :

a) The purification by molecular cloning of DNA fragments complementary to histone mRNA. The strands of cloned Psammechinus miliaris histone DNA (Clarkson et al., 1976) can be separated by electrophoresis (Gross et al., 1976b) which may allow for the preparation of highly labelled single stranded DNA complementary to histone mRNA.

b) The synthesis of a histone mRNA specific complementary DNA using viral reverse transcriptase which is the approach which has been adopted here.

The work reported in this thesis in mainly concerned with the production and purification of a histone mRNA specific cDNA probe (Woods and Fitschen, 1977). Its application to a study of histone mRNA metabolism during the first cleavage stage of the sea urchin embryo is also reported.

PART 2

THE ISOLATION AND CHARACTERIZATION OF HISTONE mRNA FROM SEA URCHIN EMBRYOS

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2.1 INTRODUCTION

During early embryonic development of the sea urchin there is a rapid rate of cell division, cleavage occurring every 30-50 minutes. With the rapid rate of DNA synthesis which is required for chromosomal replication, there is a concomitant rate of histone synthesis; in fact nuclear proteins have been shown to account for as much as 60% of the proteins synthesized during cleavage stages of which at least half are histones (Kedes et al., 1969b; Moav and Nemer, 1971). Infante and Nemer (1967) have shown that a prominent class of light polysomes was present in the sea urchin embryos, and which has been shown to synthesize histones (Kedes et al., 1969(a); Kedes et al., 1969(b); Moav and Nemer, 1971). Other workers have shown that a distinct class of rapidly labelled 9S mRNA (putative histone mRNA) was associated with these polysomes (Nemer and Infante, 1965; Kedes and Gross, 1969a; Kedes and Gross, 1969b). When 9S RNA was isolated from sea urchin polysomes and used as a template in a cell free protein synthesizing system it was shown to support the synthesis of histones and no other proteins (Gross et al., 1973a; Levy et al., 1975), indicating that in the 9S RNA preparation there are no other messengers except those coding for histones. A similar 9S mRNA isolated from S-phase HeLa cell polyribosomes has also been shown to support the synthesis of histones (Jacobs-Lorena et al., 1972; Breindl and Gallwitz, 1973).

The synthesis of histones which constitutes 25-30% of total protein synthesis (Kedes, 1976), has been shown to be largely dependent on mRNA synthesized after fertilization (Kedes and Gross, 1969(a); Skoultchi and Gross, 1973). This histone mRNA representing a majority species (70%) of the newly synthesized RNA (Nemer et al., 1974; Nemer, 1975) could therefore be labelled in vivo, making possible the isolation of highly labelled preparations of histone mRNA. The fact that neither ribosomal RNA (Gross et al., 1964) nor ribosomal RNA precursors (Giudice and Mutolo, 1969) are synthesized in detectable quantities during cleavage stages of the sea urchin embryo is of great advantage in the isolation of labelled histone mRNA preparations. Labelled 9S mRNA isolated from the light polysomes by sucrose gradient centrifugation, has been fractionated and further purified by polyacrylamide gel electrophoresis (Kedes and Gross, 1969(a); Grunstein et al., 1973; Levy et al., 1975). Gross et al. (1976) have recently fractionated and identified five individual histone mRNAs.

Labelled histone mRNA preparations have been used extensively in RNA:DNA hybridization reactions to elucidate the number (Weinberg et al., 1972) and structural organization of the histone genes (reviewed Kedes, 1976). The fact that the histone genes are reiterated 500-1000 fold (Weinberg et al., 1972) and are clustered forming a high density satellite on CsCl centrifugation (Kedes and Birnstiel, 1971) has allowed the enrichment, by actinomycin D-CsCl gradient centrifugation, of DNA coding for histones (Birnstiel et al., 1974; Weinberg et al., 1975). Kedes et al. (1975a, 1975b) have recently cloned two EcoRl restriction endonuclease fragments from this enriched histone-DNA into E.coli plasmids.

In all the work described above labelled histone mRNA preparations have been used for the identification and localization of histone genes. In this section I will describe the isolation and characterization of an unlabelled preparation of histone mRNA from sea urchin embryos to be subsequently used for the preparation of a histone mRNA specific complementary DNA.

2.2 THE SEA URCHINS

The species of sea urchin found around Cape Town and used throughout this study was Parechinus angulosus. Although sea urchins from the Atlantic and the Indian Oceans around the Cape are taxonomically the same species, differences have been found in the histones isolated from urchins obtained from the two oceans (W. Brandt and C. von Holt, personal communication). For this reason all specimens of Parechinus angulosus used in work for this thesis have been obtained from the Atlantic Coast, 30 Km north of Cape Town. Urchins were either used immediately on arrival in the laboratory or were maintained in closed system aquaria as described in Materials and methods (5.3.1). There are a number of excellent reviews on the care and handling of sea urchin eggs, embryos and adults (Harvy, 1956; Tyler and Tyler, 1966; Hinegardner, 1967; Hinegardner, 1975) and all the work with sea urchins was carried out with reference to these articles.

Parechinus angulosus has two spawning seasons, a major season in January-March and a second minor season in June-July, and at these times it was possible to obtain large numbers of gametes. In the January-March season yields as high as 25 ml of packed eggs have been obtained from a single urchin. Despite seasonal variations we have found it possible to obtain small numbers of gametes at any time of the year.

Fertilization and development of embryos

Fertilization and development was carried out as described in Materials and methods (5.3.1). Under the conditions used eggs became rapidly fertilized after the addition of sperm and the fertilization membrane usually formed within a minute. In all experiments eggs were only used if they showed greater than 95% fertilization, as measured by the appearance of the fertilization membrane. On some occasions however, it was found that a particular batch of eggs would not become fertilized. This was a characteristic of the egg suspension and was independent of the amount or source of the sperm added. The reason for this is unknown and when it happened the batch of eggs was discarded.

Fertilized eggs developed at 22°C (± 1°C) were shown to develop synchronously during early cleavage (Table 2.1). The first division takes place about 55 minutes (± 2 min) after fertilization and thereafter, at least up to the 32 cell stage, the cells divide every 25-30 minutes. The first division shows a high degree of synchrony, all the eggs of a particular batch dividing within 1-2 minutes of each other. The degree of synchrony, however, decreases with development and it can take up to 10 minutes for all the embryos in the 16 cell stage to divide to give 32 cell stage embryos. Usually at this stage about 85% of the embryos divide within the 5 minute period, 165-170 minutes after fertilization. It was not possible to determine the degree of synchrony of cell division beyond the 32 cell stage, because of the difficulty of determining the number of cells per embryo, but it was found that at any particular time after fertilization a large majority (>90%) of a batch of embryos were at the same stage of development e.g. early blastula 4 h, swimming blastula 8 h, early gastrula 20 h etc. Fig. 2.1 shows clearly the developmental stages of Parechinus angulosus and the times after fertilization during which these stages exist.

2.3 ISOLATION OF HISTONE mRNA

Messenger RNAs which contain a 3'OH terminal poly(A) tail (Edmonds et al., 1971; Lee et al., 1971; Darnell et al., 1971) can be isolated from polyribosomal RNA by chromatography on oligo(dT) cellulose (Aviv and Leder, 1972). The absence of the poly(A) tail on histone mRNA (Adesnik and Darnell, 1972) makes its purification from polyribosomal RNA more difficult.

TABLE 2.1

THE SYNCHRONY OF DEVELOPMENT OF SEA URCHIN EMBRYOS

The Table shows a) the time after fertilization at which a particular division takes place with the range found in different experiments;

b) the % of a particular batch of eggs which divide from one stage to the next during the time indicated in (a);

c) the number of cells per embryo

d) the average duration of each cell stage.

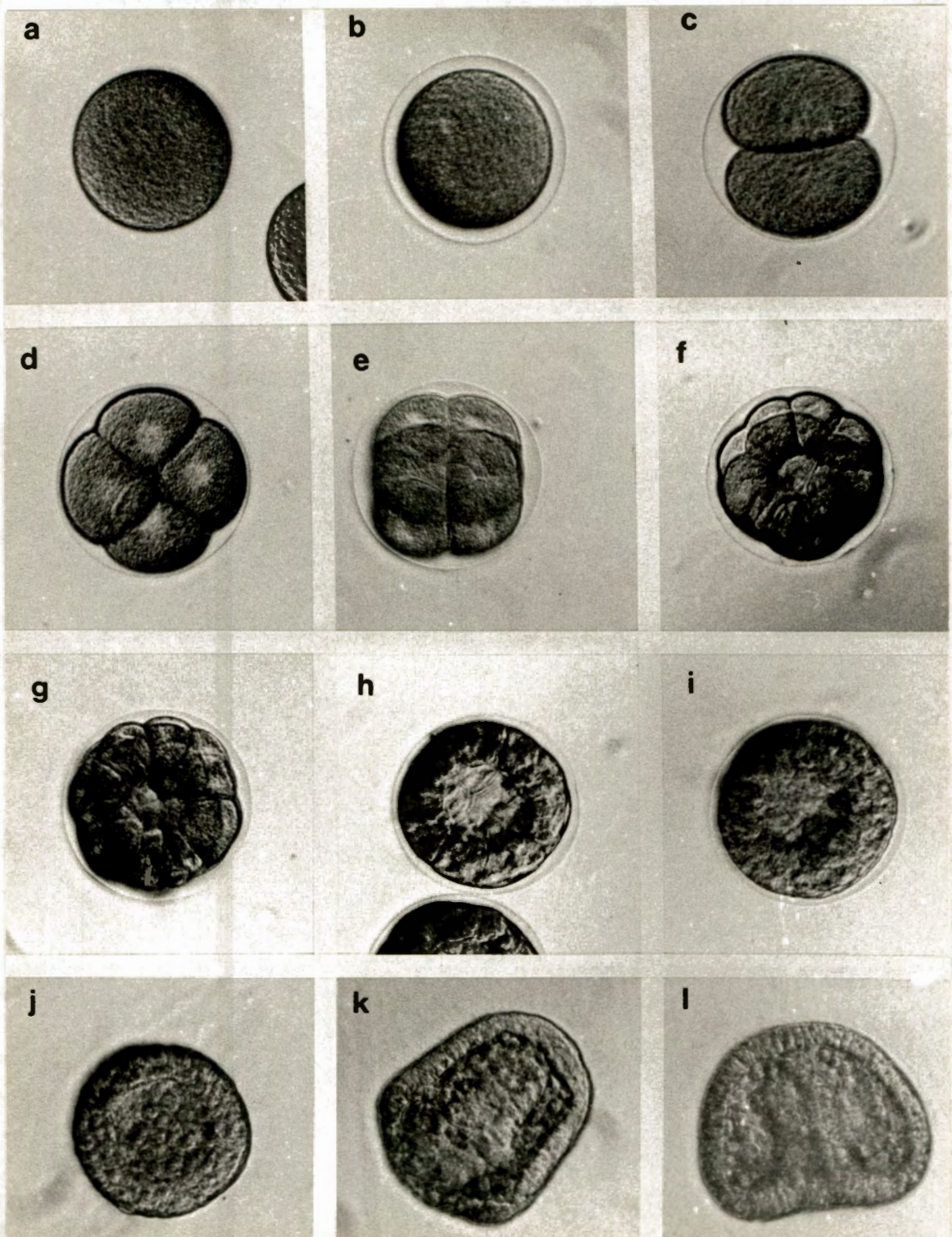
The results given are the average results of a number of experiments in which eggs have been fertilized and developed at 22°C.

	After fertilization		c)	d)
	a) Time (min)	b) % of total eggs	Stage of development	Average duration of cell stage (minutes)
Fertilization	0	98	Fertilized egg 1 cell	55
1st Division	53-57	98	2 cell	30
2nd Division	85-90	95	4 cell	30
3rd Division	115-120	93	8 cell	25
4th Division	140-145	90	16 cell	25
5th Division	165-170	85	32 cell	

FIG. 2.1

DEVELOPMENT OF THE SEA URCHIN EMBRYO

a) Unfertilized egg b) fertilized egg (30 min), c) 2-cell stage (70 min), d) 4-cell stage (1½ h), e) 8-cell stage (2 h), f) 16-cell stage (2½ h), g) morula (3 h), h) early blastula (3½ h), i) blastula (6 h), j) hatched blastula (9 h), k) early gastrula (18 h), l) late gastrula (25 h). (The times recorded represent the times after fertilization at which the photographs were taken).



The procedure used for the isolation of sea urchin 9S histone mRNA in this work involved successive sucrose density gradient fractionation of polyribosomal RNA.

2.3.1 ISOLATION OF SEA URCHIN EMBRYO POLYRIBOSOMES

The isolation of ribosomes, polyribosomes and RNA from sea urchin eggs and embryos was complicated by high levels of endogenous ribonuclease activity which have been shown by Humphreys (1969) to be present in homogenates. We have successfully isolated, using both bentonite (1 mg/ml) and polyvinylsulphate (10 µg/ml) as RNase inhibitors during homogenization (5.3.2.1), ribosome preparations containing polyribosomes in good yield (Fig. 2.2). Sedimentation analysis of these ribosome preparations on 15-30% sucrose gradients showed ribosome:polyribosome ratios of 55:45. The importance of the RNase inhibitors in the isolation is shown by the comparison of sedimentation patterns of ribosomes isolated in the presence and absence of RNase inhibitors (Fig. 2.3). The isolations carried out in the absence of RNase inhibitors show very low polyribosome:ribosome ratios. The use of bentonite in homogenization buffers did not result in decreased yields of ribosomal material.

The sea urchin embryo ribosomes sedimented to form a pellet of varying degrees of packing, ranging from an orange-coloured, loosely packed fraction, on a clear tightly packed fraction. In early isolations of embryo ribosomes very low yields were obtained (6-10 A_{260} units of ribosomal material/10^6 embryos) due to loss of the loosely packed ribosomes which constitute 60-70% of the ribosomal material. The yield of ribosomes obtained from preparative isolations was 25-30 A_{260} units of ribosomal material/10^6 embryos, and despite their orange colour, they showed typical ribosomal optical density profiles when scanned from 210-300 nm, with A_{260}/A_{280} ratios of 1.75 ± 0.05 indicating relatively pure ribosome preparations (Noll, 1969).

In all experiments ribosome pellets were suspended in 10 mM Tris-HCl pH 7.6 (sterile) and either used immediately or stored in liquid nitrogen. It was found that sea urchin ribosomes suspended in buffers containing 5 mM magnesium ions aggregated and precipitated when frozen. Analysis of ribosome preparations, suspended in Tris-HCl buffer, on sucrose gradients immediately

FIG. 2.2

SUCROSE DENSITY GRADIENT CENTRIFUGATION ANALYSIS
OF SEA URCHIN EMBRYO POLYRIBOSOMES

Sea urchin embryo polysomes (2.0 A_{260} units/gradient) were analysed on a 15-30% sucrose gradient at 420 000 x g (SW 65Ti rotor) for 30 min (5.2.2.3.1).

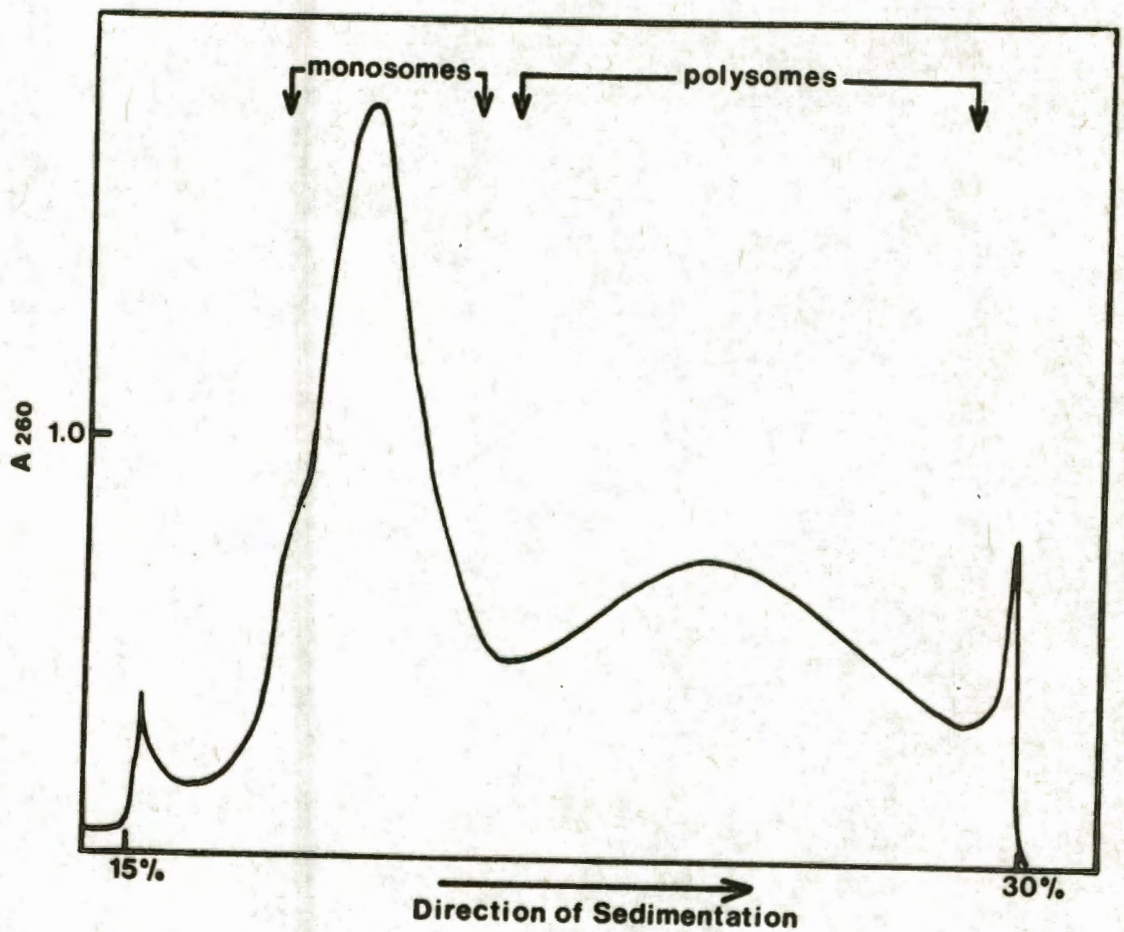


FIG. 2.3

SUCROSE DENSITY GRADIENT CENTRIFUGATION ANALYSIS OF POLYRIBOSOMES ISOLATED USING RNase INHIBITORS

Polyribosomes were isolated using :

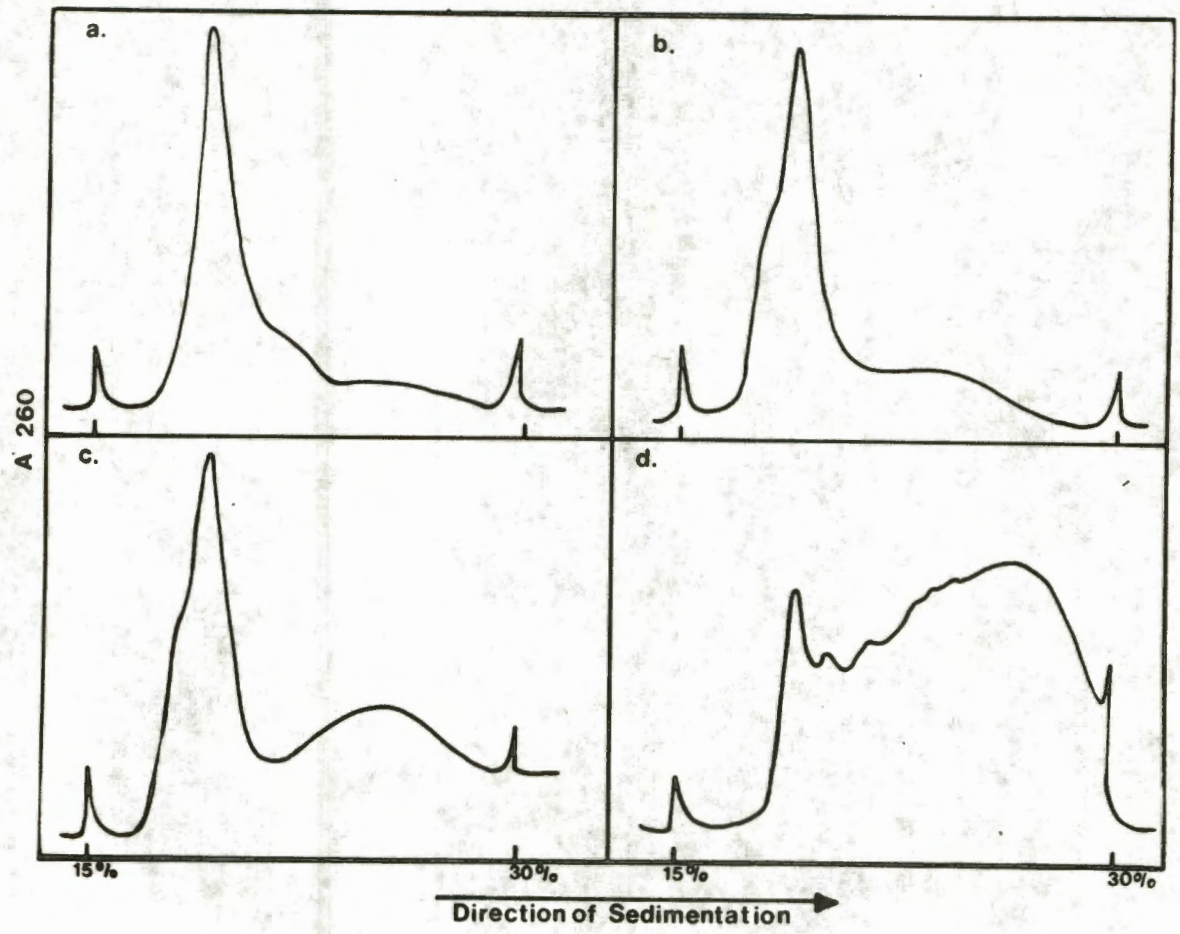
a) no RNase inhibitors

b) polyvinylsulphate 10 µg/ml

c) polyvinylsulphate 10 µg/ml and bentonite 1 mg/ml

d) rat liver polyribosomes isolated by the method of Wettstein et al. (1963).

Sucrose density gradient centrifugation was carried out as described in the legend to Fig. 2.2.



after preparation, and after storage at -100°C for various lengths of time showed that storage in the absence of magnesium did not affect the polyribosome:ribosome ratio.

2.3.2 ISOLATION OF POLYRIBOSOMAL RNA

Polyribosomal RNA was extracted with phenol from stored or freshly prepared polyribosomes as described in Materials and methods (5.3.2.2). The extractions were carried out under conditions (low ionic strength, neutral pH, water saturated phenol) which preferentially extract non-poly(A) containing RNA (poly(A)$^-$ RNA) into the aqueous phase. The poly(A) containing mRNA which does not dissociate from proteins under the conditions used, sediments with the protein gel at the phenol-water interface (Lee et al., 1971; Perry et al., 1972; Brawerman et al., 1972). This method is therefore effective in removing polyadenylated mRNA, in fact, our final preparation of 9S mRNA isolated from this sea urchin polyribosomal RNA was shown, by oligo(dT) cellulose chromatography (Aviv and Leder, 1972) (5.2.4) to contain negligible amounts of polyadenylated RNA. Under the conditions which ologo(dT) cellulose binds polyadenylated RNA a maximum of 3% of our 9S mRNA preparation bound to the column but this small amount could be accounted for by non-specific binding. The amounts of polyadenylated 9S mRNA are very low as expected from the work of Fromson and Duchastel (1975) who showed that no detectable polyadenylated 9S mRNA was synthesized during cleavage and early blastula stages in the sea urchin embryo.

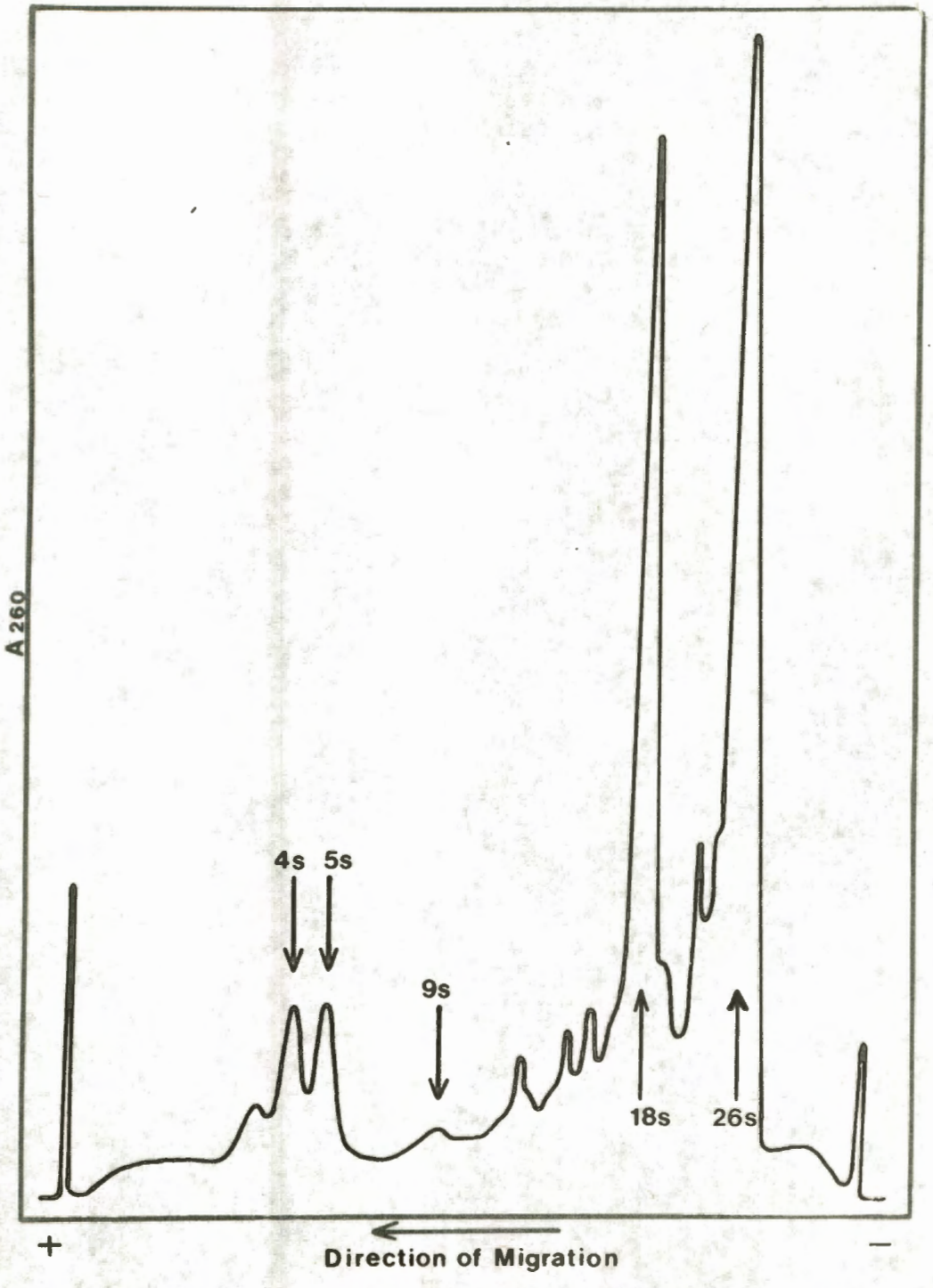
The polyribosomal RNA extractions were carried out at polyribosome concentrations of 100 A_{260} units/ml or less which resulted in RNA yields (A_{260} units) of 60 $\pm$ 5% of the starting material. The use of higher concentrations of polyribosomes for phenol extraction resulted in low yields of RNA due either to the voluminous gels at the interface or to aggregation of the RNA (Brawerman, 1973).

Analysis of polyribosomal RNA on 2.6% polyacrylamide gels (5.2.2.1) (Fig. 2.4) showed the prominent bands of 26S, 18S, 5S and 4S RNAs expected in polyribosomal RNA as well as a number of less prominent RNA bands. One of these migrated as a broad peak corresponding to 9S RNA and, as shown later, contains histone mRNA. The origin and/or function of the RNA in the other three small peaks migrating between the 18S and the 9S RNA is uncertain but they could be ribosomal

FIG. 2.4

POLYACRYLAMIDE GEL ELECTROPHORESIS OF
TOTAL SEA URCHIN POLYRIBOSOMAL RNA

Total polyribosomal RNA (2.0 A_{260}/gel) was analysed on 2.6% polyacrylamide gels (5.2.2.1) after heating RNA at 60°C for 10 min. Gels run at 5 mA/gel for 2 h.



RNA fragments resulting from specific nuclease digestion of rRNA molecules as has been found in sea urchin (Nemer and Infante, 1967) and other systems (Dalgarno and Shine, 1973; Muramatsu, 1973) (see also 3.6.1).

2.3.3 PURIFICATION OF THE 9S mRNA

The 9S RNA was purified from the total polyribosomal RNA by two successive sucrose gradient fractionation steps (Fig. 2.6). Prior to fractionation on sucrose gradients the polyribosomal RNA was heated to 60°C for 10 min and chilled rapidly to room temperature which eliminated aggregation of polyribosomal RNA (McKnight and Schimke, 1974). The importance of this heat treatment was shown by the analysis of untreated sea urchin polyribosomal RNA on 2.6% polyacrylamide gels (Fig. 2.5). The electrophoretogram shows a broad band of aggregated RNA, comprising about 30% of the total polyribosomal RNA, which barely enters the gel. Comparison of Fig. 2.4 and Fig. 2.5 show clearly the effect of the heat treatment in reducing RNA aggregation. The results indicate that the aggregation of the polyribosomal RNA is not altogether nonspecific as the amount of some species of RNA were increased relative to others by heat treatment. This is seen clearly in the relative amounts of 4S to 5S and 18S to 28S in heat treated and untreated samples. The 9S "mRNA" peak which is evident in heat treated RNA (Fig. 2.4) is absent when RNA is not heat treated (Fig. 2.5).

The 9S RNA peak present after the first sucrose gradient fractionation step (Fig. 2.6a) was pooled and recentrifuged. The 9S peak from the second centrifugation (Fig. 2.6b) was pooled eliminating the 5S RNA and the shoulder on the leading edge of the 9S peak. The yield of 9S RNA constituted 0.8 - 1.0% of the starting total polyribosomal RNA.

The 9S RNA was analysed on a calibrated sucrose gradient (SW 65Ti rotor) as described in Materials and methods (5.2.1) and sedimented as a homogeneous peak of mean sedimentation coefficient 8.8 - 9.0S. When analysed on 2.6% polyacrylamide gels (5.2.2.1) the 9S RNA (20 µg/gel) migrated as a single peak (Fig. 2.7a) but when very small amounts of RNA (4 µg) were analysed (Fig. 2.7b) it could be clearly seen that the RNA was heterogeneous. This heterogeneity was confirmed by demonstrating the existence of four major species of RNA by gradient polyacrylamide microgel electrophoresis

FIG. 2.5

THE AGGREGATION OF SEA URCHIN POLYRIBOSOMAL RNA
IN THE ABSENCE OF HEAT TREATMENT

Total polyribosomal RNA (2.0 A_{260}/gel) was analysed on 2.6% polyacrylamide gels (5.2.2.1). The RNA was not heat treated prior to electrophoresis. Gels run at 5 mA/gel for 2 hours.

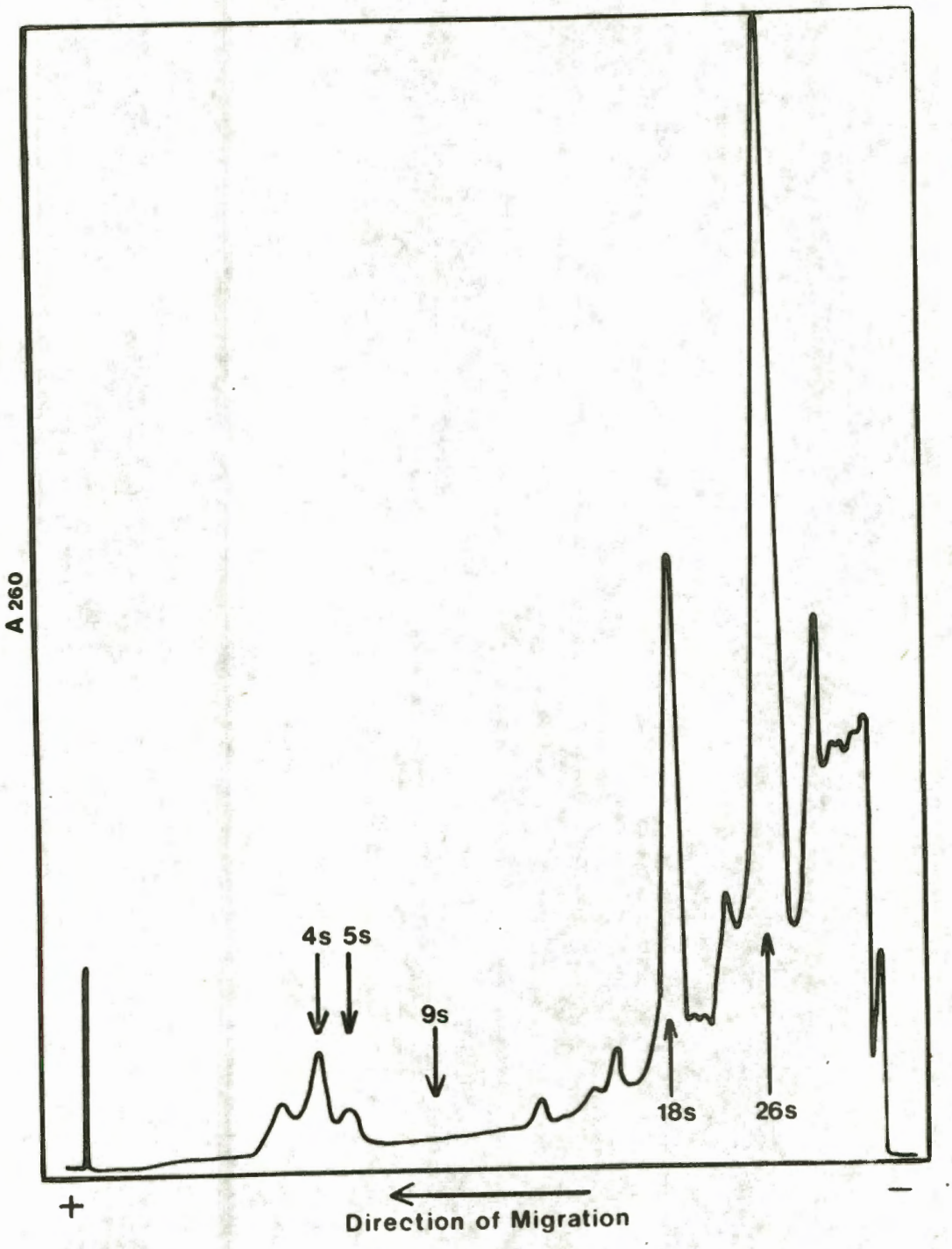


FIG. 2.6

PURIFICATION OF 9S RNA FROM TOTAL POLYRIBOSOMAL RNA BY SUCROSE DENSITY GRADIENT FRACTIONATION (5.2.1)

a) Total polyribosomal RNA fractionated on a 15-30% sucrose gradient at 280 000 x g for 17 h. Approximately 100 A_{260} units of RNA was applied per gradient.
b) The pooled 9S peak from (a) was recentrifuged at 280 000 x g for 22 h on 15-30% sucrose gradients. Both centrifugation steps were carried out in the Beckman SW 40Ti rotor at 20°C (all buffers contained SDS).

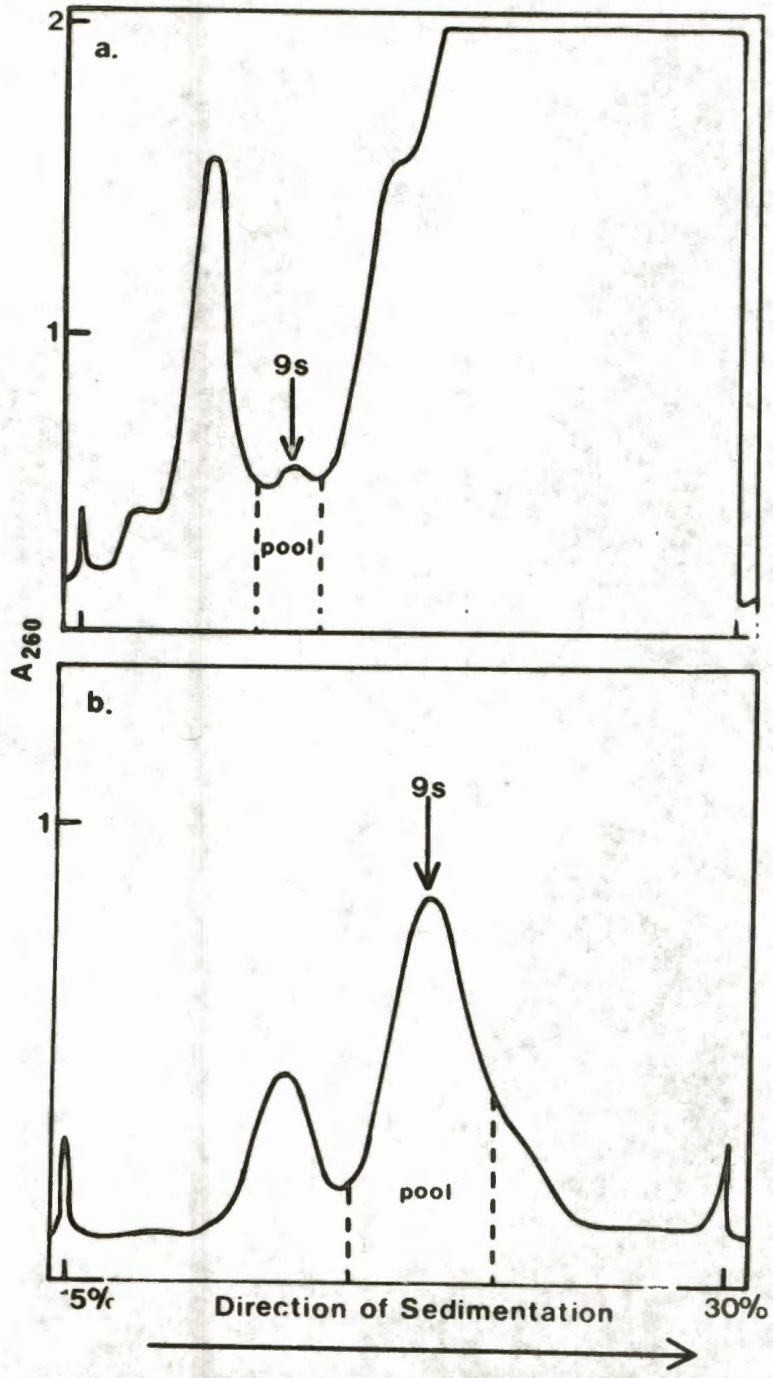


FIG. 2.7

ANALYSIS OF 9S POLYRIBOSOMAL RNA ON 2.6% POLYACRYLAMIDE GELS

a) 20 µg of 9S RNA fractionated for 2 hours at 5 mA/gel.

b) 4 µg of 9S RNA fractionated for 2 hours at 5 mA/gel

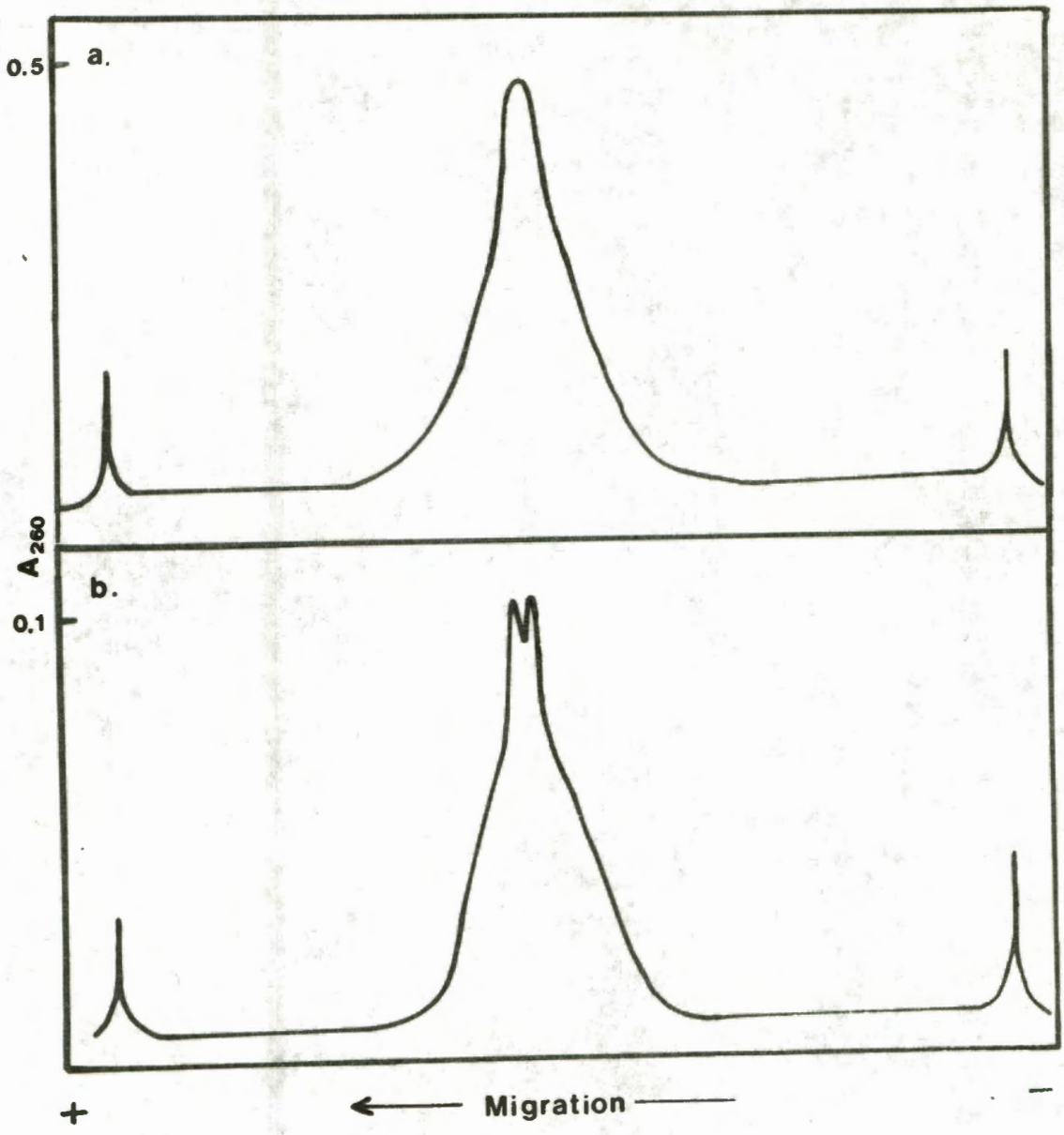
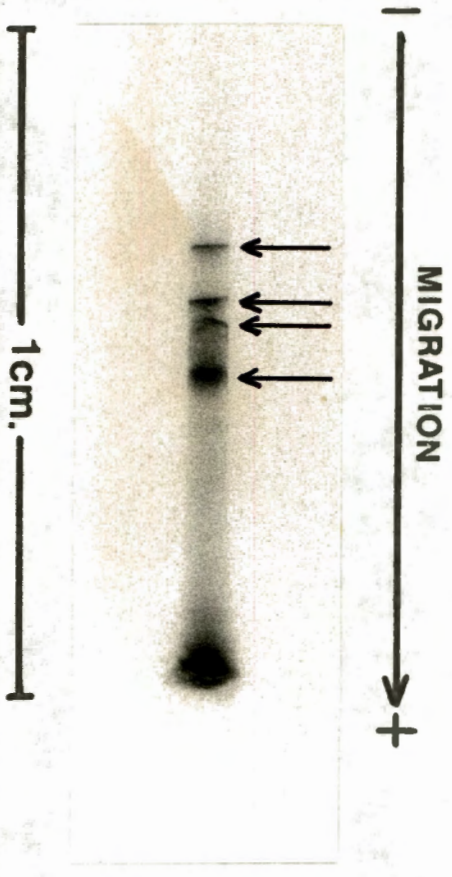


FIG. 2.8

THE ANALYSIS OF SEA URCHIN 9S POLYRIBOSOMAL RNA
ON POLYACRYLAMIDE GRADIENT MICROGELS

Approximately 1 µg of 9S RNA was applied to the microgel and electrophoresed for 1 hour at 90 µA. The gel was stained with toluidine blue, destained and photographed. The dark stained region at the bottom of the gel results from the stain being trapped in the very concentrated (~ 40%) acrylamide.



(5.2.2.2) of 9S RNA preparations (Fig. 2.8) which showed 4 bands of RNA. The existence of multiple RNA species within the 9S RNA has also been shown by electrophoresis in radioactively labelled 9S histone mRNAs (Kedes and Gross, 1969a; Weinberg et al., 1972; Grunstein et al., 1973; Kedes et al., 1975b; Grunstein and Schedl, 1976; Grunstein et al., 1976; Gross et al., 1976). The different mRNA species code for different histones when translated in cell free systems (Levy et al., 1975; Gross et al., 1976), but these workers have not excluded the presence of unlabelled rRNA contaminants in their RNA fractions. Such contamination has been found in electrophoretically purified histone mRNA fractions (Bos et al., 1976; Wu etal., 1976). Ribosomal RNA contaminants in my 9S mRNA preparation (see Section 3.6) does not allow a simple interpretation of these electrophoretic analyses.

Use of polyvinylsulphate as a RNase inhibitor

In initial 9S mRNA isolations polyvinylsulphate was used as a RNase inhibitor (Sela and Littauer, 1962;Howells and Wyatt, 1969) in all solutions. This procedure was discontinued in later isolations as it was very difficult to remove the polyvinylsulphate (a 9S polymer isolated with the 9S RNA during centrifugation) from the final preparation of 9S RNA. It was necessary to remove the polyvinylsulphate as its presence in the mRNA preparation was shown by Hayes and Gross (1969) to inhibit cell free translation. A method was developed to remove polyvinylsulphate from RNA preparations by chromatography on DEAE-Sephadex but when used on the small amounts of 9S mRNA available large RNA losses were experienced. In later experiments polyvinylsulphate was only used up to the stage where the polyribosomes were sedimented through 36% (w/w) sucrose (no polyvinylsulphate). In subsequent purification steps RNase activity was eliminated by autoclaving or treatment with diethylpyrocarbonate of all solutions and glassware.

2.4 TRANSLATION OF THE 9S HISTONE mRNA

2.4.1 THE ASCITES CELL FREE SYSTEM

The ascites cell free system was first developed about 15 years ago (Kerr et al., 1962) and has since been greatly improved and used for the translation of eukaryotic mRNAs (Housman et al., 1971; Mathews et al., 1972; Jacobs-Lorena and Baglioni, 1972; Jacobs-Lorena et al., 1972) as well as viral RNA (Mathews and Korner, 1970). The reasons for choosing the ascites

cell free system in preference to the other available systems for mRNA translation were : mice infected with Ehrlich ascites tumours were easily maintained (5.2.7.1), the cell free extract was simple to prepare (5.2.7.2), and the cell free system showed low levels of endogenous protein synthesis. An attempt was made to translate 9S mRNA in the in vivo Xenopus laevis oocyte system (Moar et al., 1971; Marbaix et al., 1971; Lane et al., 1971) but without success.

The dependence of the ascites cell free system on added (exogenous) messenger RNA is shown by the results in Fig. 2.9a, where the stimulation of protein synthesis after addition of equal amounts (5 µg) of chicken 9S globin mRNA and chicken 28S and 18S ribosomal RNA is compared. The results show clearly that the cell free system was dependent on added mRNA and was not stimulated by ribosomal RNA. Any ribosomal RNA contaminants in the mRNA preparation will therefore not be translated but may, as shown by Jacobs-Lorena and Baglioni (1972), increase the mRNA dependent protein synthetic activity. When our sea urchin 9S RNA fraction was incubated in the ascites cell free system it supported a level of protein synthesis (Fig. 2.9b) similar to chicken globin mRNA showing that this 9S RNA, isolated from sea urchin polyribosomes, contains messenger RNA.

The ascites cell free system prepared and incubated as described in Materials and methods (5.3.7) showed variations in both the levels of endogenous protein synthesis and added-mRNA dependent protein synthesis. Protein synthetic activity was determined by the TCA precipitable incorporation of [^{3}H]-lysine by the cell free system. Table 2.2 shows the protein synthetic activity of 4 typical preparations of the ascites cell free extract in the presence and absence of added sea urchin 9S mRNA. The level of endogenous protein synthesis in ascites cell free extracts was approximately 1000 dpm per incubation (Table 2.2), but some ascites cell free extracts showed very high levels of endogenous protein synthesis, probably due to inefficient preincubation. These cell free extracts however, showed equally high, if not higher, mRNA specific protein synthetic activity, in the presence of added mRNA (Table 2.3), than did extracts with low levels of endogenous protein synthesis (Table 2.2). Similar levels of mRNA specific incorporation (dpm/µg added mRNA) were obtained with added chicken globin mRNA (see Fig. 2.9) and TMV RNA.

FIG. 2.9

THE DEPENDENCE OF THE PROTEIN SYNTHETIC ACTIVITY
OF THE ASCITES CELL FREE SYSTEM ON ADDED MESSENGER RNA

a) The levels of incorporation of [^{3}H]-histidine (10 µCi/ml, 55 Ci/mmole) into proteins by the ascites cell free system after addition of 5 µg each of chicken 18S rRNA, 28S rRNA and 9S globin mRNA.

b) The incorporation of [^{3}H]-lysine (10 µCi/ml, 8 Ci/mmole) into proteins by the ascites cell free system in the presence and absence of 5 µg of sea urchin polysomal 9S mRNA (tentative histone mRNA).

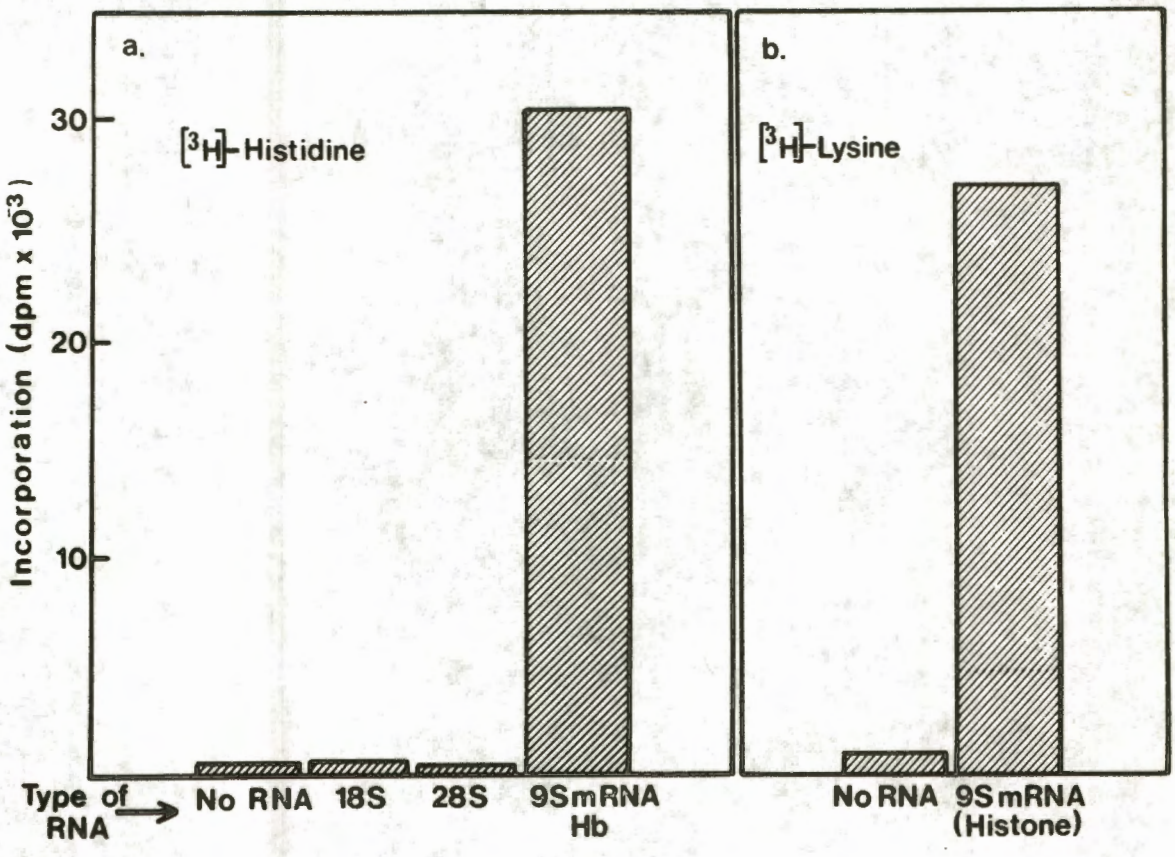


TABLE 2.2

PROTEIN SYNTHETIC ACTIVITY OF DIFFERENT ASCITES CELL FREE EXTRACTS STIMULATED BY THE ADDITION OF SEA URCHIN 9S mRNA

Incubations were carried out in 200 µl as described in Materials and methods (5.2.7.3) using 10 µCi/ml of [^{3}H]-lysine (8 Ci/mmole) and the levels of protein synthesis were determined by the amount of TCA precipitable radioactive material.

Protein synthesis TCA precipitable (dpm)		Amount 9S mRNA added µg	9S mRNA specific incorporation dpm/µg mRNA
Endogenous	With added 9S mRNA		
939	20 577	5	3927
1256	29 866	5	5722
1206	19 949	5	3748
1059	29 745	10	2868

TABLE 2.3

TRANSLATION OF SEA URCHIN 9S mRNA BY ASCITES CELL FREE EXTRACTS WHICH HAVE HIGH LEVELS OF ENDOGENOUS PROTEIN SYNTHESIS

Incubations were carried out as described in legend to Table 2.2.

Protein synthesis TCA precipitable (dpm)		Amount of added 9S RNA µg	9S mRNA specific incorporation dpm/µg mRNA
Endogenous	With added 9S mRNA		
27 926	73 363	5	9087
13 385	48 204	10	3481

TABLE 2.4

COMPARISON OF THE PROTEIN SYNTHETIC ACTIVITY OF FRESHLY PREPARED AND STORED (0°C FOR 24 h) ASCITES CELL FREE EXTRACTS

5 µg of sea urchin 9S mRNA was used in each incubation. Incubations were carried out as described in the legend to Table 2.2.

Ascites extract	Protein synthesis TCA precipitable (dpm)		9S mRNA specific incorporation dpm/µg 9S RNA
	Endogenous	With added 9S mRNA	
Freshly prepared	27 926	73 363	9087
Stored for 24 hours at 0°C	23 560	69 377	9163

Ascites cell free extracts lost their activity on freezing; this has been shown to be due to aggregation and precipitation of ribosomal material. Ascites cell free extracts retained their protein synthetic activity for 2-3 days when stored at 0°C. The results in Table 4 compare the activity of an ascites cell free extract immediately after preparation and after 24 hours storage at 0°C. The endogenous activity of the extract decreased (probably due to breakdown of endogenous mRNA) but the stimulation of protein synthesis due to added mRNA was not affected.

2.4.2 IDENTIFICATION OF THE PRODUCTS OF CELL FREE TRANSLATION

The 9S RNA isolated from the sea urchin polyribosomes stimulated protein synthesis in the ascites cell free system. In order to determine whether the proteins synthesized were in fact histones (Gross et al., 1973), it was necessary to characterize the sea urchin histones themselves.

2.4.2.1 ISOLATION AND CHARACTERIZATION OF SEA URCHIN SPERM AND EMBRYO HISTONES

An important factor to take into consideration when characterizing sea urchin histones is that the pattern of histone synthesis changes during development (Benttinen and Comb, 1971; Ruderman et al., 1974; Cohen et al., 1975; Arceci et al., 1976). Different H1 histones have been shown to be synthesized at the morula stage (H1m) and gastrula stage (H1g) in response to different messenger RNAs transcribed at the different stages (Ruderman et al., 1974). The complexity of the H1 histones varies from a single species in the early cleavage stages to three species at the gastrula stage (Arceci et al., 1971). Variations have also been shown in the types of H2A and H2B histones synthesized during cleavage and blastula stages (Cohen et al., 1975). Another important factor which must be taken into consideration when comparing histones of different tissues, or embryonic developmental stages, is post translational modifications such as methylation (Byvoet and Baxter, 1975), phosphorylation (Ord and Stocken, 1975), acetylation (Gallwitz and Sekeris, 1969) and ADP-ribosylation (Smith and Stocken, 1973).

In P. angulosus we have noted changes in the number and electrophoretic mobility of histones isolated from sperm and embryos at different developmental stages. Fig. 2.10 shows the gels and Fig. 2.11 the electrophoretograms of sperm, blastula and early gastrula histones analysed on 15% acetic acid-urea gels as described in Materials and methods (5.2.2.3).

The H1 histones present in the embryo migrated slightly faster than the sperm H1, and in the early gastrula three H1 species were evident which agrees with the results of Arceci et al. (1971). In somatic tissue (sea urchin gut) the two minor H1 bands present in the gastrula become prominent and the major H1 band is not present; the embryo H1s have been shown to have a greater lysine:arginine ratio (lysine 30 mole percent, arginine 1.4 mole percent) than sperm H1 (lysine 20 mole percent; arginine 8 mole percent) (W. Brandt personal communication).

The H2A histones in the embryo also vary from those in sperm. They migrated more slowly than sperm H2A and have been shown to contain a methionine residue which is not present in sperm H2A (W. Brandt, personal communication). Embryo H2B on the other hand migrated faster than sperm H2B. The embryo

FIG. 2.10

ANALYSIS OF SEA URCHIN SPERM
AND EMBRYO HISTONES ON 15% POLYACRYLAMIDE GELS

A. sea urchin sperm histones

B. 8 hour blastula histones

C. 17 hour early gastrula histones.

The sea urchin histones were isolated and analysed by gel electrophoresis as described in Materials and methods (5.3.6 and 5.2.2.3).

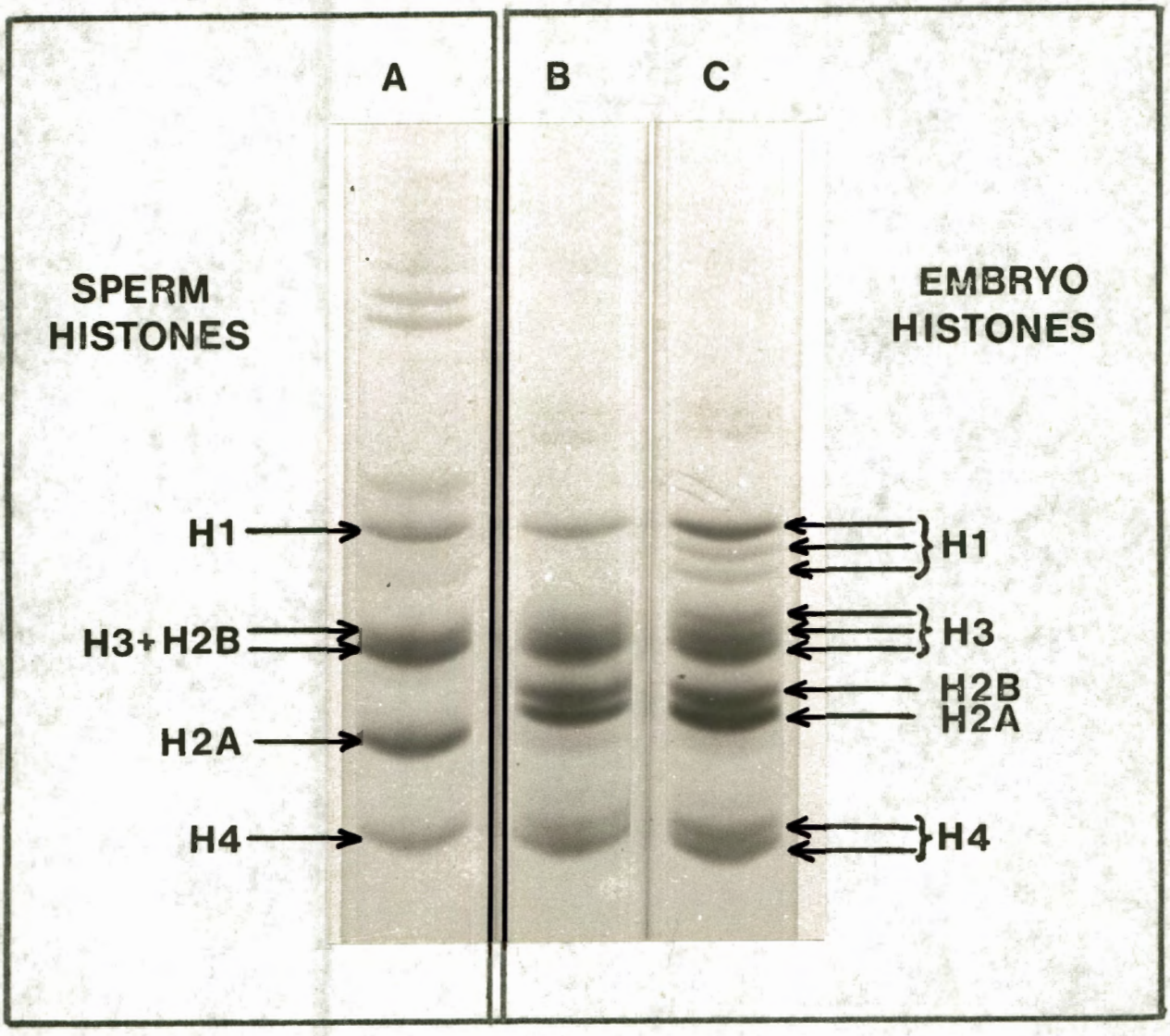
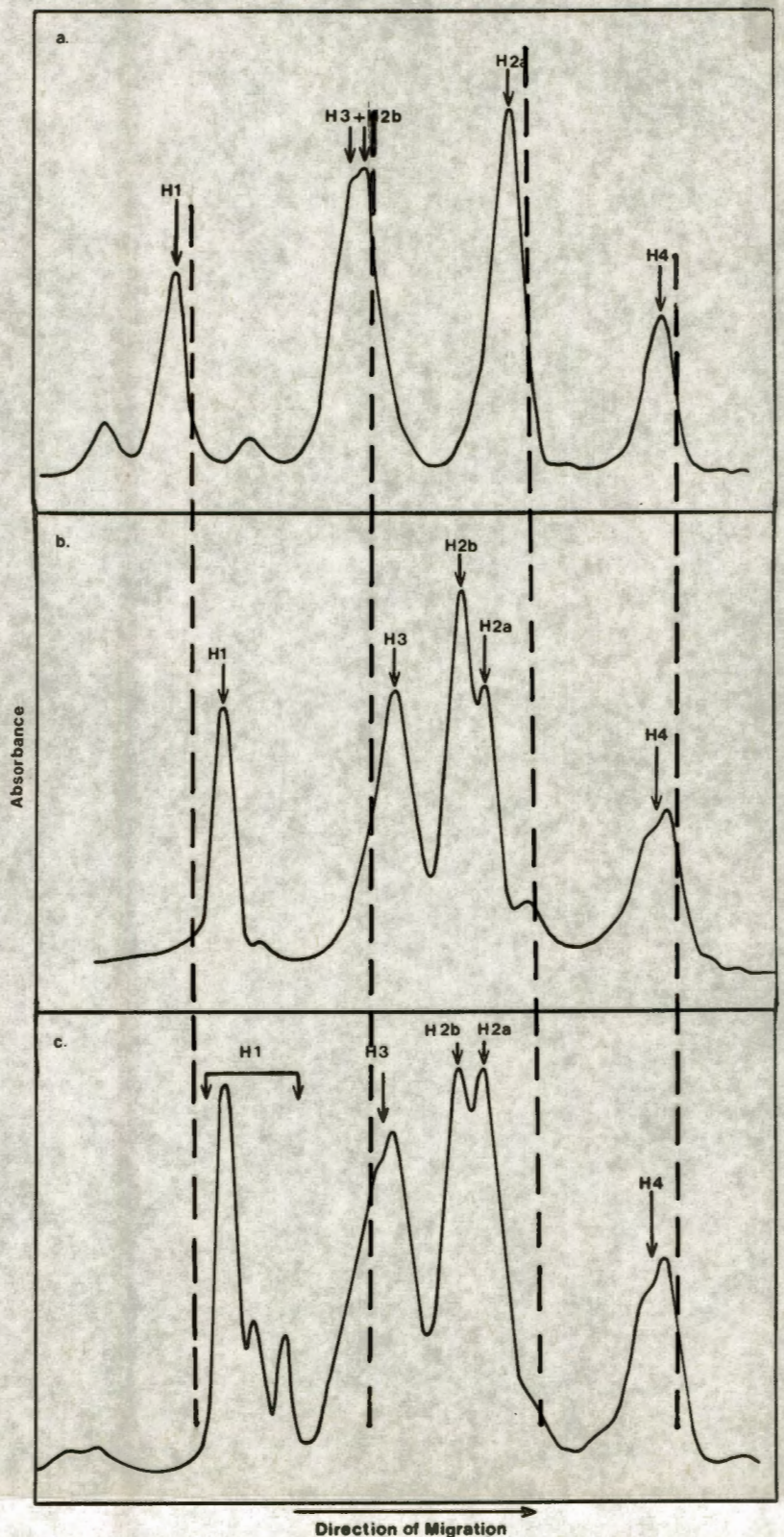


FIG. 2.11

ELECTROPHORETIC ANALYSIS OF SEA URCHIN SPERM AND EMBRYO HISTONES

Sea urchin sperm and embryo histones were isolated as described in Materials and methods and analysed on 15% polyacrylamide gels. The gels were stained with amido black and scanned (5.2.2.3) a) sperm histones b) 8 hour embryo c) 17 hour embryo.



and sperm H3 histones showed similar migration but in the embryo three species of H3 were evident. The embryonic histones contained 2 bands of H4, the faster migrating band being the same as sperm H4. The second band was probably caused by modification of the H4 by acetylation or methylation (Benttinin and Comb, 1971).

Some histone bands in polyacrylamide gels, stained with amido black, have been shown to change colour on storage in 7% acetic acid. For example, H1 (embryo), H2B and H4 bands turn brown on storage while H1 (sperm), H2A and H3 bands retain their blue colour. In some cases H2B bands have been shown to turn red on storage. This characteristic is very useful in distinguishing between different histone bands, for example the H1s of sperm and embryo can be shown to be different by this phenomenon. The physico-chemical nature of this phenomenon is however not understood.

2.4.2.2 ISOLATION OF HISTONES FROM THE ASCITES CELL FREE SYSTEM

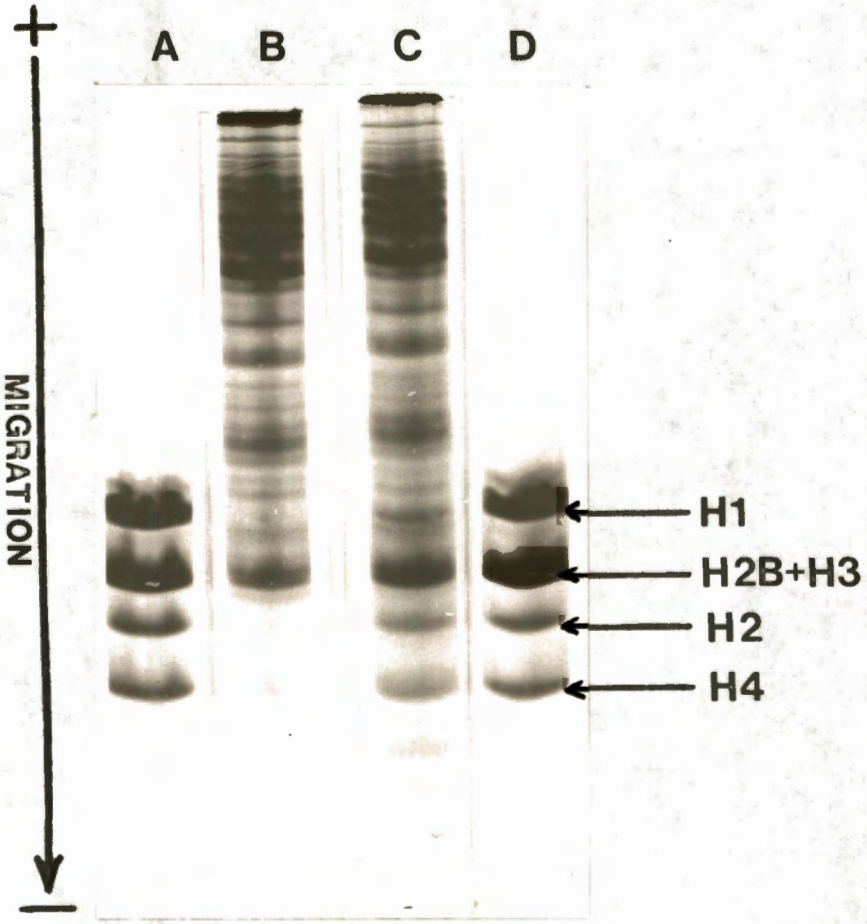
The reisolation of carrier sea urchin histones with newly synthesized products of cell free protein synthesis (labelled) could not be carried out by standard histone isolation procedures (5.3.6). These procedures rely on the histones being associated with chromatin, which can be thoroughly washed prior to the acid extraction of histones. In the cell free system however, both the carrier histones and the newly synthesized proteins would be free in solution, or possibly non-specifically bound to some component of the cell free extract. They were therefore isolated by acid extraction of the total cell free incubate with either 0.25 M HCl or 0.2 M H_2SO_4 (5.3.7.4). These two methods gave similar results with respect to the efficiency of reisolation of carrier histones, but both methods, as well as a third method involving Reinecke salt precipitation of histones (Lindh and Brandtmark, 1966), resulted in preferential losses of carrier H1 histone. These losses of H1 were more extensive in isolations involving HCl extraction and dialysis than the H_2SO_4 : acetone extraction (compare optical density tracings in Figs. 2.13 and 2.14). This result can be explained by the fact that H1 histones are more susceptible to proteolytic attack than other histones (Panyim et al., 1968).

Fig. 2.12 shows the gel patterns of H_2SO_4 : acetone extracts from the ascites

FIG. 2.12

15% ACID-UREA GEL ELECTROPHORESIS (METHOD 5.2.2.3) OF CARRIER SEA URCHIN SPERM HISTONES REISOLATED FROM ASCITES CELL FREE INCUBATES

Gels A and D show sea urchin sperm histones. Gel B shows the proteins isolated from cell free incubates (+ 20 µg carrier histone) by acid extraction followed by precipitation with 2 volumes of acetone. Gel C shows proteins isolated from cell free incubate containing 20 µg of carrier sea urchin histones by acid extraction followed by precipitation with 4 volumes of acetone.



cell free incubations (Gel C). As can be seen there were a large number of ascites proteins extracted with the carrier histones but most of these migrated more slowly than the carrier histones on gels. An attempt was made to reduce these contaminants by precipitating with only two volumes of acetone instead of four as finally used (5.3.7.4), however, almost total losses of the carrier histones resulted with no apparent reduction in the amount of ascites proteins (Gel B).

Identification of the products of cell free protein synthesis

Identification of the products of cell free protein synthesis in the presence and absence of added sea urchin 9S mRNA was carried out by fractionation of acid soluble extracts (5.3.7.4) from cell free incubates, on 15% polyacrylamide gels (5.2.2.3). Fig. 2.13 and Fig. 2.14 show the positions on gels of radioactively labelled proteins, synthesized in the cell free system, relative to carrier sea urchin sperm histones. The results shown are from experiments using different ascites cell free extracts and different histone extraction procedures (5.3.7.4). The results show similarity in both the optical density profile and, more importantly, in the position of the labelled proteins on the gels. In both cases the labelled proteins co-migrate with carrier sea urchin histones. The apparent absence of H2A synthesis in the results in Fig. 2.13 could be explained by the different migration of sperm and embryo H2A histones (see Fig. 2.11).

The results of these experiments showed that the 9S mRNA preparations, isolated from sea urchin embryo polyribosomes, contained messenger RNA coding for histones. Other workers have shown that similar 9S polyribosomal RNA fractions, isolated from sea urchin embryos, do not support the *in vitro* synthesis of any proteins except histones (Gross et al., 1973; Levy et al., 1975). The possibility however, that my 9S histone mRNA preparation was contaminated with small amounts of other mRNA molecules and/or ribosomal RNA has not been excluded.

FIG. 2.13

I. ANALYSIS OF THE PRODUCTS OF CELL FREE PROTEIN SYNTHESIS

An ascites cell free extract was incubated with 5 µg of histone mRNA as described in Materials and methods (5.3.7.3). The total TCA precipitable incorporation per incubation was 19 949 dpm in the presence of mRNA and 1 206 dpm in the absence of mRNA. Acid soluble proteins were isolated from the incubate with H_2SO_4 extraction followed by precipitation with acetone (5.3.7.4a) and the extract was analysed on 15% acid-urea gels (5.2.2.3). Gels were stained with amido black, scanned and then sliced. The gel slices were oxidized and the radioactivity determined. (——) Absorbance; (●——●) radioactivity (dpm) in presence of added mRNA; (x——x) radioactivity in the absence of added mRNA.

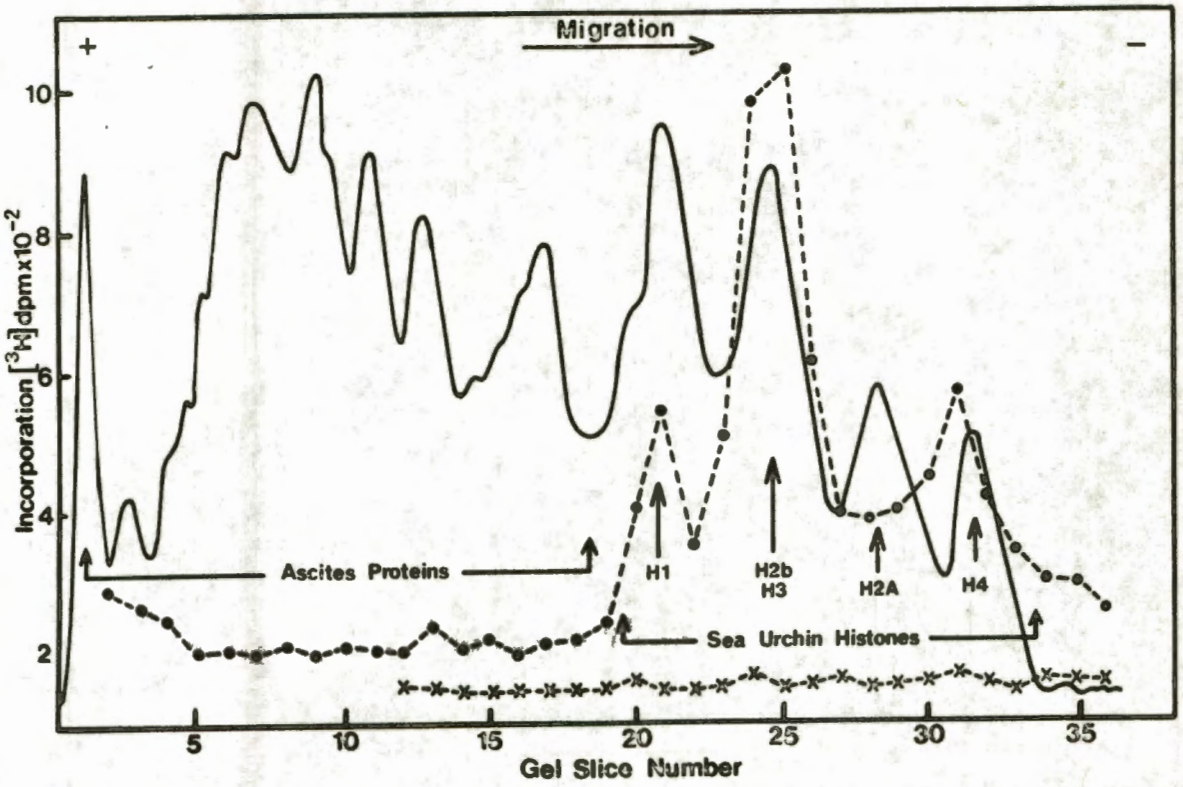
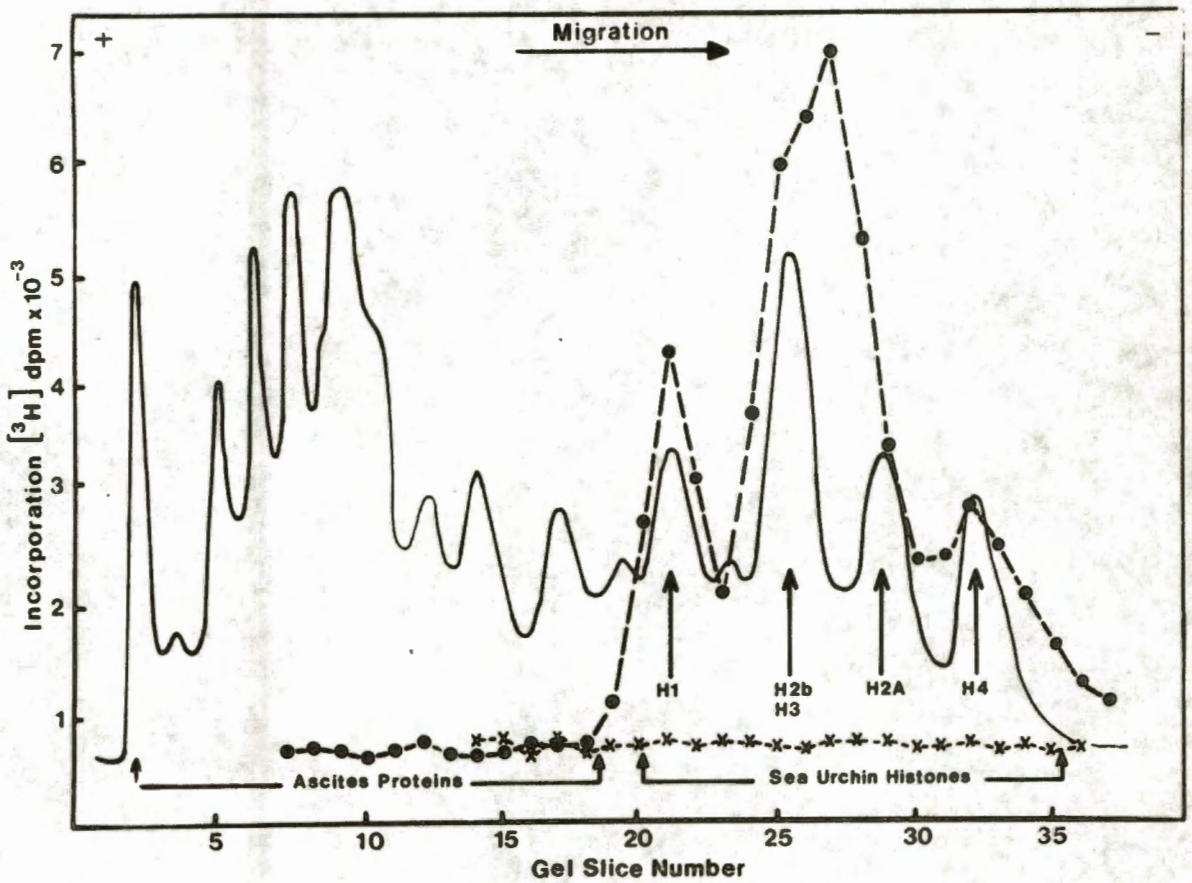


FIG. 2.14

II. ANALYSIS OF THE PRODUCTS OF CELL FREE PROTEIN SYNTHESIS

An ascites cell free extract was incubated with 5 µg of histone mRNA as described in Materials and methods (5.3.7.3). The total TCA precipitable incorporation was 73 363 dpm in the presence of added histone mRNA and 27 926 dpm in the absence of added mRNA. Acid soluble material was extracted with HCl and dialysed against 0.9 M glacial acetic acid (5.3.7.4b) and then analysed on acid-urea gels. Gels were stained with amido black, scanned and sliced. The gel slices oxidized and the radioactivity determined. (——) Absorbance; (●——●) radioactivity (dpm) in the presence of added mRNA; (x——x) radioactivity in the absence of added mRNA.



PART 3

PREPARATION OF COMPLEMENTARY DNA TO HISTONE mRNA

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3.1 INTRODUCTION

Since the discovery of reverse transcriptase in 1970 (Baltimore, 1970; Temin and Mizutani, 1970) reverse transcription of eukaryotic mRNAs has become a widely used method for preparing highly labelled complementary DNA (cDNA) probes. These have been used as tools for the elucidation of chromatin structure, gene reiteration, the hnRNA-mRNA relationship, the effect of hormones on mRNA metabolism and mRNA metabolism during embryonic development, to mention only a few of the applications. More recently cDNAs have been inserted into bacterial plasmids thus allowing the synthesis of large quantities of these DNAs.

Eukaryotic mRNAs alone are poor templates for reverse transcriptase. However the addition of oligo(dT) stimulates DNA synthesis between 10 and 60 fold depending on the mRNA being used as template (Verma et al., 1972). The same authors have shown, for example, that with globin mRNA as template the amount of DNA synthesized is 30-80% of the input RNA. Dependence on added oligo(dT) is due to the binding of the oligo(dT) to the poly(A) tail of the mRNA and thus acting as a double stranded primer for reverse transcription of the mRNA. In this manner cDNAs have been transcribed from 9S globin mRNA (Harrison et al., 1972; Ross et al., 1972; Verma et al., 1972; Lanyon et al., 1975) ovalbumin mRNA (Harris et al., 1975), fibroin mRNA (Lizardi and Brown, 1973), 14S α-crystallin mRNA (Berns et al., 1973).

Histone mRNA does not contain a 3' OH terminal poly(A) tail (Adesnik and Darnell, 1972) and therefore cannot be efficiently transcribed with reverse transcriptase. The existence of enzymes from both prokaryotic and eukaryotic sources which catalyse the addition of poly(A) to the 3' terminus of RNA molecules (Hardy and Kurland, 1966, Terzi et al., 1970; Walter and Mans, 1970; Kato and Kurokawa, 1970), have led to the isolation of enzyme preparations which have made possible the *in vitro* polyadenylation of RNA molecules (Mans and Walter, 1971; Getz et al., 1974; Mans and Huff, 1975). This made possible the reverse transcription of *in vitro* polyadenylated human ribosomal RNA (Hell et al., 1976), HeLa cell histone mRNA (Thrall et al., 1974) and chicken histone H5 mRNA (Scott and Wells, 1976).

In this section I describe the *in vitro* polyadenylation and reverse transcription of my preparation of 9S sea urchin histone mRNA followed by the purification of a histone mRNA specific complementary DNA fraction from the total synthesized cDNA.

3.2 cDNA SYNTHESIS AND RNA:cDNA HYBRIDIZATION

3.2.1 cDNA SYNTHESIS

Complementary DNA synthesis and RNA:cDNA hybridization experiments were initially carried out using the well characterized globin mRNA:cDNA system (Williamson et al., 1971; Harrison et al., 1974; Young et al., 1974). These experiments were carried out at the Beatson Institute of Cancer Research, Glasgow, under the direction of R. Williamson and P. Tolstoshev.

Human foetal globin mRNA was isolated as described in Materials and methods (5.3.3). The yield of globin mRNA obtained was approximately 1% of the total cytoplasmic RNA. Globin mRNA isolated as described has been shown, by translation in a wheat germ cell free protein synthesizing system, to direct the synthesis of human α- (55%), β- (13%) and γ-globin (32%) (Lanyon et al., 1975) and has therefore been termed globin $mRNA_{\alpha\beta\gamma}$.

When incubated with AMV reverse transcriptase (Beatson RT17) under the conditions described in Materials and methods (5.3.8) this mRNA preparation was very effective as a template for the synthesis of cDNA; the yield of cDNA isolated from the incubation was 20% of the input mRNA. The specific activity of the cDNA was calculated assuming a 50% (G+C) content in the cDNA (Young et al., 1974).

3.2.2 HYBRIDIZATION EXPERIMENTS

During the course of this thesis two types of hybridization experiments have been performed : a) titration experiments and b) kinetic experiments (Cot curves).

a) Titration experiments :

Fixed amounts of cDNA were hybridized to increasing amounts of mRNA to a cDNA Cot (Co = initial concentration of nucleic acid in mole of nucleotides per litre, and t = time in secs.) value which ensured completion of the slowest reaction. Hybridization reactions were therefore allowed to proceed to at least 10 x the $Cot_{\frac{1}{2}}$ of the mRNA:cDNA reaction ($Cot_{\frac{1}{2}}$ is defined as the conditions of concentration and time at which a RNA:DNA hybridization reaction

is half completed).

b) Kinetic experiments were performed using fixed amounts of cDNA and at least a 50 x RNA excess so that as RNA is removed from the single stranded to the double stranded state, the apparent single stranded RNA concentration does not change sufficiently to effect the kinetics of the reaction. The RNA concentration (Co) and incubation time (t) were varied and the extent of hybridization at the different values of Cot (mole sec/l) was determined by S1 nuclease assay (5.3.4.2). (Cot values were calculated using the relationship : 83 µg of nucleic acid/ml incubated for 1 hour is equivalent to a Cot of 1, (Church, 1973)). In all hybridization reactions RNA degradation was reduced by adding to each reaction an excess of E.coli RNA (0.2 - 2 mg/ml) and reactions were carried out at 43°C in the presence of 50% (v/v) formamide (Harrison et al., 1974).

When globin mRNA was hybridized to the cDNA (Fig. 3.1) a maximum of 93% of the cDNA became hybridized under conditions of RNA excess. The extent of hybridization was determined by the resistance to S_1 nuclease digestion (5.3.9.2). The remaining 7% of the cDNA which does not form hybrids even at very high RNA:DNA ratios is referred to as non-hybridizable cDNA. The presence of this fraction of non-hybridizable cDNA has been shown in other mRNA:cDNA systems but the nature of this fraction is unknown (Harrison et al., 1974). In the absence of mRNA 5-10% of the cDNA was resistant to S_1 nuclease digestion and therefore must have been double stranded. This fraction is referred to as background hybridization. The titration curve (Fig. 3.1) shows that the cDNA became saturated (93%) at an RNA:cDNA ratio of about 3 which is in close agreement with the results of Lanyon et al. (1975) working with the same system. Theoretically, under ideal conditions, the mRNA:cDNA reactions should reach saturation at a RNA:cDNA ratio of 1, but this does not occur in practice (Young et al., 1974; Lanyon et al., 1975; Weiss et al., 1976). This can be explained in part by the cDNA being smaller than the mRNA.

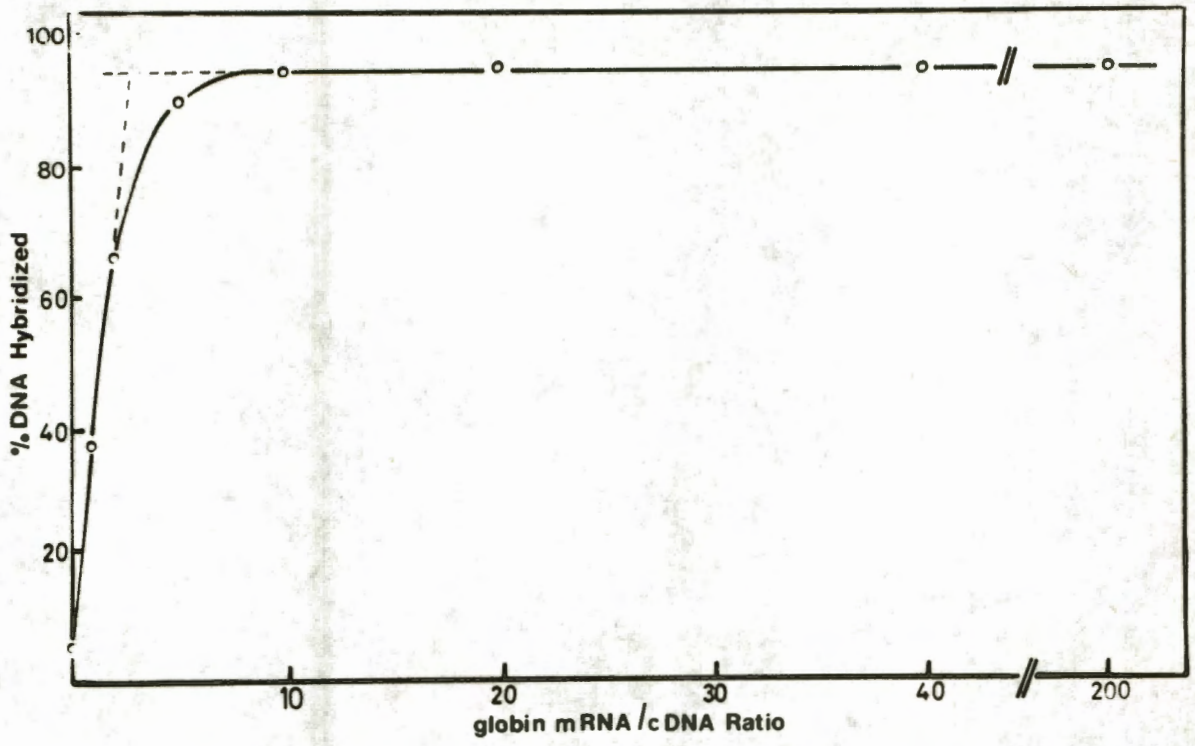
3.3 HISTONE mRNA AS TEMPLATE FOR REVERSE TRANSCRIPTASE

The presence of small amounts of polyadenylated RNA in the histone mRNA preparation was indicated by chromatography of the mRNA on oligo(dT) cellulose (5.2.4) where about 3% of the histone mRNA preparation was bound under conditions which

FIG. 3.1

HYBRIDIZATION OF GLOBIN mRNA$_{\alpha\beta\gamma}$ TO cDNA

0.2 ng of cDNA was hybridized to increasing amounts of mRNA. The reactions were incubated for 48 h which resulted in a Cot for the reaction of 0.057 sec/litre. This is greater than 10 x the Cot$_{\frac{1}{2}}$ of the globin mRNA: cDNA reaction therefore ensuring completion of the slowest reaction.



bind polyadenylated RNA (Aviv and Leder, 1972). The value of 3% however could not be taken as the absolute amount of polyadenylated RNA because during chromatography on oligo(dT) cellulose there is some degree of nonspecific binding of RNA to cellulose.

A comparison of the template activities of globin mRNA and histone mRNA when incubated with reverse transcriptase are shown in Table 3.1. The template activity of histone mRNA was very low (2%) in comparison to globin mRNA. The histone mRNA which had been passed through an oligo(dT) cellulose column showed an even lower activity (0.05%) than the total histone mRNA preparation which was a firm indication that the histone mRNA preparation from sea urchin did contain a small amount of poly(A) containing RNA.

TABLE 3.1

ACTIVITY OF REVERSE TRANSCRIPTASE WITH DIFFERENT RNA TEMPLATES

10 µl incubations containing 0.5 µg of RNA were carried out as described in Materials and methods (5.3.8). The activity was determined as the TCA precipitable radioactivity after incubation at 37°C for 90 min. The incorporation due to globin mRNA is taken as 100% activity.

RNA	Incorporation dpm	% Activity
Globin mRNA (chicken)	235 266	100%
Histone mRNA (total)	4 668	2%
Histone mRNA (poly(A)$^-$)	1 275	0.05

3.4 POLYADENYLATION AND REVERSE TRANSCRIPTION OF HISTONE mRNA

In vitro polyadenylation of the histone mRNA preparation using E.coli poly(A) polymerase and maize seedling ATP polynucleotidylexotransferase has made possible the efficient reverse transcription of the histone mRNA preparation.

3.4.1 POLYADENYLATION WITH E.COLI POLY(A) POLYMERASE

The isolation of E.coli poly(A) polymerase is described in Materials and methods (5.3.10.1). The purification of the enzyme preparation on Sephadex G-200 (Fig. 3.2) gave different results from those of Getz et al. (1974) and Hell et al. (1976). The poly(A) polymerase activity was not eluted as a single peak but was heterogeneous and when the three pooled fractions (Fig. 3.2) were tested for RNase (Fig. 3.3) it was found that the RNase activity was not in the leading edge of the poly(A) polymerase activity peak (Hell et al., 1976) but in fractions 2 and 3. A. Hell (personal communication) has obtained similar results with recent poly(A) polymerase isolations and has indicated that this may be due to the method of homogenization. Fractions 2 and 3 were pooled and used as poly(A) polymerase for the polyadenylation of histone mRNA in the presence of rat liver RNase inhibitor.

3.4.1.1 POLYADENYLATION AND REVERSE TRANSCRIPTION OF HISTONE mRNA

Histone mRNA was passed through a column of oligo(dT) cellulose (5.2.4) to remove any polyadenylated RNA. The nonpolyadenylated RNA which comprised 97% of the total mRNA preparation was analysed on 2.6% polyacrylamide gels (5.2.2.1) and migrated with the 9S histone RNA showing that no RNase degradation had taken place during oligo(dT) cellulose chromatography. The *in vitro* polyadenylation of this RNA was carried out as described in Materials and methods (5.3.10.1.2) and the RNA (polyadenylated) was isolated from the incubation mixture by extraction with phenol chloroform (5.2.7) and desalted on a Sephadex G-25 column (5.2.5). This RNA was used as a template for reverse transcriptase (Beatson RT17) under standard conditions (5.3.8) and the cDNA synthesized was isolated from the incubation mixture (5.3.8). When analysed on a 15-30% alkaline sucrose gradient the cDNA preparation sedimented as a peak of 2.9S (Fig. 3.4). There is no cDNA sedimenting in the 9S region of the gradient. The yield of cDNA obtained was approximately 0.5% of the input mRNA template. The yield was very low in comparison with the yields which Hell et al. (1976) obtained with cDNA synthesized from poly-

FIG. 3.2

FRACTIONATION OF E.COLI POLY(A) POLYMERASE ON SEPHADEX G-200

The poly(A) polymerase was applied to G-200 Sephadex (1.5 x 60 cm) and eluted at a flow rate of 10-12 ml/h with a buffer head of 15 cm. Alternate fractions eluting in the inner volume were assayed (5.3.10.1.1) for poly(A) polymerase activity and pooled as shown.

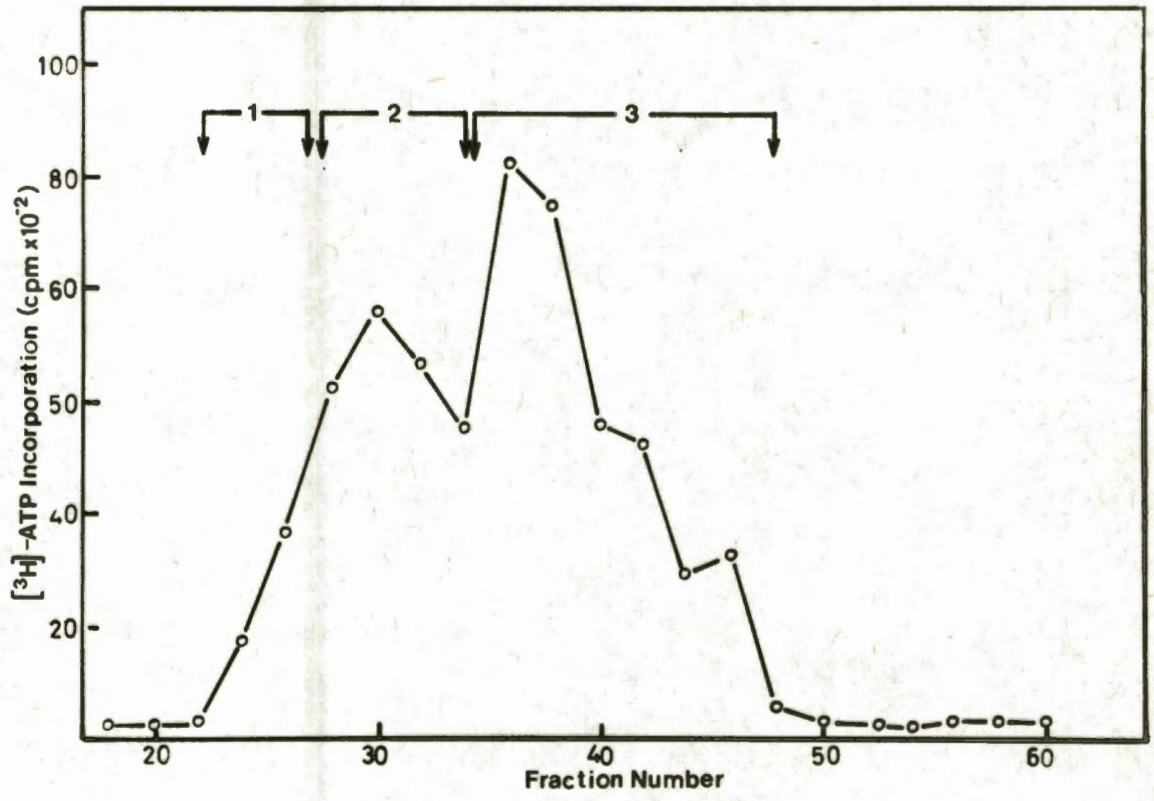


FIG. 3.3

ASSAY OF RNase ACTIVITY IN POLY(A) POLYMERASE FRACTIONS

Aliquots (10 µl) of the pooled fractions from Fig. 3.2 were incubated for 20 min with E.coli rRNA in a standard assay mixture (5.3.10.1.1). The RNA was then re-isolated from the incubation with phenol:chloroform and the RNA was analysed on 2.6% polyacrylamide gels (5.2.2.1). a) Control E.coli RNA not incubated with poly(A) polymerase (10 µg), b) E.coli RNA incubated with fraction 1 of poly(A) polymerase (11 µg), c) E.coli RNA incubated with fraction 2 (7.5 µg), d) E.coli RNA incubated with fraction 3 (7 µg).

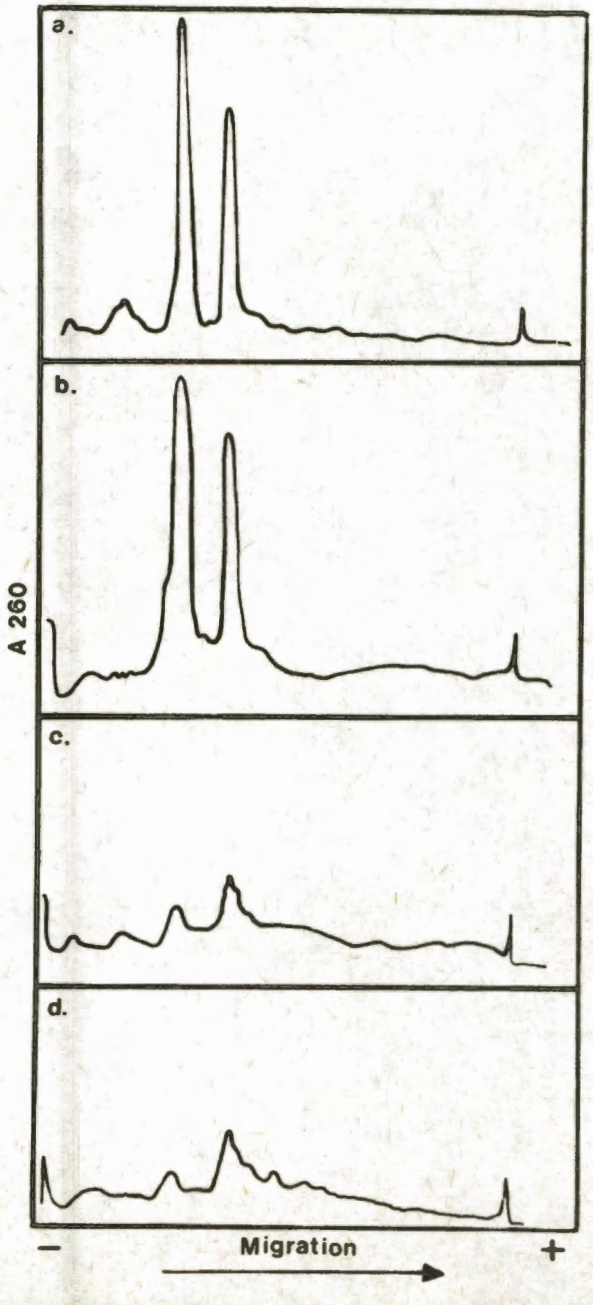
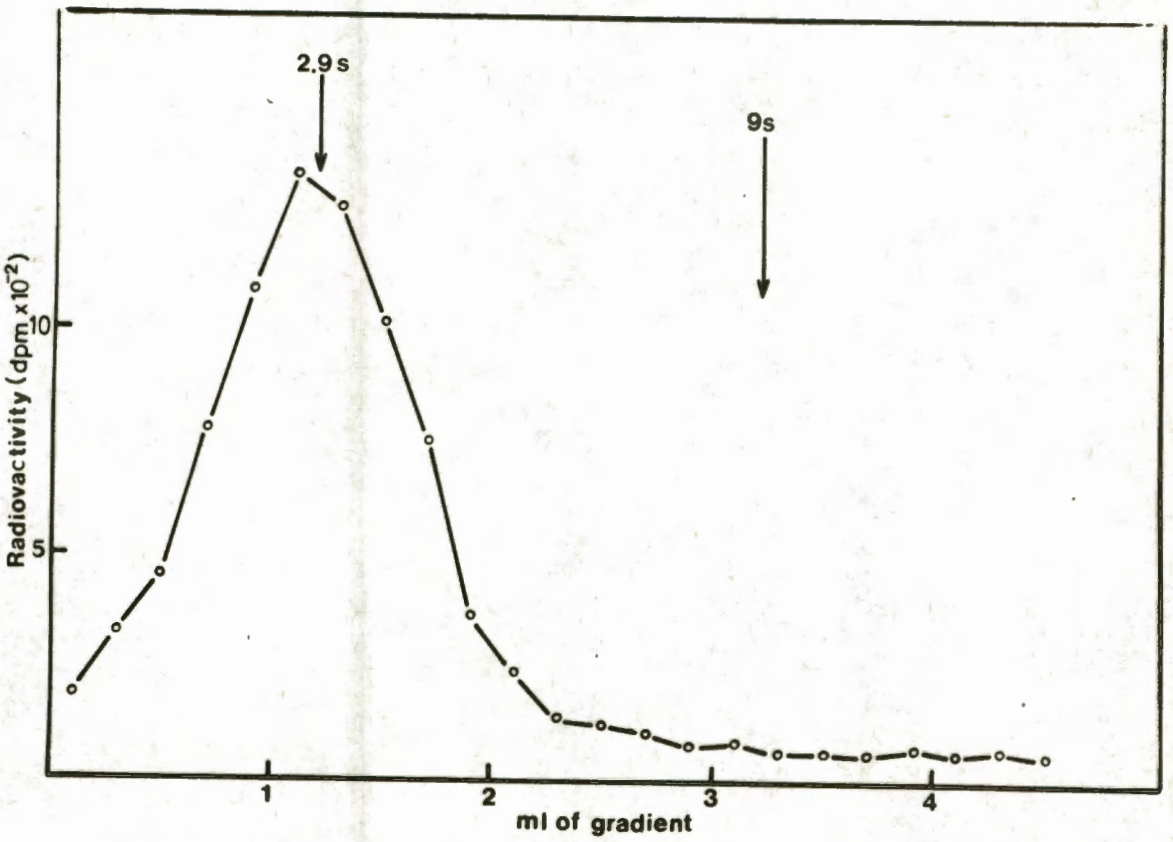


FIG. 3.4

ANALYSIS OF cDNA ON 15-30% ALKALINE SUCROSE GRADIENTS

An aliquot of the cDNA was applied to an alkaline sucrose gradient (0.9 M NaCl, 0.1 M NaOH) and centrifuged for 7 h in the SW 65Ti rotor (g_{max}). The gradient was fractionated into 0.2 ml fractions and to each fraction was added 0.2 ml of 0.1 M HCl, 0.4 ml of H_2O and 10 ml of scintillator and the radioactivity was determined.



adenylated ribosomal RNA (yields 3-5% of input rRNA). The conditions of cDNA synthesis were identical to those used for globin mRNA (5.3.8) where the yield of cDNA was 20% of the input RNA. This indicates that the low yield of cDNA obtained with the polyadenylated histone mRNA was due to inefficient polyadenylation or excessive degradation of the histone mRNA.

3.4.1.2 HYBRIDIZATION

When hybridized back to histone mRNA under conditions of RNA excess at a Cot value greater than 10, 65% of the cDNA formed hybrids with mRNA (as determined by S_1 nuclease resistance). In the absence of mRNA 5-10% of this cDNA preparation was resistant to S_1 nuclease digestion. The fact that only 65% of the cDNA hybridized may be due to either the small average size of the cDNA, or the non-hybridizable cDNA (always synthesized by reverse transcription) being a greater percentage of the total cDNA than was found with globin cDNA where reverse transcription was very efficient. The kinetics of the annealing reaction of histone mRNA to its cDNA are shown in Fig. 3.5. The reaction followed pseudo first order kinetics expected for a reaction of this type and proceeded with a $Cot_{\frac{1}{2}}$ of 4.5×10^{-2} mole sec/litre. The range of the reaction was approximately 2½ log units of Cot, characteristic of the hybridization of a single abundance class of RNA with its cDNA. An abundance class of RNA is defined as a group of different RNA species present in similar concentration (Getz et al., 1975).

Although this method of polyadenylation and reverse transcription produced a cDNA which hybridized back to mRNA there were a number of disadvantages to this method :

i) the RNase contamination in the poly(A) polymerase
ii) the low yields of cDNA
iii) the small average size of the cDNA
iv) the relatively low percentage of the cDNA which formed hybrids

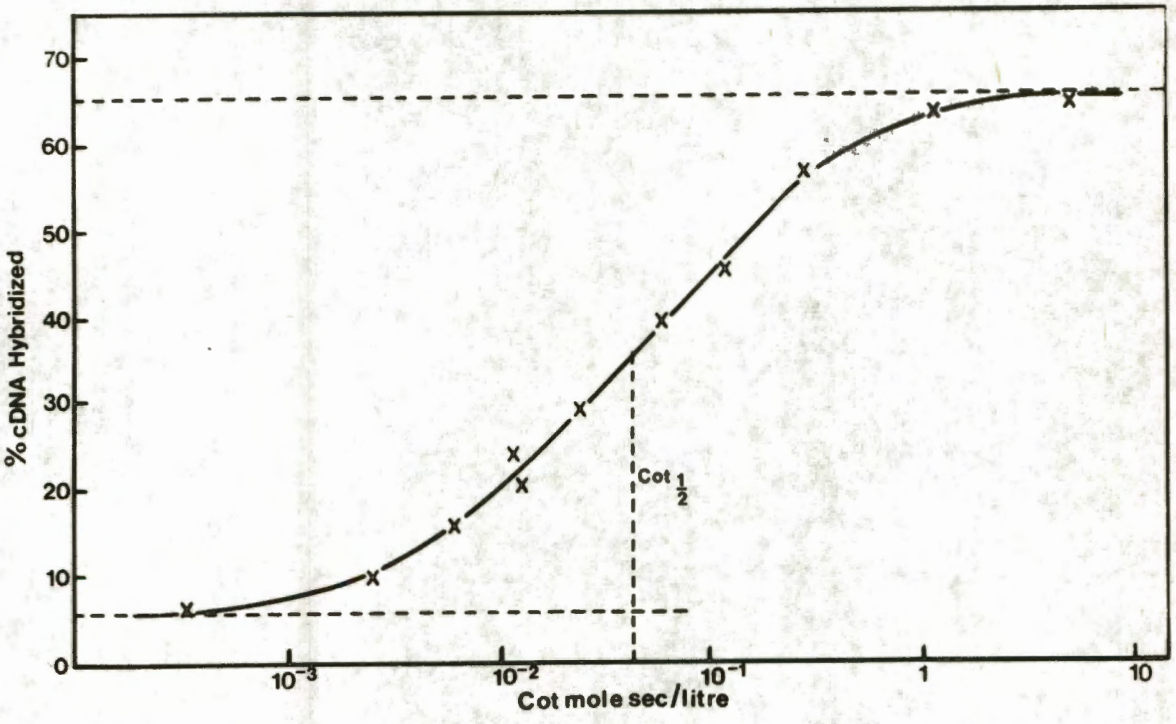
3.4.2 POLYADENYLATION WITH MAIZE SEEDLING POLYNUCLEOTIDYLEXOTRANSFERASE

In an attempt to overcome the difficulties enumerated above, a polyadenylating enzyme isolated from maize seedlings, ATP-polynucleotidylexotransferase (Walter and Mans, 1970; Mans and Huff, 1975) was used in place of E.coli poly(A) polymerase. The maize seedling enzyme has been successfully used for the polyadenylation of HeLa cell histone mRNA (Thrall et al., 1974;

FIG. 3.5

KINETICS OF THE ANNEALING OF HISTONE mRNA
TO ITS COMPLEMENTARY DNA

0.1 ng of cDNA was incubated with RNA (RNA excess at least 50 x) in 20 µl incubations. The RNA concentration in the incubate was varied from 0.25 µg-25 µg/ml and the incubation time from 0.5 - 20 h.



Stein et al., 1975) and chicken H5 histone mRNA (Scott and Wells, 1976). In both cases cDNAs, synthesized from these polyadenylated mRNAs, sedimented at 6.1 - 6.5 S (400 - 450 nucleotides) and hybridized back efficiently to the template mRNA.

3.4.2.1 ISOLATION OF MAIZE SEEDLING POLYNUCLEOTIDYLEXOTRANSFERASE

Polynucleotidylexotransferase was isolated from maize seedlings as described in Materials and methods (5.3.10.2). The isolation followed the method of Mans and Huff (1975) although their final purification of the enzyme on 2-20% glycerol gradients was omitted. The final purification step used was fractionation of the enzyme preparation on DEAE-cellulose (Fig. 3.6) which was included to remove RNase activity from the enzyme preparation (Mans and Huff, 1975). However, when 9S histone mRNA was incubated with the enzyme under the conditions described by Mans and Huff (1975) most of the mRNA was degraded. The RNA degradation was decreased substantially by including in the incubation mixture 10 units/ml of rat liver RNase inhibitor (Roth, 1956) (Fig. 3.7). The large peak of A_{260} absorbing material migrating slightly faster than 4S (Figs. 3.7, 3.9) is due to ATP complexed in the presence of magnesium (3.4.2.3). The replacement of BSA with rat liver RNase inhibitor in the incubation was shown also to substantially increase the apparent activity of the polynucleotidylexotransferase (Table 3.2). However, no difference in the activity of the enzyme was evident in the presence or absence of rat liver RNase inhibitor.

TABLE 3.2

ACTIVITY OF POLYNUCLEOTIDYLEXOTRANSFERASE
IN PRESENCE OF BSA OR RAT LIVER RNase INHIBITOR

200 µl incubations were carried out as described in Materials and methods (5.3.10.2.1). In assays when rat liver RNase inhibitor (RNase inhib) was absent BSA was added to a final concentration of 1 mg/ml (Mans and Huff, 1975).

Incubation time	Incorporation (dpm)		Apparent stimulation with RNase inhib.
	+ RNase inhib.	+ BSA	
30 min	4249	2738	1.55 x
60 min	5879	3257	1.80 x

FIG. 3.6

PURIFICATION OF MAIZE SEEDLING POLYNUCLEOTIDYLEXOTRANSFERASE ON DEAE-CELLULOSE

The enzyme preparation was applied to the DEAE-cellulose column and eluted with a linear $(NH_4)_2SO_4$ gradient. The column fractions were assayed for enzyme activity and the active peak was pooled.

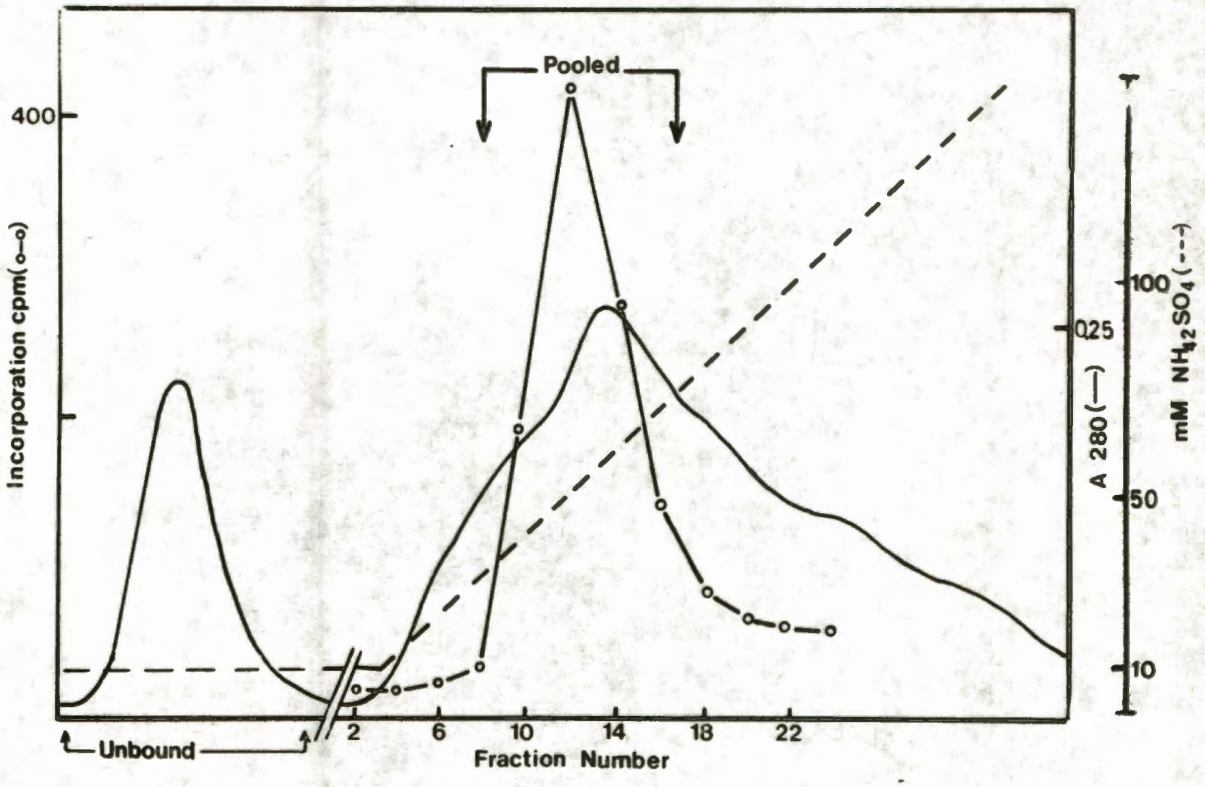
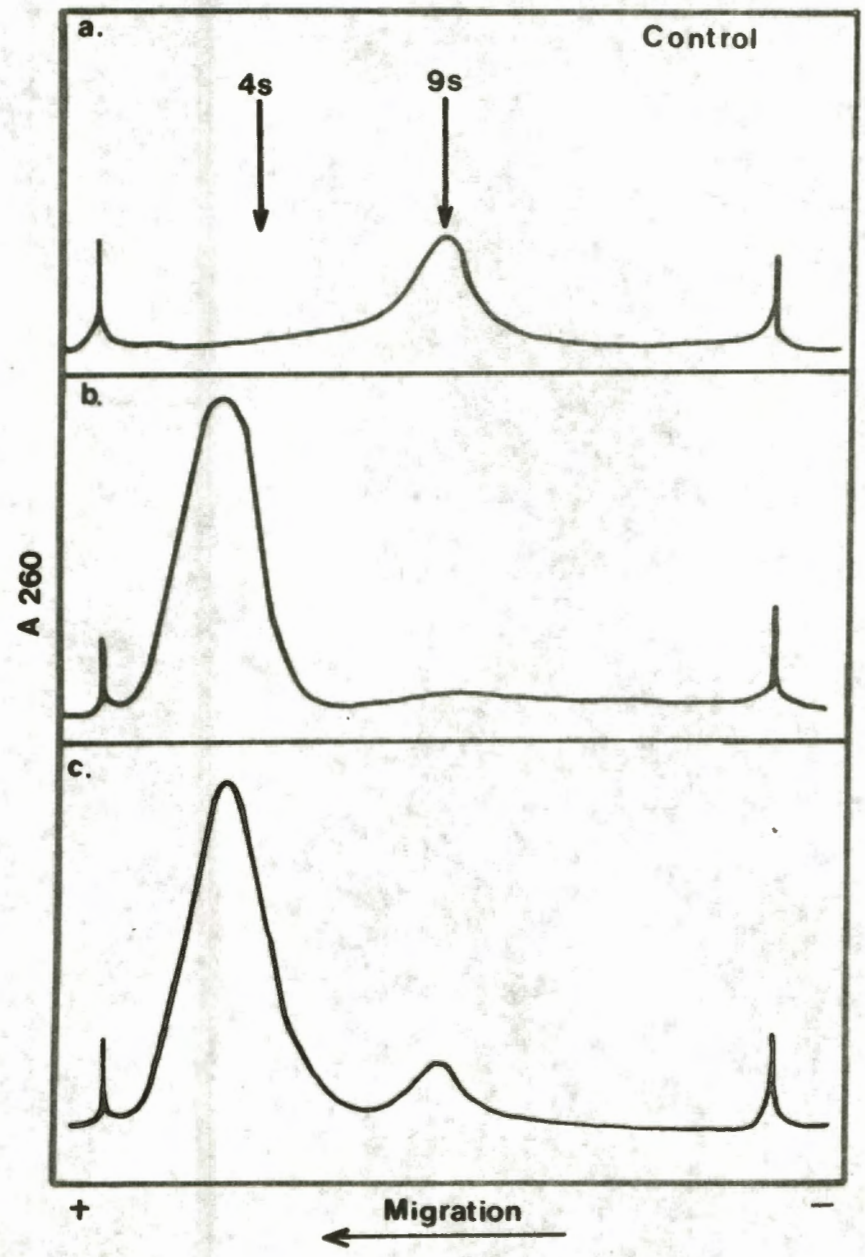


FIG. 3.7

PRESENCE OF RNase ACTIVITY IN ENZYME PREPARATION
AND THE EFFECT OF RAT LIVER RNase INHIBITOR

Histone mRNA was incubated with polynucleotidylexotransferase under standard conditions (5.3.10.2.2) in the presence and absence of rat liver RNase inhibitor and then analysed on 2.6% polyacrylamide gels (5.2.2.1). a) Control 10 µg of histone mRNA, b) 10 µg of histone mRNA incubated in the absence of rat liver RNase inhibitor, c) 10 µg of histone mRNA incubated in the presence of rat liver RNase inhibitor.



3.4.2.2 CONDITIONS OF POLYADENYLATION

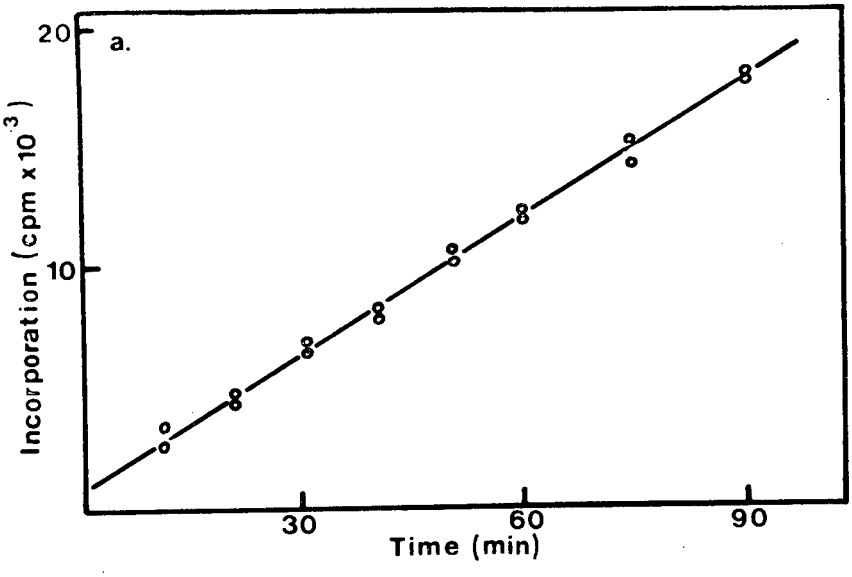
For the preparative polyadenylation of histone mRNA it was necessary to establish suitable conditions (Mans and Huff, 1975). As described above, the inclusion of rat liver RNase inhibitor reduced RNase digestion and was included in all preparative incubations. A suitable time for incubation was determined by carrying out incubations for varying lengths of time in the standard assay mixture (5.3.10.2.1) using E.coli RNA (Fig. 3.8a). The results showed that the incorporation was proportional to the time of incubation, at least up to 90 min of incubation. This did not necessarily mean that 90 minutes was an ideal incubation time as two criteria had to be met : maximum polyadenylation and minimum RNase digestion of the mRNA. When histone mRNA was incubated with the enzyme for different lengths of time and the products analysed on 2.6% polyacrylamide gels (5.2.2.1) it was found that there was a reduction in the yield of 9S mRNA as the incubation time was increased from 60 min to 90 min with no apparent increase in the amount of ^{3}H -polyadenylated mRNA (Fig. 3.9). A time of 60 minutes was therefore used for subsequent preparative polyadenylations.

To achieve the most efficient polyadenylation, experiments were carried out under conditions of enzyme excess. These conditions were determined by carrying out standard incubations with 20 µl of enzyme (90 µg of enzyme protein) and increasing amounts of yeast tRNA and E.coli rRNA (Fig. 3.8b). As may be expected conditions of RNA excess were reached with less tRNA (5-10 µg) than E.coli rRNA (40-80 µg). This can probably be explained by the fact that there would be a greater number of tRNA than E.coli rRNA molecules per unit weight of RNA, therefore making available a greater number of 3'OH termini as enzyme primers. E.coli rRNA however was a better primer for the enzyme than tRNA, as under conditions of RNA excess the use of E.coli rRNA resulted in about 2.5 x the incorporation obtained with tRNA (Fig. 3.8b). When the activity of the enzyme was compared using E.coli rRNA and histone mRNA as primers the results indicated that histone mRNA was a better primer than E.coli rRNA for polyadenylation. The reason for this apparent specificity of the enzyme for different RNA primers is unknown. A titration was not carried out using histone mRNA due to the large amounts of RNA required but the results of Fig. 3.8b indicated that if histone mRNA was incubated under standard conditions using 5 µg of mRNA/20 µl of enzyme preparation the incubation would be in conditions of enzyme excess.

FIG. 3.8

CONDITIONS FOR POLYADENYLATION WITH POLYNUCLEOTIDYLEXOTRANSFERASE

a) Incubation of enzyme (40 µl) with E.coli rRNA (20 µg) under standard conditions for varying lengths of time. (Conditions of enzyme excess).
b) Incubation (60 min) of enzyme (20 µl) with increasing amounts of E.coli rRNA and yeast tRNA under standard conditons of incubations.
The activity in both a) and b) was determined by the incorporation of [^{3}H]-AMP into TCA-precipitable material.



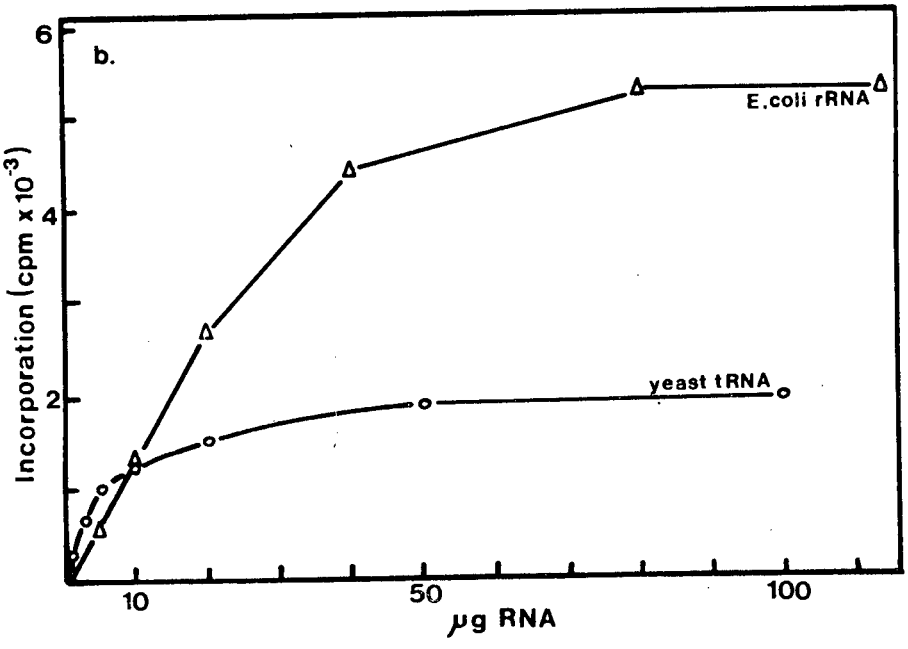
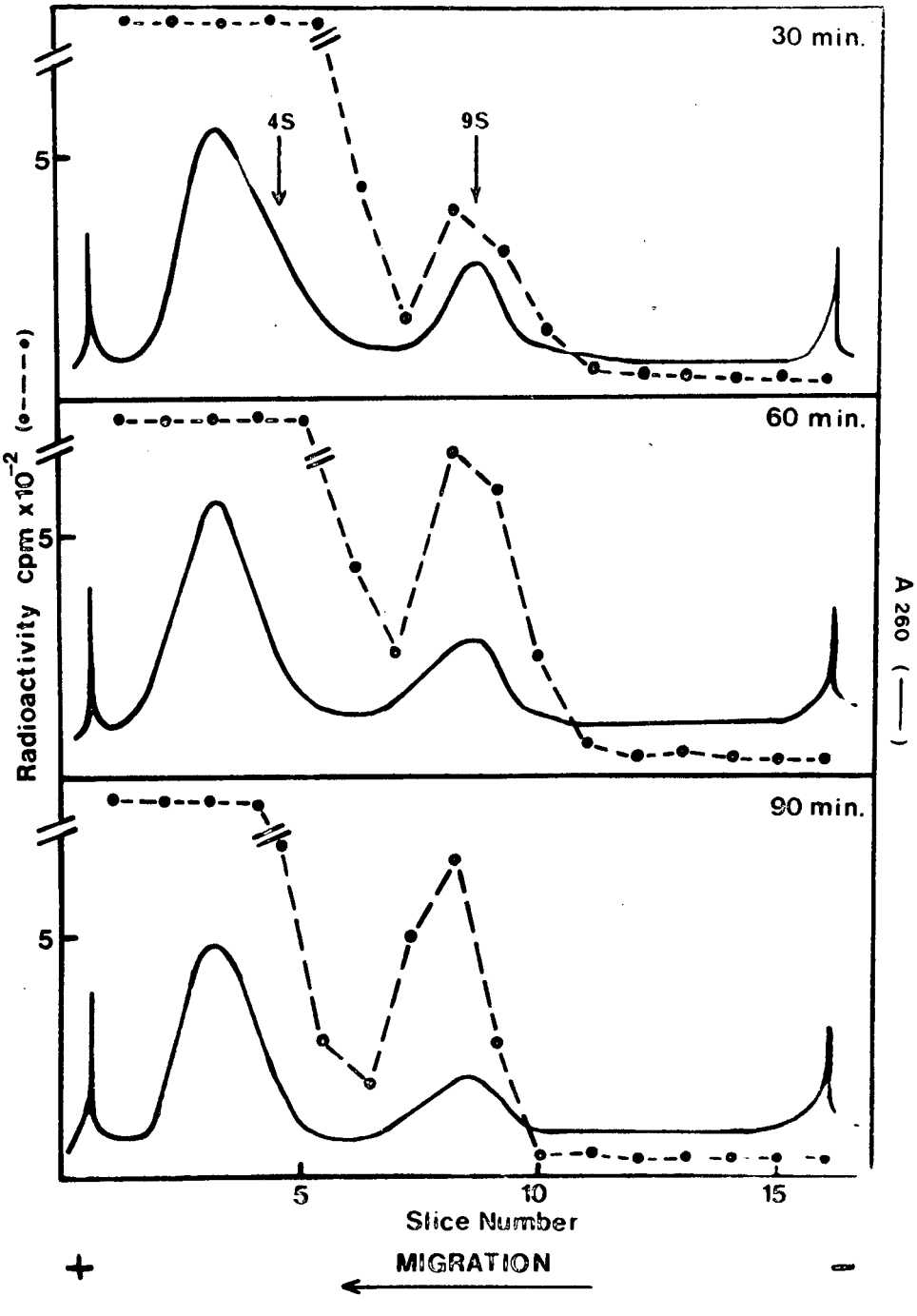


FIG. 3.9

ANALYSIS OF PRODUCTS OF POLYADENYLATION
AFTER INCUBATION FOR DIFFERENT TIMES

Standard assays were set up as described in Materials and methods (5.3.10.2.1) containing 10 µg of histone mRNA and incubated for different times. After incubation the RNA was isolated from the incubate with phenol: chloroform (5.2.7) and analysed on 2.6% polyacrylamide gels. The A_{260} and radioactive peaks were determined as described (5.2.2.1).



3.4.2.3 COMPLEXING OF ATP

In Fig. 3.7 and 3.9 large peaks of A_{260} absorbing and labelled material were evident migrating slightly faster than 4S. This was initially confusing, but was shown to be due to Mg^{++}-ATP complexes which were isolated with the RNA after polyadenylation.

An investigation of this phenomenon showed that under the conditions of incubation as much as 84% of the [^{3}H]-ATP was in the form of complexes which were excluded from Sephadex G-25 (5.2.5). This is in agreement with the results of Storer and Cornish-Brown (1976) who showed that in the presence of Mg^{++} ions 80% of ATP is in the form of complexes. When only [^{3}H]-ATP (as purchased) was passed through Sephadex G-25 as much as 77% was excluded indicating that the [^{3}H]-ATP was in the form of complexes before it was even added to the incubation (in the presence of 0.1 M EDTA less than 13% of [^{3}H]-ATP was excluded from Sephadex G-25).

The method of isolation of the RNA from incubation mixtures involved the initial dilution of the incubate in NETS (containing 1 mM EDTA) buffer before phenol:chloroform extraction (in the presence of NETS buffer however as much as 36% of the [^{3}H]-ATP from a standard incubation mixture was excluded from Sephadex G-25). The results of the polyacrylamide gel analyses showed that the Mg-ATP complexes migrated slightly faster than tRNA. Exclusion from G-25 indicates that the size of the complexes was greater than 5000 daltons which corresponds to a minimum of about 10 ATP molecules/complex.

The complexing of ATP is an important factor to take into consideration when using labelled nucleoside triphosphates as precursors for DNA and RNA synthesis. If a large percentage of the nucleotides are in the form of complexes they will not be available to the polymerases thus making necessary the use of high concentrations of labelled nucleotides. A recent report by Karin Mölling (1976) explains that this has in fact been the case with incubations utilizing reverse transcriptase, where workers, unaware of this phenomenon, have used very high [^{3}H]-nucleoside triphosphate concentrations in order to obtain full length cDNA transcripts (Efstratiadis et al., 1975; Weiss et al., 1976; Kacian et al., 1976).

3.4.2.4 PREPARATION OF POLYADENYLATED HISTONE mRNA

Histone mRNA (100 µg) was polyadenylated with the maize seedling polynucleotidylexotransferase and reisolated as described in Materials and methods (5.3.10.2.2). In order to remove any degraded mRNA produced by RNase digestion during incubation, the polyadenylated RNA was fractionated on a 15-30% sucrose gradient (5.2.1) as shown in Fig. 3.10a. The RNA sedimenting in the 9S region was pooled, precipitated with ethanol and dissolved in distilled water. When analysed on a 2.6% polyacrylamide gel (5.2.2.1) it migrated as a peak corresponding to 9S RNA and showed little contamination with low molecular weight polyadenylated RNA (Fig. 3.10b). This 9S polyadenylated RNA was used as template for the synthesis of cDNA using AMV reverse transcriptase.

3.4.3 SYNTHESIS OF cDNA TO POLYADENYLATED mRNA

Using in vitro polyadenylated RNAs as templates two preparations of reverse transcriptase, one from Dr. G. Birnie (Beatson RT17) and one from Dr. J.W. Bear G1776) were used for the synthesis of cDNA (see 5.3.8). The cDNA preparations produced using the two enzymes varied even though the same preparation of polyadenylated mRNA was used as template.

In both cases the yields of cDNA were substantially higher than those obtained using the histone mRNA polyadenylated with the E.coli poly(A) polymerase (3.4.1.1). Using the Beatson RT17 enzyme the yield of cDNA ($cDNA_1$) was approximately 5-7% of the input polyadenylated RNA. This is in close agreement with the results of Hell et al. (1976) who obtained yields of 3-5% using in vitro polyadenylated RNA. Using the Beard enzyme however, yields of cDNA ($cDNA_2$) as high as 15-20% of the input RNA were obtained. The different activities of the two enzyme preparations may be explained by the different methods of isolation of the two enzyme preparations. The Beatson enzyme (RT17) was isolated by the method of Kacian et al. (1971) whereas the Beard enzyme (G1776) was isolated by a modified procedure which increased the yields (6-10 x) and stability of the reverse transcriptase (Tsiapalis et al., 1976).

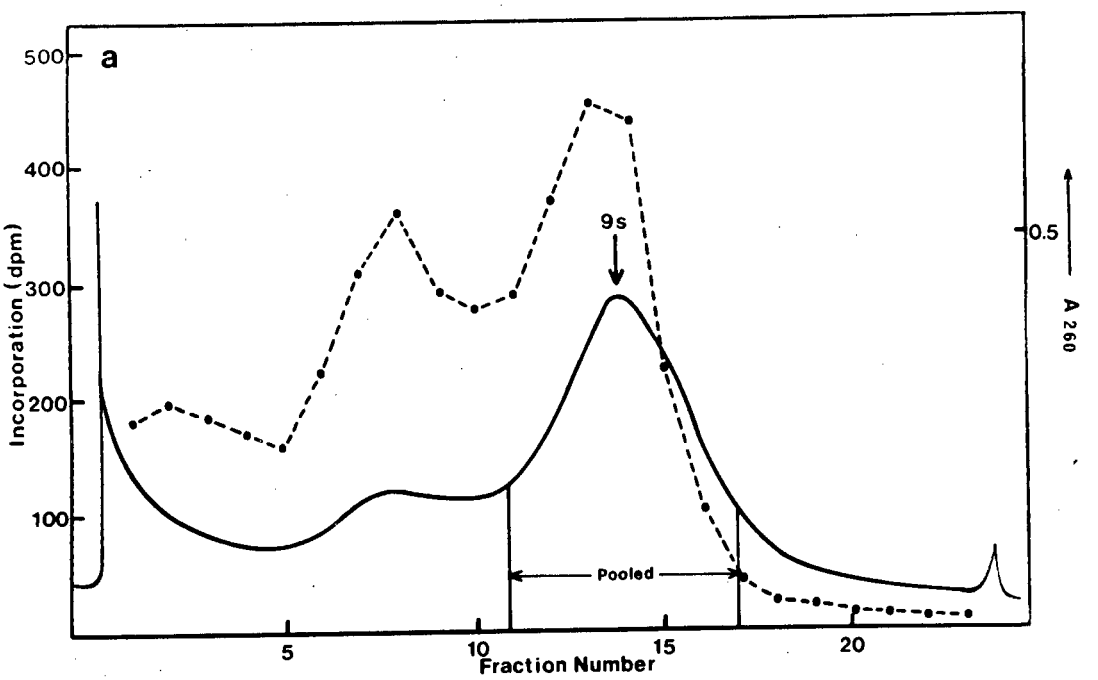
Analysis of the two cDNA preparations on 15-30% alkaline sucrose gradients showed that $cDNA_1$ migrated at 3.6S and $cDNA_2$ migrated at 5.2S (Fig. 3.11). Uisng the relationship of Studier (1965) ($S^{o}_{20w} = 0.0528\ M^{0.400}$) the sedimentation coefficients of the cDNA preparations corresponded to DNA

FIG. 3.10

ISOLATION AND CHARACTERIZATION OF
9S IN VITRO POLYADENYLATED HISTONE mRNA

a) Histone mRNA polyadenylated in vitro was fractionated on a 15-30% sucrose gradient and the 9S material was isolated. (——) A_{260}, (●---●) radioactivity.

b) Analysis of 9S polyadenylated histone mRNA on 2.6% polyacrylamide gel. (——) A_{260}, (●----●) radioactivity.



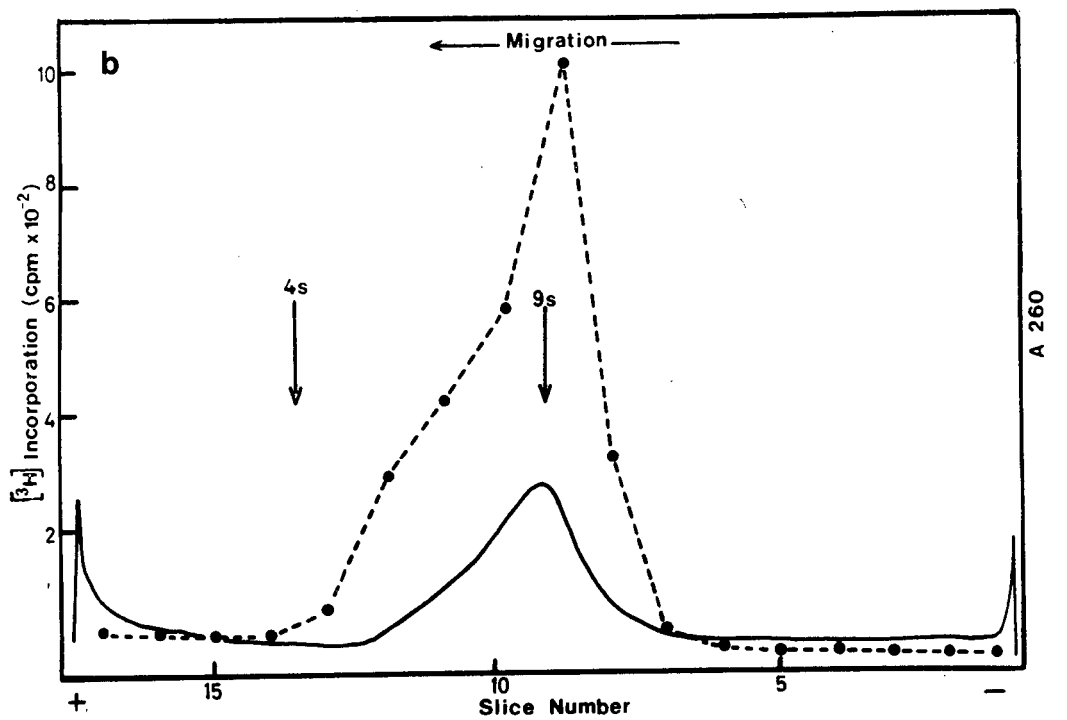
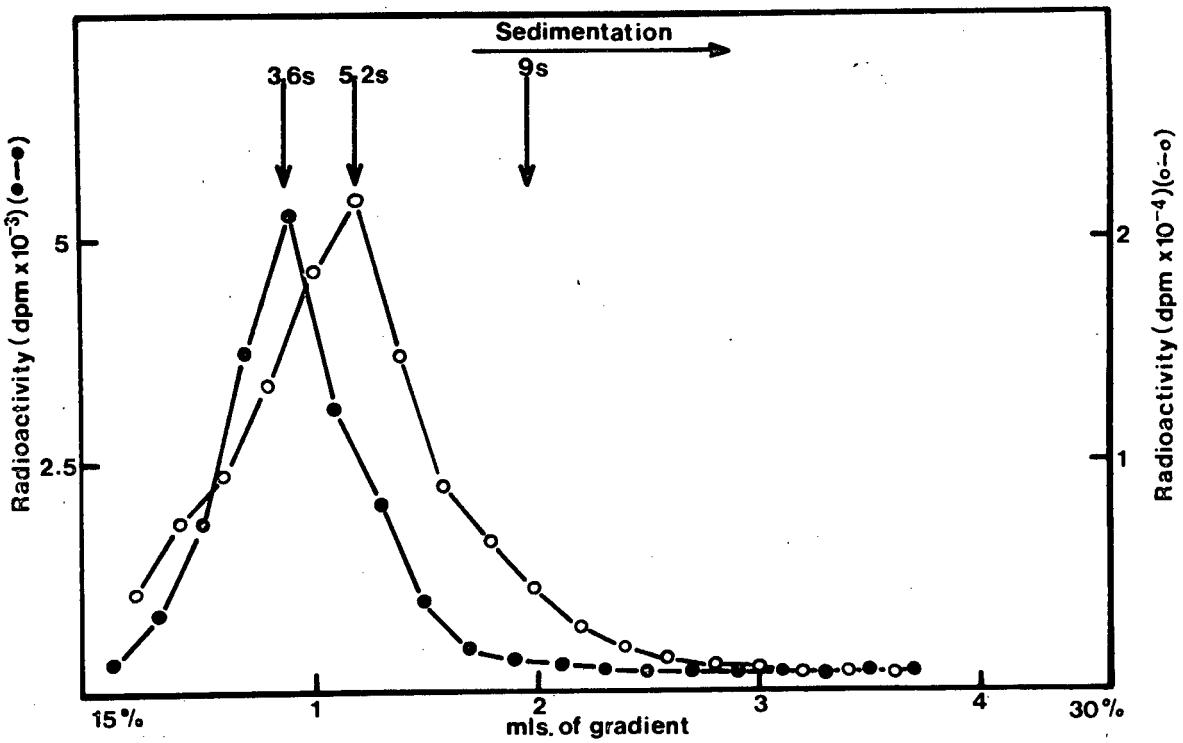


FIG. 3.11

ANAYLSIS OF cDNA ON 15-30% ALKALINE SUCROSE GRADIENTS

cDNA on alkaline sucrose gradients (0.9 M NaCl, 0.1 M NaOH) was centrifuged for 5 hours in the SW 65Ti rotor(g_{max}) and fractionated into 0.2 ml fractions. To each fraction was added 0.2 ml of 0.1 M HCl, 0.4 ml H_2O and 10 ml of scintillator. ●——● $cDNA_1$; o——o $cDNA_2$.



of approximately 115 and 228 nucleotides in length respectively.

3.5 HYBRIDIZATION OF cDNA

When these two cDNA preparations were tested for their ability to hybridize back to histone mRNA (500 x RNA excess), 63.5% of $cDNA_1$ hybridized whereas 72% of $cDNA_2$ hybridized. In both cases the background hybridizations were between 5 and 8% of the cDNA. The differences in the size of the two cDNA preparations probably contributed to the different levels of hybridization. Another contributing factor may have been that, with lower yields of $cDNA_1$ the nonhybridizable cDNA synthesized during reverse transcription would be a greater percentage of the total cDNA preparation.

The kinetics of the hybridization reactions of the two cDNA preparations to histone mRNA are shown in Fig. 3.12. Both cDNA preparations hybridized with pseudo first order reaction kinetics (second order) (Young and Paul, 1973) the range of the reactions being about 2½ log Cot units. An ideal pseudo first order reaction has a range of 1½ log units but reactions between RNA and DNA are affected by the variations in the size of the cDNA molecules (Young et al., 1974). The globin mRNA:cDNA reaction ranges over approximately 2½ log units (Getz et al., 1975) which is characteristic of the hybridization of a single abundance class of RNA with cDNA.

Although both cDNA preparations hybridized with pseudo first order kinetics the $Cot_{\frac{1}{2}}$ values of the two reactions were different; $cDNA_1$ hybridized with a $Cot_{\frac{1}{2}}$ of 0.042 mole sec/litre whereas $cDNA_2$ hybridized with a $Cot_{\frac{1}{2}}$ of 0.027 mole sec/litre. This variation in reaction rate is explained by the different sizes of the two cDNA preparations. Wetmur and Davidson (1968) showed that the rate of reassociation of mouse DNA fragments was roughly proportional to the square root of the fragment size. Comparison of the sizes and $Cot_{\frac{1}{2}}$ values of the two cDNA preparations (Table 3.2) showed that this relationship was followed under the conditions of RNA:cDNA hybridization used and explains the variations in $Cot_{\frac{1}{2}}$ obtained for $cDNA_1$ and $cDNA_2$ hybridization reactions.

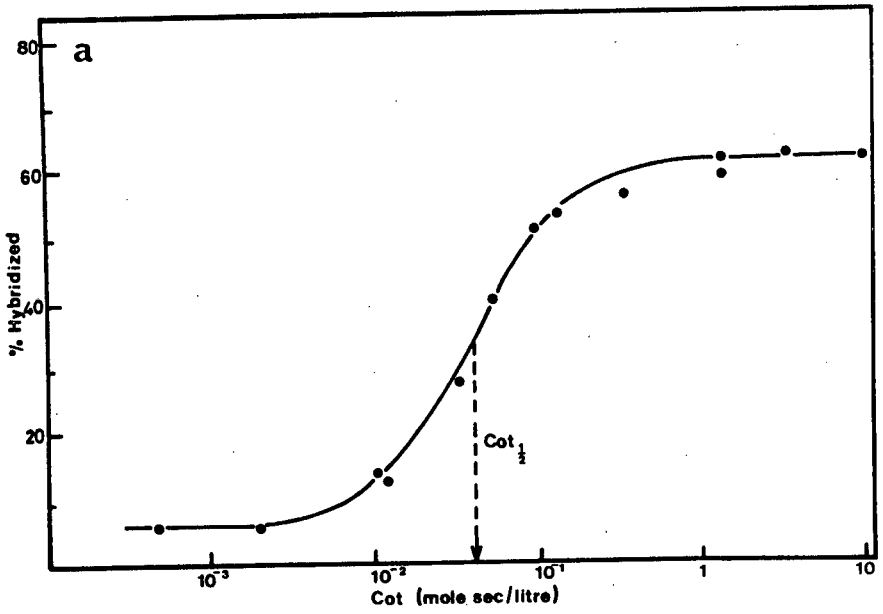
FIG. 3.12

KINETICS OF HYBRIDIZATION OF HISTONE mRNA:cDNA

Reactions were carried out using 0.1 ng of cDNA and RNA excess (at least 50 x) in 20 µl incubations. mRNA concentration was varied from 0.25 - 25 µg/ml and the time of incubation was varied from 0.5 - 20 hours. The extent of hybridization was determined by S_1 nuclease assay (5.3.9.2).

a) Hybridization of $cDNA_1$ to histone mRNA

b) Hybridization of $cDNA_2$ to histone mRNA.



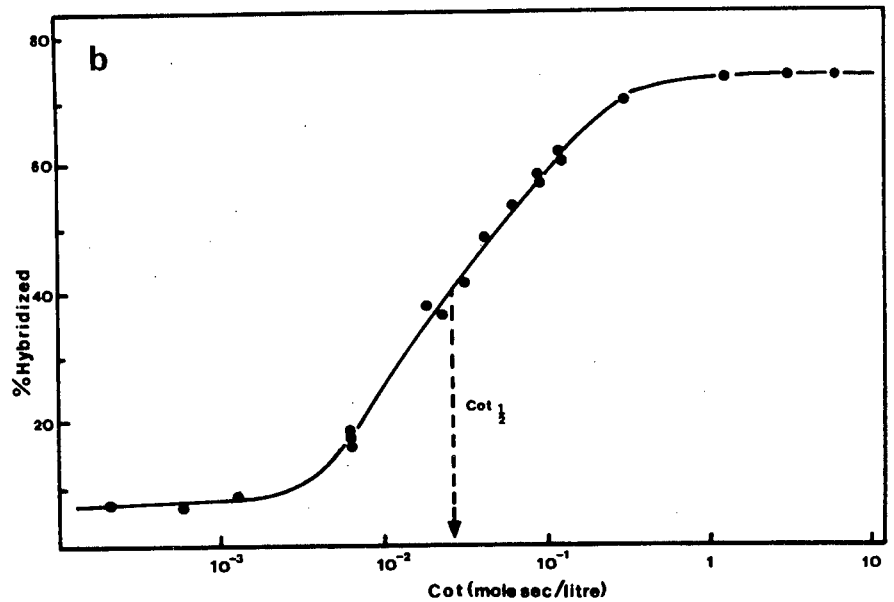


TABLE 3.2

COMPARISON OF TWO COMPLEMENTARY DNA PREPARATIONS

The relationship of Wetmur and Dividson (1968) that the rate of reassociation is proportional to $(size)^{\frac{1}{2}}$ is shown to hold under the conditions of RNA:cDNA hybridization used.

Complementary DNA	Size (nucleotides)	$Cot_{\frac{1}{2}}$ (mole sec/l)	$Cot_{\frac{1}{2}} \times (size)^{\frac{1}{2}}$
$cDNA_1$	115	0.042	0.450
$cDNA_2$	288	0.027	0.458

3.5.1 NUCLEOTIDE COMPLEXITY OF HISTONE mRNA

Under conditions of RNA excess the rate of hybridization of RNA to cDNA (when measured in terms of Cot) has been shown to be proportional to the nucleotide complexity (base sequence complexity) of the RNA (Birnstiel et al., 1972; Young and Paul, 1973; Young et al., 1974). Nucleotide complexity of the RNA is defined as the number of nucleotides of unique DNA transcribed to give rise to the RNA population.

With this information it was therefore possible, using the mouse globin mRNA:cDNA reaction as a standard, to calculate the nucleotide complexity of the histone mRNA and therefore the cDNA preparations. Mouse globin mRNA is comprised of α and β globin mRNA (1:1) and therefore has a total nucleotide complexity of 1200 nucleotides (Williamson et al., 1971). When hybridized under the same conditions (43°C, 50% (v/v) formamide, 0.5 M NaCl) as used for the histone mRNA:cDNA reactions the globin mRNA:cDNA reaction takes place with a $Cot_{\frac{1}{2}}$ of 4×10^{-3} mole sec/l (Young et al., 1974). The nucleotide complexity of the histone mRNA can therefore be calculated as follows :

$$\frac{Cot_{\frac{1}{2}}\ (\text{histone})}{\text{complexity (histone)}} = \frac{Cot_{\frac{1}{2}}\ (\text{globin})}{\text{complexity (globin)}}$$

Before calculating the complexity of the histone mRNA is was necessary to take into consideration the different sizes of the histone cDNA and globin cDNA molecules. As shown earlier, the rate of hybridization is proportional to the square root of the size of the cDNA. The measurements of the kinetics

of the globin mRNA:cDNA reaction were performed with a cDNA of 330 nucleotides in length (Young et al., 1974). It is possible therefore to convert the $Cot_{\frac{1}{2}}$ of the two histone mRNA:cDNA reactions to a value of Cot corresponding to a cDNA of length 330 nucleotides.

$$cDNA_1 \qquad 0.042 \times \left(\frac{115}{330}\right)^{\frac{1}{2}} = 0.0248 \text{ mole sec/l}$$

$$cDNA_2 \qquad 0.027 \times \left(\frac{288}{330}\right)^{\frac{1}{2}} = 0.0252 \text{ mole sec/l}$$

The close agreement of the two calculated values for $Cot_{\frac{1}{2}}$ of the histone mRNA:cDNA reaction again shows that the relationship between $(\text{size})^{\frac{1}{2}}$ and rate of hybridization (Wetmur and Davidson, 1968) holds for RNA:DNA hybridization as well as the reannealing of DNA.

Using the modified $Cot_{\frac{1}{2}}$ value for the histone mRNA:cDNA (330 nucleotides) reaction the approximate nucleotide complexity of the histone mRNA was calculated to be 7500 nucleotides. Variation in the (G+C) content between the globin mRNA and histone mRNA (Wetmur and Davidson, 1968) have not been taken into consideration for this calculation of nucleotide complexity.

The theoretical nucleotide complexity of sea urchin histone mRNA was calculated as 2000 - 2500 nucleotides assuming that 5 messenger RNAs are present coding for the 5 classes of sea urchin histones. The experimentally determined complexity of the histone mRNA preparation was about 3 x this theoretical value. Similarly Stein et al. (1975) have found a complexity higher than expected with HeLa cell histone mRNA. This meant either that there were more than 5 histone mRNAs present (approximately 17) or that the histone mRNA was contaminated with other types of RNA which would be represented by complementary sequences in the cDNA.

The presence of more than 5 histones has been demonstrated by a number of workers. Ruderman et al., (1974) and Arceci et al. (1976) have shown that different H1 histones were present at different stages of embryonic development. Similar variations in the H1 histones have been shown in P. angulosus (see 2.4.2.1). Cohen et al. (1975) have demonstrated variations in the electrophoretic mobility of the H2A and H2B histones synthesized at different developmental stages and recently Strickland et al. (1977a,b) have shown by amino acid sequence analysis that there are at least two types of H2B histones in sperm of P. angulosus. Further variations have been shown

in the amino acid sequences of H2B histones isolated from embryos and somatic tissue (gut) of P. angulosus (W. Brandt personal communication). The presence of all these histone variants at different stages does not necessarily mean that the histone mRNA preparation isolated from sea urchin embryos at a particular stage contains mRNAs for all these different histones. It does however indicate that the histone mRNA preparation may contain more than five different messengers coding for histones.

3.6 CONTAMINATION OF HISTONE mRNA

During the cleavage stages of the sea urchin from which the histone mRNA preparation was isolated (see 2.3) it has been shown that histone mRNAs are the predominant species among polyribosome associated mRNAs (Kedes and Gross, 1969a, 1969b; Nemer, 1975). These results, together with those of Gross et al. (1973) and Levy et al. (1975), who showed that 9S RNA isolated from cleavage stage sea urchin embryos supported the synthesis of histones and no other proteins, indicated that any contamination of histone mRNA with other mRNAs would be low if present at all.

Another possible contaminant of histone mRNA was ribosomal RNA which may be present in 9S RNA fractions as a result of degradation of rRNA during isolation. Any rRNA contaminants in the histone mRNA preparation would have been polyadenylated with the histone mRNA molecules making them suitable templates for reverse transcriptase thus resulting in a cDNA preparation containing DNA complementary to rRNA. To test this the cDNA was incubated under hybridizing conditions with a vast excess of sea urchin ribosomal RNA. It was shown (Table 3.3) that as much as 84% of the hybridizable cDNA

TABLE 3.3

HYBRIDIZATION OF cDNA TO mRNA AND RIBOSOMAL RNA

0.2 ng of $cDNA_1$ was incubated under hybridizing conditions with excess histone mRNA and rRNA (1 µg) in a final volume of 20 µl. Tubes were incubated for 48 h.

RNA	% cDNA hybridized	% of Hybridizable cDNA
mRNA	62.5	100
ribosomal RNA	52.5	84

hybridized to the ribosomal RNA preparation. The rRNA used in these experiments was the fraction of total polysomal RNA which sedimented faster than 18S on sucrose gradients. The possibility that this high molecular weight fraction of polysomal RNA contained histone mRNA sequences could not be excluded. Kabat (1975) has shown that rabbit 9S globin mRNA was complexed with ribosomal RNA when isolated from reticulocyte polyribosomes and was present in both the 18S and 28S RNA fractions on sucrose gradients. To eliminate this possibility sea urchin ribosomes were dissociated with EDTA into subunits (Tashiro and Siekevitz, 1965) which were fractionated on sucrose gradients and then used for the isolation of large and small ribosomal subunit RNA.

3.6.1 ISOLATION OF RIBOSOMAL SUBUNIT rRNA

Sea urchin ribosomes and subunits were isolated as described in Materials and methods (5.3.4.1). The ribosomal subunits sedimented in sucrose gradients at 23S (small) and 41S (large) (Fig. 3.13) and not as the standard 40S and 60S subunits. Similar results however were obtained by Tashiro and Siekevitz (1965) with EDTA dissociated liver ribosomes where the subunits sedimented at 28S and 46S. These low sedimentation rates of the subunits can be explained by extreme variations in the conformation of the subunits in the absence of magnesium ions.

The ribosomal subunits were pooled as shown in Fig. 3.13 and the RNA was isolated from the pooled fractions (5.2.7). Analysis of the isolated RNA on sucrose gradients gave the results shown in Fig. 3.14. The small subunit RNA (Fig. 3.14a) contains three species of RNA, an 18S RNA fraction as well as two other RNAs migrating at 12S and 14S. (Only small amounts of 28S RNA were evident). Similar peaks of 12S and 14S RNA were also evident in preparations of total polyribosomal RNA (see Fig. 2.4). The possibility that the 12S and 14S RNAs were mRNA molecules, isolated with the small ribosomal subunits in the form of ribonucleoprotein particles (Infante and Nemer, 1968), seemed unlikely due to the high 14S + 12S : 18S ratio. The ratio of yields of large subunit RNA to small subunit RNA was approximately 2.5 which is the same as the ratio of 26S : 18S rRNA (Parish, 1972). This indicated that the 12S and 14S were derived from the 18S rRNA by specific cleavage during isolation. Nemer and Infante (1967) showed such breakdown of 18S rRNA isolated from Strongylocentrotus purpuratus resulting in a 13S

FIG. 3.13

ISOLATION OF RIBOSOMAL SUBUNITS ON 15-30% SUCROSE GRADIENTS

Sea urchin ribosomes were dissociated by suspension in buffer containing 10 mM EDTA and fractionated on 15-30% sucrose gradients (SW 40Ti) for 7 h. 20 A_{260} units of ribosomal material were applied to each gradient.

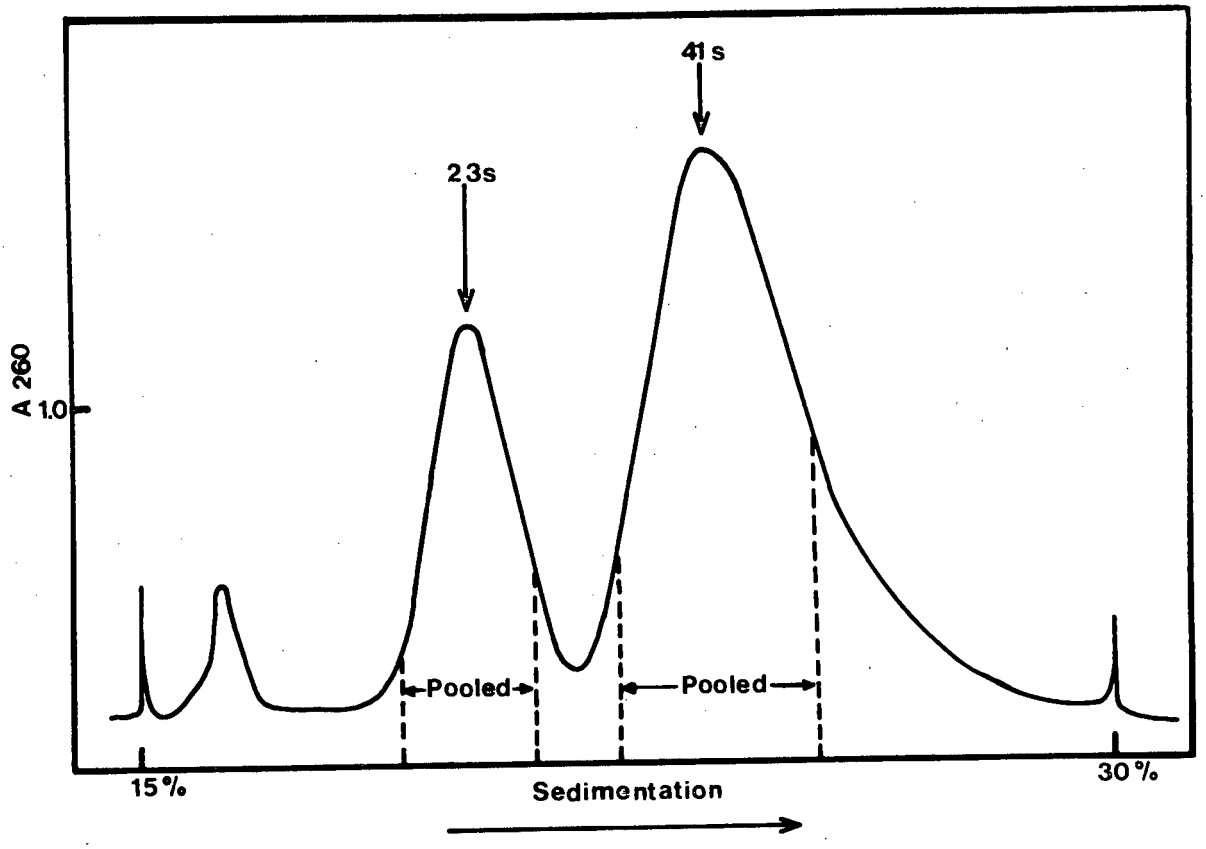


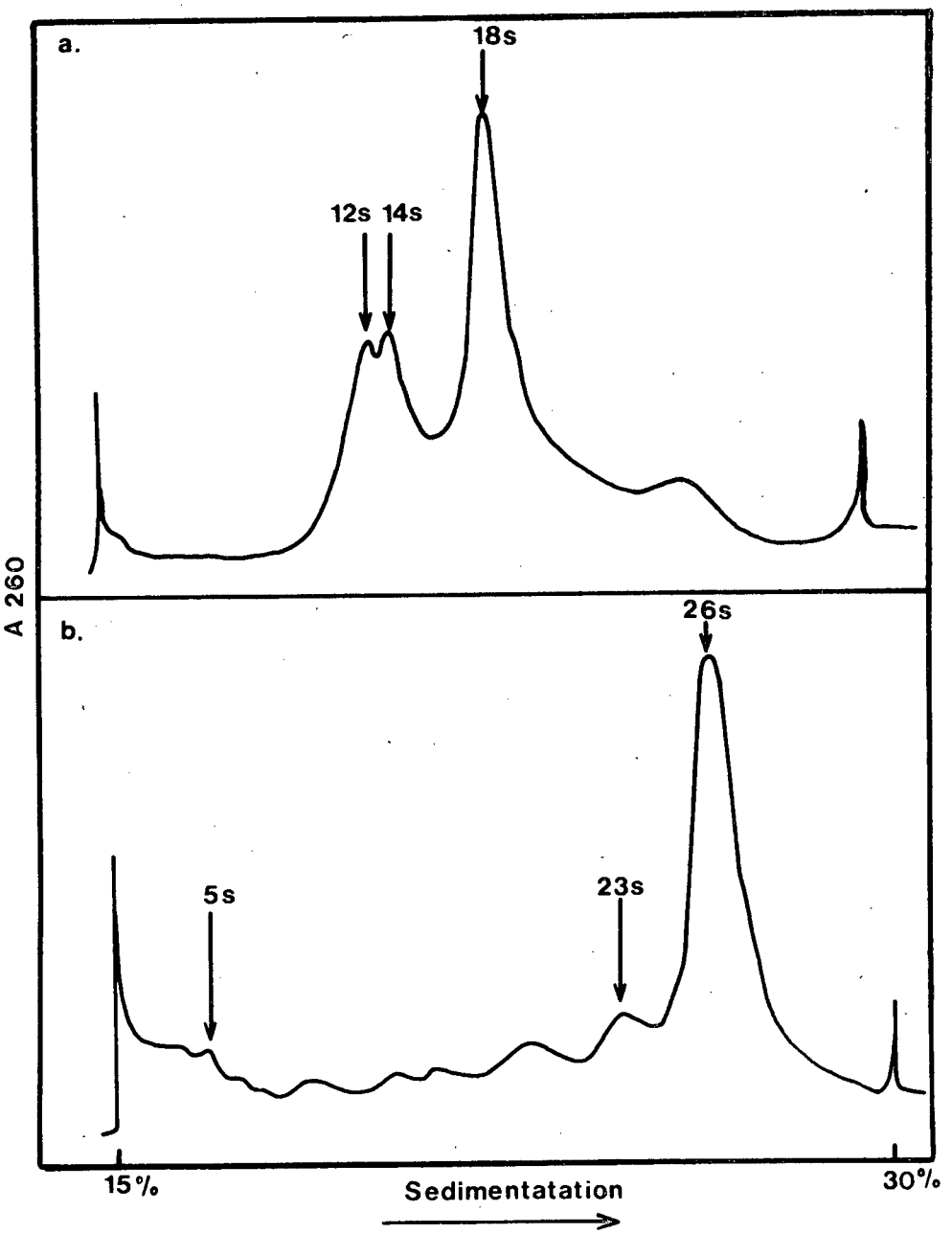
FIG. 3.14

ANALYSIS OF RIBOSOMAL SUBUNIT RNA ON 15-30% SUCROSE GRADIENTS

Approximately 2.5 A_{260} units of each RNA preparation was applied to a 15-30% sucrose gradient (SW 65 Ti) and centrifuged for 3 hours.

a) small subunit RNA

b) large subunit RNA.



RNA fraction which they presumed to be two fragments. This was not evident in the rRNA of other species of sea urchin they studied and was characteristic of the rRNA of the egg and early developmental stages.

The large subunit RNA (Fig. 3.14b) included a peak of 26S RNA as well as a number of peaks of lower molecular weight RNA; three of which correspond to the 18S, 14S and 12S rRNA of the small subunit. The 5S rRNA was present as well as a small amount of RNA sedimenting between the 12S rRNA and the 5S RNA which probably represented 26S rRNA degradation products.

3.6.2 HYBRIDIZATION OF cDNA TO RIBOSOMAL SUBUNIT RNA

When the cDNA preparations were incubated under hybridizing conditions with 5000 fold excess of large and small subunit RNA it was found that 83-85% of the hybridizable cDNA became hybridized to both RNA preparations (Table 3.4).

TABLE 3.4

HYBRIDIZATION OF cDNA TO SUBUNIT RNA

$cDNA_1$ and $cDNA_2$ were incubated with large and small subunit RNA in vast RNA excess.

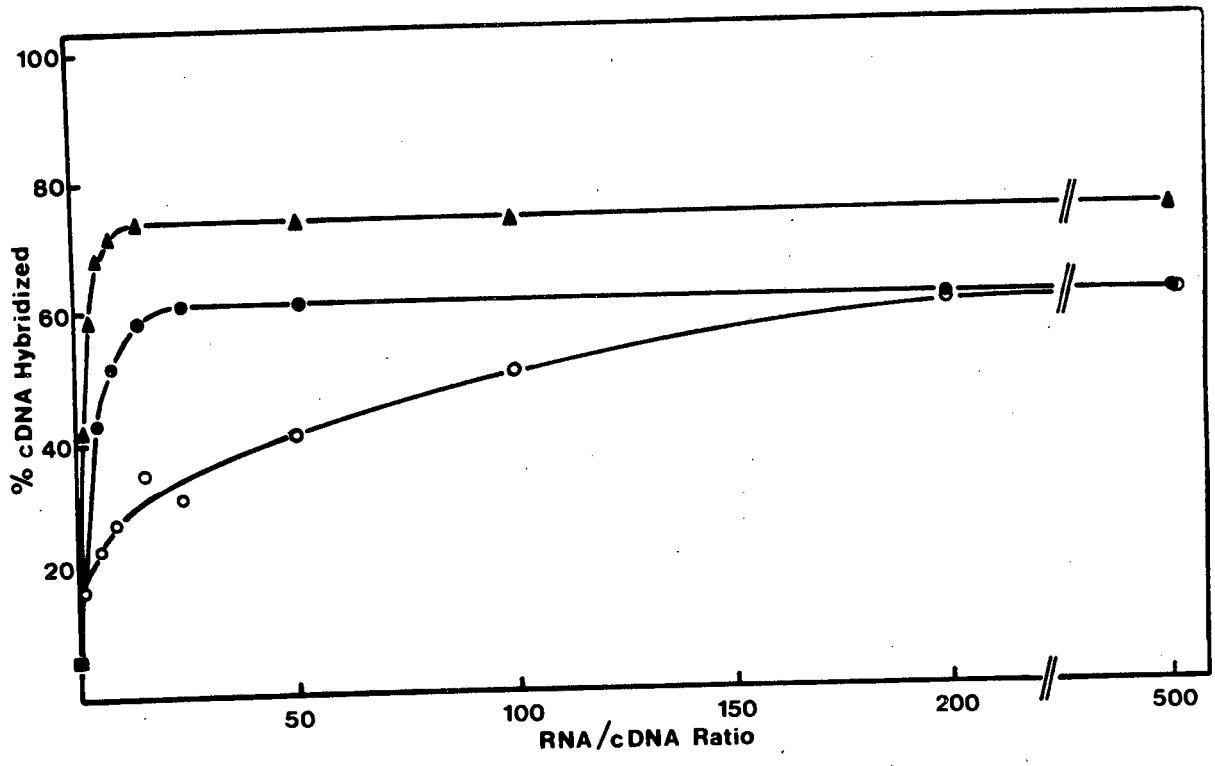
RNA	% cDNA Hybridized		% of Hybridizable cDNA	
	$cDNA_1$	$cDNA_2$	$cDNA_1$	$cDNA_2$
mRNA	63.5	72.0	100	100
large subunit RNA	53.0	61.4	83.5	85.3
small subunit RNA	52.5	60.8	82.6	84.4

When $cDNA_2$ was titrated against increasing amounts of large and small subunit RNA (Fig. 3.15) it was found that the cDNA was saturated (61-62%) with the RNA of the large subunit at a very much lower RNA:cDNA ratio (10-15) than was required to cause saturation with the small subunit RNA (approximately 200). It was therefore evident that the histone mRNA preparation was extensively contaminated (85%) with RNA derived from the large ribosomal subunit which became polyadenylated and reverse transcribed. These results indicate therefore that only 15% of the histone mRNA preparation is authentic histone mRNA. The fact that the cDNA preparation hybridized to saturation with the small subunit RNA preparation at high RNA:cDNA ratios, as well as

FIG. 3.15

TITRATION OF cDNA TO RIBOSOMAL SUBUNIT RNA

0.5 ng of cDNA was incubated with increasing amounts of histone mRNA (▲——▲), large subunit RNA (●——●) and small subunit RNA (o——o). (■) Background hybridization. Reactions were incubated to a Cot of 0.3 (minimum 100 h incubation) and the hybrids analysed by S_1 nuclease digestion.



the monophasic nature of the large subunit RNA:cDNA titration showed that the small subunit RNA fraction was contaminated with large subunit RNA. It also indicated that the histone mRNA preparation contained little if any contamination with 18S RNA.

The fact that the histone mRNA preparation was contaminated with large subunit RNA was not altogether unexpected. It has been shown in a number of systems that the large ribosomal RNA (26-28S) contains a number of hidden "nicks", which result, after heat treatment (60°C for 5 min), in the release of partially degraded fragments of rRNA (Muramatsu, 1973). The contamination of the histone mRNA with fragments of 26S RNA was probably caused by the heat treatment to which the polyribosomal RNA was subjected prior to sucrose density gradient fractionation (see 2.3.3). This heat treatment was however necessary to eliminate aggregation of the polyribosomal RNA (see Fig. 2.4 and 2.5).

3.6.3 KINETICS OF THE RIBOSOMAL RNA:cDNA REACTION

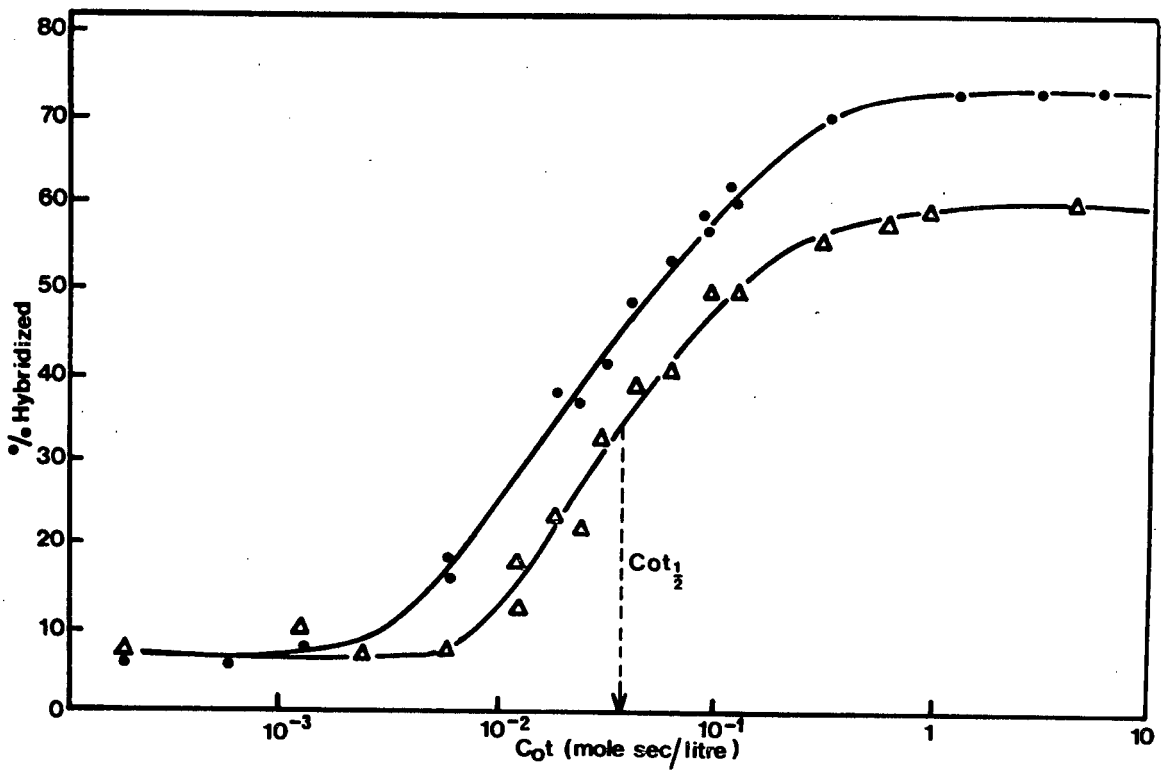
The reaction between cDNA and large ribosomal subunit RNA (Fig. 3.16) was shown to take place with pseudo first order reaction kinetics and the apparent $Cot_{\frac{1}{2}}$ of the reaction was 0.036 mole sec/l. Using the globin mRNA: cDNA (330 nucleotides) system as a standard (Harrison et al., 1974) and assuming the molecular weight of the sea urchin 26S rRNA to be 1.4×10^6 daltons (Parish, 1972) the theoretical $Cot_{\frac{1}{2}}$ (Hell et al., 1976) for the 26S rRNA : cDNA (330 nucleotides) reaction was calculated as approximately 0.014 mole sec/l. The $Cot_{\frac{1}{2}}$ obtained for the reaction (0.036 mole sec/l) was for a probe of length 288 nucleotides, which when converted to the $Cot_{\frac{1}{2}}$ which would have resulted from a cDNA probe of length 330 nucleotides gave a value of 0.034 mole sec/l. Comparison of this $Cot_{\frac{1}{2}}$ value with the calculated $Cot_{\frac{1}{2}}$ of the reaction (0.014 mole sec/l) indicated that less than 50% of the large ribosomal subunit RNA could hybridize to the cDNA i.e. less than 50% of the large ribosomal subunit RNA was represented by complementary DNA sequences. This RNA fraction probably corresponds to fragments of the 26S RNA which were small enough to contaminate the original 9S histone mRNA preparation. Specific cleavage of the 26S rRNA (Muramatsu, 1973) would explain this result (see above).

Published work describing the isolation and fractionation of sea urchin histone mRNAs and its hybridization to DNA has involved the use of <u>in vivo</u>

FIG. 3.16

KINETICS OF THE HYBRIDIZATION OF cDNA:rRNA

Reactions were carried out using 0.1 ng of cDNA and RNA excess (at least 50 x). 20 µl incubations contained 0.25 - 25 µg/ml of RNA and were incubated for 0.5 - 20 h. The extent of hybridization was determined by S_1 nuclease digestion. ●——● mRNA:cDNA; Δ——Δ large subunit RNA:cDNA.



labelled histone mRNA (Kedes and Gross, 1969a; Kedes and Birnstiel, 1971; Weinberg et al., 1972; Grunstein et al., 1973; Levy et al., 1975; Gross et al., 1976). The absence of ribosomal RNA synthesis during cleavage stages of the sea urchin embryonic development (Gross et al., 1964) allowed the isolation of labelled mRNA free of labelled rRNA. This did not mean that the labelled mRNA preparations were not contaminated with unlabelled rRNA. Recently Wu et al. (1976) have mentioned that their labelled histone mRNA was extensively contaminated with unlabelled rRNA but indicated that these contaminants were 5S and 18S rRNA. A similar contamination of HeLa cell histone mRNA with 18S rRNA, even after fractionation of the histone mRNA by preparative SDS-gel electrophoresis has recently been described by Bos et al. (1976). In contrast to the results of these authors, as well as results reported here, Stein and coworkers however have reported the preparation of cDNA to HeLa cell histone mRNA (Thrall et al., 1974) which did not contain sequences complementary to 5S, 18S or 28S ribosomal RNA (Stein et al., 1975) even though as mentioned earlier the apparent complexity of their histone cDNA was higher than expected.

3.7 PURIFICATION OF HISTONE mRNA SPECIFIC cDNA

The contamination of the histone mRNA specific cDNA (histone cDNA) with cDNA complementary to rRNA made the cDNA unsuitable as a probe for histone mRNA. It was therefore necessary either to attempt to isolate sea urchin histone mRNA free of rRNA contaminants (but see above) or to isolate the histone mRNA specific cDNA from a total cDNA preparation. This problem was approached by the purification of the histone-cDNA.

3.7.1 HYDROXYLAPATITE CHROMATOGRAPHY

Hydroxylapatite chromatography is a well established tool for the fractionation of nucleic acids (Bernardi, 1971). At low concentrations of phosphate buffer (0.03 M) at 60°C both RNA and DNA are bound to hydroxylapatite but when the concentration of phosphate buffer is increased to 0.12 M, RNA, double stranded DNA and RNA:DNA hybrids remain adsorbed to the column, but single stranded DNA is eluted, whereas at 0.4 M phosphate buffer all nucleic acids are eluted from hydroxylapatite (Smith, 1973). This technique therefore appeared to provide a method for the purification of histone cDNA from the total cDNA preparation by the removal of cDNA complementary to rRNA in the form of rRNA:cDNA hybrids.

Initial experiments with hydroxylapatite gave very low yields of cDNA (± 20%) but this was overcome by passing native DNA (M.lysodeikticus) through the column prior to fractionation of RNA:cDNA hybrids. Under these conditions the yields of cDNA from hydroxylapatite columns was in all cases greater than 90%. If denatured, sheared DNA (M.lysodeikticus) was used instead of native DNA to presaturate the column the yields of cDNA were however only about 70%.

When RNA:cDNA hybrids were analysed by hydroxylapatite chromatography it was found in all experiments that the percentage of cDNA which eluted as double stranded material (hybridized) was about 11% higher than when similar hybridization reactions were assayed by S_1 nuclease digestion (see Table 3.5).

TABLE 3.5

ANALYSIS OF HYBRIDIZATION REACTIONS BY S_1 NUCLEASE DIGESTION AND HYDROXYLAPATITE FRACTIONATION

Hybridization reaction	% cDNA hybridized	
	S_1 nuclease digestion	Hydroxylapatite fractionation
cDNA : mRNA	72.0	83.5
cDNA : rRNA	61.4	72.8
cDNA : no RNA	6.8	17.0

This result may be explained by the presence of tails of single stranded cDNA remaining attached to double stranded regions. On hydroxylapatite chromatography these "tails" would be eluted with the double stranded cDNA whereas with S_1 nuclease digestion they would be removed from the double stranded fraction. For this reason, apart from the simplicity of the method, the S_1 nuclease assay is a very much more suitable method for the assay of hybridization reactions.

Typical elution profiles of cDNA:RNA hybrids and cDNA alone are shown in Fig. 3.17. Of the cDNA 2-3% was eluted with 0.03 M phosphate

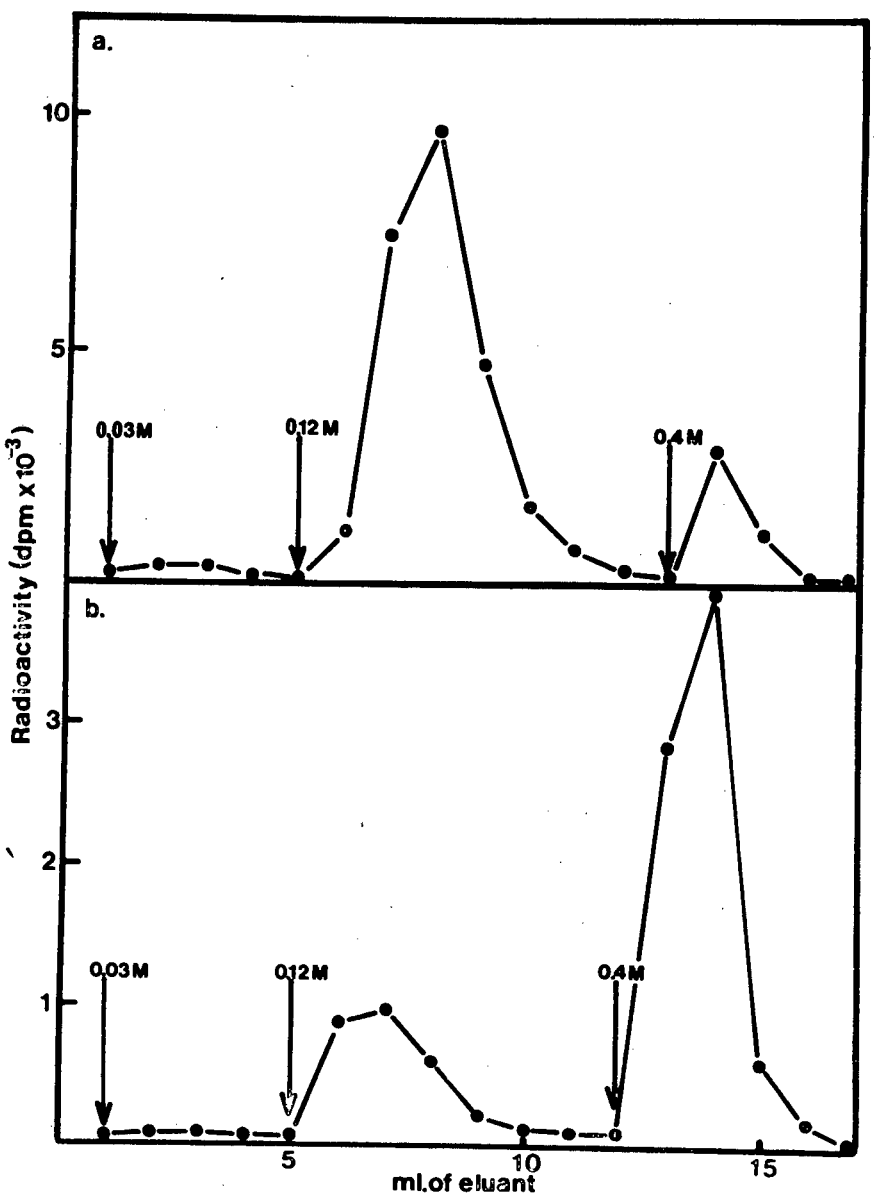
FIG. 3.17

FRACTIONATION OF RNA:cDNA HYBRIDS ON HYDROXYLAPATITE

Hydroxylapatite chromatography was performed essentially as described in Materials and methods (5.2.6) except that the samples were applied and eluted stepwise in 1 ml fractions. The radioactivity in each fraction was determined.

a) cDNA incubated under hybridizing conditions in the absence of RNA.

b) cDNA incubated with a vast excess (2000 x) of large ribosomal subunit RNA.



buffer in both experiments. Similar amounts of cDNA were eluted with 0.03 M phosphate buffer in all hydroxylapatite chromatography experiments.

3.7.2 EFFICIENCY OF HYBRIDIZATION OF cDNA AFTER HYDROXYLAPATITE CHROMATOGRAPHY

To test the extent of cDNA hybridization after fractionation on hydroxylapatite an aliquot of cDNA was hybridized to histone mRNA and the hybridization mixture was fractionated on hydroxylapatite (5.2.6). The double stranded material was pooled and the cDNA in this fraction (83.5% of total cDNA) was reisolated and hybridized back to histone mRNA. The extent of hybridization was determined by S_1 nuclease digestion and 82.5% of the cDNA showed nuclease resistance. If one considers that 83.5% of the cDNA was eluted as double stranded cDNA despite the fact that only 72% was double stranded as determined by S_1 nuclease digestion (see Table 3.5) it can be concluded that the maximum possible S_1 nuclease resistance of the purified cDNA preparation was 86%. The cDNA was therefore hybridizing very efficiently (96%).

3.7.3 REMOVAL OF COMPLEMENTARY DNA to rRNA from the cDNA

The first attempt at the purification of histone cDNA involved the removal of the cDNA complementary to rRNA by hybridization of large subunit RNA to the cDNA followed by fractionation on hydroxylapatite and isolation of the non-hybridized single stranded cDNA. This fraction, approximately 25% of the total cDNA, contained the histone cDNA as well as the non-hybridizable cDNA. The possible existence of self complementary regions in the histone cDNA could result in loss of histone cDNA by isolation with the double stranded cDNA:rRNA hybrids on hydroxylapatite. However it was unlikely in view of the results shown in Table 3.5 where 11% of the total cDNA preparation is shown to be histone mRNA specific when analysed by <u>both</u> hydroxylapatite and S_1 nuclease digestion. The cDNA isolated from this single stranded fraction was incubated under hybridizing conditions with histone mRNA and rRNA and it was found that 34% of the cDNA hybridized to histone mRNA. The percentage of cDNA which hybridized to rRNA was only 5-10% above the values of background hybridization (cDNA:no mRNA). The low levels of hybridization to histone mRNA were not unexpected as, of the fraction of cDNA which did not hybridize to rRNA ($\pm$ 38% of total), only about 30% was hybridizable to mRNA, the remaining 70% being non-hybridizable cDNA (see Fig. 3.15). The fact that slightly more than 30% hybridized to the histone mRNA preparation can

be accounted for by the presence of a small percentage of complementary rRNA still present in the cDNA preparation.

3.7.4 REMOVAL OF NON-HYBRIDIZABLE cDNA

In order to increase the percentage of purified cDNA which hybridized to histone mRNA the non-hybridizable fraction of cDNA was removed from the cDNA preparation. This was achieved by hybridizing the partially purified cDNA (3.7.3) back to histone mRNA and then isolating, from the hybridization mixture, the double stranded cDNA:mRNA hybrids by hydroxylapatite chromatography. Of the cDNA eluted from the hydroxylapatite columns 60-64% eluted as single stranded material and 36-40% as double stranded material. The cDNA reisolated from the double stranded fraction is referred to as histone cDNA (histone mRNA specific cDNA).

When hydridized back to histone mRNA only 49% of histone cDNA became S_1 nuclease resistant (Table 3.6). This level of hybridization of the histone cDNA was surprising as the cDNA was isolated from a fraction of double stranded material. Factors contributing to this low percentage hybridization could have been the small average size of the histone cDNA (2.7S, 70 nucleotides) determined on alkaline sucrose gradients as described in legend to Fig. 3.11) as well as variations in the percentage hybridization evident by hydroxylapatite chromatography and S_1 nuclease assay (see Table 3.5). The cDNA was partially degraded during the hybridization, hydroxylapatite fractionation and cDNA reisolation steps (the starting cDNA had an average size of 5.2S). The very low yields (7-15 ng, 5-10% of input cDNA) of histone cDNA made the isolation of the larger histone cDNA fraction by alkaline sucrose gradients, impractical. Purified cDNA to β-globin mRNA (Old et al., 1976) has also been shown to exhibit low levels of hybridization (50-60%) when the cDNA was of small average size (P. Tolstoshev, personal communication).

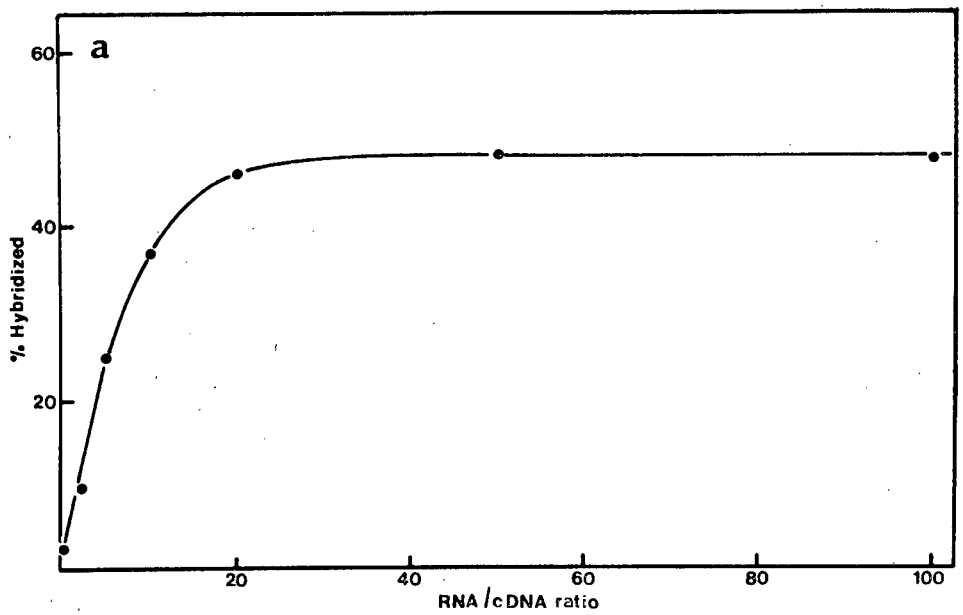
3.7.5 HYBRIDIZATION OF HISTONE cDNA

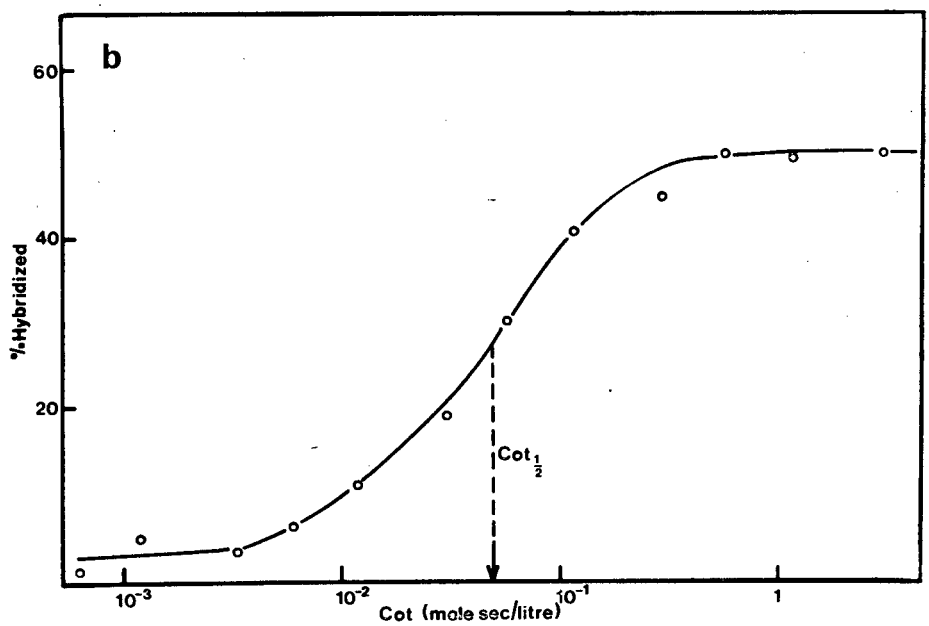
When the purified histone cDNA was allowed to hybridize with increasing amounts of histone mRNA (Fig. 3.18a) the reaction reached saturation (49%) at a RNA:cDNA ratio of approximately 12. If the assumption was made that only 15% of the histone mRNA preparation was authentic histone messenger (see section 3.6.2) the effective RNA:cDNA ratio could be taken as approximately 2 when cDNA saturation is reached.

FIG. 3.18

HYBRIDIZATION OF HISTONE cDNA : HISTONE mRNA

a) Titration of histone cDNA using 0.2 ng of cDNA and increasing amounts of histone mRNA. Reactions were carried out in volumes of 2 µl and were incubated for 14 days to take the reactions to a Cot of 0.4 mole sec/litre.
b) Kinetics of the hybridization reaction between histone cDNA and histone mRNA. 0.1 ng of histone cDNA was incubated with histone mRNA to increasing Cot values in 20 µl incubations.





The results of the hybridization of histone cDNA to the histone mRNA preparation at increasing values of Cot are shown in Fig. 3.18b. The reaction proceeded with an apparent $Cot_{\frac{1}{2}}$ of 0.0513 mole sec/litre. If it was assumed, from the results in section 3.6, that only 15% of the histone mRNA preparation was in fact histone mRNA the apparent $Cot_{\frac{1}{2}}$ of the reaction must be converted to a value of 0.0077 mole sec/litre. To calculate the nucleotide complexity of the histone cDNA preparation this $Cot_{\frac{1}{2}}$ was converted to correspond to a probe of 330 nucleotides in length in order to allow comparison with the globin mRNA:cDNA standard (see 3.5.1). Using this value (0.0035 mole sec/litre) the estimated nucleotide complexity of the cDNA was 1050 nucleotides which corresponds to about half the expected complexity of a cDNA complementary to histone mRNA (2000-2500 nucleotides). This could be explained if the amount of authentic histone mRNA in the RNA preparation used in hybridization experiments was higher than the assumed value of 15% (3.6.2). Preferential degradation of histone messenger RNA may have taken place during polyadenylation which would have decreased the percentage of histone mRNA in the polyadenylated RNA preparation used for reverse transcription (resulting in low yields of histone mRNA specific cDNA).

TABLE 3.6

PURIFICATION OF HISTONE mRNA SPECIFIC cDNA

Total and purified cDNA were hybridized to histone mRNA and rRNA in conditions of RNA excess (2000 x) and the extent of hybridization was determined by S_1 nuclease resistance.

Hybridization reaction	% cDNA hybridized	% of hybridizable cDNA	% of hybridizable cDNA specific for mRNA
cDNA : mRNA	72	100	15
cDNA : rRNA	61	85	
cDNA : no RNA	7	10	
Histone cDNA : mRNA	49	100	84
Histone cDNA : rRNA	8	16	
Histone cDNA : no RNA	2	4	

A second indication that the estimate of 15% was too low was the calculated RNA:cDNA ratio required for cDNA saturation (see above). It was unlikely that a probe of the size of the histone cDNA (70 nucleotides) would reach saturation at such a low RNA:cDNA ratio (Weiss et al., 1976). It has been shown that only 15% of the hybridizable cDNA preparation was complementary to non-ribosomal RNA or putative histone mRNA. To relate this value to the amount of authentic histone mRNA in the histone mRNA preparation a number of assumptions have been made, inter alia that the mRNA and rRNA were reverse transcripted at equal rates and that there was no preferential degradation of histone mRNA.

The histone cDNA showed only 8% hybridization to ribosomal RNA. This corresponds to only 16% of the total hybridizable histone cDNA. These results showed therefore that the fraction of cDNA which was specific for histone mRNA had been increased from 15% of the hybridizable cDNA to 84% in the purified histone cDNA preparation (Table 3.6) making the histone cDNA very much more suitable as a probe for the qualitative and quantitative analysis of histone mRNA in sea urchin eggs and embryos.

PART 4

SEA URCHIN MATERNAL HISTONE mRNA

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4.1 INTRODUCTION

As discussed earlier (1.1.4) the maternal mRNA stored in the unfertilized sea urchin egg plays an important role in the early embryonic development of the sea urchin. The mechanism, however, by which this maternal mRNA becomes activated after fertilization is unknown and probably involves a number of different control mechanisms for different proteins (see 1.1.4). The availability of a probe for histone mRNA (3.7) has allowed an investigation into the localization and utilization of a specific class of mRNA before and after fertilization.

4.2 RNA AND PROTEIN SYNTHESIS IN P. ANGULOSUS

Prior to an investigation into the maternal mRNA metabolism in P. angulosus it was necessary to characterize the RNA and protein synthetic activities in the unfertilized egg and early cleavage stages of embryonic development.

4.2.1 PROTEIN SYNTHESIS

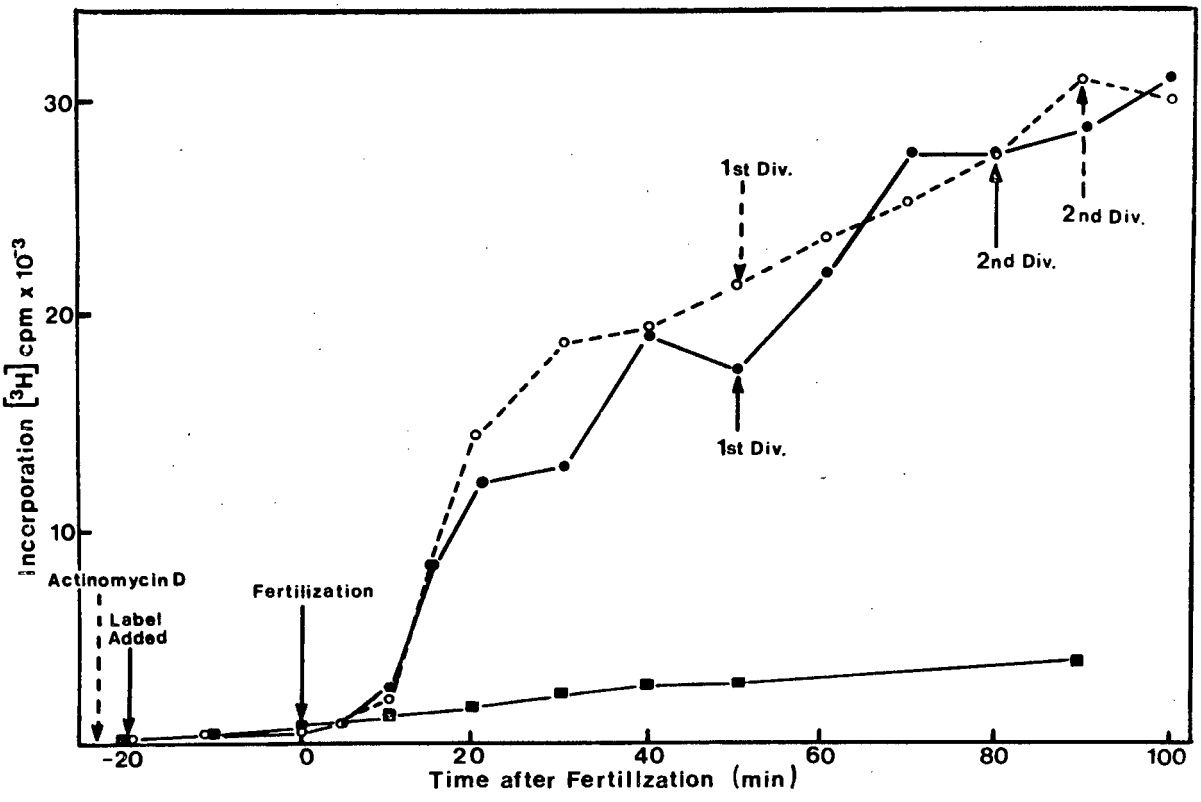
To determine the protein synthetic activity in unfertilized and fertilized eggs, eggs were preincubated with a [^{3}H]-labelled amino acid mixture (5 µCi/ml) for 20 minutes, fertilized and the protein synthetic activity at different times after fertilization determined (hot TCA precipitable radioactivity). The results are shown in Fig. 4.1 together with the protein synthetic activity in the presence of actinomycin D (actinomycin D (25 µg/ml) was added to the unfertilized eggs 10 minutes prior to addition of the label i.e. 30 minutes prior to fertilization). Low levels of protein synthesis are present in the unfertilized egg but upon fertilization, after a lag period of about 5 minutes, there is a rapid increase in the rate of protein synthesis.

The rates of protein synthesis are approximately the same in the presence and absence of actinomycin D. The presence of the actinomycin D however results in a slight delay in cleavage as has been observed by other workers (see Giudice, 1973). The concentration of actinomycin D used, 25 µg/ml, has been shown to inhibit at least 85% of RNA synthesis of the sea urchin embryo; the product of actinomycin D-resistant RNA synthesis being localized in the

FIG. 4.1

PROTEIN SYNTHESIS IN SEA URCHIN EGGS AND EMBRYOS

Eggs and embryos were incubated at a concentration of 10^5 eggs/ml in the presence of 5 µCi/ml of [^{3}H]-amino acid mixture. At the times indicated 100 µl aliquots (10^4 eggs) were removed and the hot TCA precipitable incorporation determined. Actinomycin D (25 µg/ml) was added 30 min before fertilization. The protein synthetic activity was determined in unfertilized eggs (■——■), fertilized eggs (●——●) and fertilized eggs in the presence of actinomycin D (o----o).



4S region (Gross and Cousineau, 1964; Gross et al., 1964; Guidice, 1973). If this RNA synthesis was terminal addition on tRNA then these experiments are indicative of the protein synthetic activity during the first and second cleavage stages being totally dependent on maternal mRNA templates.

4.2.2 RNA SYNTHESIS

The rate of RNA synthesis in P. angulosus eggs and embryos, measured in terms of total TCA precipitable [^{3}H]-uridine incorporation, is shown in Fig. 4.2. Eggs were fertilized and allowed to develop after preincubation in the presence of 10 µCi/ml of [^{3}H]-uridine and at the times indicated aliquots were removed and the TCA precipitable radioactivity was determined. The low levels of incorporation in the unfertilized egg may be nonspecific or may be attributed to the terminal addition on tRNA required for the low levels of protein synthesis in the unfertilized egg. An important factor to take into consideration when comparing the RNA synthetic activity in unfertilized and fertilized eggs is the increased permeability of the eggs to labelled nucleosides after fertilization (Piatigorsky and Whiteley, 1965). The TCA precipitable incorporation of [^{3}H]-uridine up to the fourth cleavage is low, thereafter there is a rapid increase in rate of RNA synthesis. It must be realized that a large percentage of this incorporation is due to terminal CCA addition on tRNA (Glisin and Glisin, 1964; and see below).

To determine if newly synthesized RNA was entering the cytoplasm during the first cleavage stage fertilized eggs were incubated in the presence of [^{3}H]-uridine (50 µCi/ml) for 40 min after fertilization and cytoplasmic RNA was isolated (phenol extraction 5.2.7) from a post mitochondrial supernatant prepared from the embryos as described in Materials and methods (5.3.11.1). The RNA was fractionated on sucrose gradient (5.2.1) and the radioactivity in gradient fractions was determined. The results (Fig. 4.3.a) show that radioactivity is found associated with the 4-5S RNA fraction but there is no evidence of any labelled RNA in the regions greater than 4S even at the high [^{3}H]-uridine concentration used. The incorporation into the 4S RNA can probably be explained by the terminal addition onto tRNA described by Glisin and Glisin (1964). When embryos were incubated to the 8 cell stage (120 min) however, in the presence of a lower

FIG. 4.2

RNA SYNTHESIS IN SEA URCHIN EGGS AND EMBRYOS

Sea urchin eggs and embryos were incubated at a concentration of 10^5/ml in the presence of [^{3}H]-uridine (10 µCi/ml). At the times indicated two 100 µl aliquots were removed and the TCA precipitable incorporation determined. (■----■) unfertilized eggs; (o——o) embryos.

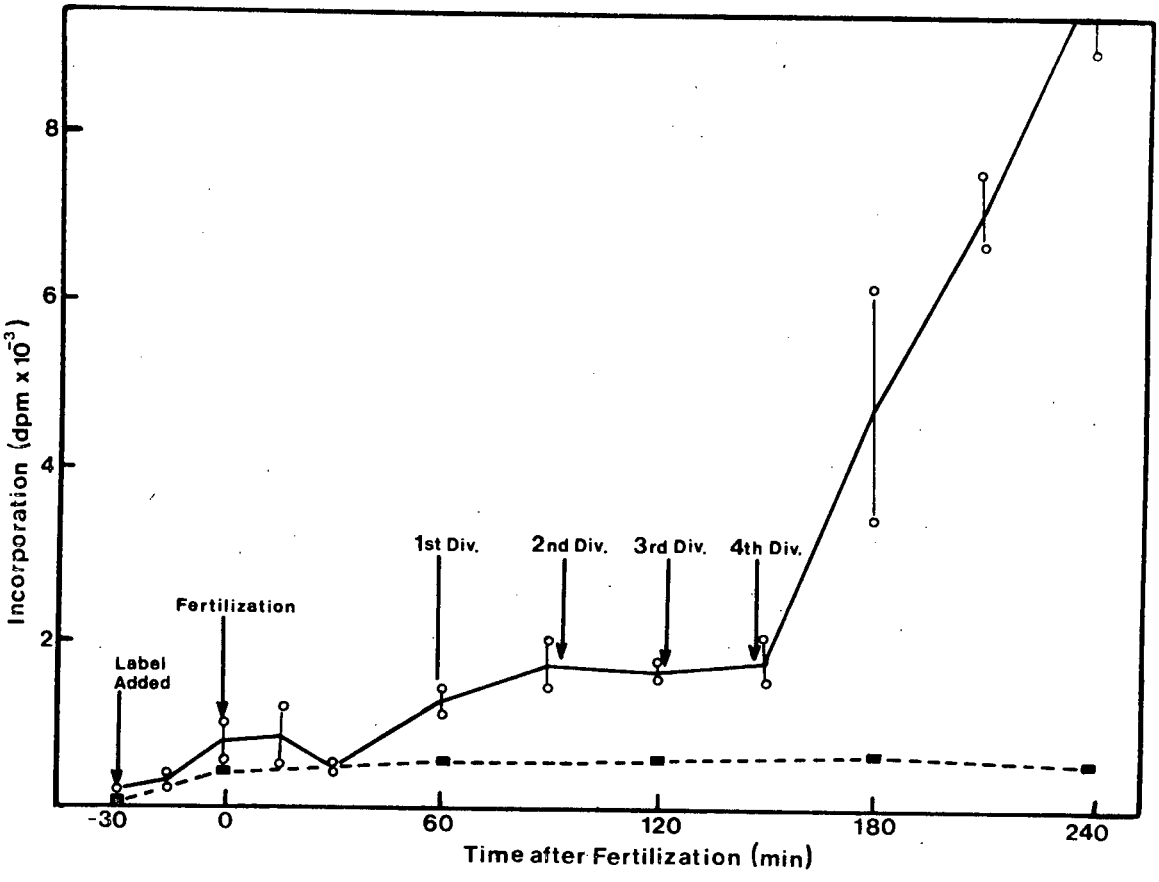
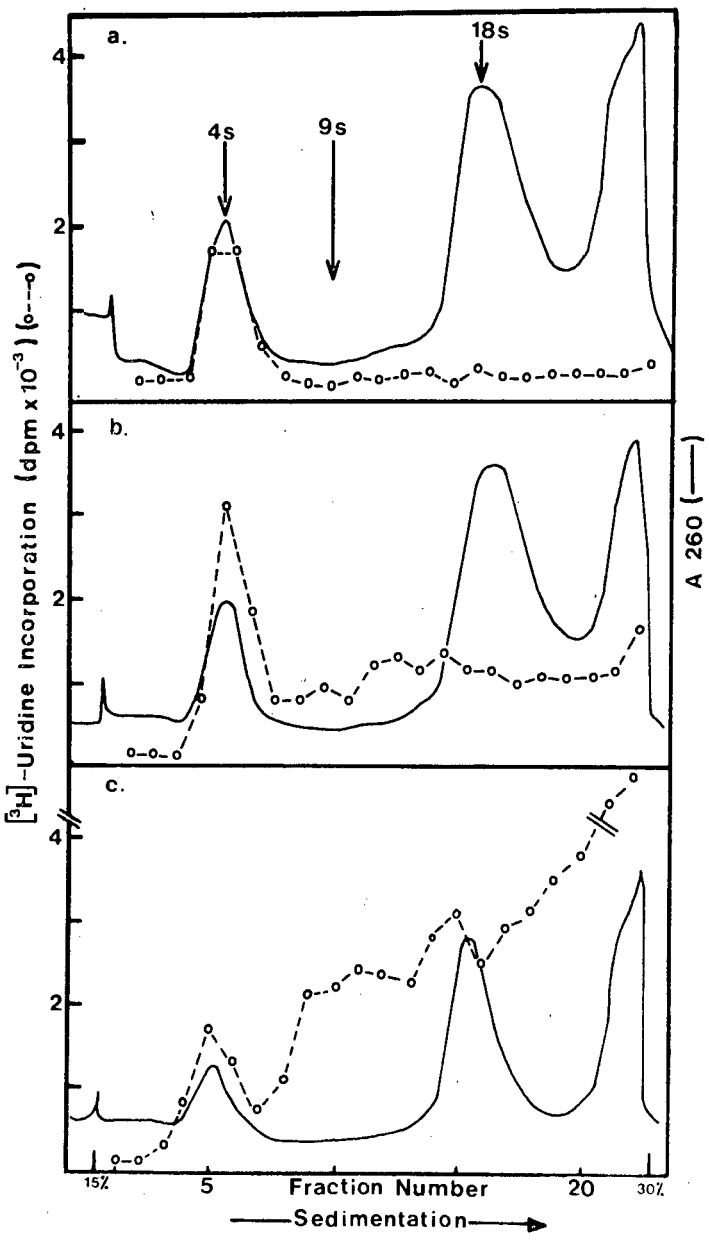


FIG. 4.3

RNA SYNTHESIS IN SEA URCHIN EMBRYOS

Sea urchin embryos (10^5/ml) were incubated in the presence of [^{3}H]-uridine at varying concentrations and the cytoplasmic RNA was isolated and analysed in sucrose gradients. Eggs were fertilized and incubated with uridine for varying times :

	Number of eggs	H-uridine	Length of incubation
a)	5×10^5	50 µCi/ml	40 min
b)	5×10^5	20 µCi/ml	120 min
c)	3×10^5	10 µCi/ml	240 min



concentration of [^{3}H]-uridine (20 µCi/ml), radioactively labelled RNA was detectable in the RNA sedimenting faster than 4S (Fig. 4.3b). Similarly, RNA isolated from early blastula embryos (240 minutes) which had been incubated with 10 µCi/ml of [^{3}H]-uridine contained labelled RNA which migrated faster than 4S (Fig. 4.3c). In this case the labelled RNA in the >4S region was far in excess of the labelled 4S RNA. Different concentrations of [^{3}H]-uridine were used in these three experiments for reasons of economy.

The fact that synthesis of high molecular weight RNA is detectable at the 8 cell and early blastula stages when embryos were incubated with lower concentrations of [^{3}H]-uridine than were used in the experiment with first cleavage stage embryos (see above) is convincing evidence that RNA synthesis during the first cleavage stage is negligible if present at all. The absence of detectable RNA synthesis coupled with the high levels of protein synthetic activity in the presence and absence of actinomycin D (4.2.1) indicate that in the first cleavage stage of sea urchin embryos all the protein synthetic activity is dependent on maternal mRNA. Ruderman and Gross (1974) have shown that both maternal and newly synthesized mRNAs have been shown to be involved in protein synthesis during the later cleavage stages.

4.3 CYTOPLASMIC LOCALIZATION OF HISTONE mRNA

To investigate the cytoplasmic localization of histone mRNA, RNA was isolated from fractionated post mitochondrial supernatants prepared from eggs and embryos (5.3.11.1) and titrated with the histone cDNA preparations (3.7) under hybridizing conditions.

4.3.1 PREPARATION AND FRACTIONATION OF CYTOPLASMIC CONSTITUENTS

The post mitochondrial supernatants from eggs and embryos were prepared and fractionated as described in Materials and methods (5.11.1). Centrifugation of post mitochondrial supernatants on 15-30% sucrose gradients was performed at 20 000 rpm (71 000 x g) using the SW 40Ti rotor (Fig. 4.4) and not at the rotor maximum of 40 000 rpm (284 000 x g). It was found that when post mitochondrial supernatants were centrifuged through the sucrose gradients at 40 000 rpm there was a total breakdown of ribosomes

to ribosomal subunits (Fig. 4.4). This phenomenon can be explained by pressure-induced dissociation of ribosomes as described by Baierlein and Infante (1974). Ribosomes moved into the gradient before the onset of the pressure-dissociation (Fig. 4.4).

For the analysis of the RNA in subcellular constituents post mitochondrial supernatants were fractionated into the following fractions (see Fig. 4.5) :

1. Post-RNP fraction (~0 - 20S)
2. RNP fraction (~20 - 70S) (Gross et al., 1973b)
3. Ribosomes ~80S
4. Polyribosomes >80S

(Pelleting of polyribosomes was prevented by inclusion into the sucrose gradients of a cushion of 50% (w/v) sucrose).

In the unfertilized egg only 15-17% of the ribosomes are shown to be in the polyribosomal fraction whereas in the early blastula approximately 40% of the ribosomes sediment in the polyribosomal fraction. Rinaldi and Monroy (1969) have shown similar distributions of ribosomes at different stages.

Analysis of the ribosome to polyribosome ratios during the first cleavage stage are shown in Table 4.1. There is an increase in the number of polyribosomes at 30 minutes after fertilization but the results indicate that there is a levelling off of the ribosome to polyribosome ratio during the latter half of the first cleavage. This may be associated with the metaphase arrest of protein synthesis during the first cleavage (Fry and Gross, 1970a).

TABLE 4.1

POLYRIBOSOMES AND RIBOSOMES DURING FIRST CLEAVAGE

Time after fertilization	% of ribosomes in polysomes
(unfertilized egg)	15-17%
30 min	25-27%
40 min	25-26%
55 min	24-25%
240 min (early blastula)	38-40%

FIG. 4.4

FRACTIONATION OF POST MITOCHONDRIAL SUPERNATANTS
ON 15-30% SUCROSE GRADIENTS (SW 40Ti ROTOR)

Sucrose gradients were prepared as described in Materials and methods (5.2.1) and ~ 20 A_{260} units of unfertilized egg post mitochondrial supernatant (5.3.11.1) were applied to each gradient. Gradients were centrifuged at the speeds and for the times indicated.

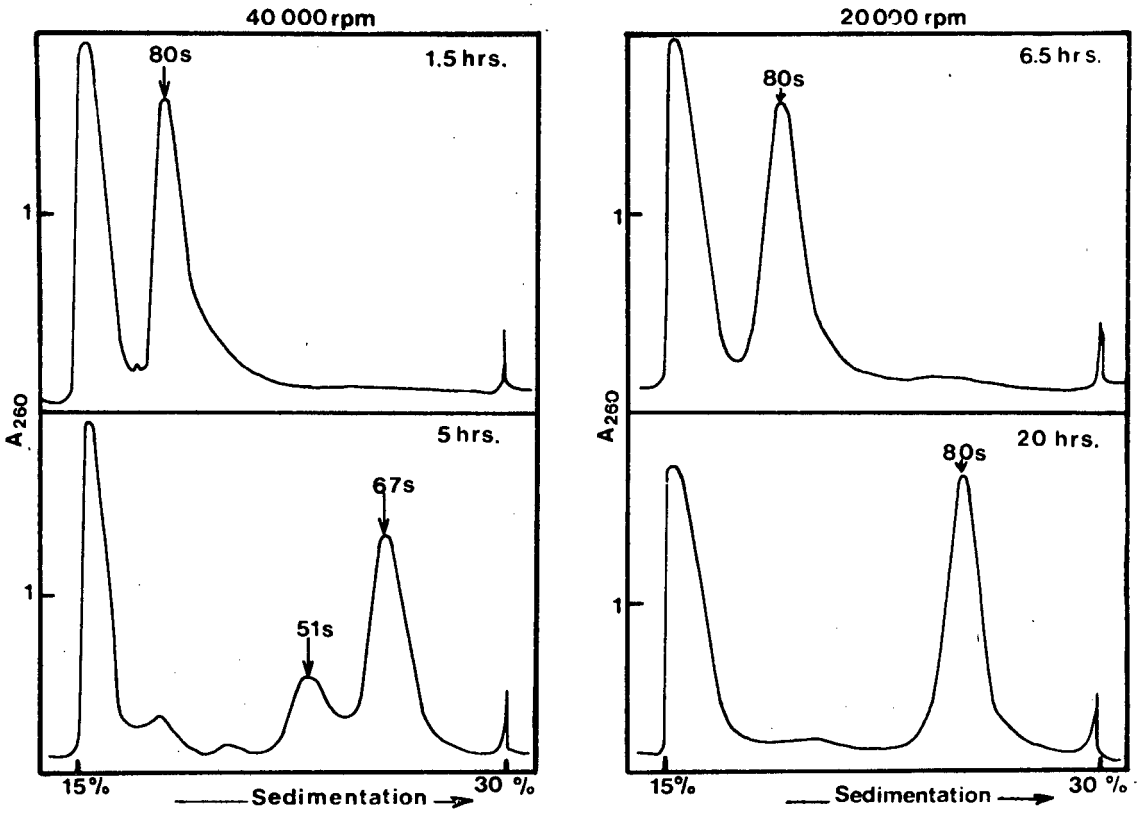
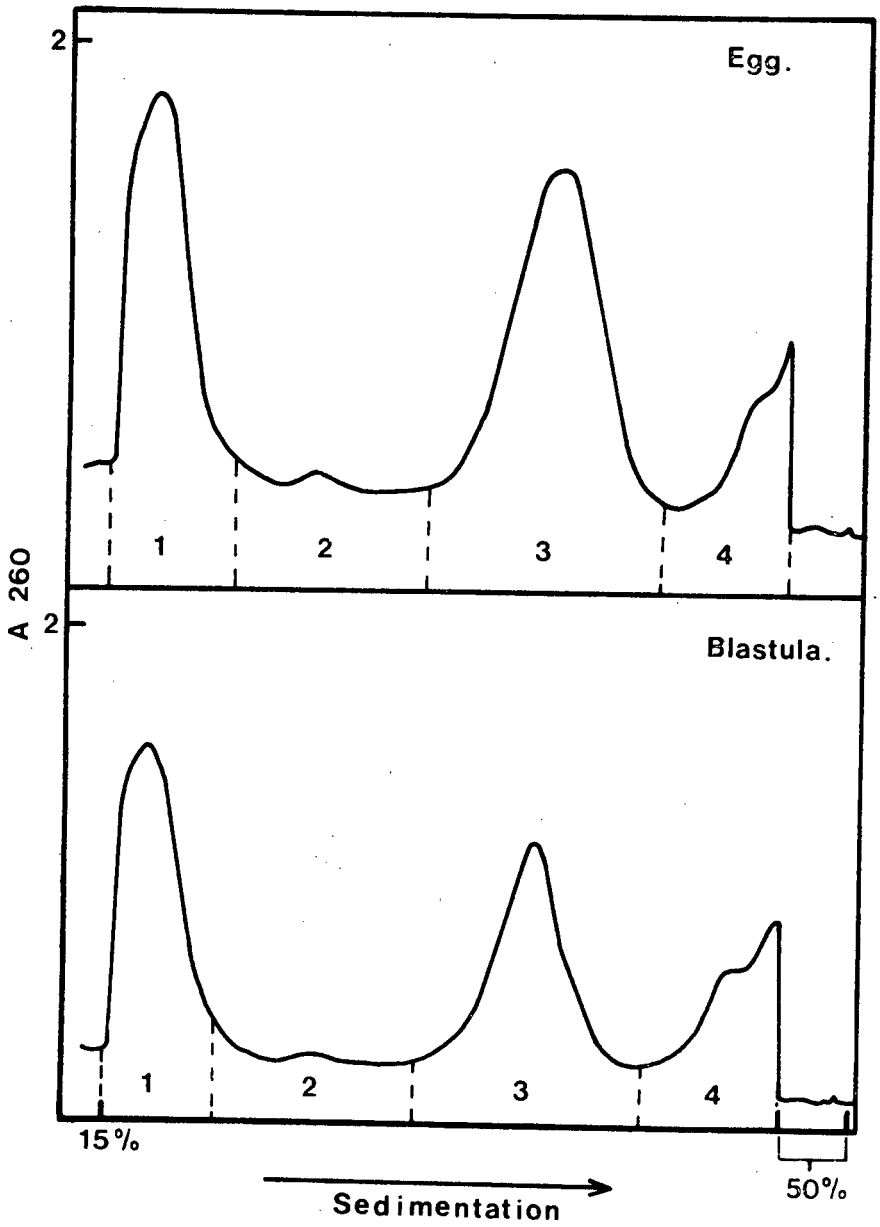


FIG. 4.5

SUCROSE GRADIENT FRACTIONATION OF POST MITOCHONDRIAL SUPERNATANTS

Post mitochondrial supernatants isolated as described in Materials and methods were fractionated on 15-30% sucrose gradients (SW 40Ti) containing a 1 ml cushion of 50% sucrose (sucrose solution in TKM buffer). An aliquot of post mitochondrial supernatant was applied to each gradient and centrifuged at 71 000 x g (20 000 rpm) for 20 h at 0-2°C. Gradients were fractionated and pooled into 4 fractions as shown on the gradient profile.



4.3.2 IDENTIFICATION OF HISTONE mRNA IN BLASTULA EMBRYOS

The histone mRNA preparation from which the histone cDNA was prepared, was isolated from polyribosomes of early blastula stage embryos (2.3). For this reason the efficiency of the histone cDNA probe for the detection of histone mRNA, was tested by hybridizing the histone cDNA to RNA isolated from sub-cellular fractions of early blastula stage embryos (it was known that the polyribosomes contain histone mRNA). The post mitochondrial supernatant of early blastula stage embryos was fractionated (see Fig. 4.5) and RNA was isolated from the fractions as described in Materials and methods (5.3.11.2). When histone cDNA was hybridized to the RNA of the four fractions under conditions of RNA excess (RNA:cDNA ratio 10 000) histone mRNA was shown to be present in the RNP and polyribosomal fractions and possibly in very small amounts in the ribosomal fraction. (When hybridized to ribosomal RNA at this RNA:cDNA ratio (10 000:1) approximately 20% of the cDNA became hybridized).

When the RNA of the RNP and polyribosomal fractions were titrated against histone cDNA (Fig. 4.6) the polyribosomal RNA saturated the histone cDNA at a RNA:cDNA ratio of approximately 150-200 whereas RNA:cDNA ratios of approximately 500-700 were required for the RNP RNA fraction (values of saturation were estimated from inflection points). The amount of authentic histone mRNA in the RNA used in the control titration (histone mRNA:cDNA) in Fig. 4.6 has been estimated as 15% (see 3.6.2) although the results in section 3.7.5 indicate that it may be higher. Using this value it can be calculated, by comparing the control histone mRNA/cDNA titration curve and the blastula RNA/cDNA titration curves, that approximately 1% of the polyribosomal RNA and 0.25% of the RNP RNA is histone mRNA. The values of the RNA:cDNA ratios at saturation estimated graphically were in close agreement with values determined mathematically using linear regression analyses (determined by the least squares method) of the initial points of the hybridization curve.

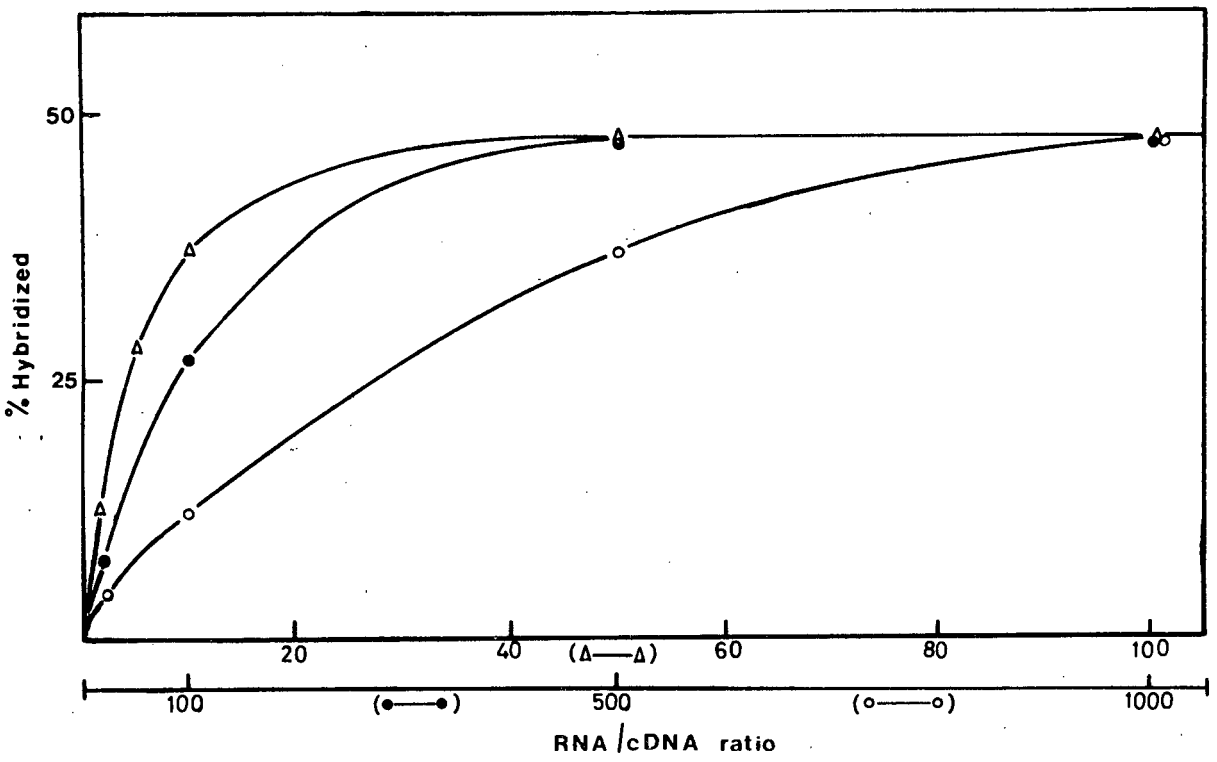
The presence of histone mRNA in the RNP fraction RNA at the blastula stage indicates that this may be a remnant of the maternal histone mRNA as Dworkin and Infante (1976) have shown that the newly synthesized histone mRNA was found exclusively in the polyribosomal fraction and not in the RNP particles.

The results of these experiments showed that the histone cDNA could be used effectively as a probe for the quantitative determination of histone mRNA. More accurate quantitation from the results of hybridization experiments however awaits a method for determining the percentage of the histone mRNA preparation which is authentic histone mRNA. The method at this stage allows excellent

FIG. 4.6

DETECTION OF HISTONE mRNA IN BLASTULA EMBRYOS

0.2 ng of histone cDNA was incubated with increasing amounts of histone mRNA (Δ——Δ), blastula polyribosomal RNA (●——●) (fraction 4 RNA) and blastula RNP RNA (o——o) (fraction 2 RNA). The RNA:cDNA ratios used were 0-100 for histone mRNA and 0-10 000 for blastula RNA (only 0-1 000 plotted).



comparison of the amounts of histone mRNA present in different RNA preparations.

4.3.3 LOCALIZATION OF MATERNAL HISTONE mRNA

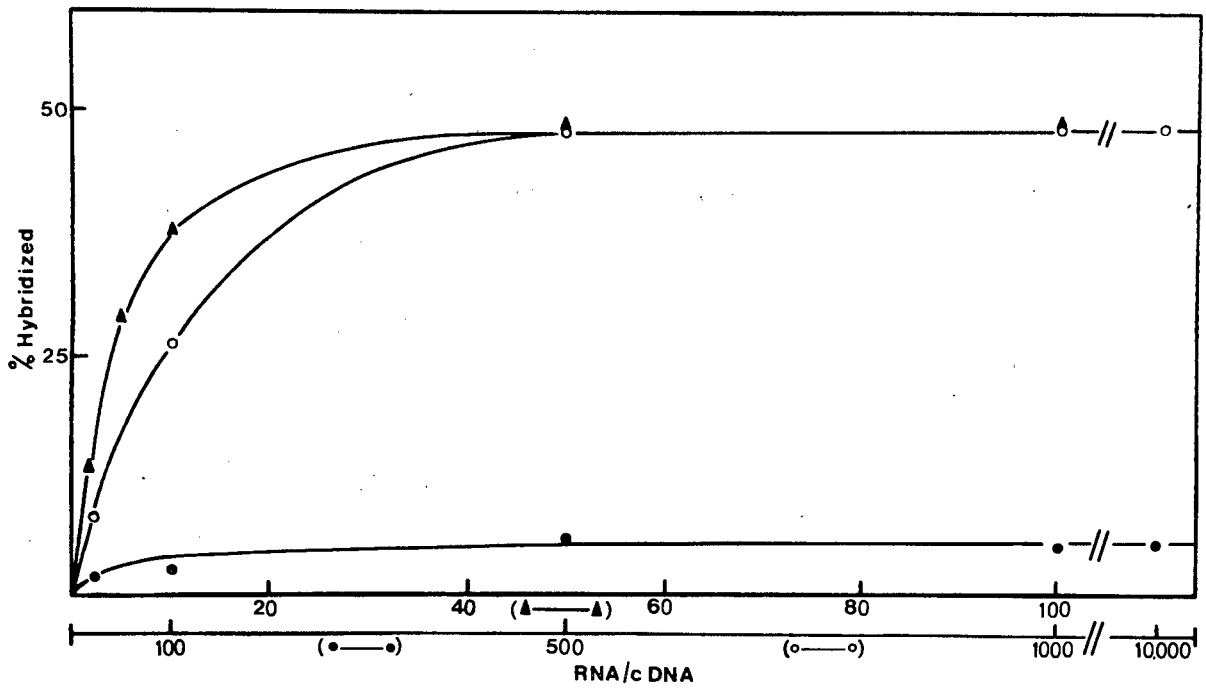
The results of Gross et al. (1973) and Skoultchi and Gross (1973) showed that more than 95% (probably all) of the maternal mRNA in the unfertilized egg was localized in the post mitochondrial supernatant. All the investigations described here were directed to determine the localization of maternal mRNA in post mitochondrial supernatant fractions, and the possible existence of maternal mRNA in other fractions has not been investigated.

A post mitochondrial supernatant was prepared from unfertilized eggs and fractionated on sucrose gradients as shown in Fig. 4.5 (5.3.11.1) followed by isolation of the RNA from the four fractions shown. The presence of histone mRNA was examined by titrating increasing amounts of RNP particle and polyribosomal RNA to histone cDNA. (The post-RNP and ribosomal fraction RNA were shown not to contain histone mRNA by the absence of hybrid formation at a RNA:cDNA ratio as high as 10 000:1). Titrations were carried out over the range of RNA:cDNA ratios 0-10 000. The results (Fig. 4.7) showed that in the unfertilized egg there is no histone mRNA associated with the polyribosomes as even at the RNA:cDNA ratio of 10 000:1 the extent of hybridization was at background levels (cDNA:rRNA $\longrightarrow$ ~8% hybridization). The RNP fraction RNA however hybridized to the histone cDNA saturating the cDNA at a RNA:cDNA ratio of approximately 200-220 (Fig. 4.7). The result shows therefore that all the cytoplasmic histone mRNA in the unfertilized egg is localized in the RNP fractions. By comparison of the cDNA:RNP RNA curve with the cDNA:mRNA curve it can be estimated that approximately 1% of the RNA in this fraction is histone mRNA. For calculations of the concentration of histone mRNA in RNA preparations it is required that the amount of authentic histone mRNA in the mRNA preparation is known to allow standardization of the mRNA:cDNA titration which is used as a reference. In the calculations used here the amount of authentic histone mRNA in the mRNA preparation has been taken to be 15% as determined earlier (3.7.5) and if this value is correct the above result of 1% is accurate to within $\pm$ 0.1%. It must be realized however, as indicated earlier, that the percentage of authentic histone mRNA may be higher than 15% which would result in a higher estimate of the amount of histone mRNA in the RNA preparation described above (in the order of 3% if 30% authentic histone mRNA present).

FIG. 4.7

LOCALIZATION OF MATERNAL HISTONE mRNA IN UNFERTILIZED EGG

0.2 ng of histone cDNA was hybridized to increasing amounts of RNA isolated from fractions 2 and 4 of the unfertilized egg post mitochondrial supernatant (see Fig. 4.5) and to histone mRNA. Histone cDNA was hybridized to histone mRNA over a range RNA:cDNA ratios 0-100 and to post mitochondrial supernatant RNA fractions over a range of RNA:cDNA ratios of 0-10 000. (▲——▲) histone mRNA:cDNA; (o——o) RNP RNA (fraction 2) : cDNA; (●——●) polyribosomal RNA (fraction 4) :cDNA.



The results of these experiments are in agreement with those of Gross et al. (1973) and Skoultchi and Gross (1973) who demonstrated the RNP localization of the histone mRNA by cell free translational and competition hybridization experiments. The absence of histone mRNA in the polysomal fraction has been demonstrated more conclusively however by the histone cDNA:RNA hybridization experiments as the methods used by the above authors would not detect the extremely low concentrations of histone mRNA in the RNA preparation detectable by the RNA:cDNA titration techniques (in the order of 10^{-5}%, Humphries et al., 1976). Davidson (1976) has estimated that approximately 4-8% of the maternal mRNA in the unfertilized egg is histone mRNA. The RNP fraction containing the maternal histone mRNA was shown in this laboratory to also contain ribosomal RNA (by gel electrophoresis) probably as a result of ribosomal subunits in the RNP fraction (it was not possible however to determine the percentage of this RNP RNA which was rRNA). This means that not all the RNA in the RNP fraction is maternal mRNA, thus the percentage of the maternal mRNA which is histone mRNA would be greater than 1% (approximately 1% of the total RNP fraction RNA is histone mRNA).

4.4 UTILIZATION OF MATERNAL HISTONE mRNA DURING FIRST CLEAVAGE

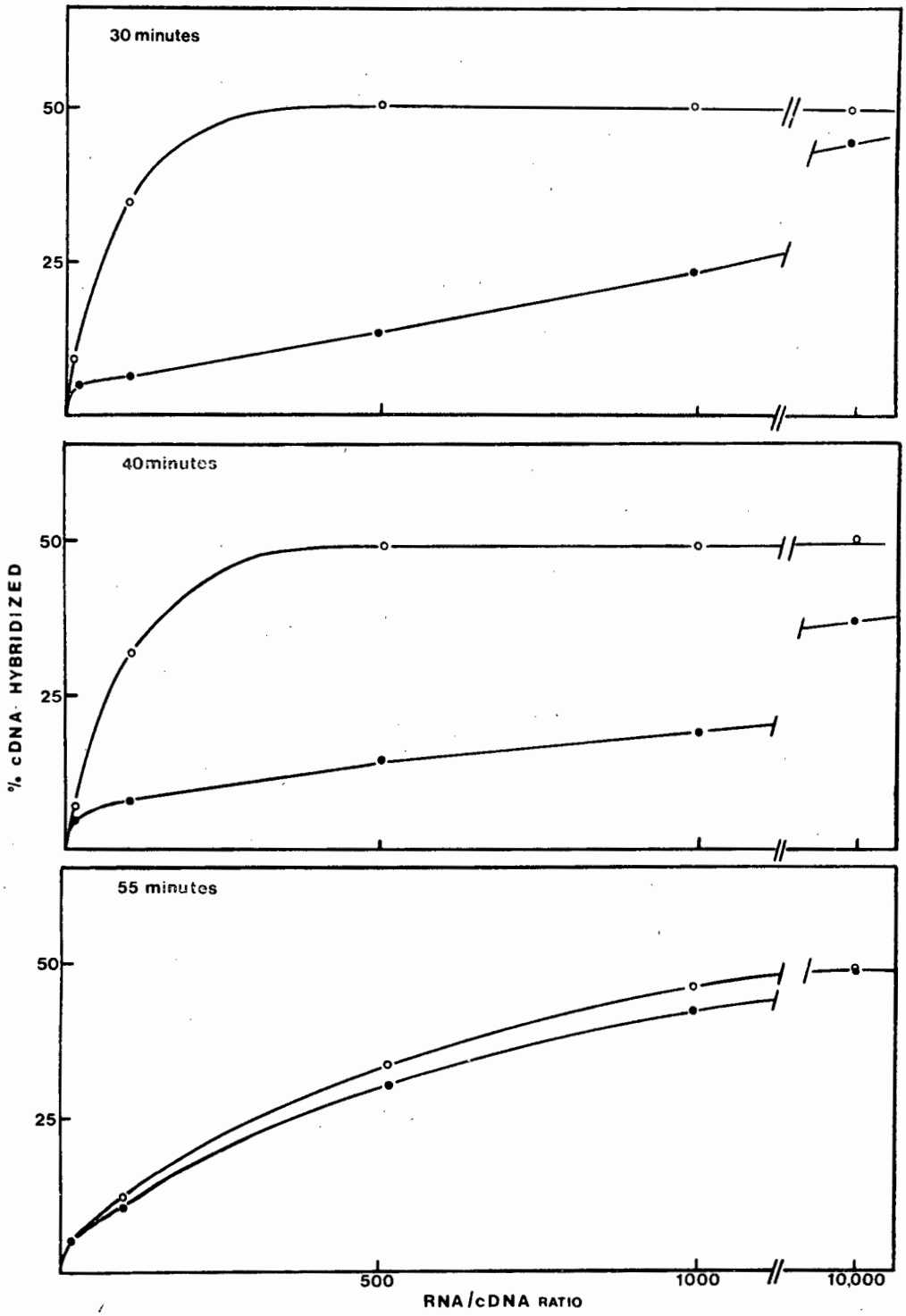
To determine if the maternal histone mRNA, stored in the RNP particles of the unfertilized egg, was utilized during the first cleavage stage and if so at what stage during the cell cycle, RNA fractions prepared at different times after fertilization were titrated against histone cDNA.

Batches of fertilized eggs were allowed to develop for 30 minutes, 40 minutes and 55 minutes. The embryos taken at 55 min were in the middle of the first cleavage, approximately 50% of the embryos being already divided to 2 cell stage. The embryos at this stage were therefore in telophase which is the period of active DNA synthesis (Hinegarder et al., 1964; Fansler and Loeb, 1969). Post mitochondrial supernatants were prepared and fractionated on sucrose gradients as described in Materials and methods (5.3.11.1) (see Fig. 4.5) and the RNA was isolated from the RNP and polyribosomal fraction and used in hybridization titration experiments with histone cDNA. The results of these experiments are shown in Fig. 4.8. As described above the histone mRNA in the cytoplasm of the unfertilized egg is located exclusively in the RNP fraction and even at RNA:cDNA

FIG. 4.8

THE UTILIZATION OF MATERNAL HISTONE mRNA

Histone cDNA (0.2 ng) was hybridized to increasing amounts of RNA isolated from the RNP and polysomal fractions of sea urchin embryos at different stages after fertilization. (o——o) histone cDNA hybridized to RNP fraction RNA; (●——●) histone cDNA hybridized to polyribosomal fraction RNA.



ratios of 10 000:1 no histone mRNA was detectable in the polyribosomal fraction (Fig. 4.7). 30 minutes after fertilization however, the picture was slightly different (Fig. 4.8). The amount of histone mRNA in the RNP fraction does not appear to have changed significantly but small amounts of histone mRNA were shown to be present in the polyribosomal fraction, constituting less than 0.02% of the RNA (Table 4.2) (cDNA was not saturated even at the RNA:cDNA ratio of 10 000:1). 40 minutes after fertilization the position was similar (Fig. 4.8) but even less histone mRNA was present in the polyribosomal fraction. 55 minutes after fertilization however, with the cells in telophase, the cytoplasmic distribution of the histone mRNA had changed drastically. A large fraction of the maternal histone mRNA had been transferred from the RNP fraction to the polyribosomes (Fig. 4.8). The results indicate that at this stage approximately 0.15% of the polyribosomal RNA and 0.20% of the RNP RNA was histone mRNA (see Table 4.2).

TABLE 4.2

SUBCELLULAR LOCALIZATION OF HISTONE mRNA

Time after fertilization minutes	Subcellular fraction			
	Ribonucleoprotein particles		Polyribosomes	
	RNA:cDNA ratio at saturation	% histone mRNA	RNA:cDNA ratio at saturation	% histone mRNA
0 (unfertilized)	200-220	1 ± 0.1	No hybridization	0
30	200-220	1 ± 0.1	> 10 000	< 0.02
40	200-220	1 ± 0.1	> 10 000	< 0.02
55	800-1200	0.2 ± 0.05	1000-1500	0.15 ± 0.05
240 (blastula)	500-700	0.35 ± 0.06	150-200	1.1 ± 0.2

(± values represent the range as calculated from the graphically determined RNA:cDNA ratios).

In conjunction with the experiments described above (4.2) these experiments demonstrate conclusively that maternal histone mRNA is utilized during the first cleavage stage of the sea urchin embryo. The existence of histone mRNA in the polyribosomes which is indicative of histone synthesis, together with the results of Ruderman and Gross (1974) who showed that histones are synthesized during first cleavage, argue against the presence of a stockpile of maternal histones (Cognetti et al., 1974). Benttinen and Comb (1971) have also shown that detectable quantities of histone cannot be isolated from the cytoplasm of unfertilized egg.

The fact that the amount of histone mRNA in the polyribosomes is markedly increased when the cells are in telophase (Fig. 4.8) (55 minutes after fertilization) which has been shown to be associated with DNA synthesis (Nemer, 1962; Hinegardner et al., 1964; Fansler and Loeb, 1969) supports the findings of Kedes et al. (1969b) and Ruderman and Gross (1974) that histone synthesis is tightly coupled to DNA synthesis. The small amounts of histone mRNA present in the polyribosomal fraction 30 and 40 minutes after fertilization are possibly responsible for histone synthesis which would be coupled with the small amount of DNA synthesis shown to take place in the fertilized egg prior to fusion of the pronuclei (Hinegardner et al., 1964; Fansler and Loeb, 1969).

These results are in agreement with the hypothesis that histone and DNA synthesis are coupled and show conclusively that in the first cleavage stage the initiation of histone synthesis involves a translational control mechanism requiring the activation and transport of the maternal histone mRNA to the polyribosomes at a specific stage during the cell cycle. This is the first direct demonstration of the mobilization of a specific maternal mRNA fraction after fertilization in the sea urchin embryo.

PART 5

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5.1 MATERIALS

5.1.1 GENERAL MATERIALS

AMV reverse transcriptase was generously supplied by Dr. J.W. Beard (Life Science Inc., Florida) and Dr. G. Birnie (Beatson Institute of Cancer Research, Glasgow). Chicken 9S globin mRNA and rRNA were isolated in this laboratory by Dr. C. Boyd. Mouse ascites tumour cells were obtained from Mrs. V. McConnell of the Department of Biophysics, Groote Schuur Hospital, Cape Town.

All radioactive materials were obtained from the Radiochemical Centre, Amersham. Unlabelled ATP and GTP, deoxyribonucleoside triphosphates, Micrococcus lysodeikticus DNA, S_1 nuclease (A. oryzae), creatine phosphate and dithiothreitol were obtained from Miles Laboratories Inc. Penicillin G, streptomycin sulphate, HEPES*, bentonite, Dowex chelating resin, DEAE cellulose and puromycin were obtained from Sigma Chemical Co.; N,N,N',N'-tetramethyl-ethylenediamine and polyvinlysulphate from Eastman Kodak Co.; unlabelled amino acids from Nutritional Biochemicals Co.; oligo$(dT)_{12-18}$ and oligo(dT) cellulose from Collaborative Research; actinomycin D from P.L. Biochemicals; rat liver RNase inhibitor from Searle Biochemicals; creatine kinase from Boehringer Mannheim (Germany); PPO scintillator and BBS-3 solubilizer from Beckman Instruments; hydroxylapatite (Biogel HTP) from Biorad Laboratories and diethylpyrocarbonate, formamide and dichlorodimethylsilane from Merck (Germany). All chemicals not further described were Analar (or equivalent) grade.

Sea urchins, Parechinus angulosus, were collected from intertidal pools 30 Km north of Cape Town (Atlantic Ocean).

All solutions were prepared using glass, double distilled water.

Where RNase free conditions were necessary all glassware and solutions (except sucrose solutions (5.1.2.2) were sterilized by autoclaving or treatment with diethylpyrocarbonate (~0.05%)).

*HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid

5.1.2 TREATED MATERIALS

5.1.2.1 BENTONITE

Bentonite was prepared essentially as described by Fraenkel-Conrat (1966). 10 g of bentonite (Sigma) were suspended in distilled water with continual stirring for 24 h, centrifuged at 450 x g for 5 min (2 000 rpm, SS 34 rotor) and the supernatant decanted. The supernatant was recentrifuged at 12 000 x g for 20 min (10 000 rpm, SS 34 rotor) and the supernatant discarded. The pellet was resuspended in TNM buffer (0.2 M NaCl, 5 mM Mg acetate, 10 mM Tris-HCl pH 7.5) and resedimented at 12 000 x g. The pellet was suspended in TNM buffer to give a final bentonite concentration of 50 mg/ml. The suspension was autoclaved and stored at $4^{o}C$.

5.1.2.2 RNase FREE SUCROSE SOLUTIONS

Sucrose solutions were made up using RNase free sucrose (Miles Laboratories), by dissolving the sucrose to the required concentration in the necessary buffer. The solution was rendered RNase free by addition of diethylpyrocarbonate to a final concentration of 0.05% (v/v) (for details of diethylpyrocarbonate as a RNase inhibitor see Ehrenberg et al., 1976). The diethylpyrocarbonate was removed from the solution by boiling for 20-30 min and after cooling the solution was made up to the original volume by addition of sterile distilled water (autoclaved). The sterile sucrose solutions were used on the day of preparation only.

5.1.2.3 SILICONIZED GLASSWARE

Tubes were scrubbed with detergent (Vim), rinsed thoroughly with water and dried. After immersion in a solution of 1% dichlorodimethylsilane in carbontetrachloride tubes were baked in an oven at $150^{o}C$ for approximately 3 h. The tubes were then resiliconized as above, rinsed with distilled water, boiled in distilled water containing 0.1% diethylpyrocarbonate and then dried in an oven.

Capillary tubes were siliconized as above except that they were only washed thoroughly with distilled water prior to treatment.

5.1.2.4 FORMAMIDE

Before being used in hybridization experiments formamide was deionized by treatment with Bio-Rad mixed bed resin AG 501-X8D (20-50 mesh) as follows (P. Tolstoshev, personal communication).

Approximately 1 g of resin was added to 50 ml of formamide and the mixture was shaken at 4°C for 2 h and then filtered to remove the resin. The deionized formamide (conductivity less than 10 μmho) was stored at -20°C in the dark for up to three months.

5.1.2.5 DOWEX CHELATING RESIN

Dowex chelating resin (Sigma), dry mesh 50-100, was prepared for use as described by P. Tolstoshev and R. Williamson (personal communication).

The resin (30-40 g) was suspended in distilled water and the pH was measured (pH 10 or higher). The mixture was neutralized to pH ~7 by addition of concentrated HCl and then allowed to stand with occasional stirring (the pH rises). The water was decanted and the resin resuspended in fresh distilled water and neutralized to pH ~7. This procedure was repeated until the pH was stable at approximately 7; this can take a couple of days as the pH rises slowly.

Once the resin was stabilized at pH 7 it was washed thoroughly (in a column) with distilled water to remove all traces of the acid. The resin can be stored in distilled water at 4°C for 3-4 months.

5.1.2.6 BUFFERS

NETS buffer	-	0.1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 7.4 and 0.2% SDS.
NTS buffer	-	0.5 M NaCl, 10 mM Tris-HCl pH 7.4 and 0.5% SDS.
TS buffer	-	10 mM Tris-HCl pH 7.4 and 0.5% SDS
TKM buffer	-	50 mM Tris-HCl pH 7.8, 0.24 M KCl and 5 mM $MgCl_2$
TNM buffer	-	10 mM Tris-HCl pH 7.5, 0.2 M NaCl and 5 mM Mg acetate
Hybridization buffer	-	0.5 M NaCl, 25 mM HEPES, 10 mM EDTA pH 6.8, 50% (v/v) formamide
Nuclease assay buffer	-	70 mM sodium acetate, 2.8 mM $ZnSO_4$, 140 mM NaCl pH 4.5

5.2 GENERAL METHODS

5.2.1 SUCROSE DENSITY GRADIENT CENTRIFUGATION

All sucrose gradient centrifugation was carried out through 15-30% sucrose gradients either in the Beckman SW 65Ti rotor or SW 40Ti rotor. Centrifugation was performed in either a Beckman L2-75B ultracentrifuge or a Beckman L5-65 ultracentrifuge.

Rotor	Max rpm	Gravitational force at r max	Capacity/tube	Length of tube
SW 65Ti	65 000	420 000 x g	5 ml	5.1 cm
SW 40Ti	40 000	284 100 x g	14 ml	9.52 cm

(All g values reported in this thesis represent g max).

Preparation and analysis of sucrose gradients

Sucrose gradients were prepared using a Beckman Density Gradient Former with sterile sucrose solutions (5.1.2) and when gradients were centrifuged at 20°C the gradient buffers contained 0.2% SDS. Sucrose gradients were analysed (and fractionated if required) using an Isco Density Gradient Fractionator (Model 640) with the $A_{254\ nm}$ continuously monitored using an Isco Absorbance monitor (Model UA5).

Preparation of standard RNA sedimentation markers

Ribosomal RNA isolated from rat liver by the method of Kirby (1971) was analyzed using the Beckman Model E analytical ultracentrifuge and sedimentation coefficients of the two major RNA peaks were determined as 27.53 and 16.27 S (this analysis was carried out by Dr. A. Polson, Department of Microbiology, U.C.T.). These were used as large molecular weight markers. Yeast tRNA isolated by the method of Holley et al. (1961) was used as a 4S marker. For sucrose gradient analysis the markers were mixed to give a tRNA:rRNA ratio ($A_{260\ nm}$) of 1:3.

FIG. 5.1

CALIBRATION OF 15-30% SUCROSE GRADIENTS IN SW 65Ti ROTOR

Marker RNAs were applied to the sucrose gradients in a final volume of 100 µl and centrifuged at 65 000 rpm for different times. Gradients were analysed by upward displacement with 50% (w/v) sucrose, at a flow rate of 1 ml/min, through an optical flow cell. The optical density was monitored at 254 nm and recorded at a chart speed of 150 cm/hour. The position of the peaks on the scan (measured in cm from start of gradient) was plotted against sedimentation coefficient x time (hours) of centrifugation (S x t). Circles represent the relative migration of 4S tRNA, triangles 16.35 rRNA and squares 27.5S rRNA. (——) calibration at 20°C; (----) calibration at 0°C.

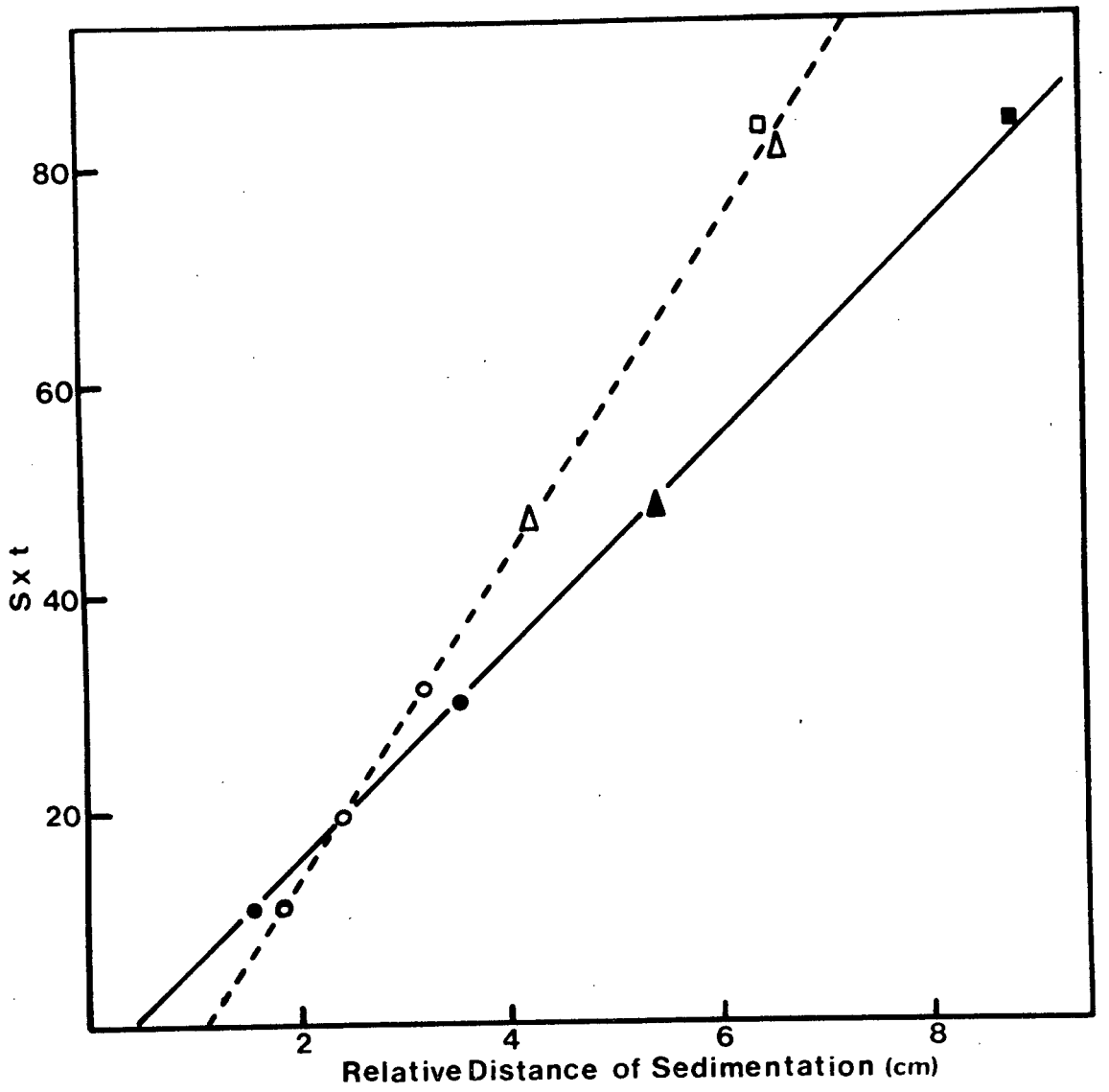
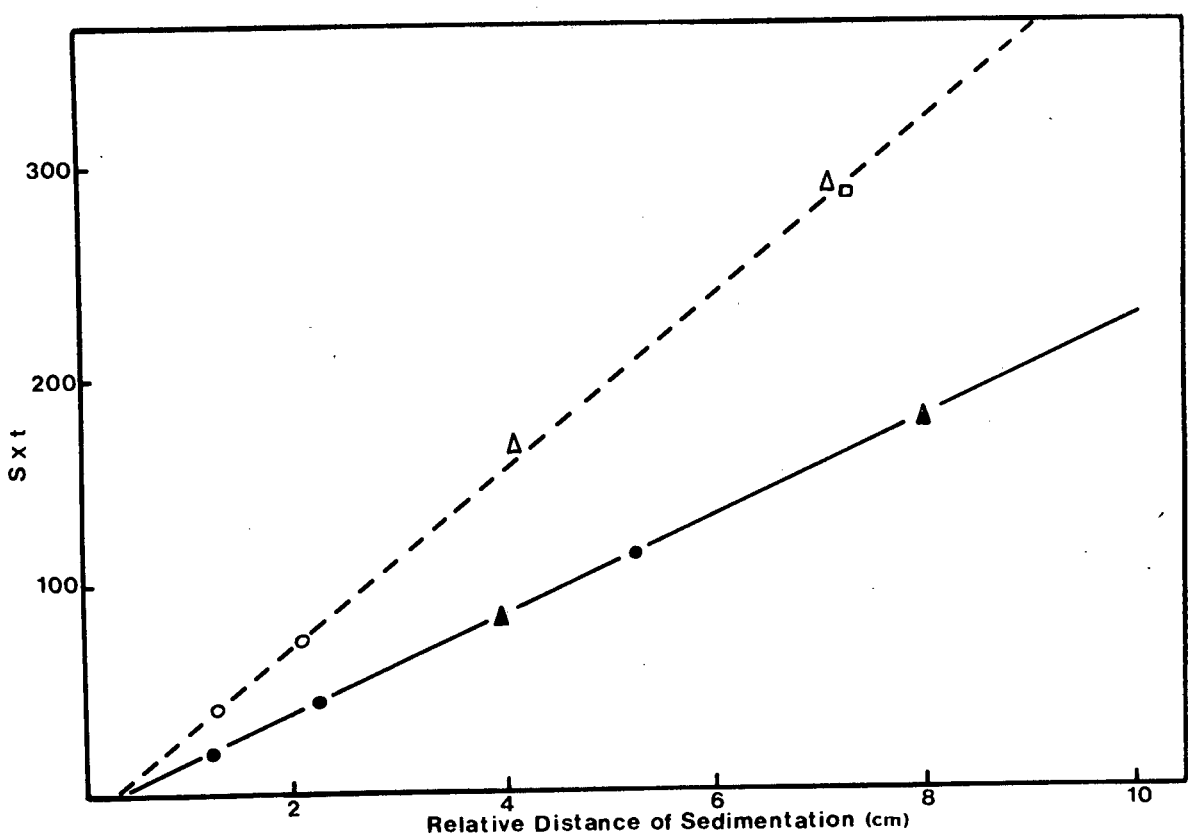


FIG. 5.2

CALIBRATION OF 15-30% SUCROSE GRADIENTS IN SW 40Ti ROTOR

Marker RNAs were applied to the sucrose gradients in a final volume of 500 µl and centrifuged at 40 000 rpm for different times. Gradients were analysed by upward displacement with 50% (w/v) sucrose at a flow rate of 1 ml/min through an optical flow cell. The optical density was monitored at 254 nm and recorded at a chart speed of 60 cm/h. The calibration curves were plotted as described in the legend to Fig. 5.1.



Centrifugation

Centrifugation was carried out at maximum rotor speed. Time of centrifugation was taken as the period of operation of the rotor at plateau speed including initial acceleration period of the rotor.

Calibration curves

Calibration curves were prepared (Fig. 5.1 and Fig. 5.2) by centrifuging the marker RNAs for different times and then measuring the distance of migration of the different peaks (recorded as mm of chart recorder paper). The distance of migration was plotted against the sedimentation coefficient x time (hours) of centrifugation (S x t).

5.2.2 POLYACRYLAMIDE GEL ELECTROPHORESIS

5.2.2.1 GEL ELECTROPHORESIS OF RNA ON 2.6% GELS

Electrophoresis of RNA was carried out essentially as described by Loening (1967) on 2.6% polyacrylamide gels.

The following solutions were made up :

A. 15% (w/v) acrylamide, 0.75% (w/v) N,N-methylenebisacrylamide
B. EP buffer (4.36 g Tris-HCl pH 7.8, 4.68 g NaH_2PO_4, 0.37 g EDTA/litre)
C. 10% ammonium persulphate

Gels were prepared by mixing 6 ml of A, 28.5 ml of B, 0.3 ml of C, and 30 µl of N,N,N',N'-tetramethylethylenediamine (TEMED). The mixture was poured into 10 x 0.6 cm perspex tubes (sealed at one end with parafilm) and about 200 µl of distilled water was carefully layered onto the surface of the acrylamide. Gels were allowed to polymerize for 30 min and, after puncturing the parafilm at the bottom of the tubes, were pre-electrophoresed at 5 mA/gel for 1 hour (Shandon disc electrophoresis apparatus) using EP buffer containing 0.2% SDS as tray buffer.

RNA samples were dissolved in EP buffer 0.2% SDS, heated to 60°C for 10 min, chilled rapidly, and applied to the surface of the gel. The amount of RNA

applied to each gel varied from 1-2 $A_{260\ nm}$ units for total polyribosomal RNA to as little as 0.1 $A_{260\ nm}$ units for a single species RNA such as 9S histone mRNA. Samples were run into gels at 15 volts for 15 min and then electrophoresed at 5 mA/gel for 2 h (room temperature).

Gels were analysed immediately after electrophoresis by scanning at 260 nm using a Varian Techtron UV-VIS spectrophotometer (Model 635) with gel scanning attachment. The S values assigned to different peaks on gel scans are based on the migration of 4S (yeast tRNA), 9S (histone mRNA) and 26S and 18S (sea urchin rRNA).

Analysis of radioactivity in 2-6% gels

2.6% gels were too fragile to be sliced with a gel slicer and were therefore sliced manually. The gel was placed on a flat surface and a piece of absorbent paper towel was lowered onto the gel. Due to the "sticky" nature of the gel it became stuck to the paper. The paper, with gel attached, was then cut into the required slices with a pair of scissors. The slices were dried, oxidized (Packard Tri-Carb Sample Oxidizer) and the radioactivity determined.

5.2.2.2 GRADIENT MICROGEL ELECTROPHORESIS OF RNA

9S histone mRNA was analysed on gradient polyacrylamide microgels using the method of Neuhoff (1973).

The following solutions were prepared :

A. 0.86 g Tris
8 ml H_2O
0.063 ml TEMED
3.6 N H_2SO_4, to pH 8.8
H_2O to 10 ml

B. 20 g acrylamide
0.4 g bis acrylamide
3.75 mg $K_3Fe(CN)_6$
H_2O to 37.5 ml

immediately examined using a Zeiss stereomicroscope and photographed. The RNA bands diffused out of the gels fairly rapidly (1-2 hours) so it was necessary to photograph them soon after destaining.

5.2.2.3 GEL ELECTROPHORESIS OF HISTONES

Polyacrylamide gel electrophoresis of histones was performed according to the method of Panyim and Chalkley (1969), in 15% acrylamide gels containing 2.5 M urea and 0.9 M acetic acid.

The following solutions were made up :

A. 60% (w/v) acrylamide, 0.4% (w/v) N,N'-methylenebisacrylamide
B. 4% (v/v) N,N,N',N'-tetramethylethylenediamine (TEMED) in 43.2% (v/v) glacial acetic acid
C. 0.2% (w/v) ammonium persulphate in 4 M urea
D. 0.9 M glacial acetic acid (tray buffer).

All solutions were stored at 4°C except solution C which was prepared immediately before use.

Gels were prepared by mixing solutions A, B and C in the ratio 2:1:5. The mixture was degassed under vacuum for 1 min and poured quickly (to about 9 cm) into 10 x 0.5 cm glass tubes, which were sealed at the lower end with parafilm. About 200 µl of distilled water was carefully layered onto the polyacrylamide. Polymerization was allowed to occur for 1-2 hours at room temperature. Gels were pre-electrophoresed in a Shandon disc electrophoresis apparatus for 3½ hours at 2 mA per tube using solution D as tray buffer.

Histone samples, dissolved in 8 M urea - 0.5 M mercaptoethanol, were allowed to stand at room temperature for 1 h (minimum) or were heated to 80°C for 2 min before being applied to the gel. Samples containing 20-40 µg of histones were applied to each gel and electrophoresis was carried out for 3½ h at 2 mA/gel. Gels were removed from the tubes and stained with 0.1% (w/v) amido black, 7% (v/v) acetic acid for ½ h and then destained in a Shandon transverse disc destainer using 25% ethanol, 7% acetic acid. Gels were scanned using a Vitatron TLD 100 densitometer (615 nm filter).

Analysis of radioactivity in 15% gels

Gels were cut into 1 mm slices using a gel slicer. The slices were then dried, oxidized (Packard Tri-Carb Sample Oxidizer 306) and the radioactivity determined.

5.2.3 DETERMINATION OF RADIOACTIVITY

Radioactivity was determined using either a Beckman model LS-250 or a Packard Tri-Carb model 3385 liquid scintillation counter. Samples were counted in standard scintillation vials (glass or plastic) using toluene based scintillators. Radioactivity determinations of dried samples, such as dried Millipore filters, were carried out in 10 ml of scintillator containing 0.5% PPO (2,5-diphenyloxazole) in toluene. Aqueous samples were solubilized by the inclusion of 10% BBS-3 solubilizer (Beckman Instruments) into the above scintillation mixture (10% BBS-3 solubilized up to 1 ml of water/10 ml of scintillator). Solid samples were oxidized using a Packard Tri-Carb Sample Oxidizer (model 306) and the radioactivity was determined in 10 ml of Monophase 40 scintillator (Packard).

Counts per minute (cpm) were converted to disintegrations per minute (dpm) using a suitable quench correction curve prepared using Packard and Beckman standard quenched samples. The quench correction curves were stored in a bench top computer (Wang 700) program (program written by E. Lee).

5.2.4 OLIGO(dT) CELLULOSE CHROMATOGRAPHY

Oligo(dT) cellulose chromatography was carried out essentially as described by Aviv and Leder (1972).

Oligo(dT) cellulose (Collaborative Research) (0.25 g) was suspended in distilled water containing 0.1% diethylpyrocarbonate and poured into a 5 ml syringe containing a pad of glass wool. The cellulose was allowed to settle and was washed with 5 ml of 0.1 M NaOH and then with 50 ml of NTS buffer (0.5 M NaCl, 10 mM Tris-HCl pH 7.4, 0.5% SDS). The RNA sample was dissolved in NTS buffer and applied to the column and the unbound, non-poly(A) containing RNA was eluted with 15-20 ml of NTS buffer. The column was then washed with 5-7 ml of TS buffer (10 mM Tris-HCl pH 7.4, 0.5% SDS) to elute the poly(A) containing RNA.

C. 35 mg ammonium persulphate to 50 ml in 2% (v/v) Triton X-100 in H_2O

Bath buffer.

3.0 g Tris, 14.4 g glycine to 500 ml in H_2O (pH 8.4)

D. 1. 60% (w/v) sucrose
2. 24 mg ammonium persulphate to 10 ml in 2% (v/v) Triton X-100 in H_2O
3. 0.86 g Tris
8 ml H_2O
3.6 N H_2SO_4, to pH 8.8
H_2O to 10 ml

To prepare solution D, mix 7 parts (1) to 2 parts (2) to 1 part (3).

All stock solutions were stored for prolonged periods at 4°C except for solution (C) which was prepared fresh each week.

Sulphuric acid-washed 5 µl capillary pipettes (Drummond Scientific Co.) were used for gradient microgel electrophoresis. Before filling, the middle of the capillary was marked with a felt-tip pen. The capillary was then dipped into solution C, and filled up to this mark by capillary attraction. Immediately afterwards it was held in a stock solution mixture A/B (one part A to 3 parts B) and filled to the tip of the capillary by capillary attraction. The capillaries were then placed vertically in a small beaker containing 2-4 mm of solution D. The gels were allowed to polymerize overnight at room temperature in a moist chamber. The gradient gels were removed from the solution D, un-polymerized solution was removed from the top of the gel using a finely drawn out glass capillary and replaced by the RNA sample (1 mg/ml; about 4-8 mm of capillary available for sample (0.625 - 1.25 µl)).

Electrophoresis was performed for 60 min starting at 90 µA. Sample migration was towards the anode.

The gels were extruded from the capillary tube with air pressure and stained in 0.2% (w/v) toluidene blue, 10% (v/v) glacial acetic acid for 15 min. They were then destained in 7.5% (v/v) glacial acetic acid for 5-10 min and

5.2.5 DESALTING ON SEPHADEX G-25 COLUMNS BY CENTRIFUGATION

Sephadex G-25 (medium) was allowed to swell in distilled water containing 0.1% diethylpyrocarbonate (minimum 3 hours at room temperature) and then stored at 4°C.

A 5.0 ml plastic syringe (Galaxy RSA) was filled with the Sephadex G-25 and placed into a centrifuge tube of suitable size so that the syringe was supported only by its upper rim and did not touch the bottom of the centrifuge tube. It was centrifuged at approximately 200 x g for 2-3 minutes to remove excess water from the Sephadex. This was repeated until no more water was eluted by centrifugation. The sample, usually 100-200 µl, was applied to the Sephadex column which was then centrifuged in a siliconized centrifuge tube at 200 x g for 2-3 minutes to elute the desalted material (100-200 µl).

5.2.6 HYDROXYLAPATITE CHROMATOGRAPHY

Hydroxylapatite chromatography of RNA:DNA hybrids was carried out as described by Old et al. (1976) and in detail by P. Tolstoshev (personal communication).

5.2.6.1 PREPARATION OF HYDROXYLAPATITE

25 g of hydroxylapatite (Biogel HTP) was suspended in approximately 100 ml of 1 M sodium phosphate buffer pH 6.8 and allowed to settle. The buffer was decanted and the hydroxylapatite was resuspended in 100 ml of 0.16 M sodium phosphate buffer pH 6.8 and allowed to settle. The hydroxylapatite was resuspended in 0.16 M sodium phosphate buffer pH 6.8 and boiled for 15 min. After cooling the hydroxylapatite was washed once with distilled water and then twice with 0.03 M sodium phosphate buffer pH 6.8 and stored in this buffer at 4°C. (At all stages of preparation the resuspension of the hydroxylapatite was carried out by gently swirling the flask).

5.2.6.2 PREPARATION OF THE COLUMN

1-2 ml of hydroxylapatite suspension was pipetted into a small glass jacketed column maintained at 60°C and washed with 5-10 ml of 0.03 M sodium phosphate

buffer pH 6.8. 100-200 µg of native DNA (M.Lysodeikticus) was applied and washed into the column with 0.03 M sodium phosphate buffer pH 6.8. The column was washed with 5 ml of 0.12 M sodium phosphate buffer pH 6.8 followed by 10 ml 0.4 M and 10 ml of 0.03 M sodium phosphate buffer pH 6.8.

In all cases the buffer was allowed to equilibrate to 60°C before entering the hydroxylapatite. If necessary the buffers were forced through the column of hydroxylapatite with air pressure.

5.2.6.3 FRACTIONATION OF HYBRIDS

The sample, hybridized as described in section 5.3.9, was diluted 20 fold with 0.15 M NaCl (this diluted out the formamide and stabilized the hybrids) and loaded onto the hydroxylapatite column in 1 ml aliquots (each aliquot was allowed to equilibrate to 60°C before entering the column). The column was washed with 6-10 ml of 0.03 M sodium phosphate buffer pH 6.8 and the eluant collected. Single stranded material was eluted from the column with 6-8 ml of 0.12 M sodium phosphate buffer pH 6.8 and double stranded material with 0.4 M sodium phosphate buffer (all steps were carried out at 60°C).

5.2.6.4 REISOLATION OF cDNA

The required fraction, either the double or single stranded fraction, was desalted, after addition of 100 µg of E.coli rRNA as carrier, on a Sephadex G-25 column (2.5 x 25 cm). The volume of the desalted fraction (12-15 ml) was reduced to approximately 5 ml by lyophilization, made 0.33 M with respect to NaOH and incubated at 37°C for 16-20 h. The incubation mixture was neutralized with HCl, 50 µg of E.coli rRNA was added as carrier and the cDNA was precipitated by addition of 2 volumes of ethanol (-20°C overnight). The cDNA and carrier were dissolved in distilled water and desalted on a Sephadex G-50 chelex column (5.3.8).

5.2.7 PHENOL EXTRACTIONS

Phenol extractions of total RNA were performed using essentially the method of Perry et al. (1972). The samples from which RNA was to be extracted were suspended in NETS buffer (0.1 M NaCl, 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.2% SDS) and to this was added an equal volume of a 1:1 mixture of phenol:chloroform saturated with ANE buffer (10 mM sodium acetate, 0.1 M NaCl, 1 mM EDTA). The mixture was shaken at room temperature (22°C) for 30 min and then centrifuged at 16 000 x g for 10 min (~ 17°C). The aqueous phase was removed and re-extracted with an equal volume of phenol:chloroform (1:1). The phenol extractions were repeated until no white protein gel was evident at the aqueous:phenol interface.

The RNA was precipitated from the aqueous phase by addition of one fifth volume of 4 M NaCl and 2 volumes of 96% ethanol (-20°C for 16 h). The RNA was pelleted by centrifugation at 16 000 x g for 10 min and washed with 75% ethanol and dried under vacuum.

5.3 SPECIFIC METHODS

5.3.1 MAINTENANCE OF SEA URCHIN ADULTS AND EMBRYOS

5.3.1.1 COLLECTION AND MAINTENANCE OF SEA URCHINS

The maintenance and handling of sea urchin adults and embryos was carried out essentially as described by Hinegardner (1967, 1975).

Urchins were maintained in the laboratory in closed system aquaria, using natural sea water at 17°C ± 2°C. The water was continually filtered through glass wool-charcoal filters which were replaced every 3-5 days and about every 2 weeks 20-30 litres of the water was replaced with fresh sea water (aquaria contained a maximum of 1 urchin per three litres of sea water for long term maintenance). The urchins were fed on finely chopped kelp.

5.3.1.2 SEXING OF SEA URCHINS

It is generally difficult to determine the sex of sea urchins by external characteristics (Hinegardner, 1975). In P.angulosus the sexes can be distinguished with a certain degree of confidence, by examining the external appearance of the gonophores. The male gonophores are situated on the top of small papilla whereas the female gonophore is in a slight depression on the body surface. The sexing was carried out by placing the urchin in a beaker of sea water and examining the appearance of the gonophores under a dissecting microscope (10 x magnification).

5.3.1.3 COLLECTION OF GAMETES

Spawning was induced by introducing 0.5 M KCl into the coelomic cavity. This was done in one of two ways : either 0.5 ml of KCl was injected through the peristomatous membrane into the coelomic cavity, or the specimens were cut open and about 0.2 ml of KCl was poured onto each gonad. Both methods were successful in stimulating spawning but the latter usually gave better yields of gametes especially when urchins were used out of their spawning season. The advantage of the first method was that by careful

injection of KCl only one gonad can be stimulated to spawn and the urchin can be kept alive and used in other experiments. Eggs were collected by inverting stimulated females over 100 ml beakers filled with filtered (Whatman No. 1) sea water. Concentrated sperm was collected by inverting the male in a petri dish (sperm were not diluted until immediately before use (Hinegardner, 1967)).

During artificial spawning the urchins often exude small amounts of fluid from the digestive tract and therefore the eggs were always washed 3-4 times in filtered sea water. This was carried out by initially suspending the eggs in about 10 volumes of sea water and passing them through 2 layers of cheese-cloth which removes faecal pellets, spines etc. Eggs were then allowed to settle by gravity sedimentation and resuspended in fresh sea water; this was repeated three times. When small numbers of eggs were being used (2-5 x 10^6 eggs) they were washed as above but the sedimentation of the eggs was speeded up considerably by gentle centrifugation in a bench centrifuge. This did not affect the development of the eggs. Eggs were counted by suspending them in a known volume of sea water and taking a small aliquot, 20-50 µl, which was counted on a slide with a 1 mm grating. By this method accurate determinations ($\pm$ 5%) could be made of the number of eggs present. Rough estimates of the number of eggs could be made by measuring the volume of packed eggs (gravity sedimentation) and we found that a conversion factor of two ml/10^6 eggs gave reasonable estimations.

5.3.1.4 FERTILIZATION AND DEVELOPMENT OF EMBRYOS

Fertilization was achieved by adding diluted sperm (50 x diluted) to an egg suspension (10^2-10^5 eggs/ml) at about 1 ml sperm/100 ml egg suspension. Fertilized eggs were incubated at 22°C in Millipore filtered sea water containing 100 µg/ml of penicillin G and 50 µg/ml streptomycin sulphate. Embryos were maintained in suspension with sufficient aeration at all stages of development. In all experiments only batches of eggs showing greater than 95% fertilization (determined by presence of fertilization membrane) and normal development were used. (Millipore filtered sea water : 0.45 µ filter).

5.3.2 ISOLATION OF HISTONE mRNA

5.3.2.1 ISOLATION OF SEA URCHIN EMBRYO POLYRIBOSOMES

The isolation procedure described follows closely the method of Gross et al. (1973). Sea urchin embryos were allowed to develop (5.2.1.4) to the early blastula stage (4 h after fertilization). Embryos were allowed to settle (gravity sedimentation) and the excess sea water was removed by aspiration. All further procedures were carried out at 0-4°C. Embryos were washed twice in Millipore filtered sea water, by resuspension and centrifugation at 30 x g (500 rpm SS 34 rotor) for 1 min, twice in TNM buffer (0.2 M NaCl, 5 mM Mg acetate, 10 mM Tris-HCl pH 7.5) containing 10 µg/ml of polyvinylsulphate and finally pelleted at 450 x g (2000 rpm SS 34 rotor) for 5 min. Pelleted embryos were suspended in 3-4 volumes of TNM buffer containing 10 µg/ml polyvinylsulphate and 1 mg/ml of bentonite (5.1.2.1) and homogenized with a Dounce glass homogenizer (10 strokes, tight pestle). The homogenate was centrifuged at 14 500 x g (11 000 rpm SS 34 rotor) for 15 min and the post mitochondrial supernatant was carefully aspirated avoiding the lipid layer on the surface. The post mitochondrial supernatant was carefully layered on a 12.5 ml cushion of RNase free 36% (w/w) sucrose in TNM buffer (5.1.2.2) and centrifuged at 361 000 x g for 1 h (60 000 rpm Beckman 60Ti rotor). Immediately at the end of the run the supernatant was aspirated and the ribosomal pellet was very carefully washed with about 2 ml of 10 mM Tris-HCl pH 7.5, to remove excess 36% (w/w) sucrose. Polyribosomes were suspended in a small volume of 10 mM Tris-HCl pH 7.5 by gentle homogenization to a concentration of 200-400 $A_{260\ nm}$ units/ml.

Yields of ribosomes were determined by the absorbance at 260 nm. Concentrations were estimated using the relationship : 1 $A_{260\ nm}$ unit/ml of polyribosomes is equivalent to 90 µg/ml of polyribosomes (Sarkar et al., 1973).

5.3.2.2 ISOLATION OF POLYRIBOSOMAL RNA

Phenol extractions were based on the method of Brawerman (1973). Stored or freshly prepared polyribosomes were diluted 4 x with sterile distilled water to give a polyribosome concentration of 50-100 $A_{260\ nm}$ units/ml and buffer concentration of 2.5 mM Tris-HCl pH 7.5. The preparation was made 0.5% with respect to SDS and an equal volume of water saturated phenol was

added. The mixture was shaken at room temperature for 30 minutes then centrifuged at 16 000 x g (Sorval HB4 rotor, 10 000 rpm) for 10 min at 20°C. The aqueous phase was carefully removed, avoiding the protein interphase, and the phenol phase was re-extracted with an equal volume of sterile distilled water containing 0.5% SDS as described above. The aqueous phases were combined and the RNA precipitated at -20°C overnight by the addition of 1/5th volume of 4 M NaCl and 2.5 volumes of ethanol (96%). The precipitated RNA was pelleted by centrifugation at 16 000 x g (HB4 rotor, 10 000 rpm) for 10 min at 0°C, dried under vacuum and dissolved in sterile 10 mM Tris-HCl pH 7.5, 0.5% SDS.

The $A_{260\ nm}$ absorption of the RNA solution could be determined after suitable dilution and the RNA concentration estimated using the relationship: 1 $A_{260\ nm}$ unit/ml is equivalent to 40 µg RNA/ml (Sarkar et al., 1973).

5.3.2.3 ISOLATION OF SEA URCHIN POLYRIBOSOMAL 9S RNA

The isolation of sea urchin polyribosomal 9S RNA was based on the method of Gross et al. (1973). Polyribosomal RNA was dissolved in 10 mM Tris-HCl pH 7.5, 0.5% SDS to a concentration of 200-250 A_{260} units/ml, heated to 60°C for 10 min and then chilled rapidly to room temperature. 0.5 ml aliquots (100 A_{260} units). were applied to 15-30% sucrose gradients, in 10 mM Tris-HCl pH 7.5, 0.5% SDS, and centrifuged at 284 000 x g for 17 h at 20°C (Beckman SW 40Ti rotor). Gradients were analysed (5.2.1) and the peak of 9S RNA was pooled, made 0.8 M with NaCl and precipitated with 2 volumes of ethanol. This 9S RNA fraction was further purified by a second sucrose gradient fractionation (as above) with centrifugation for 22 h. The final 9S RNA was precipitated with ethanol, dissolved in sterile distilled water and stored at -100°C.

5.3.3 ISOLATION OF HUMAN FOETAL GLOBIN mRNA

These isolations were carried out at the Beatson Institute of Cancer Research, Glasgow. Neonatal reticulocytes were obtained from cord exchange blood from infants with Rhesus incompatibility. Reticulocytes were washed in buffered saline and stored as a pellet at -70°C.

The mRNA isolation was carried out essentially as described by Lanyon et al. (1975). Two volumes of 1 mM $MgCl_2$ were added to frozen reticulocytes and

the cells were thawed rapidly and homogenized in a Dounce homogenizer (2-3 strokes, tight pestle). The homogenate was centrifuged at 16 000 x g for 10 min and the supernatant was removed and made 0.5% with respect to SDS. An equal volume of phenol:chloroform 1:1 was added and the mixture shaken at room temperature for 20 min and then centrifuged at 27 000 x g for 5 min. The supernatant was re-extracted with phenol:chloroform as above. The RNA was precipitated from the combined supernatants by addition of one fifth volume of 4 M NaCl and 2 volumes of ethanol (-20°C for 2 h). The RNA was pelleted by centrifugation at 10 000 x g for 10 min, dissolved in NETS buffer and reprecipitated with ethanol. The RNA was dissolved in NTS buffer and fractionated on oligo(dT) cellulose (5.2.4). The poly(A) containing RNA was precipitated with ethanol at -20°C overnight. The precipitated RNA was pelleted, washed with 75% ethanol, and dissolved in 1-2 ml of sterile distilled water and stored at -20°C.

5.3.4 ISOLATION OF SEA URCHIN RIBOSOMAL RNA

5.3.4.1 ISOLATION OF RIBOSOMAL SUBUNITS

Sea urchin embryos were allowed to develop to the early gastrula stage (18 h) as described earlier (5.3.1) and ribosomal pellets were prepared as described in section 5.3.2.1. Pelleted ribosomes were suspended in a small volume of 50 mM Tris-HCl pH 7.5, 10 mM EDTA, 10 µg/ml polyvinyl-sulphate. The presence of the 10 mM EDTA resulted in dissociation of the ribosomes into subunits (Tashiro and Siekevitz, 1965).

The subunits were fractionated on 15-30% sucrose gradients in 50 mM Tris-HCl pH 7.5, 1 mM EDTA (sterile 5.1.2.2) by centrifugation at 284 000 x g for 7 hours (SW 40Ti rotor). After centrifugation the fractions containing the large and small ribosomal subunits were precipitated with 2 volumes of ethanol (-20°C, 4 h).

5.3.4.2 ISOLATION OF RNA FROM SUBUNITS

The precipitated subunits were dissolved in NETS buffer and the RNA was extracted using the phenol:chloroform method described in section 5.2.7.

The RNA was precipitated by addition of one fifth volume of 4 M NaCl and 2 volumes of ethanol (-20°C overnight). The RNA was pelleted, washed with 75% ethanol (10 000 x g, 10 min) and then dissolved in a small volume of distilled water.

5.3.5 ISOLATION OF E.COLI RIBOSOMAL RNA

E.coli ribosomes which were pelleted during the isolation of E.coli poly(A) polymerase (5.3.10.1) were suspended in NETS buffer and the RNA was extracted with phenol:chloroform as described in section 5.2.7. The RNA was precipitated with ethanol and washed as described above (5.3.4.2).

5.3.6 ISOLATION OF SEA URCHIN HISTONES

Sea urchin sperm was washed 2 x with Millipore filtered sea water by centrifugation at 12 000 x g for 5 min (10 000 rpm SS 34). Sea urchin embryos at the required stage of development were washed 2 x in Millipore filtered sea water by centrifugation at 30 x g for 2 min.

Pelleted tissue was suspended in 5 volumes of a solution - SSC (0.15 M NaCl, 0.015 M Na citrate) and homogenized with 10 strokes of the tight Dounce homogenizer and centrifuged at 3 000 x g for 10 min (5 000 rpm SS 34 rotor). The pelleted material was then washed by repeated homogenization (30 ml) and centrifugation at 3 000 x g as follows:

3 x in SSC
1 x in SSC 0.2% TRITON 100
2 x in SSC
1 x in 0.3 M NaCl, 0.15 M Na citrate
2 x in SSC
2 x in absolute ethanol
2 x in SSC

The histones were extracted by homogenizing the pellet in 0.25 M HCl and allowing the homogenate to stand at 0°C for 30 min. The extract was centrifuged for 10 min at 3 000 x g and the supernatant removed and dialysed for 24 h against 3 changes of distilled water, and then freeze dried. The yields of protein were determined by weighing.

5.3.7 TRANSLATION OF MESSENGER RNA

A cell free protein synthesizing system was prepared from Ehrlich ascites tumour cells essentially as described by Jacobs-Lorena and Baglioni (1972) and used for the translation of messenger RNA.

5.3.7.1 MAINTENANCE OF ASCITES TUMOUR CELLS

Ehrlich ascites tumour cells were a gift from V. McConnell of the Department of Biophysics and Bioengineering, Groote Schuur Hospital, Cape Town. Ascites cell cultures were maintained by transplanting ascites tumour cells every 10-14 days as follows : 2-5 ml of ascitic fluid were removed from the peritoneal cavity of an ascites tumour infected mouse ("bloody" tumours were rejected) using an 18 gauge needle. The ascites cells were pelleted by centrifugation for 5 min at 200 x g at room temperature. The pelleted cells were washed three times by repeated suspension and centrifugation with 10 volumes of sterile physiological saline. The final cell pellet was suspended in 2 volumes of sterile saline and 0.2 ml of this cell suspension was injected (26 gauge needle) intraperitoneally into uninfected albino mice. Ascites tumour cells for the preparation of the cell free extract were harvested 10-14 days after transplantation.

5.3.7.2 PREPARATION OF ASCITES CELL FREE EXTRACT

Ascitic fluid (10-15 ml) was extracted from ascites tumour infected mice and centrifuged at 200 x g for 5 min and washed 4 x in 10 volumes of sterile saline (200 x g, 5 min) at room temperature. Cells were pelleted at 1000 x g for 5 min and suspended in 2.5 volumes of medium A ($0^{o}C$) (15 mM KCl, 1.5 mM Mg acetate, 6 mM mercaptoethanol, 10 mM Tris-HCl pH 7.5) and allowed to swell for 5 min at $0^{o}C$. All further steps were carried out at $0-4^{o}C$. Cells were homogenized in a glass Dounce homogenizer (50 strokes, tight pestle) and then immediately made isotonic by addition of 1/10th volume of 10 x medium B (Medium B : 120 mM KCl, 5 mM Mg acetate, 6.0 mM mercaptoethanol, 20 mM Tris-HCl pH 7.5). The homogenate was centrifuged at 30 000 x g for 15 min and the supernatant (S_{30}) removed. The S_{30} was incubated at $37^{o}C$ for 50 min in the presence of 1 mM ATP, 0.2 mM GTP, 0.1-0.5 mM of each amino acid, 2 mg/ml creatine phosphate and 0.2 mg/ml creatine kinase. During incubation a clear gelatinous material was produced which was removed by centrifuging at 30 000 x g for 15 min. The supernatant was removed and applied to a Sephadex G-25 column (2.5 x 30 cm), equilibrated with Medium B, and the material eluting in the excluded volume

was pooled and used as the ascites cell free extract. The protein content of the extracts varied from 4-8 mg/ml. Cell free extracts were stored on ice for up to 3 days.

5.3.7.3 THE CELL FREE PROTEIN SYNTHETIC SYSTEM

Incubations were carried out in a final volume of 200 µl containing 120 µl of the ascites cell free extract, 20 µl of master mix (see below) and the remaining 60 µl was made up with mRNA, unlabelled amino acids (final conc. 0.1-0.5 mM) and the labelled amino acid dissolved in distilled water. The composition of the master mix was : 0.24 M KCl, 6 mM mercaptoethanol, 80 mM Tris-HCl pH 7.5, 10 mM ATP, 2 mM GTP, 20 mg/ml creatine phosphate and 2 mg/ml creatine kinase. This solution was made up by first preparing the buffer (KCl, mercaptoethanol and Tris-HCl) which was used to dissolve the rest of the constituents (the ATP and GTP were first dissolved in a very small volume of 5 mM NaOH). Radioactive amino acids were added to the incubations to give a final concentration of 10 µCi/ml and incubated at 30°C for 60 min.

In order to determine TCA precipitable radioactivity reactions were stopped by addition of 200 µl of 10% TCA containing 1 mg/ml unlabelled amino acid (i.e. either lysine or histidine depending which was added as labelled precursor) and tubes were allowed to stand at 0°C for 30 min. The TCA precipitates were then heated to 90°C for 15 min and filtered onto Millipore filters which were dried and the radioactivity determined.

5.3.7.4 ISOLATION OF HISTONES FROM ASCITES CELL FREE EXTRACTS

20 - 25 µg of sea urchin sperm histones isolated as described (5.2.3.4) were added to ascites cell free incubation mixtures(after incubation) to act as carrier.

Histones were reisolated from cell free extracts by one of two methods :

a) H_2SO_4 extraction followed by acetone precipitation
b) HCl extraction followed by dialysis

a. The ascites cell free incubate, containing carrier histones, was made 0.2 M with H_2SO_4 and allowed to stand at 0°C for 30 min. The extract was centrifuged at 12 000 x g for 10 min (SM 24 rotor, 10 000 rpm) and the supernatant was removed and to it were added 4 volumes of acetone and allowed to stand at -20°C for 1 h. The precipitate was pelleted by centrifugation at 8 000 x g

for 2 min and washed twice with acetone (-20°C) and the final pellet was dried under vacuum. The dried protein was dissolved in 8 M urea 0.5 M mercaptoethanol and heated to 80°C for 2 min prior to electrophoresis (5.2.3.5).

b. The ascites cell free incubation mixture containing carrier histones was made 0.25 M with HCl and allowed to stand at 0°C for 30 min. The extract was centrifuged at 30 000 x g for 15 min and the supernatant removed and dialysed against 0.9 M acetic acid for 1-2 hours (dialysis was carried out in micro-dialysis chambers, E. Schütt, Göttingen, Germany). The dialysate was made 8 M with respect to urea (addition of solid urea) and 0.5 M with respect to mercaptoethanol and heated to 80°C for 2 min prior to electrophoresis (5.2.3.5).

5.3.8 SYNTHESIS OF COMPLEMENTARY DNA

cDNA was synthesized essentially as described by Harrison et al. (1974). The incubation mixture contained : 50 µg/ml mRNA (template) 500 µM each of dATP, dTTP and dGTP, 0.1 mCi/ml [^{3}H]-dCTP (23 Ci/mmole), 5 A_{260} units/ml oligo(dT)$_{12-18}$, 50 µg/ml actinomycin D, 100 units/ml rat liver RNase inhibitor, 50 mM Tris-HCl pH 8.2, 50 mM KCl, 10 mM dithiothreitol, 5 mM magnesium acetate and reverse transcriptase as described below.

Two preparations of reverse transcriptase were used for cDNA synthesis :
1) a gift from Dr. G. Birnie of the Beatson Institute of Cancer Research, Glasgow. The reverse transcriptase preparation, RT17, was used at the recommended concentration of 100 µl/ml incubation.
2) a gift from Dr. J.W. Beard of Life Science Inc., Florida. This preparation (G1776) of specific activity 38811 units/mg protein was used at a concentration of 1000 units/ml (one unit of enzyme activity is defined as the incorporation of one nanomole of dTMP into an acid-insoluble product in 10 minutes at 37°C).

For the synthesis of cDNA 100 µl incubation mixtures were set up as follows:

Nucleotide mixture :

5 mM dATP	10 µl
5 mM dGTP	10 µl
5 mM dTTP	10 µl
[^{3}H]-dCTP (23 Ci/mmole, 1 mCi/ml)	100 µl.

This nucleotide mixture was freeze dried and to it was added : 20 µl of 5 x RT buffer (250 mM KCl, 250 mM Tris-HCl pH 8.2, 25 mM magnesium acetate, 50 mM dithiothreitol), 5 µl actinomycin D (1 mg/ml), 5 µl oligo$(dT)_{12-18}$ (10 A_{260} units/ml), 10 µl rat liver RNase inhibitor (100 units/ml) and the reverse transcriptase and RNA were added with water to a final volume of 100 µl.

The mixture was incubated at 37°C for 90 min. The reaction was stopped by addition of 20 µl of 100 mM EDTA and 100 µg of E.coli RNA was added as carrier. The incubate was applied to a Sephadex G-50 column (1 x 25 cm) containing a 1 cm pad of Dowex chelating resin (G-50-chelex column) equilibrated and eluted with 50 mM NaCl, 10 mM HEPES pH 7.0 buffer (1 ml fractions collected). The radioactivity in 5-10 µl aliquots of each fraction was determined and the labelled material eluting with the void volume (cDNA) was pooled (3-5 ml). After addition of 20 µg of M.lysodeikticus DNA as carrier it was made 0.33 M with respect to NaOH and incubated at 37°C for 16-20 hours. 0.5 ml of 1 M HEPES and 5 drops of phenol red were added to the incubate and it was neutralized using 2 M HCl (neutral point was salmon pink). 100 µl of E.coli RNA was added as carrier and the cDNA was precipitated by addition of 1/20th volume of 4 M NaCl and 2 volumes of ethanol (-20°C overnight). The cDNA was pelleted in a siliconized Corex tube by centrifugation at 16 000 x g for 20 min and dissolved in 0.5 ml of distilled water. The cDNA was desalted on a G-50 chelex column equilibrated with distilled water and 1.0 ml fractions were collected. The radioactivity in 5-10 µl aliquots of each fraction was determined and the fractions containing the radioactive cDNA were pooled and stored at -20°C.

5.3.9 RNA:cDNA HYBRIDIZATIONS

5.3.9.1 HYBRIDIZATION REACTIONS

Hybridizations were carried out essentially as described by Getz et al. (1975). The required amounts of RNA (in distilled water) and 2-5 µg of E.coli rRNA were placed into small (3 ml) siliconized glass tubes and freeze dried. The amount of cDNA necessary for a set of reactions was treated likewise. The cDNA was then dissolved in hybridization buffer (see below) and aliquots of this mixture were used to dissolve the lyophilized RNA samples. The hybridization mixtures (2-20 µl) were sealed in siliconized

glass capillaries (5.1.2.3).

Hybridization buffer (0.5 M NaCl, 25 mM HEPES, 0.5 mM EDTA, pH 6.8, 50% (v/v) formamide) was prepared as follows : 4 x concentrated buffer was prepared, passed over Dowex chelating resin, treated with diethylpyrocarbonate, autoclaved, and mixed with 2 parts deionized formamide (5.1.5), and 1 part sterile distilled water.

Capillaries containing hybridization mixtures were heated to 80°C for 2 min and then incubated at 43°C. Reactions were stopped by immersing the capillaries into ethanol at -20°C.

5.3.9.2 S_1 NUCLEASE ASSAY

The contents of each capillary were flushed out with 250 µl of nuclease assay buffer (70 mM sodium acetate, 2.8 mM $ZnSO_4$, 140 mM NaCl pH 4.5) containing 10 µg/ml of heat denatured M.lysodeikticus DNA. 100 µl of S_1 nuclease (A. oryzae) (2500 units/ml in nuclease assay buffer) was added and the mixture incubated at 37°C for 90 minutes. A 100 µl aliquot of the incubate was removed and the radioactivity determined (total dpm). A 200 µl aliquot of the incubate was removed and to it was added 50 µl of a 1 mg/ml RNA soln. and 50 µl of 6 N perchloric acid. The mixture was chilled at 0°C for 30 min and then centrifuged at 12 000 x g for 2 min. A 200 µl aliquot of the supernatant was removed and the radioactivity was determined (soluble dpm). From the soluble and total dpm the percentage of the cDNA hybridized was calculated using the formula $100 - (0.75 \times \frac{\text{soluble dpm}}{\text{total dpm}})$.

5.3.10 IN VITRO POLYADENYLATION OF RNA

5.3.10.1 ISOLATION OF E.COLI POLY(A) POLYMERASE

The method used for the isolation of poly(A) polymerase from E.coli was described by Getz et al. (1974) and carried out at the Beatson Institute of Cancer Research.

30 g of frozen mid-log E.coli cells strain MRE 600 (MRE, Porton) were suspended in 25 ml of TSM buffer (10 mM Tris, 3 mM succinic acid, 10 mM $MgSO_4$, pH 8.0) containing 10 µg/ml DNase I (Worthington). After addition of 1.7 ml

of 10 x TSM buffer and 60 g of acid washed glass beads (0.11 mm) cells were disrupted in a Waring blendor at full speed for 12-15 min (the mixture was maintained below 4°C at all times). The homogenate was centrifuged at 27 000 x g for 30 min (HB4 rotor, 13 000 rpm). Puromycin (Sigma) was added to the supernatant to a final concentration of 30 µg/ml and incubated at 32°C for 15 min. The incubate was diluted to 100 ml with TSM buffer (0°C) and solid $(NH_4)_2SO_4$ was added to 27% saturation at 0°C. The mixture was stirred at 0°C for 3 min and then centrifuged at 20 000 x g for 10 min (SS 34 rotor, 13 000 rpm). The $(NH_4)_2SO_4$ concentration of the supernatant was increased to 55% saturation (addition of solid $(NH_4)_2SO_4$) and stirred for 3 min at 0°C. The precipitate was pelleted by centrifugation at 20 000 x g for 10 min and dissolved in 100 ml of TSM buffer. The precipitation with $(NH_4)_2SO_4$ was repeated twice, first taking the material precipitated between 27% and 50% saturation and then the material precipitating between 27% and 46% saturation. The final pellet was dissolved in 25-30 ml of TSM buffer and dialysed for 16 h against three changes of TSM buffer. The dialysed sample was centrifuged at 150 000 x g for 2 h and the pellet was suspended in TSM buffer and centrifuged at 150 000 x g for 90 min. The pellet was then suspended in TSM buffer containing 0.6 M $(NH_4)_2SO_4$ (pH 7.4) and centrifuged at 150 000 x g for 3 h. The top four fifths of the supernatant (30 ml), containing the poly(A) polymerase activity, was removed and adjusted to 40% saturation (at 0°C) with $(NH_4)_2SO_4$. The precipitate was pelleted by centrifugation at 20 000 x g for 10 min and dissolved in 30 ml of TSM buffer, 0.6 M $(NH_4)_2SO_4$ and the $(NH_4)_2SO_4$ precipitation was repeated. The final pellet was dissolved in 1.5 ml of TSM buffer containing 0.6 M $(NH_4)_2SO_4$, 2 mM dithiothreitol and applied to a Sephadex G-200 column (1.5 x 60 cm) equilibrated with the same buffer and eluted collecting 1.5 ml fractions. The fractions eluting in the inner volume were assayed for poly(A) polymerase activity as described below. The active fractions were pooled and the enzyme precipitated by addition of $(NH_4)_2SO_4$ to 40% saturation (0°C). The precipitated material was pelleted by centrifugation at 20 000 x g for 10 min and dissolved in 0.5 ml of TSM buffer containing 0.6 M $(NH_4)_2SO_4$, 2 mM dithiothreitol and 0.5 ml of sterile glycerol was added.

5.3.10.1.1 STANDARD ASSAY CONDITIONS

The standard assay mixture contained in a final volume of 200 µl : 10 mM Tris, 3 mM succinic acid (pH 8.0), 20 mM $MgSO_4$, 16 mM $MnCl_2$, 0.5 mM [^{3}H]-ATP

(10 µCi/ml), 20 µg of E.coli rRNA and 10 µl of enzyme solution. Incubations were at 37°C for 20 min and the TCA precipitable radioactivity was determined.

5.3.10.1.2 CONDITIONS OF PREPARATIVE POLYADENYLATION

The conditions used for the polyadenylation of histone mRNA varied slightly from the standard assay conditions as follows : Incubations were carried out in a final volume of 200 µl containing 10 mM Tris, 3 mM succinic acid (pH 8.0), 2 mM $MnCl_2$, 40 mM $MgSO_4$, 1 mM ATP (unlabelled), 10 units per ml of rat liver RNase inhibitor, 10 µg mRNA and 25 units of poly(A) polymerase (1 unit of poly(A) polymerase catalyses the incorporation of 100 pmoles of AMP into acid insoluble material in 20 min at 37°C under standard assay conditions). The reaction mixture was incubated at 37°C for 20 min and the reaction was stopped by addition of 1 ml of NETS buffer. The RNA was extracted with phenol:chloroform (1:1) and precipitated with ethanol (5.2.7) and dissolved in sterile distilled water.

5.3.10.2 ISOLATION OF MAIZE SEEDLING POLYNUCLEOTIDYLEXOTRANSFERASE

Polynucleotidylexotransferase was isolated from maize seedlings essentially as described by Mans and Huff (1975).

Maize seedlings (Zea mays, cv. golden bantam) were raised in constant temperature (25°C), constant humidity (60-70%) plant growth rooms. 4 day old shoots (200 g) were suspended in 300 ml of buffer A (100 mM Tris:HCl pH 8.0, 0.1 mM $MgCl_2$, 50 mM mercaptoethanol and 5% glycerol) and homogenized in a Waring blendor for 5 min at 4°C. The homogenate was passed through 2 layers of cheese cloth and then centrifuged at 143 000 x g for 90 min (Type 40 rotor, 35 000 rpm). The supernatant was decanted and made 40% with respect to $(NH_4)_2SO_4$ (by dropwise addition of saturated $(NH_4)_2SO_4$) followed by stirring at 0°C for 30 min. The precipitate was pelleted by centrifugation at 10 000 x g for 10 min. The precipitate was dissolved in 40 ml of buffer B (2.5 mM Tris:HCl pH 8.0, 1 mM mercaptoethanol, 5% glycerol) and applied to a Sephadex G-50 column (2.5 x 90 cm) equilibrated with 10 mM $(NH_4)_2SO_4$ in buffer B. The material eluting with the excluded volume was collected and pooled and applied to a DEAE cellulose (0.8 meq/g) column (2.5 x 30 cm) equilibrated with 10 mM $(NH_4)_2SO_4$ in buffer B. (The DEAE

cellulose was prepared as described by Peterson and Sober (1962)). After flushing the column with 10 mM $(NH_4)_2SO_4$ in buffer B (400 ml) at a flow rate of 40 ml per hour, the material bound to the column was eluted with a 1 litre gradient of 10-400 mM $(NH_4)_2SO_4$ in buffer B at a flow rate of 40 ml/hour. The A_{280} was monitored continuously (Isco Absorbance Monitor model UA5) and 10 ml fractions were collected. Alternate fractions were assayed for enzyme activity (5.3.10.2.1) and the fractions containing the exotransferase activity were pooled and precipitated by addition of solid $(NH_4)_2SO_4$ to a final concentration of 75%. The precipitate was pelleted by centrifugation at 10 000 x g for 10 min, then dissolved in 5 ml of buffer B and dialysed for 10 h at 0°C against 3 changes of buffer B. The final preparation was stored at -20°C.

5.3.10.2.1 STANDARD ASSAY CONDITIONS

Standard assays were carried out essentially as described by Mans and Huff (1975).

Incubations were carried out in a final volume of 200 µl containing 70 mM Tris HCl pH 8.8, 10 mM dithiothreitol, 1 mM [^{3}H]-ATP (10 µCi/ml), 1 mM $MnCl_2$, 10 units/ml rat liver RNase inhibitor, 0.8 A_{260} units/ml yeast tRNA or E.coli rRNA and the required volume of enzyme preparation. Incubation was for 90 min at 30°C and the enzyme activity was determined as the TCA precipitable radioactive material.

5.3.10.2.2 CONDITIONS OF PREPARATIVE POLYADENYLATION

The conditions of the preparative polyadenylation were the same as in the standard assay. 1 ml reactions were carried out containing 200 µl of enzyme (4.5 mg protein/ml) and 100 µg of histone mRNA. The reaction mixture was incubated at 30°C for 1 h. A 50 µl aliquot was removed to determine acid precipitable incorporation and to the remainder of the incubate was added 5 ml of NETS buffer and 1 ml of 100 mM EDTA. The RNA solution was extracted with phenol:chloroform (1:1) at room temperature and the RNA precipitated with 2 volumes of ethanol at -20°C overnight (5.2.7).

5.3.11. ISOLATION OF SEA URCHIN EGG AND EMBRYO RNA FRACTIONS

5.3.11.1 PREPARATION AND FRACTIONATION OF POST MITOCHONDRIAL SUPERNATANTS

Sea urchin eggs and embryos were obtained and allowed to develop as described in part 5.3.1. At the required stage of development eggs or embryos were pelleted by centrifugation at 650 x g for 2 min and washed twice in TKMS buffer (0°C) (50 mM Tris-HCl pH 7.8, 0.24 M KCl, 5 mM $MgCl_2$, 0.25 M sucrose) by suspension and centrifugation (650 x g, 2 min) and then pelleted at 4 000 x g for 5 min. The pellet was suspended in 3-4 volumes of TKMS containing 1 mg/ml bentonite and homogenized with a Dounce homogenizer (10 strokes, tight pestle). The homogenate was centrifuged at 16 000 x g for 15 min and the post mitochondrial supernatant carefully removed. Under these conditions of isolation the post mitochondrial supernatant had an absorption of 100-150 A_{260} units/ml.

Sucrose gradients (15-30% in TKM., SW 40Ti) were prepared as described in Part 5.2.1 with the slight modification that a 1.0 ml cushion of 50% (w/v) sucrose (in TKM) replaced the bottom 1 ml of the sucrose gradient. The sample, approximately 20 A_{260} units of the post mitochondrial supernatant (in 0.5 ml), was applied to each gradient and centrifuged at 71 000 x g (20 000 rpm) for 20 h at 0-2°C. The gradients were analysed and fractionated as described in Part 5.2.1 and divided into four fractions (see Fig. 4.5).

5.3.11.2 ISOLATION AND PREPARATION OF RNA

To each of the gradient fractions (1-4) were added two volumes of ethanol and the material (RNA and proteins) allowed to precipitate at -20°C for approximately 16 h. The precipitate was pelleted by centrifugation at 16 000 x g for 10 min, dried under vacuum and then dissolved in NETS buffer. The RNA was isolated by phenol:chloroform extraction and precipitation with ethanol as described in Part 5.2.7 and dissolved in distilled water. The RNA was desalted by passing it through Sephadex G-25 (5.2.5) and then stored in distilled water at -20°C.

5.3.12 IN VIVO LABELLING OF RNA AND PROTEIN IN SEA URCHIN EMBRYOS

Sea urchin eggs and sperm were obtained and embryos allowed to develop as described in Part 5.2.1. [^{3}H]-labelled amino acids (amino acid mixture, Amersham TRK 440) or [^{3}H]-uridine (50 Ci/mmole) were added to unfertilized eggs (10^5 eggs/ml) 20 minutes prior to fertilization. Immediately after addition of label (zero time) and at various time intervals thereafter aliquots (100 µl = 10^4 eggs) were removed and precipitated with TCA as described below.

TCA precipitation was performed as follows : 100 µl aliquots of eggs or embryos were added to 2 ml of 0.5% SDS at room temperature and mixed. To this was added 2 ml of 10% TCA and tubes were placed on ice (0^{o}C) for 30 min. To determine the amount of RNA synthesis the precipitated material was filtered onto glass fibre filters (Whatman) and the radioactivity determined. To determine the protein synthetic activity TCA precipitates were incubated at 90^{o}C for 15 min prior to collection and washing of the TCA precipitates on glass fibre filters.

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