RNA SYNTHESIS AND PROCESSING IN ISOLATED HELA CELL NUCLEI

by

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Abbreviations

The abbreviations used in this work are as laid down in the Biochemical Journal Instructions to Authors (Biochem. J. (1978) 169, 5;27), with the following additions:-

Ado-Met	S-adenosyl methionine
BSS	Balanced salt solution
DNase I	Deoxyribonuclease I
DRB	5,6 dichloro-1-β-D ribofuranosyl-benzimidazole
DTT	Dithiothreitol
DMSO	Dimethyl sulphoxide
Hepes	4-(2-hydroxyethyl)-1-piperazine-ethane sulphonic acid
hnRNP	heterogeneous nuclear ribonucleoprotein
HSB	High salt buffer
ISB	Isotonic salt buffer
K.b.	Kilobase
mRNP	messenger ribonucleoprotein
NTP	Nucleoside triphosphate
PPLO	Pleuropneumonia-like organism
PPO	2,5 diphenyloxazole
RSB	Reticulocyte standard buffer
SDS	Sodium dodecyl sulphate
SSC	0.15 M NaCl, 0.015 M sodium citrate
TEMED	N,N,N',N',tetramethylethylenediamine

CONTENTS

Page

Page

Page

Page

List of figures

Page

List of tables

Summary

Isolated HeLa cell nuclei have been characterised in terms of their ability to transcribe, process and transport RNA.

In terms of transcription, it was found that all three RNA polymerases were active in the isolated nuclei. The size and nuclear location of the products of RNA polymerase I and III suggested that transcription by these polymerases was occurring normally in vitro. However, the RNA synthesised by RNA polymerase II was found to be much smaller than expected from the reported size of HeLa cell transcription units, when analysed in denaturing gradients. This was in contrast to the results of Sarma et al (1976) which showed that the size of RNA synthesised by RNA polymerase II in isolated HeLa cell nuclei is large when analysed under non-denaturing conditions. A number of possible reasons for this small size of RNA were examined. The results obtained indicate that this small size of RNA polymerase II product was probably not due to:-

i. A slow elongation rate by RNA polymerase II resulting in a "nascent transcript profile."
ii. Degradation of RNA in the isolated nuclei.
iii. The absence of nuclear and cytoplasmic factors during incubation of the nuclei.
iv. Degradation of the DNA template during isolation and incubation of nuclei.

It was found, however, that the state of the chromatin template was important in determining the size of RNA transcribed. Thus, addition of acetyl CoA to isolated nuclei, which acetylated the histones, caused an increase in the size of RNA polymerase II product.

On the other hand methylation of histones with Ado-Met *in vitro* was correlated with a decrease in the size of RNA.

It was also found that the ionic content of the incubation medium affected the size of RNA transcript synthesised by RNA polymerase II. In particular, substituting 90 mM $(NH_4)_2SO_4$ for 75 mM KCl in the incubation medium increased the size of RNA. This effect is discussed in terms of recent results which suggest that the small size of RNA polymerase II transcript commonly observed *in vitro* might be due to premature termination of transcription.

The small RNA transcribed by RNA polymerase II *in vitro* appears to be stable. However, hnRNA prelabelled *in vivo* is reduced in size during incubation of isolated nuclei. Some of this RNA is released from the nuclei during incubation. This release of RNA was examined to determine whether it represented the specific transport of mRNA. Although the size of released RNP particles, their lability, and the size of released RNA were consistent with mRNA transport, other features were more consistent with the leakage of hnRNP particles. The released RNA resembled hnRNA in terms of binding to poly(U) Sepharose, and the protein associated with the released RNA were similar to hnRNP particle proteins. Although the released RNA had messenger activitiy in a wheat germ cell free translation system, it is not possible to rule out mRNA contamination as the cause of this stimulation.

It therefore appears that the isolated nuclei system of Sarma *et al* (1976) may not be ideal either for examining the transcription or the processing and transport of mRNA. On the other hand, the results obtained in the present study suggest ways in which this

system might be modified in order to achieve full length transcription by RNA polymerase II *in vitro*, and study the processing and transport of these transcripts.

INTRODUCTION

Areas of study in biochemistry can generally be divided into one of two categories:- functional and structural studies. Although an oversimplification, this division may best be illustrated by work in enzymology, in which studies of the primary and higher order structure of an enzyme have complemented kinetic and metabolic studies to give a broad picture of the mechanism of action of an enzyme and its role in vivo. In the field of gene expression, the distinction is not so clear cut. The determination of the structure of DNA was a major factor in the determination of its function. However, in general, research into the genetic or informational aspects of gene expression may be independent of research into the structure or mechanisms of the physical entities involved.

Possibly the two most important breakthroughs in the study of eukaryotic gene expression in recent years exemplify this distinction. On the one hand, the way in which DNA is packaged in the nucleus by coiling round beads of histones has been established with great success using many diverse techniques. On the other hand, the arrangement of genetic information within nuclear DNA, and the discovery of non-coding intervening sequences within the coding sequences of particular genes has radically altered the understanding of eukaryotic gene expression.

However, many of the problems arising from these discoveries now seem to lie at the interface between the structural and functional. For instance, the known structure of chromatin raises questions about the transcription of information from the chromatin template. The discovery of non-contiguous genes poses many problems concerning the processing of transcripts from these genes, and the selection of

particular sequences for transport to the cytoplasm. Research into these problems may require both an analysis of the informational content of transcripts, and of the structural mechanisms of processing and transport. In the present study, a system with the potential for examining some of these questions *in vitro* has been characterised.

1.1. Chromatin Structure

1.1.1. The nucleosome concept

Within the past five years the establishment of the nucleosomal subunit model of chromatin structure has led to a better understanding both of higher order chromatin packaging and of chromatin transcription. The work which culminated in the formulation of the nucleosome concept was a number of parallel studies using widely differing techniques:-

Electron microscopical examination of chromatin from eukaryotic nuclei revealed linear arrays of spherical "v" bodies, 70Å in diameter, connected by 15Å strands (Olins and Olins, 1974). Histone H_1-depleted chromatin was also found to have a beaded appearance, the beads being called "nucleosomes" (Oudet *et al*, 1975).

In parallel with the E.M. studies, a repeating subunit structure of chromatin was deduced by self digestion of rat liver chromatin in isolated nuclei by endogenous Ca^{2+}/Mg^{2+} dependent nuclease (Hewish and Burgoyne, 1973). Discrete sizes of DNA degradation products, being multiples of 180-230 base pairs were observed. Using micrococcal nuclease, a repeat length of 205 base pairs was detected (Noll, 1974a).

A repeating unit of histones and DNA was also postulated (Kornberg, 1974), following cross-linking studies on histones dissociated from chromatin (Kornberg and Thomas, 1974). Thus, the basic nucleosome model of a repeating structure of nucleosome beads containing an octamer of the four main histones (H_{2A}, H_{2B}, H_3 and H_4) in association with $\sim$ 200 base pairs of DNA wasproposed. Since then, the resolution of this structure has become much more detailed.

1.1.2. The nucleosome core

Nucleases have been used with great success to probe the structure of the nucleosome. Precise measurements of the repeat length of DNA after brief digestion with micrococcal nuclease have been achieved by comparing the sizes of fragments generated with standards of known size. In rat liver (Axel et al, 1975) and duck reticulocyte chromatin (Sollner-Webb and Felsenfeld, 1975) an array of fragments with a repeat length of 185 base pairs was generated by this enzyme. This length, however, varies between species (Compton et al, 1976; Lohr et al, 1977) and tissues (Thomas and Thompson, 1977), and within single cell types (Kornberg, 1977).

Further digestion of nuclei or of isolated nucleosome monomer particles resulted in a particle containing 140 base pairs of DNA, termed the "core particle" (Sollner-Webb and Felsenfeld, 1975). A "beads-on-a-string" model was put forward in which nucleosome cores containing 140 base pairs of DNA are linked by a variable length (roughly 60 base pairs) of nuclease sensitive DNA (Sollner-Webb and Felsenfeld, 1975; Axel et al, 1975). This core-length of DNA is invariant between species (Morris, 1976; Lohr et al, 1977).

Using neutron and X-ray scattering techniques, results were obtained consistent with a model of the nucleosome core as an oblate structure of height 50Å diameter 110Å, and the DNA confined to 2 annuli on the outside of the core, at the top and bottom of the particle (Pardon et al, 1977; Richards et al, 1977). X-ray crystallography of nucleosome cores also indicates a flat particle of dimensions 110 x 110 x 57Å (Finch et al, 1977). The particles are divided into two layers, consistent with $\sim$ 1.3/4 turns of DNA wound round the outside of the flattened cylindrical particle.

1.1.3. Internal structure of the nucleosome core

Further information about the structure of the nucleosome core has been obtained by analysis of the products of limit digests of chromatin with various nucleases. Extensive digestion of chromatin with micrococcal nuclease gives rise to fragments evenly spaced at 10 base pair intervals from 40-140 base pairs (Camerini-Otero et al, 1976). Analysis of the DNase I limit digest products on denaturing gels also reveals a pattern of fragments, multiples of 10 bases in length, generated by single strand nicks within the nucleosome core (Noll, 1974b; Camerini-Otero et al, 1976). DNase II (Sollner-Webb et al, 1976) and rat liver endogenous Ca^{2+}/Mg^{2+} dependent nuclease (Simpson and Whitlock, 1976b) also generate fragments by single strand nicks with a repeat length of 10 bases.

These results suggest a regular pattern of nuclease susceptible sites within the nucleosome core. It has been possible to map these susceptible sites by 5' end labelling of nucleosome core particles. These experiments indicate that the nuclease cleavage sites are distributed symmetrically around a dyad axis in the nucleo-

some (Simpson and Whitlock, 1976a). This element of two-fold symmetry in the nucleosome has been incorporated into the model of Finch et al (1977), such that one superhelical turn of DNA wound round the nucleosome core consists of 80 base pairs. The possibility of this two-fold symmetry in the histone octamer itself has led to the proposal that the nucleosome not only contains a dyad axis, but is capable of unfolding into two symmetrical "half nucleosomes", during replication or transcription (Weintraub et al, 1976).

1.1.4. The role of histone H_1

Most of the work described above has used isolated nucleosome cores, which contain an octamer of the four major histones and 140 base pairs of DNA. Histone H_1 is liberated from nucleosomes by micrococcal nuclease action when the DNA size is reduced from 200 base pairs to 140 base pairs (Noll and Kornberg, 1977; Whitlock and Simpson, 1976). Therefore, it seems likely that histone H_1 is associated with the nuclease susceptible "linker" DNA between nucleosomes, since selective removal of H_1 with high salt renders this DNA more sensitive to nuclease attack (Noll and Kornberg, 1977). Particles containing the nucleosome histone octamer, 160 base pairs of DNA and one molecule of histone H_1 have been isolated ("chromatosomes") (Simpson, 1978a). The properties of these particles suggest that there are two full turns of DNA around the core particle, with H_1 found outside the DNA-inner histone complex. Therefore, it is still not certain whether the structure of chromatin is a "bead and bridge" type as seen in chromatin spreads in very low salt under the electron microscope (Olins and Olins, 1974; Oudet et al, 1975), or

whether all the DNA is actually wound around the nucleosome cores, with very little "linker" DNA.

The role of histone H_1 seems to be related to higher order structures of chromatin. Chromatin isolated from nuclei in the absence of Mg^{2+} ions appears under the electron microscope as 100Å diameter filaments, which are probably linear arrays of nucleosome cores in contact with one another. In the presence of Mg^{2+} ions, the filaments condense into a supercoiled solenoidal structure with a pitch of 110Å and a diameter of 300Å (Finch and Klug, 1976). Neutron diffraction analysis of chromatin also points to a solenoidal structure of this size (Carpenter et al, 1976). Histone H_1 has been implicated in the compaction of oligonucleosomes, perhaps by linking of non-adjacent nucleosomes (Christiansen and Griffith, 1977) or by connecting the spacer regions on either side of a single nucleosome (Hardison et al, 1977). H_1 is capable of stabilising oligonucleosomes containing 8 or more nucleosomes in higher order structural units, which appear under the electron microscope as spherical structures and have been termed "super-beads" (Renz et al, 1977). The relationship of superbeads to the solenoidal conformation of oligonucleosomes mentioned above is not clear, but differences in structure could arise during preparation due to rearrangement of histones.

1.1.5. The structure of transcriptionally competent or active chromatin

Since the formulation of the nucleosome model of chromatin structure, a lot of work has been directed towards studying the structure of transcriptionally active chromatin. Most estimations of the extent of the eukaryotic genome existing in nucleosomes give a figure of 85% or more. It is, therefore, possible that up to 15% of

the nuclear DNA may not exist in this type of subunit structure, but may exist in a different conformation, or even not be bound to histones. Since the figure of 15% is similar to the fraction of the genome transcribed, as estimated by the most sensitive measurements of sequence complexity of nuclear RNA (Chickaraishi et al, 1978; Pederson, 1978), it is important to ascertain whether the transcriptionally active regions of the genome have a nucleosomal structure.

One approach to this question has been to determine whether active gene sequences occur in isolated nucleosomal subunits. Rat liver mononucleosomes were found to contain most, if not all, the sequences in total nuclear DNA, and all of the sequences expressed as poly(A)$^+$ mRNA (Lacy and Axel, 1975). Similarly, mononucleosome subunits formed by DNase II action on human lymphocyte chromatin were found to contain poly(A)$^+$ mRNA sequences in the same concentration as in total nuclear DNA (Kuo et al, 1976).

Specific mRNA sequences have also been detected in isolated nucleosome particles; e.g. globin mRNA sequences (Lacy and Axel, 1975) and ovalbumin mRNA sequences (Garel et al, 1977).

Information about the nucleosomal character of individual genes undergoing transcription has come from electron microscopical studies on low salt chromatin spreads (Miller and Beatty, 1969). It is possible in such spreads from suitable tissues to discern individual active transcription units and identify their ribosomal or non-ribosomal nature by the characteristics of the lateral ribonucleoprotein fibril arrays (Laird et al, 1976; Foe et al, 1976). It has been reported that non-ribosomal transcription units have a beaded

nucleosomal appearance in Oncopeltus fasciatus embryos (Laird et al, 1976; Foe et al, 1976) and Drosophila embryos (Laird and Chooi, 1976) which is similar to the inactive regions of the chromatin. Histones have been positively identified in these active non-ribosomal transcription units using immunochemical techniques (McKnight et al, 1977). However, no nucleosomal structures are seen on more closely transcribed chromatin, such as the loops of amphibian oocyte lampbrush chromosomes (Franke et al, 1976) and the silk fibroin transcription unit of Bombyx mori silk gland (McKnight et al, 1976). Thus, it may be that the transition from smooth to beaded morphology in non-nucleolar chromatin seen during maturation of amphibian oocytes represents a spectrum of degrees of unfolding of the chromatin dependent upon the packing density of RNA polymerase molecules (Scheer, 1978).

Nucleosomes are not seen in the familiar "Christmas tree" arrays of fibrils on densely packed ribosomal transcription units (Foe et al, 1976; Laird et al, 1976). On the other hand, purified oocyte nucleolar chromatin contains histones (Higashinakagawa et al, 1977). Moreover, nuclease digestion experiments indicate that ribosomal genes do have a repeating subunit structure (Reeves, 1976; Mathis and Gorovsky, 1976). It is therefore possible that histones are present on transcribed ribosomal DNA, but in a modified, extended conformation, such that although the nucleosome structure is not recognisable ultrastructurally, a nuclease sensitive repeating structure is maintained.

Although DNase I attacks total chromatin DNA progressively to a limit digest of 95-100% acid solubility, it has been found that brief digestion of nuclei with this enzyme selectively attacks active gene sequences. Brief digestion of avian erythrocyte nuclei with

DNase I preferentially solubilises globin sequences, but not ovalbumin sequences (Weintraub and Groudine, 1976). Conversely, 70% of the ovalbumin sequences in oviduct nuclei are digested by DNase I when only 10% of the total DNA is solubilised (Garel and Axel, 1976).

Further work with DNase I digestion of oviduct nuclei has shown that the genes coding for low abundance class messenger sequences are digested at the same rate as ovalbumin sequences (Garel et al, 1977). The number of elongating polymerase molecules/ovalbumin gene has been determined to be at least 5 in oviduct nuclei (Bellard et al, 1977). A much lower packing density of polymerase molecules (less than one/gene) would be expected on the genes coding for low abundance mRNA than on the ovalbumin genes, leading to the conclusion that DNase I susceptibility is not due to the presence of RNA polymerase or lateral RNP fibrils, but may be caused by an active conformation of the chromatin (Garel et al, 1977). Thus, it seems likely that the difference in conformation detected by DNase I reflects a history of, or a potential for, transcription. For instance, globin genes are still preferentially sensitive to DNase I in transcriptionally inactive mature avian erythrocytes (Weintraub and Groudine, 1976), and thus maintain an active conformation reflecting a past history of transcription.

There are conflicting reports as to whether the increased sensitivity of active sequences to DNase I is retained in isolated nucleosomes prepared by micrococcal nuclease digestion. Globin sequences are preferentially digested in 115 mononucleosomes from avian erythrocytes (Weintraub and Groudine, 1976), but isolated nucleosomes from oviduct nuclei do not retain an active conformation

(Garel and Axel, 1976). It has been suggested that the differences reported above are the result of more extensive digestion with micrococcal nuclease in the latter case, leading to the removal of a postulated "active conformation factor" (Villeponteaux et al, 1978).

Whilst DNase I apparently recognises a difference in conformation within nucleosomes containing active regions of the genome, and hydrolyses them, it has also been discovered that DNase II preferentially shears active sequences between nucleosomes. A fraction enriched in active sequences can then be purified on the basis of its solubility in 2 mM $MgCl_2$ (Gottesfeld et al, 1974). This "template-active" fraction contains 11% of total rat liver chromatin DNA and is enriched 6-7 fold in transcriptionally active sequences. The fraction is also enriched in non-histone proteins and nascent RNA, which is probably responsible for the solubility of the fraction in 2 mM $MgCl_2$ (Gottesfeld and Butler, 1977).

1.2. Modification of histones

The conformational differences between active and inactive chromatin could be a result of the various post-synthetic modifications of histones which occur. At the present these are known to include acetylation, phosphorylation, methylation, ADP-ribosylation.

1.2.1. Acetylation of histones

Histones H_1, H_{2A} and H_4 are acetylated at their amino-terminal serine residue. This modification occurs early in histone biosynthesis, when the nascent chains are still attached to polysomes. It is essentially irreversible, and N-acetyl serine is a permanent modification common to all histone H_4 molecules (Ruiz-Carrillo et al, 1975).

The acetylation of internal amino acids occurs in all four of the nucleosome core histones, and takes place in the nucleus. Acetylation sites in H_4 are lysines at positions 5, 8, 12 and 16; in H_3 at positions 9, 14, 18 and 23; in H_{2B} at positions 8, 35, 39 and 47, and in H_{2A} at position 5 (Isenberg, 1979). These acetylation sites appear to be conserved between species.

Metabolic studies of histone acetylation have shown that, whilst the N-terminal acetyl group is stable, the internal acetyl groups turnover very rapidly (Jackson et al, 1975).

For some time it has been suggested that histone acetylation might play a role in modification of chromatin structure, and in particular, affect its template function in RNA synthesis (Allfrey et al, 1964). Indirect support for this has been provided by a number of correlations between histone acetylation and levels of RNA synthesis in many different cell types. Thus, there is an increase in histone acetylation in mitogen stimulated lymphocytes (Pogo et al, 1966), regenerating liver cells (Pogo et al, 1968) and in numerous cell types stimulated by hormones (Ruiz-Carrillo et al, 1975). Conversely, inactivation of avian erythrocyte nuclei is accompanied by a progressive decrease in the proportion of acetylated histones (Ruiz-Carrillo et al, 1974).

More direct evidence for a role of acetylation in the conformation of active chromatin has been obtained recently. Using the chromatin fractionation technique of Gottesfeld et al (1974) (see section 1.5.5) an increased proportion of acetyl groups has been found in the "active" fraction of Drosophila chromatin (Levy-Wilson et al, 1977) and trout testis chromatin (Davie and Candido, 1978). Micro-

coccal nuclease has also been employed to release a fraction of trout testis chromatin enriched in transcribed DNA sequences, and also containing high levels of multiacetylated histone H_4 as well as acetylated H_3, H_{2A} and H_{2B} (Levy-Wilson et al, 1979).

Many recent studies on histone acetylation have made use of the discovery that sodium butyrate leads to hyperacetylation of histones H_3 and H_4 (Riggs et al, 1977). Sodium butyrate has been shown to inhibit the deacetylation of histones both in vivo (Boffa et al, 1978; Reeves and Candido, 1978) and in vitro (Boffa et al, 1978; Sealy and Chalkley, 1978; Candido et al, 1978), without affecting the rate of acetylation (Candido et al, 1978; Vidali et al, 1978). Thus, acetyl groups rapidly accumulate on the histones, but only in the particular sites which are acetylated under normal conditions. This has provided a useful tool for studying the effects of these modifications on chromatin structure.

In this way, it has been shown that the DNA in HeLa cell nuclei containing hyperacetylated chromatin is digested much more rapidly by DNase I than in control nuclei (Simpson, 1978b; Vidali et al, 1978). The histones remaining after limited digestion of these nuclei are depleted in multiacetylated histones H_3 and H_4 (Vidali et al, 1978). DNase I also selectively releases ^3H-acetate labelled H_3 and H_4 from avian erythrocyte nuclei under conditions known to preferentially degrade globin genes (Vidali et al, 1978).

1.2.2. Phosphorylation of histones

Whilst acetylation is restricted to the nuclease core histones and is correlated with transcriptional activity, phosphorylation of all histones occurs and appears to have a number of diverse

functions. Thus, phosphorylation of histones occurs at three times:- S phase of the cell cycle, mitosis, and after hormonal stimulation of cells. In each case, different specific serine and threonine residues are phosphorylated (Isenberg, 1979).

The phosphorylation of histone H_1 in Physarum occurs just before the synchronous nuclear diversion which occurs in this organism, and has been proposed as a "mitotic trigger" (Bradbury et al, 1974). In mammalian cells, 2 types of H_1 phosphorylation have been identified; one occurring during interphase, and the other related to mitosis (Gurley et al, 1978). Thus one proposed function of H_1 phosphorylation is the condensation of chromatin during mitosis. Phosphorylation of H_3 has also been implicated with mitotic condensation and H_1 and H_{2A} with heterochromatin condensation (Gurley et al, 1978).

Histone phosphorylation also correlates with S-phase of the cell cycle, and a role in deposition of newly synthesised histones on replicating DNA has been suggested (Jackson et al, 1975).

The phosphate groups of the nucleosome core histones turnover much more rapidly than those of histone H_1 (Jackson et al, 1975). Phosphorylated histones have been preferentially isolated in a "template active" chromatin fraction of Drosophila chromatin (Levy-Wilson et al, 1977), and therefore a role in transcription may also be possible.

1.2.3. Methylation of histones

The lysine groups of histones H_3 and H_4 may be methylated. This modification is specific for lysine residues in positions 9 and 27

in H_3, and lysine 10 in H_4 (Isenberg, 1979). This modification occurs late in S phase and in G2 phase, probably after chromatin replication (Borun et al, 1973; Thomas et al, 1975). Histone methyl groups turnover slowly and are of unknown function, although a role in chromatin condensation during mitosis has been suggested (Thomas et al, 1975; Honda et al, 1975).

1.2.4. ADP-ribosylation of histones

Nuclei contain an enzyme activity capable of forming a polymer of poly(ADP-ribose) from NAD^+ (e.g. Hayaishi and Ueda, 1977). Most of this polymer is found bound to histones, particularly histone H_1, probably by an ester linkage to glutamate or aspartate carboxyl residues.

The role of this modification is not clear, although a role in DNA replication or repair seems more likely than a role in transcription (Hayaishi and Ueda, 1977). More recently, a role in chromatin condensation has been postulated, based on the formation of histone H_1 dimers by this modification (Stone et al, 1977), and the maximum activity of the enzyme in oligonucleosomes of 8-10 nucleosomes (Butt et al, 1979), a similar size to the "superbeads" of chromatin observed by Renz et al (1977).

2. Eukaryote transcriptional apparatus

2.1. Eukaryote RNA polymerases

Whilst prokaryotes appear to have only one type of RNA polymerase, with a relatively simple structure (Chamberlin, 1976; Burgess, 1976), there are a number of RNA polymerase classes with

different functions in eukaryotes, none of which are structurally analogous to prokaryote RNA polymerase.

2.1.1. Classes of eukaryote RNA polymerases

The different RNA polymerase classes in eukaryotes were originally detected and classified according to the order of elution from DEAE-Sephadex, into classes I, II and III (Roeder and Rutter, 1969, 1970). These different classes of enzymes were found to have different properties with regard to salt optima and divalent cation requirements *in vitro*. Thus, from most, but not all organisms, class I has a salt optimum of $\sim$ 0.05M $(NH_4)_2SO_4$ and a preference for Mn^{2+}, class II has a salt optimum of 0.13M $(NH_4)_2SO_4$ and a preference for Mn^{2+} ions, and class III typically has a broad salt optimum of 0.05M - 0.20M and a preference for Mn^{2+} ions (Roeder, 1976; Beebee and Butterworth, 1977). A major breakthrough in defining the separate classes of enzyme was the discovery that the fungal bicyclic peptide, α-amanitin, specifically inhibits class II RNA polymerases at low concentrations (Kedinger *et al*, 1970; Lindell *et al*, 1970).

RNA polymerase III is generally sensitive to high concentrations of the drug, whereas RNA polymerase I is insensitive (Weinmann and Roeder, 1974). The drug has its effect by binding to the RNA polymerase, rather than the template, and blocks elongation (Cochet-Meilhac and Chambon, 1974).

2.1.2. The functions of eukaryote RNA polymerases

Particularly by applying α-amanitin sensitivity in conjunction with the salt and divalent cation requirements to studies with isolated nuclei, it has been possible to localise and determine

the function of each polymerase class. Class I enzymes are restricted to the nucleolus, whilst classes II and III are found in the nucleoplasm (Roeder and Rutter, 1970).

The synthesis of ribosomal RNA in isolated nuclei and nucleoli is optimal at low ionic strength and with magnesium ions and is insensitive to α-amanitin, and is therefore synthesised by class I enzymes (Novello and Stirpe, 1969). The synthesis of heterogenous RNA in isolated nuclei is optimal at high ionic strength and in the presence of manganese ions (Widnell and Tata, 1966; Novello and Stirpe, 1969), and is sensitive to α-amanitin in low concentrations (Weinmann and Roeder, 1974). It was therefore deduced that hnRNA is synthesised by RNA polymerase II, and following from the proposal that some hnRNA molecules are mRNA precursors (see section 3.1), it has been presumed that mRNA sequences are transcribed by RNA polymerase II. More recently, this has been directly demonstrated by showing the α-amanitin sensitivity of the de novo synthesis of particular mRNA sequences in isolated nuclei (e.g. silk fibroin mRNA sequences, Suzuki and Giza, 1976; ovalbumin mRNA sequences, Roop et al, 1978; lysozyme, Nguyen-Huu et al, 1978; histone, Detke et al, 1978).

The sensitivity to α-amanitin of 4.5S precursor tRNA and 5S ribosomal RNA synthesis in vitro matches that of isolated RNA polymerase III, and they are therefore presumed to be synthesised by RNA polymerase III in vivo (Price and Penman, 1972; Weinmann and Roeder, 1974; Marzluff et al, 1974; Weinmann et al, 1976).

2.2. Methods for studying eukaryote transcription in vitro

The assays from which the salt optima and divalent cation

requirements were obtained for the RNA polymerase classes involve transcription of purified DNA with purified polymerases. These transcriptional events, however, are non-specific and the RNA polymerases, particularly classes I and II, tend to initiate synthesis at single strand nicks under these conditions (Beebee and Butterworth, 1977). The search for a useful system with which to study eukaryote transcription *in vitro* has therefore tended to examine more intact templates or homologous cell free RNA synthesis systems.

2.2.1. Transcription of chromatin by E. coli RNA polymerase in vitro

Early studies in this field suggested that the transcription of isolated chromatin by *E. coli* polymerase produced RNA specific for the cell from which the chromatin was isolated. However, these studies were only able to detect highly repetitive sequences, and were in fact no more specific than the transcription of the corresponding deproteinised DNA (Reeder, 1973). More recent studies have probed for specific gene transcription with cDNA to particular mRNA species, and have indicated that *E. coli* RNA polymerase can preferentially transcribe abundant mRNA sequences specific to the cell of origin of the chromatin; e.g. globin sequences from erythrocyte chromatin (Axel *et al*, 1973; Gilmour and Paul, 1973), ovalbumin sequences from oviduct chromatin (Tsai *et al*, 1976) and histone sequences (Stein *et al*, 1975). However, the type of experiment is unreliable since it is difficult to control the presence of endogenous RNA in the chromatin preparation which will also be detected by the probe.

As a measure to eliminate the problem of endogenous RNA contamination, the use of mercurated nucleotides (Dale and Ward, 1975)

in the transcription system, followed by separation of de novo transcripts on sulphydryl-columns has been tried. However, this method has its own artefactual problems, particularly that of aggregation of RNA on the column, leading to retention of endogenous RNA (Konkel and Ingram, 1977; Crouse et al, 1976). It was also found using this method that E. coli RNA polymerase could transcribe endogenous RNA to give a "minus strand" RNA copy (Zasloff and Felsenfeld, 1977a and b; Giesecke et al, 1977). This "anti-sense" RNA could then act as a template for de novo transcription of "sense" RNA (Pays et al, 1979). Thus, previous studies are suspect both from the presence of endogenous RNA, and the possibility that this RNA might itself have been the template for E. coli RNA polymerase transcription.

2.2.2. Transcription of chromatin and DNA by eukaryote RNA polymerases

Systems involving the transcription of chromatin by eukaryotic RNA polymerases promise to yield more information about the transcription of specific genes and the role of particular RNA polymerase classes in this transcription.

Successful results have been reported using such techniques in the past two years. For instance, exogenous Xenopus RNA polymerase III will selectively and accurately transcribe the 5S RNA genes of isolated Xenopus oocyte chromatin (Parker and Roeder, 1977). Similarly, the 5S RNA genes of HeLa cell chromatin are selectively transcribed by added RNA polymerase III (Yamamoto et al, 1977b). The transcription of 5S RNA genes by RNA polymerase III does not require an intact chromatin template, since 5S RNA is produced from the transcription of a plasmid containing 5S DNA by exogenous RNA polymerase III

in the presence of a soluble extract from Xenopus oocytes (Ng et al, 1979). Deproteinised adenovirus DNA is also faithfully transcribed by RNA polymerase III in the presence of a soluble extract from KB cells (Wu, 1978; Weil et al, 1979a). The nature of the factors in these extracts which direct the faithful transcription by RNA polymerase III is not yet known. It has recently been shown that adenovirus sequences can be selectively transcribed by RNA polymerase II in a similar reconstituted system (Weil et al, 1979b).

2.2.3. Transcription of chromatin by endogenous RNA polymerases

The initial observation that isolated nuclei can synthesise RNA in vitro (Weiss and Gladstone, 1959), has led to the use of isolated nuclei to study various aspects of RNA transcription and processing.

Isolated nuclei have been particularly useful for studies determining the effect on transcription of various factors which would not be feasible in vivo. An outstanding example of this was the elucidation of the functions of the three RNA polymerase classes using the drug α-amanitin (Lindell et al, 1970; Kedinger et al, 1970; Weinmann and Roeder, 1974) (see section 2.2.1.). This drug does not readily enter the living cell, making any similar inhibition studies difficult in vivo. More recently, the transcription of specific mRNA sequences by RNA polymerase II has been demonstrated by the α-amanitin sensitivity of their synthesis in isolated nuclei (e.g. silk fibroin mRNA, Suzuki and Giza, 1976; lysozyme, Nguyen-Huu et al, 1978; ovalbumin, Roop et al, 1978; and histone mRNA sequences, Detke et al, 1978). In most of these examples the newly synthesised RNA was separated from old transcripts by incorporating mercurated nucleotides

into the RNA synthesised *in vitro*, and isolating this RNA on sulphydryl columns (Dale and Ward, 1975).

This technique has been widely used to demonstrate the specific synthesis of mRNA sequences in isolated nuclei. For instance, apart from the mRNA sequences mentioned above, globin (Fodor and Doty, 1977; Orkin and Swerdlow, 1977, 1978), immunoglobin K light chain (Smith *et al*, 1976), vitellogenin (Panyim *et al*, 1978) and α-2-globulin mRNA sequences (Chan *et al*, 1978) have been demonstrated to be present in the population of de novo transcripts of the corresponding nuclei at a concentration many orders of magnitude higher than expected for random transcription of the genome.

Studies on transcription by RNA polymerase I and III have not required such elaborate technology due to the relative homogeneity of their products. Thus, the transcription of precursor tRNA of discrete 4-5S size by endogenous RNA polymerase III has been shown (Marzluff *et al*, 1974; McReynolds and Penman, 1974; Weinmann and Roeder, 1974). Ribosomal 5S RNA is also transcribed faithfully in isolated nuclei by endogenous RNA polymerase III (Yamamoto *et al*, 1977a, 1978).

There is also good evidence that 45S precursor ribosomal RNA is synthesised correctly by RNA polymerase I in isolated nuclei and nucleoli, by the criteria of size (Udvardy and Seifert, 1976) and sequence (Reeder and Roeder, 1972).

2.3. Transcription units

As will be described later, it has become important, particularly in eukaryotes, to distinguish between sequences in the

genome which code for proteins when the corresponding mRNA is translated, and "transcription units." The former correspond to genetically defined structural genes, whereas the latter may best be operationally defined as the region of DNA in the genome between functioning start and stop signals for RNA polymerase. Transcription of such a region will generate a "primary transcript." Therefore, a transcription unit could be identified either by examining the DNA template or the RNA primary transcript.

2.3.1. Prokaryote transcription units

It has been possible to define regions of the prokaryote genome which contain structural gene sequences and are flanked by promotor and terminator sequences at the 5' and 3' end of the mRNA sequence. In these cases, the primary transcript of the transcription unit is the mRNA, which indeed is translated whilst still being transcribed. It is known that some mRNAs are polycistronic (i.e. more than one structural gene is contained within the transcription unit). Moreover, the transcription of such a transcription unit containing related genes is typically controlled at the level of restriction or promotion of initiation, as predicted by the operon theory of Jacob and Monod (1961).

2.3.2. Eukaryote transcription units

2.3.2.1. Ribosomal transcription units

Using a number of techniques, eukaryote ribosomal transcription units have been well characterised.

Early kinetic studies identified the 45S primary precursor

of ribosomal RNA (Scherrer and Darnell, 1962), and this was shown to contain both 28S and 18S native ribosomal RNA (Maden, 1971). The gene spreading technique showed that the length of the ribosomal transcription unit, as visualised as the well known "Christmas-tree" array, was in good agreement with the size of the primary precursor rRNA (Miller and Hamkalo, 1972).

The topology of the ribosomal transcription unit has been probed by kinetic methods, U.V. mapping and enzymatic mapping of the precursor rRNA. Despite previous results to the contrary, the available evidence supports an organisation of the ribosomal transcription unit as:- 5' end-transcribed spacer - 18S rRNA - transcribed spacer - 5.8S rRNA - 28S r RNA - non-transcribed spacer (Hackett and Sauerbier, 1975; David amd Wellauer, 1976). The arrangement of ribosomal transcription units within the genome is one of clustered, multiple copies in tandem array, each transcription unit separated by non-transcribed spaces.

Intervening sequences have been detected in some of the rRNA genes in Drosophila (Glover and Hogness, 1977; Wellauer and David, 1977), but have not been found in the rDNA of other organisms.

2.3.2.2. 5S RNA Transcription units

The 5S RNA genes in Xenopus have been well characterised, and are found in tandem clusters on most of the chromosomes. Oocyte and somatic-type genes of Xenopus laevis and Xenopus borealis have been isolated, and some repeat units have been sequenced (Federoff and Brown, 1978; Miller et al, 1978; Korn and Brown, 1978). A comparison of sequences flanking different types of Xenopus 5S genes

with Drosophila and yeast 5S genes and adenovirus 2 VA genes, revealed sequence homologies which are potential recognition signals for initiation and termination of transcription of these genes by RNA polymerase III (Korn and Brown, 1978).

There is evidence that 5S RNA is transcribed as a larger precursor molecule, extended at the 3' end by about 15 nucleotides in Drosophila (Rubin and Hogness, 1975; Jacq et al, 1978) and 8 nucleotides in rat liver and HeLa cells (Hamada et al, 1979).

2.3.2.3. tRNA Transcription units

Eukaryote tRNA genes are also repetitive and clustered in the genomes of animals that have been studied (e.g. Drosophila, Yen et al, 1977; Xenopus, Clarkson et al, 1978).

In yeast, some, but not all, tRNA genes are interrupted by a very short intervening sequence (Valenzuela et al, 1978) which is transcribed as part of a precursor molecule (Hopper et al, 1978; Knapp et al, 1978; O'Farrell et al, 1978).

Apart from inserts, eukaryote tRNAs are transcribed as longer precursor molecules which sediment at $\sim$ 4.5S, compared to 4S for mature tRNA, indicative of a difference in length of 15-35 nucleotides (Burdon, 1975). Using Xenopus oocyte nuclei to transcribe cloned yeast tyrosine tRNA genes, a primary transcript containing a 5' leader sequence as well as the intervening sequence, was identified, and it was found that the CCA 3' end of this tRNA is not encoded in the DNA, but added post-transcriptionally (de Robertis and Olsen, 1979).

2.3.2.4. Transcription units for hnRNA and mRNA

The determination of the size of transcription units from

which mRNA is transcribed has not been possible by the analysis of precursor molecules. Such studies have shown that the hnRNA population is kinetically complex, and that there is not a simple precursor-product relationship between hnRNA and mRNA (see 3.1.2.1). Therefore, other techniques have been used to study hnRNA and mRNA transcription units.

Using very short radioactive labelling times, a "nascent transcript profile" of HeLa cell non-ribosomal RNA was obtained (Derman et al, 1976). From this, a size range of primary transcript products of 2-20 kilobases was calculated, with roughly one half of these greater than 5 kilobases and one half less than 5 kilobases in size.

U.V. irradiation mapping (Sauerbier and Hercules, 1978) of the target size of the nascent hnRNA population also showed about 50% of transcripts to exceed 6-8 kilobases (Goldberg et al, 1977b).

Analysis of the target size of total HeLa cell mRNA suggests an average transcription unit size of 4-7 kilobases, overlapping considerably with the hnRNA target size. In comparison, the number average size of HeLa mRNA is 1-5 kilobases (Darnell, 1976a).

Similarly, in mouse myeloma cells the U.V. target sizes of ten predominant mRNA species ranged from 2-14 times the size of the mature mRNA (Giorno and Sauerbier, 1978). Thus, these results prove that mRNA is in general derived from larger transcription units, and hence from a larger primary transcript, and are suggestive that these primary transcripts constitute a part of the hnRNA population.

Details about the average length and distribution of non-ribosomal transcription units has also come from analysis of chromatin

spreads from suitable tissues, particularly from insects. In Oncopeltus fasciatus (milkweed bug) embryos, a range of non-ribosomal transcription units with a mean length of 22 kilobases was observed (Foe et al, 1976). These transcription units were all apparently solitary and spaced apart an average distance of greater than 20 kilobases, as opposed to being in tandem arrays like ribosomal genes. Similarly, in Drosophila melanogaster embryos a mean of 18 kilobases for the length of non-ribosomal transcription units was measured, and these arrays were also isolated from other fibre arrays (Laird and Chooi, 1976).

2.3.2.5. Transcription units for specific mRNAs

Adenovirus transcription units

The techniques outlined above have been used to define transcription units within the adenovirus-2 genome. Nascent chain analysis combined with a detailed restriction map of the genome allowed the initiation sites of four early transcription units of 2-6 kilobases long to be located (Evans et al, 1977; Craig and Raskas, 1976). The major late transcription unit of 28 kilobases long was also mapped using this method (Bachenheimer and Darnell, 1975; Weber et al, 1977; Evans et al, 1977), and the technique of U.V. mapping (Goldberg et al, 1977a). These results showed that the entire right hand 80-85% of the genome comprises this one continuous transcription unit, read in a rightward direction late in infection.

There are at least 14 different adenovirus mRNAs produced late in infection. Working out the organisation of these mRNA sequences within the major late transcription unit was the first

demonstration of non-contiguous genes. It was found by electron microscopical visualisation of mRNA-DNA hybrids (Berget et al, 1977; Chou et al, 1977), and RNA oligonucleotide analysis (Gelinas and Roberts, 1977; Klessig, 1977), that the 5' terminus of each mRNA contained ~ 200 nucleotides not contiguous with the major portion of the mRNA sequence. All these 5' terminal sequences are complementary to three distinct, distant sites on the genome, which map at positions 16.6, 19.6 and 26.6 on a left to right scale of 0-100.

The late mRNAs can be grouped into five 3' coterminal groups, such that within each group from 2-4 different 5' junctions exist between the tripartite leader and the body of the mRNA (Ziff and Fraser, 1978; Nevins and Darnell, 1978b). The transcription and processing of this transcription unit will be described later (section 3.1.4).

2.4. Organisation of specific gene sequences within the eukaryote genome

The transcription unit analysis described for the adenovirus genome has not been widely applicable to specific eukaryote mRNA transcription units. On the other hand, the advent of recombinant DNA technology along with other techniques has provided a means of preparing highly purified mRNA sequences and genomic sequences, and determining the organisation of the former within the latter.

2.4.1. Globin genes

Intervening sequences in mouse β-globin were detected by cloning β-globin genomic fragments, and using this cloned DNA for "R-loop mapping" with globin mRNA to visualise an intervening sequence

of 550 base pairs in a similar position to the rabbit gene (Tilghman et al, 1978a). Direct sequencing of this cloned DNA established the position of the intervening sequence to be after amino acid 104. Since then, the entire coding regions, intervening sequences and flanking regions of the clone have been sequenced (Konkel et al, 1978). This cloned DNA contains the mouse β-globin major gene and contains the large intervening sequence of 646 base pairs detected previously, and one of 116 base pairs between the codons for amino acids 30 and 31. Comparison of cloned mouse and rabbit β globin genes shows that the rabbit gene also has two inserts in the same position as in the mouse gene, but showing considerable evolutionary divergence of the intervening sequences (van der Berg et al, 1978).

Similarly, the mouse α-globin gene has two inserts in positions analogous to the β genes, although of smaller size (Leder et al, 1978). On the other hand, the β-globin major inserted sequences are found only in the β-globin genes, not in the α-genes or elsewhere in the genome (Miller et al, 1978).

2.4.2. Ovalbumin genes

The presence of at least six intervening sequences in the ovalbumin gene was inferred from a number of studies comparing cloned ovalbumin cDNA and genomic DNA fragments (Garapin et al, 1978; Mandel et al, 1978; Breathnach et al, 1978; Dugaiczik et al, 1978). Recent work on a cloned chick genomic DNA fragment containing the complete ovalbumin gene, including the 5' terminal leader sequence, has confirmed the existence of 7 intervening sequences, and shown the minimal size of the ovalbumin transcription unit to be 7.7 kilobases, about four times the size of the mature mRNA (Gannon et al, 1979).

2.4.3. Immunoglobulin genes

Immunoglobulin genes appear to be exceptional in that, apart from containing intervening sequences, there is evidence that different gene regions are rearranged during development. Cloned DNA fragments containing light chain V region sequences from the embryonic mouse genome were sequenced and found to code for most of the V region, including a small insert, from the leader sequence to amino acid 97 or 98 (Tonegawa et al, 1978; Lenhard-Schuller et al, 1978; Seidman et al, 1978). The remaining 10-15 codons of the V region (called the J segment), and the entire C region were not present in the cloned fragments.

In contrast, a complete light chain gene from mouse myeloma cell DNA contained the V, J and C regions, with a continuous amino acid coding sequence running across the V/J boundary (Brack et al, 1978). The C region is separated from the J region by a large 1.25 kilobase pair intervening sequence. Therefore, it appears that during development of immunoglobulin producing cells, one type of (C + J) region is brought into association with one of a number of related V regions by reorganisation of the genome (Seidman et al, 1978).

An immunoglobulin γ heavy chain constant region has also been cloned, and the presence of 2 intervening sequences visualised by R-loop mapping, with a smaller one also deduced from restriction mapping (Sakano et al, 1979). Sequencing of the cloned DNA has shown that the four separated coding regions in the DNA correspond exactly to the three homologous protein domains in the heavy chain protein constant region (CH_1, CH_2 and CH_3), and the 14-residue "hinge" region

between CH_1 and CH_2. These domain regions have evolved from a similar sequence, but have different functions in the immunoglobulin molecule. It has been proposed that the coding of functional domains bounded by splice sites allows the genome to combine these protein domains in any combination (Clark, 1979).

3. hnRNA

3.1. hnRNA as mRNA precursor

For many years it has been generally accepted that a proportion of hnRNA may be a precursor of mRNA (Lewin, 1975a and b), although without any direct evidence. A number of different types of experiment have been employed to establish this precursor-product relationship.

3.1.1. Labelling kinetics of hnRNA and mRNA

As described above, the hnRNA population is kinetically complex (e.g. Herman and Penman, 1977), and only a small proportion of the nucleotides present in hnRNA enters the cytoplasm (Brandhorst and McConkey, 1974; Herman and Penman, 1977). Therefore, it has not been possible to establish a simple precursor-product relationship between the total hnRNA and mRNA populations by simple "pulse-chase" procedures.

3.1.2. Polyadenylation of hnRNA and mRNA

A second line of research has focussed on the fact that a portion of both hnRNA and mRNA molecules contain a polyadenylate tract (Edmonds et al, 1971; Lee et al, 1971) located at the 3' terminus (Nakazato et al, 1973). In briefly labelled HeLa cells, the poly(A)

"tail" isolated from nuclear and cytoplasmic RNA is the same size (200-250 nucleotides).

From these results, the working hypothesis was adopted that some poly(A) terminated hnRNA molecules were precursor to poly(A) terminated mRNA. Experiments designed to show that nuclear poly(A) is transported to the cytoplasm made use of the fact that the synthesis of hnRNA and mRNA has the same sensitivity to actinomycin D inhibition (Penman et al, 1968; Perry and Kelley, 1970), whereas cordycepin (3' deoxyadenosine) was reported to have no effect on hnRNA synthesis, but appeared to block mRNA labelling by inhibition of polyadenylation (Darnell et al, 1973).

Attempts to determine the proportion of nuclear poly(A) transported to the cytoplasm by measuring the kinetics of accumulation of nuclear and cytoplasmic poly(A) showed that nuclear poly(A) accumulated for many hours with identical kinetics to hnRNA, and it was concluded that much of the nuclear poly(A) decays in the nucleus (Perry et al, 1974; Brandhurst and McConkey, 1975).

However, part of this conclusion is based on a calculated turnover rate of mRNA in the cytoplasm as being highly stable and having two kinetic components with half lives of 7 and 24 hours (Singer and Penman, 1973). Since then, a short lived mRNA component has been detected with a half life of one hour (Puckett et al, 1975, 1976). Taking this component into account, results consistent with the conservation of poly(A) from nucleus to cytoplasm have been obtained (Puckett et al, 1975, 1976).

3.1.3. Methylation of hnRNA and mRNA

Methyl groups were detected in eukaryotic mRNA in two types

of nucleotide structures:- blocked 5' termini ("caps") of the general type $m^7G^{5'}ppp^{5'}N'mpN''p$ .... , and internal N^6-methyl adenosine separate from the cap (Perry and Kelley, 1974).

"Cap" structures

The cap structures appearing in mRNA are either $m^7G^{5'}ppp^{5'}N'p$ (cap 0), $m^7G^{5'}ppp^{5'}N'mpN''p$ (cap I) or $m^7G^{5'}ppp^{5'}N'mpN''mp$ ... (cap II), where N'm and N"m are methylated at the 2'OH group of the ribose moiety (Perry et al, 1975; Perry and Kelley, 1976). mRNAs from all the eukaryotes studied so far are capped, as are all the individual eukaryotic mRNAs studied so far (Shatkin, 1976). HeLa cell mRNA contains ~ 1 cap structure/mRNA molecule (Salditt-Georgieff et al, 1976).

Two main functions have emerged for the cap structure, mainly from studies with "decapped" mRNA, and cap analogues. The cap appears to facilitate mRNA entry into the initiation complex with eukaryotic ribosomes, and may also have a protective role against degradation of mRNA by cellular nucleases (Filipowicz, 1978).

The 5' terminus of at least 40-60% of mouse L cell and HeLa cell hnRNA is also capped with cap I structures, but not cap II (Perry and Kelley, 1976; Salditt-Georgieff et al, 1976). The pathways of formation of this cap have been deduced by analysis of the 5' termini of the hnRNA population (Schibler and Perry, 1976). It is proposed that initiation of hnRNA synthesis occurs exclusively with a purine, and that 5' pyrimidine termini are formed by internal cleavage. Caps are then added both to internal sites and to the 5' terminus of the primary transcript (Schibler and Perry, 1976).

Kinetic analysis indicates that the cap I structures of mRNA are derived from the cap I structures of hnRNA, and that most of the hnRNA caps are conserved during processing (Perry and Kelley, 1976). Comparison of cap-containing sequences in mRNA and hnRNA showed a similar sequence composition of hnRNA and mRNA caps, also consistent with a precursor-product relationship between hnRNA and mRNA caps (Schibler and Perry, 1977).

Internal m^6-adenosine

There is also evidence that the internal m^6-adenovirus which occurs in some, but not all, mRNAs, is derived from m^6-adenosine in hnRNA. It is not yet clear, however, whether some (Perry and Kelley, 1976), or all (Schibler and Perry, 1977) of these methylated residues in hnRNA are transported to the cytoplasm.

3.2. Sequence organisation of hnRNA and mRNA

3.2.1. Sequence complexity of hnRNA and mRNA

Calculation of the sequence complexity of nuclear RNA can be used to estimate the proportion of the genome transcribed. These results show that, for instance, 20% of the single copy rat liver nuclear DNA is transcribed, corresponding to a sequence complexity of roughly 4×10^8 nucleotides, whilst the complexity of rat kidney, spleen and thymus hnRNA is roughly 2×10^8 nucleotides (Chickaraishi et al, 1978). In contrast, the sequence complexity of rat liver mRNA is estimated to be $5\text{-}6 \times 10^7$ nucleotides (Savage et al, 1978). Similar studies in other mammalian tissues also show that the sequence complexity of hnRNA is at least 5 times more complex than the corresponding mRNA (e.g. Jacquet et al, 1978; Kleiman et al, 1977).

The sequence complexity of an mRNA population gives an indication of the percentage of the genome expressed, and hence the number of genes of average length expressed. In higher eukaryotes, typically 1-5% of the genome is expressed in the mRNA population, corresponding to 10-30,000 average mRNA length genes (e.g. Kiper, 1979).

3.2.2. Sequence homology between hnRNA and mRNA sequences

A number of experiments have demonstrated homology between some mRNA sequences and hnRNA sequences. The poly(A)-adjacent sequences in HeLa cell steady state hnRNA contain all the mRNA sequences, but in addition, 30% of these hnRNA sequences are not homologous to mRNA (Herman et al, 1976). Approximately 65% of pulse-labelled poly(A)-adjacent hnRNA sequences are also homologous to the 3' termini of mRNA (Herman, 1979). Similarly, in mouse embryonal carcinoma cells, the poly(A) adjacent sequences in steady state nuclear RNA correspond primarily to polysomal mRNA sequences, despite the much greater complexity of the poly(A)$^+$ hnRNA population (Jacquet et al, 1978). Little homology in the poly(A)$^-$ hnRNA population and mRNA was found in these cells.

Homology between mRNA and hnRNA in mouse L cells was demonstrated by hybridisation of genomic DNA complementary to mRNA ("mDNA") to various pulse-labelled hnRNA fractions (Hames and Perry, 1977). The extent of homology between different classes of hnRNA and mRNA (see section 3.1.1) suggested that a substantial proportion of the large rapidly labelled hnRNA molecules are potential precursors of mRNA, whilst the small poly(A)$^+$ hnRNA may consist of partly processed derivatives of these molecules, enriched in mRNA sequences.

3.3. Precursors of specific mRNAs

3.3.1. Adenovirus-2 late mRNA precursors

The mapping of the major late adenovirus transcription unit has already been described (section 2.3.2.5). In particular, all the late mRNAs from this region of the genome share the same primary transcript (Goldberg et al, 1978), and contain the common spliced tripartite leader sequences which map at the 5' end of the transcription unit (Evans et al, 1977; Weber et al, 1977). It has been shown that the capped 5' terminus of late adenovirus nuclear and mRNA is coincident with the promoter region of the transcription unit (Ziff and Evans, 1978). Furthermore, this study showed the presence of caps in large unspliced poly(A)$^+$ adenovirus nuclear RNA.

The late mRNAs can be grouped into five 3' coterminal groups, such that within each group 2-4 different 5' junctions exist between the leader and the body of the mRNA (Nevins and Darnell, 1978b). Roughly 20% of each of five different regions of the late primary transcript is conserved on transport from the nucleus to the cytoplasm, suggesting that each primary transcript gives rise to one of the five groups of 3' coterminal mRNAs (Nevins and Darnell, 1978b). On the other hand, conservation of nuclear poly(A)$^+$ RNA approaches 100%, and addition of poly(A) at one of the five possible sites appears to be the first step in the selection of the particular mRNA formed from each primary transcript (Nevins and Darnell, 1978a). This polyadenylation occurs after cleavage of the primary transcript, rather than at a site of RNA polymerase termination (Nevins and Darnell, 1978a), and this cleavage is performed before completion of transcription, even though complete transcription appears to be necessary for correct processing (Goldberg et al, 1978).

The polyadenylation of the late adenovirus major primary transcript precedes any splicing events (Nevins and Darnell, 1978a). Analysis of nuclear poly(A)$^+$ RNA reveals the expected five primary unspliced poly(A)$^+$ RNAs (Nevins, 1979). Following the fate of the largest of these, formed by polyadenylation at the most promotor-distal site, and the precursor of the fibre mRNA, three discrete sized processing intermediates were discovered (Nevins, 1979). The kinetics of formation of these intermediates were consistent with a sequential precursor-product relationship from a 26 kilobase RNA to 11.5 KB RNA to 6.3KB RNA to a 3.2 KB nuclear RNA the size of native fibre mRNA. All these processing steps occurred in the nucleus, with the primary product having a half-life of 6 minutes, and the others with half-lives of roughly 10 minutes each, resulting in an observed time of 30 minutes for transport to the cytoplasm of adenovirus mRNA (Nevins, 1979).

3.3.2. Globin mRNA precursors

For a number of years the presence of globin mRNA sequences in nuclear RNA was implied from studies on the translation of hnRNA *in vitro*, and the probing of steady state hnRNA with cDNA to globin mRNA. Despite the misleading nature of these early experiments, recent evidence has confirmed the presence of such globin sequence-containing molecules and shown them to be physical precursors of globin mRNA. Thus, a 14-15S globin mRNA precursor was detected in mouse foetal liver cells (Ross, 1976), Friend cells (Curtiss and Weissman, 1976) and mouse spleen (Kwan *et al*, 1977). In none of these studies was a larger precursor found, although a rapidly processed primary transcript might not have been detected under the conditions of labelling and analysis used in these experiments.

Other studies of this type detected a precursor larger than 15S, of ~ 27S (Bastos and Aviv, 1977; Strair et al, 1978).

The existence of a large 27S globin precursor RNA is in doubt, however, since of the three RNA species which bind to immobilised globin cDNA, only the 15S and 10S RNA bind to cloned β-globin cDNA, whereas 27S RNA appears in the non-bound fraction (Haynes et al, 1979). It has therefore been suggested that the 27S material is a precursor to a mRNA which commonly contaminates globin cDNA.

The β-globin 15S RNA precursor is an equivalent length to that of the β-globin gene region which maps between the 5' and 3' termini of globin mRNA (Konkel et al, 1978). Electron microscopy of hybrids formed between purified 15S RNA and cloned β-globin genes does not indicate any looped out intervening sequences, as opposed to hybrids of globin mRNA with the β-globin gene which reveal the large intervening sequence (Tilghman et al, 1978b). The existence of both of the β-globin gene intervening sequences in 15S nuclear RNA has now been demonstrated (Smith and Lingrel, 1978; Kinniburgh et al, 1978).

3.3.3. Immunoglobulin precursor mRNAs

Cloned cDNA to immunoglobin light chain mRNA was used to probe myeloma cell nuclear RNA sedimented through denaturing gradients (Gilmore-Herbert and Wall, 1978). Three complementary size classes of RNA sedimenting at 40, 24 and 13S were found, and a sequential precursor-product relationship was demonstrated between these RNAs, and from the 13S nuclear RNA to 13S immunoglobulin mRNA. The size of 40S hnRNA is in good agreement with the U.V. mapped transcription unit size of 9.6 kilobases (Gilmore-Herbert et al, 1978).

Moreover, the nuclear RNA transcription unit target size is the same as the mature mRNA U.V. inactivation target size, confirming the precursor-product relationship between the 40S RNA and the mature mRNA, and suggesting that the 40S RNA is the primary transcript of this mRNA transcription unit.

3.4. Processing of hnRNA

The examples presented above suggest that capping and polyadenylation are rapid events which may occur at the 5' and 3' end of the primary transcript. Subsequent processing events may remove intervening sequences from the primary transcript, whilst conserving both the 5' and 3' terminal sequences.

One approach to understanding the "splicing" reactions which remove intervening sequences from precursor mRNAs has been to sequence the boundaries between message and intervening sequences. A comparison of these boundary sequences has revealed considerable homology between the junctions in eukaryotic and viral mRNA genes (Catterall et al, 1978; Konkel et al, 1978; Dawid and Wahli, 1979). However, the consensus sequences established for the 5' and 3' junctions of intervening sequences are not compatible with a model for splicing involving base pairing between the boundary regions at either end of an insert.

3.5. Distinctive sequences in hnRNA

3.5.1. Double stranded sequences

Double stranded sequences can be isolated from hnRNA after ribonuclease treatment (Jelinek and Darnell, 1972). The complementary regions appear to be separated by either a few nucleotides, forming a

"hair-pin" structure, or several thousand nucleotides, forming a double-stranded "stem" and single stranded "loop" structure (Jelinek et al, 1974). HeLa cell hnRNA of various size classes consists of ~ 3% double stranded sequences, suggesting that the larger hnRNA molecules contain several double stranded regions.

There is good evidence that these double stranded loops in hnRNA occur in vivo, since they are present in isolated hnRNA particles (Calvet and Pederson, 1977), and can be crosslinked by bifunctional reagents in living cells (Calvet and Pederson, 1979).

The function of these double stranded regions is not clear. Double stranded regions of this type are not found in mRNA, but the presence of sequences in mRNA complementary to double stranded hnRNA has led to the hypothesis that these regions are cleaved at the "loop" region in hnRNA to form mRNA containing one strand of the double stranded stem (Naora and Whitelam, 1975; Ryskov et al, 1976).

3.5.2. Oligo(A) sequences

Apart from post-transcriptionally added poly(A) at the 3' terminus, some hnRNA molecules contain internal regions of oligoadenylate which are transcribed from the DNA template (Nakazato et al, 1974). These regions comprise approximately 25 uninterrupted AMP residues, possibly bordered by GCC at the 3' end and GPyPy at the 5' end (Venkatesan et al, 1979). These regions are not found in poly(A)$^+$ hnRNA and appear to be absent from mRNA (Venkatesan et al, 1979). On the other hand, abundant oligo(A)-adjacent sequences in the nucleus are also found in the abundant mRNA class (Kinniburgh et al, 1976b). On this basis, a model in which the oligo(A) acts as a primer for

polyadenylation after cleavage at the 3' end of the oligo(A) region has been proposed (Edmonds et al, 1976). However, although mammalian DNA contains many d(A)d(T) rich regions (Shenkin and Burdon, 1974; Flavell et al, 1977), these regions have not been found at putative polyadenylation sites in adenovirus genes (Ziff and Evans, 1978), globin genes (Konkel et al, 1978) or ovalbumin genes (Gannon et al, 1979).

3.5.3. Oligo(U) in hnRNA

Oligo uridylate-rich stretches have also been found in hnRNA (Molloy et al, 1974) and mRNA (Korwek et al, 1976) from HeLa cells. In hnRNA these regions do occur in poly(A)$^+$ molecules, apparently located near the 5' terminus (Molloy et al, 1974). These (U)-rich regions appear to be roughly 30 nucleotides long and may be present more than once in large hnRNA molecules. In sea urchin embryos, oligo(U)-rich regions are only found in the oligo(A) and poly(A) containing classes of hnRNA (Dubroff, 1977). The possibility of formation of double or treble-stranded regions between oligo(U) and oligo(A) or poly(A) regions has been discussed, either as a means of compaction, or of "looping-out" of processing sites (Dubroff, 1977; Kish and Pederson, 1977).

4. Ribonucleoprotein particles

4.1. Heterogeneous nuclear ribonucleoprotein particles

4.1.1. Biochemical studies of heterogeneous ribonucleoprotein particles

There is a large body of biochemical evidence concerning the association of hnRNA with protein. These studies have been concerned with the analysis of the protein and RNA of ribonucleoprotein particles

(hnRNP particles) extracted from purified nuclei by a variety of methods. In general, these extraction methods can be divided into two categories:-

(i) Extraction from intact nuclei suspended in pH 8.0 buffer, by diffusion, in the manner pioneered by Georgiev's group (Samarina et al, 1968).

(ii) Disruption of nuclei, followed by fractionation into nucleoplasmic, chromatin and nucleolar fractions. Nuclei are typically disrupted by sonication (e.g. Bhorjee and Pederson, 1973) although other methods of disruption have also been used. It is necessary to distinguish between these two types of method, since conflicting results have been obtained by groups using different methods.

4.1.2. Properties of hnRNP particles

In general, RNP particles extracted from animal tissue nuclei by diffusion into pH 8.0 buffer have a relatively homogeneous sedimentation coefficient of 30-40S (Samarina et al, 1968; Lukanidin et al, 1971; Martin et al, 1973; Beyer et al, 1977). The addition of rat liver cytosolic RNase inhibitor to the pH 8.0 buffer enables larger particles with a heterogeneous sedimentation behaviour of 30->200S to be extracted (Samarina et al, 1968; Lukanidin et al, 1971).

Particles isolated in the "nucleosol" fraction after disruption of nuclei also have a heterogeneous size range of 40-250S (Pederson, 1974; Augenlicht and Lipkin, 1976; Liautard et al, 1976a), although particles extracted by disruption of nuclei from tissues with high levels of endogenous RNase are often 30-40S (Louis and Sekeris,

1976) unless cytosolic RNase inhibitor is present (Northemann et al, 1978).

However, particles extracted by both methods have a number of diagnostic properties in common:

When hnRNP particles are fixed with formaldehyde or glutaraldehyde they have a characteristic buoyant density in CsCl of 1.40 g/cm^3 (e.g. Samarina et al, 1968; Pederson, 1974), which distinguishes them from chromatin and ribosomal particles. This density corresponds to a protein to RNA ratio of 4:1 using the formula of Spirin (1969), although chemical estimates now suggest that this ratio is higher, approaching 10:1 (Stevenin and Jacob, 1974; Gross et al, 1977).

Other distinguishing features of these particles are their disruption by sodium deoxycholate, which does not affect ribosomes (Stevenin et al, 1972; Faiferman et al, 1971), and their resistance to EDTA, which dissociates polysomes (Faiferman et al, 1970; Pederson, 1974).

Mild RNase treatment in vitro of large RNP particles results in the formation of 30-40S "monoparticles" (Samarina et al, 1968; Pederson, 1974), and on this basis the large particles have been termed "polyparticles" in a manner analogous to polysome structure. Thus, the 30-40S particles extracted from rat liver nuclei in the absence of RNase inhibitor may be derived from native polyparticles by endogenous RNase action. The extraction of monoparticles from cultured cell nuclei with low endogenous ribonuclease activity requires a higher temperature, presumably to increase the digestion of native polyparticles (Pederson, 1974).

4.1.3. The RNA in hnRNP particles

4.1.3.1. hnRNA in hnRNP particles

It has been proved that nuclear hnRNP particles contain hnRNA by a number of criteria.

(i) The kinetics of labelling of hnRNA and the RNA in hnRNPs are similar, and the labelling of both is inhibited by high levels of actinomycin D (Pederson, 1974; Gross et al, 1977). The labelling of hnRNP RNA is also inhibited by low levels of α-amanitin (Louis and Sekeris, 1976; Gross et al, 1977).

(ii) The RNA extracted from nuclear RNP particles has many characteristics of hnRNA, including base composition (Ducamp and Jeanteur, 1973; Samarina and Georgiev, 1973), sequence complexity (Alonso et al, 1978), and polyadenylation (Ducamp and Jeanteur, 1973; Kish and Pederson, 1975). The RNA from large polyparticles is often large (e.g. 20-60S in non-denaturing gradients) (Faiferman et al, 1970, 1971; Pederson, 1974), and there is an apparent correlation between hnRNP size and RNA size. On the other hand, RNA from 30-40S particles isolated in the absence of RNase inhibitor is often very small, with a size of 4-8S (Northemann et al, 1978; Maundrell and Scherrer, 1979).

mRNA sequences have been detected in amphibian oocyte hnRNP particles (Sommerville and Malcolm, 1976). In ascites cells, most, if not all of the cytoplasmic mRNA sequences can be detected in 30S monoparticles (Kinniburgh et al, 1976a). In rat liver, mRNA sequences were detected in both 30S and 80S-150S RNP particles, with a higher proportion of coding RNA sequences in the 30S monoparticles

than in the larger particles, suggesting that the regions of hnRNA in polyparticles which are exposed to nuclease action are depleted in mRNA sequences (Alonso et al, 1978).

On the other hand, unusual sequences characteristic of hnRNA, have been detected in hnRNP particles. These include double stranded sequences (Calvet and Pederson, 1977, 1979) and oligo(A) sequences (Kish and Pederson, 1975; Kinniburgh and Martin, 1976b). Poly(A) is present in polyparticles (Ducamp and Jeanteur, 1973) but is released as a poly(A) protein complex during mild RNase treatment (Quinlan et al, 1974; Kish and Pederson, 1975). (U)-rich residues are also found associated with this poly(A)-protein complex although it is disputed as to whether the association is artefactual (Quinlan et al, 1977) or functional (Kish and Pederson, 1977).

4.1.3.2. Small nuclear RNA in hnRNP particles

The presence of a long-lived, low molecular weight RNA in hnRNP particles was postulated by Sekeris and Niessing (1975). This RNA has been detected by a number of groups (Diemel et al, 1977; Northeman et al, 1977; Guiomont-Ducamp et al, 1977), and shown to contain a number of small discrete RNA species in the 5S-8S size range. These RNAs co-electrophorese with some of the previously identified small nuclear RNA's (snRNA's) (Ro-choi and Busch, 1974; Zieve and Penman, 1976). Control experiments indicate that these RNAs are integral components of the hnRNP particles (Howard, 1978; Gallinaro and Jacob, 1979), and that they are not degradation products of hnRNA (Seifert et al, 1979). It appears that some of these small RNAs are hydrogen bonded to hnRNA (Flytzanis et al, 1978; Jelinek and Leinwand, 1978).

4.1.4. The proteins associated with hnRNP particles

The analysis of the proteins associated with hnRNP particles has been confused by the different methods of preparation and analysis used by different groups. Moreover, such studies rely for their validity on a firm proof that these particles are not formed by the artefactual binding of proteins to RNA during extraction.

4.1.4.1. The reality of hnRNP particles

The reality of hnRNP particles has been inferred indirectly from a number of studies which have demonstrated the unique nature of the major proteins bound to hnRNA compared to major chromatin, ribosomal and nucleoplasmic proteins (Bhorjee and Pederson, 1973; Pederson, 1974; Gallinaro-Matringe and Jacob, 1974). However, more recent studies have revealed some identity between hnRNP, non-histone chromatin and "nucleosol" proteins, although the major hnRNP proteins were not found in these other fractions (Stevenin *et al*, 1975, 1978).

Artefactual binding of nuclear proteins to hnRNA was not detected by Pederson (1974), but was by Zawislak *et al* (1974). More recent evidence in favour of the *in vivo* reality of hnRNA particles comes from the resemblance under the E.M. of extracted hnRNP particles to perichromatin RNP fibrils observed in situ (Devilliers *et al*, 1977). However, there is no biochemical evidence that extracted hnRNP particles contain the same proteins as the ribonucleoprotein particles observed *in vivo* (e.g. Monneron and Bernard, 1969; Puvion and Bernhard, 1975).

The original work of Georgiev's group on monoparticles from rat liver showed only one hnRNP protein, with a molecular weight of

40,000 in pH 4.5 urea polyacrylamide gel electrophoresis (Samarina et al, 1968). Using one dimensional SDS polyacrylamide gel electrophoresis, many more proteins can be discriminated, due to entry of many more proteins into the gel (Gallinaro-Matringe and Jacob, 1974), and the single 40,000 molecular weight band can itself be resolved into five distinct bands (Northemann et al, 1978). Despite this technical progress, there has also been controversy regarding the number of proteins found on SDS gels. This has been resolved to some extent by comparison of the extraction methods used by different groups. In general, monoparticles extracted by diffusion from intact nuclei in the absence of RNase inhibitor have a more simple protein composition than polyparticles.

4.1.4.2. The protein composition of monoparticles

The major protein of 40,000 molecular weight found by Georgiev's group (and called "informatin") has recently been resolved by 2-dimensional gel electrophoresis into 12 major proteins in the 30-45,000 molecular weight range, when analysed in rat liver monoparticles (Karn et al, 1977). Monoparticles from HeLa cell nuclei were found to have six major proteins in the 30-45,000 molecular weight range, and to contain only three other genuinely associated proteins (Beyer et al, 1977). These six major proteins were termed "core proteins" by analogy with the nucleosome core particles of chromatin. Similarly, Martin's group found 2-4 major proteins between 30-45,000, and no others, in 30S monoparticles from Taper ascites cells (Billings and Martin, 1978).

4.1.4.3. The protein composition of polyparticles

The protein composition of the total hnRNP polyparticle population is more heterogeneous than that of the monoparticles derived by the action of endogenous ribonuclease. The group of major proteins in the molecular weight range 30-45,000 is found in polyparticles from all the cell types studied (e.g. Pederson, 1974; Beyer et al, 1977; Karn et al, 1977; Gallinaro-Matringe and Jacob, 1975). Apart from these, a large number of higher molecular weight components can be seen, which appear to be species specific (Pederson, 1974; Beyer et al, 1977). Many of these proteins appear to be confined to hnRNP particles (Stevenin et al, 1978), and many are still present after high salt washes of the particles (Pederson, 1974; Gallinaro-Matringe and Jacob, 1975; Kumar and Pederson, 1975). Recent papers using 2-dimensional electrophoretic techniques have further resolved the polyparticle proteins such that 40-80 different protein species have been identified (Patel and Holoubek, 1977; Pagoulatos and Yaniv, 1979), with the major 30-40,000 proteins themselves resolved into about 15 subspecies (Maundrell and Scherrer, 1979; Lelay et al, 1979).

4.1.5. The structure of hnRNP particles

The experiments described above have generated a number of models of hnRNP structure to explain their findings. The original work of Georgiev's group led to the proposal that hnRNA was associated with a number of globular monomer particles ("informoferes") in a polysome-like "string-of-beads" (Samarina and Georgiev, 1968; Samarina et al, 1973). However, RNase treatment, or increasing salt

treatment of monoparticles, led to a sequential release of proteins, with no 30S protein particle remaining after the treatment, implying that the hnRNA is an integral part of the monomer particles (Stevenin and Jacob, 1972, 1974). Thus, a "folded ribonucleoprotein strand" model was proposed. This has been modified by others to include a structural small RNA (Sekeris and Niessing, 1975), and to locate mRNA and oligo(A) sequences within the folded monomer particles, double stranded sequences between them, and poly(A) in a separate particle (Kinniburgh et al, 1976c).

Another type of model has been suggested based on analogies with nucleosome structure (Beyer et al, 1977; Karn et al, 1977). In particular, it is proposed that the major 30-45,000 molecular weight proteins have a packaging role analogous to the four main nucleosome core histones (Beyer et al, 1977; Karn et al, 1977). Electron microscopy of nascent RNP fibrils suggests that long RNA molecules interact with protein to form repeating 200-240Å particles with equal spacing and size (McKnight and Miller, 1976; Malcolm and Sommerville, 1974). In this model, the minor high molecular weight proteins of polyparticles would correspond to non-histone chromatin proteins, and might include the proteins involved in post-transcriptional processing.

However, despite the appeal of this model, recent results suggest that the monomers of polyparticles are heterogeneous both in size and protein composition (Stevenin et al, 1976; Gattoni et al, 1978), and that extracted monoparticles of simple protein composition may be formed by artefactual rearrangement due to extensive endogenous RNase action (Stevenin et al, 1979b). The counter-proposal of this group has been a model in which the monoparticles have a core of the

major "α-class" (nuclease resistant) proteins, surrounded by a variable amount of "β-class" (nuclease sensitive) proteins, accounting for their size heterogeneity. Thus, the monoparticles, whilst having a simple core, may be more plastic than nucleosomes, having a composition which may vary during the processing of hnRNA (Stevenin et al, 1979b).

4.1.6. Functions of hnRNP proteins

Following the discovery of intervening non-coding sequences in structural genes, there is renewed interest in the role of hnRNP proteins in post-transcriptional processing. One line of research has involved the search for possible processing enzymes within the hnRNP particles.

Endonuclease activity in rat liver 30S monoparticles capable of digesting hnRNA (Niessing and Sekeris, 1970), or autodegradation (Molnar and Samarina, 1976) has been reported. An endonuclease capable of cleaving double stranded regions of hnRNA has also been found in hnRNP particles (Bajszar et al, 1978; Rech et al, 1979).

Two distinct poly(A) synthetase activities on a separate ribohomopolymer synthetase activity in 30S monoparticles have been found (Niessing and Sekeris, 1972, 1973). Also, "capping" enzymes have been discovered in these particles (Molnar et al, 1978).

However, in many of these cases, similar enzyme activities have been found in higher concentration in the nucleosal or chromatin fractions, which could indicate either contamination of the particles, or reflect a transitory association between these processing enzymes and the hnRNP particles.

4.2. Informosomes

The concept of "informosomes", or non-polysome bound cytoplasmic mRNP particles was first derived from the study of free messenger-like RNP particles in the cytoplasm of early fish embryos (Spirin, 1969). These were postulated to be a stored, non-translated form of mRNA. Similar particles have since been isolated from the post-polysomal supernatant fraction of many tissues. However, the problem of separating these particles from cosedimenting ribosomes, ribosomal subunits and other contaminants has not been fully resolved. For this reason, the study of informosomes has been restricted, to some extent, to the study of particles of discrete size containing particular mRNAs (e.g. globin 20S mRNP, Gander et al, 1973), or the use of other, not well characterised, purification methods, such as affinity chromatography on oligo(dT) cellulose (Jeffery, 1977; Jain et al, 1979).

4.2.1. Characteristics of informosomes

As with hnRNP particles and polysomal mRNP particles, the free cytoplasmic mRNP particles have a characteristic buoyant density in CsCl of approximately 1.40 g/cm^3, corresponding to a protein to RNA ratio of at least 4:1 (e.g. Gander et al, 1973; Baglioni, 1974; Bag and Sarkar, 1975; Liautard et al, 1976a). The total cytoplasmic particles have a heterogeneous sedimentation behaviour with a range of 10-100S (Spirin, 1969; Spohr et al, 1970).

4.2.2. The RNA of informosomes

The RNA of informosomes has many of the characteristics of polysomal mRNA. It has a similar heterogeneous size range to mRNA,

which differs from ribosomal and tRNA (Spohr et al, 1970). The RNA is rapidly labelled, and there is a transition of some of this labelled RNA into translating polysomes (Spohr et al, 1970; MacLeod, 1975).

The RNA from informosomes can stimulate incorporation of amino acids into protein in cell free protein synthesis assays (Martin et al, 1979b) and globin mRNA was detected in free 20S RNP particles in avian erythroblasts by this method (Gander et al, 1973; Civelli et al, 1976). Actin mRNA was similarly detected in a free cytoplasmic mRNP fraction from chick embryo muscles (Bag and Sarkar, 1975).

4.2.3. The relationship of informosomal RNA to polysomal mRNA

The studies described above do not indicate whether all of the informosomal RNA is translatable mRNA, and how informosomal mRNA sequences are related to polysomal mRNA sequences.

The kinetic studies mentioned above showed that in a "pulse-chase" experiment, some, but not all, of the label in informosomal RNA was transferred to polysomal mRNA (Spohr et al, 1970; MacLeod, 1975). Only a small fraction of the steady state labelled informosomal RNA is chasable into the polysomes, with the bulk decaying with an intrinsic specific half life (Mauron and Spohr, 1978). These results are inconsistent with a simple precursor-product relationship between free and polysomal mRNA. A number of alternative models for the relationship between free and polysomal mRNP have been suggested, which incorporate either a "storage" function for informosomes, or postulate a dynamic equilibrium between free and polysomal mRNA (e.g. Buckingham et al, 1976; MacLeod, 1975).

Most recently, Scherrer has incorporated both alternatives into a scheme in which there are two components of free mRNPs. A short-term repressed component in equilibrium with polysomal mRNP, with an intrinsic half-life of decay, and a long-term repressed component (Maundrell et al, 1979). This latter component would not be present in polysomes and could have the storage functions conceived in the original informosome concept of Spirin (1969). Since the specific activity of polysomal globin mRNA rises more rapidly than that of free globin mRNA in avian erythroblasts, a third component of "transport mRNP", the rapid transport form of mRNA from nucleus to cytoplasm, is presumed to be relatively small in these cells (Maundrell et al, 1979).

Therefore, it would appear that free mRNP particles are a heterogeneous population with a number of functions. Attempts to characterise the mRNA in these populations demonstrates this complexity further. In avian erythroblasts the sequence complexity of the polysomal mRNA is equivalent to approximately 240 different mRNAs, whereas the free mRNA contains about 2,000 in RNAs including the polysomal sequences (Maundrell et al, 1979). On the other hand, free mRNPs in Taper ascites cells contain mRNA sequences equivalent to about 400 mRNAs, compared to 9,000 mRNA sequences in polysomal mRNA (Martin et al, 1979b). The abundant free mRNA sequences are a subset of the abundant polysomal mRNAs suggesting that in these cells, unlike avian erythroblasts, there are few long-term repressed "storage" mRNPs. For this reason, other studies of labelling kinetics and protein composition of free mRNPs in these two cell types may not be strictly comparable.

4.2.4. Proteins of free mRNPs

Unlike the major proteins associated with hnRNP particles which are conserved between species, very little similarity has been found between the informosomal proteins of different cell types, possibly for the reason that informosomes in different tissues may play different roles. In general, total free mRNPs appear to have a simple protein composition. For example, five major proteins on HeLa informosomes were found with molecular weights of 78K, 35K and three from 15-25K (Liautard et al, 1976). In Ehrlich ascites cells 6 proteins were detected, with three major bands of 78K, 52K and 34K (Barrieux et al, 1975), whilst chick embryo muscle free mRNPs had seven proteins, with three major proteins of 80K, 75K and 49K molecular weight (Bag and Sarkar, 1975). In contrast, in rabbit reticulocytes, two major informosome size classes of 15S and 20S have up to 30 proteins of 22-100K molecular weight associated with each, with major proteins in the 20-30K range (Huynh-Van-Tan et al, 1978). Purified 20S free cytoplasmic globin mRNP particles have a major protein of 48K, and many of 20-30K, none of which are present in larger free mRNP particles from the same cells. The complexity of reticulocyte free mRNP proteins may be connected with the high sequence complexity of the mRNA in this population, compared to the low complexity of ascites cell free mRNA (see 4.2.3).

4.3. Polysomal mRNP particles

When polysomes are disrupted in vivo by temperature shock or puromycin, or in vitro with EDTA or puromycin, the mRNA is released as a RNP particle with a characteristic buoyant density of 1.40 $g.cm^{-3}$.

These particles can be separated from ribosomal subunits by sucrose density gradient centrifugation when the sedimentation coefficient is significantly different from them (e.g. the 15S particle containing globin mRNA - Morel et al, 1973). Otherwise, other methods such as oligo(dT)-cellulose chromatography (Lindberg and Sundquist, 1974; Sundquist et al, 1977a and b), and electrophoresis (Liautard and Kohler, 1976; Van der Marel et al, 1975) have been employed.

4.3.1. Proteins associated with polysomal mRNPs

A comparison of high-salt-washed mRNP particles from many cell types indicates a simple protein composition common to most cell mRNPs, with two major proteins of 72-78K and 50K molecular weight (e.g. HeLa cells - Kumar and Pederson, 1975; Liautard et al, 1976; Ehrlich ascites cells - Barrieux et al, 1975, 1976; rat liver - Blobel, 1973; rabbit reticulocytes - Ernst and Arnstein, 1975; duck reticulocytes - Gander et al, 1975). The analysis of individual polysomal mRNP species also reveals the presence of these two proteins (e.g. globin mRNP (Gander et al, 1975; Vincent et al, 1977), histone mRNP (Liautard and Jeanteur, 1979)).

When polysomal mRNPs are prepared in low salt, a number of other proteins are also found, and also appear to be common to mRNPs, from different tissues and species (e.g. major proteins of 55K-60K, 65K-68K and 120K-130K molecular weight in HeLa cells (Kumar and Pederson, 1975; Van der Marel et al, 1975), KB cells (Lindberg and Sundquist, 1974; Sundquist et al, 1977a and b), Ehrlich ascites cells (Barrieux et al, 1976; Jeffery, 1977), and duck reticulocytes (Morel et al, 1971)).

5. The transport of RNA from nucleus to cytoplasm

The estimated nuclear pore channel size from diffusion studies (Paine et al, 1975a and b) is much smaller than the diameter of mRNP particles. Ultrastructural studies suggest that a conformational change from a granular to an elongated fibrillar morphology of RNPs occurs during transport through the nuclear pores (Stevens and Swift, 1966; Monneron and Bernhard, 1969; Daneholt et al, 1976). A change in size of the nuclear pore diameter during transport has not been observed (Severs, 1978). It might therefore be expected that the transport of RNP is not simply a passive diffusion process of mRNPs available for transport. Again, it is possible that the selection of sequences for transport to the cytoplasm is controlled at the level of the nuclear pore by specific cytoplasmic proteins, as proposed by Lichtenstein and Shapot (1976).

6. Aims

It can be seen from this introduction that there are many questions about eukaryotic gene expression which remain unanswered.

For example, very little is yet known about the organisation of transcription units within eukaryotic DNA. On the other hand, a full understanding of the transcription of eukaryotic DNA must also consider the physical interaction of RNA polymerase with the chromatin template.

Similarly, the processing of RNA transcripts within the nucleus requires understanding both at the level of the primary sequence of the RNA, and of the role of RNP particles in this processing. Again, the way in which specific RNA sequences are selected for transport to the cytoplasm has yet to be explained at either the primary sequence level, or at the level of the physical movement of RNP particles through the nuclear pores.

As with many biochemical problems, a suitable cell-free system, retaining the ability to transcribe, process and transport RNA could be used to tackle some of these questions. In the present study, isolated HeLa cell nuclei were characterised in terms of their suitability for examining some of these questions. The original aim of this characterisation was to determine whether the large RNA transcribed by RNA polymerase II in isolated HeLa cell nuclei, reported by Sarma *et al* (1976), were primary transcripts. This would present a unique opportunity to analyse the primary transcription products of eukaryotic transcription units. Furthermore, an

investigation of the reasons why this RNA was not being processed or transported *in vitro*, might throw light on the mechanisms of processing and transport of mRNA.

METHODS AND MATERIALS

MATERIALS

1. Chemicals

1.1. Reagents

Most of the reagents used were Analar reagents supplied by B.D.H. Chemicals Ltd., Poole, Dorset, except for the following:-

4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes)	
2-Amino-2-hydroxymethyl propane-1,3-diol (Tris)	
Dithiothreitol (DTT)	
S-adenosylmethionine (Ado-Met)	
cAMP	
(NAD^+)	Sigma London Chemical Co. Ltd.,
heparin	Kingston-upon-Thames,
DNase I	Surrey.
Bovine serum albumin	
Spermidine trichloride	
Coomassie Brilliant Blue G250	
Toluene	
2,5-diphenoxazole (PPO)	Koch-Light Laboratories Ltd.,
Triton X-100	Colnbrook, England
Trichloracetic acid	
2-mercaptoethanol	
Penicillin	Glaxo Pharmaceuticals,
Streptomycin	London
Calf Serum	Biocult Laboratories Ltd.,
M.E.M. Amino Acids	Paisley, Scotland.
M.E.M. Vitamins	

Trypsin	Difco Laboratories, Michigan, U.S.A.
ATP disodium salt	
GTP "	P-L Biochemicals Inc.,
CTP "	Milwaukee, Wisconsin.
UTP "	
acetyl CoA, lithium salt	
yeast tRNA	Boehringer Mannheim,
α-amanitin	West Germany
Actinomycin D	Calbiochem Ltd., Hereford, England.
Sephadex G25	Pharmacia, Uppsala, Sweden.
poly(U) Sepharose	
poly(A) Sepharose	
oligo(dT) cellulose (T_4)	Collaborative Research Inc., 1365 Main Street, Waltham, Mass.
Hyamine hydroxide	Fisons Scientific Apparatus, Loughborough, Leics, England.
Formamide (Fluka)	Fluoreschem Ltd., Derbyshire, England.
Selectron Filters	Scheicher & Schiell GmbH, D-3354, W. Germany.
Whatman 3MM filter discs	H. Reeve-Angel & Co. Ltd.,
Whatman GF/C filter discs	London, England.

1.2. Radiochemicals

[5,6-^{3}H] uridine	45 Ci/mmol	1 mCi/ml	Radiochemical
[5,6-^{3}H] uridine 5' triphosphate, ammonium salt	41 Ci/mmol	1 mCi/ml	Centre, Amersham.
L-[4,5-^{3}H] leucine	48 Ci/mmol	1 mCi/ml	
[6-^{3}H] thymidine	27 Ci/mmol	1 mCi/ml	
[1-^{14}C] acetyl-coenzyme A	58.7 mCi/mmol	10 μCi/ml.	

2.1. Solutions

Balanced Salts Solution (BSS)

0.116M NaCl, 5.4 mM KCl, 1 mM $MgSO_4$, 1 mM NaH_2PO_4, 1.8 mM $CaCl_2$ and 0.002% (w/v) phenol red. The pH was adjusted to 7.0 with 5.6% (w/v) $NaHCO_3$.

Isotonic salt buffer (ISB) (Kish and Pederson, 1975)

0.15M NaCl, 0.0015M $MgCl_2$, 0.01M Tris-HCl, pH 7.4.

Reticulocyte standard buffer (RSB)

0.01M NaCl, 0.0015M $MgCl_2$, 0.01M Tris-HCl, pH 7.4.

High salt buffer (HSB) (Penman, 1966)

0.5M NaCl, 0.05M $MgCl_2$, 0.01M Tris-HCl pH 7.4.

2.2. Solutions used for the isolation and incubation of nuclei

Homogenisation medium (HM) (Sarma et al, 1976a) (Detke, 1978)

0.3M sucrose, 2 mM $Mg(OAc)_2$, 3 mM $CaCl_2$, 10 mM Hepes-KOH pH 7.6 0.1% Triton X-100, 0.5 mM DTT.

Solution A

2M sucrose, 5 mM $Mg(OAc)_2$, 10 mM Hepes-KOH pH 7.6, 0.5 mM DTT.

Solution B

25% (v/v) glycerol, 5 mM $Mg(OAc)_2$, 50 mM Hepes-KOH pH 7.6, 5 mM DTT.

Incubation salt solution (IM)

150 mM KCl, 5 mM $Mg(OAc)_2$.

METHODS

1. Cell Culture

1.1.1. Growth of cells

HeLa cells (Gey et al, 1962) were maintained as monolayers in rotating 80 oz clear glass Winchester bottles (House and Wildy, 1965). The growth medium was the Glasgow modification of Eagle's minimal essential medium supplemented with 10% (v/v) calf serum (Busby et al, 1964), penicillin (100 units/ml) and streptomycin (100 µg/ml). Bottles were seeded with 2×10^7 cells in 180 ml growth medium and grown in an atmosphere containing 5% (v/v) CO_2 at 37°C.

1.1.2. Subculture of cells

Cells were subcultured by removal from the glass with a solution of trypsin and ethylenediaminetetraacetic acid (EDTA). The cell monolayer was washed with 10 ml of a solution made up of four volumes "Versene" solution (0.6 mM EDTA, 0.17M NaCl, 3.4 mM KCl, 10 mM Na_2HPO_4, 2.4 mM KH_2PO_4) and one volume trypsin (0.25% w/v trypsin, 10.5 mM NaCl, 1.0 mM sodium citrate, 0.002% (w/v) phenol red, pH 7.8) at 37°C. The monolayer was then treated with a further 10 ml of trypsin/versene solution until opaque, at which point the solution was poured off, leaving approximately 1 ml of solution on the monolayer. As soon as the cell layer began to peel off the glass surface, 10 ml of growth medium was added, and the cells were shaken into suspension. The cell density of the suspension was measured and used to subculture further bottles.

1.1.3. Contamination Checks

All sterile media and passaged cells were checked regularly for contaminating microorganisms. Aliquots were grown on blood agar plates and in brain-heart infusion broth at 37°C to detect bacterial contamination. Yeast and fungal contamination was monitored by incubating aliquots of media in Sabouraud's medium at 32°C. Infection by mycoplasma was checked by examining PPLO agar plates seeded with cells and incubated at 37°C.

1.2. Radioactive labelling of cells

1.2.1. Labelling with [5,6-^{3}H]uridine

Growing cells were labelled by reduction of the growth medium volume to 50 ml and addition of 100 µCi of [5,6-^{3}H]uridine (45 Ci/mmol) to give 2 µCi/ml. Labelling time was usually 10 minutes

When inhibition of labelling of precursor ribosomal RNA was required, cells were pretreated for 20 minutes before labelling with actinomycin D at a final concentration of 0.04 µg/ml. This concentration completely inhibits ribosomal RNA synthesis in HeLa cells, whereas hnRNA synthesis is only inhibited by approximately 30% (Penman et al, 1968).

1.2.2. Labelling of cells with [6-^{3}H] thymidine

HeLa cells were seeded at a density of 2 x 10^6 cells in 50 ml of growth medium. 24 hours later, the volume of growth medium was reduced to 20 ml, and [6-^{3}H]thymidine (27 Ci/mmol) was added to a final concentration of 2.5 µCi/ml. After 48 hours of cell growth, [6-^{3}H]thymidine was again added at a final concentration of 2.5 µCi/ml. Cells were harvested after 72 hours of cell growth.

1.3. Harvesting of cells

The culture medium was decanted and the cell monolayer was washed twice with approximately 50 ml of ice-cold Balanced Salts Solution (BSS) (see Materials). Cells were then scraped into 10 ml of cold BSS with a rubber wiper and collected by centrifugation at 800 g for 10 minutes at 4°C. The cells were washed twice by resuspending in 10 pellet volumes of BSS and centrifugation as described previously.

2. Preparation of Nuclei

2.1.1. Method of Sarma et al (1976) for purified nuclei

A method for isolation and incubation of HeLa cell nuclei, in which conditions are optimised for RNA synthesis in vitro, was used (Sarma et al, 1976).

Cells which were harvested and washed in BSS, were resuspended in homogenisation medium (HM) (0.3M sucrose, 2 mM Magnesium acetate, 3 mM $CaCl_2$, 10 mM Hepes - KOH pH 7.6, 0.1% Triton X-100, 0.5 mM dithiothreitol (DTT)) at a density of 1.3×10^7/ml. After leaving for 10 minutes at 4°C, cells were broken in a stainless steel Dounce homogeniser (clearance 0.002" diameter) (Penman, 1969). 5 strokes were usually sufficient to obtain complete cell breakage, as monitored by phase contrast microscopy.

In later experiments, $CaCl_2$ was omitted from the homogenisation medium, following the observation that inclusion of the salt reduced transcriptional activity of nuclei and increased the adherence of cytoplasmic material to nuclei (Detke et al, 1978).

The cell homogenate was mixed with an equal volume of

solution A (2M sucrose, 5 mM magnesium acetate, 10 mM Hepes - KOH pH 7.6, 0.5 mM dithiothreitol), and 10 ml of this mixture was layered over 27 ml of solution A in a cellulose nitrate tube. This was centrifuged for 45 minutes at 52,800g(av)(Rav 11.8 cm) at 4°C in the Beckman SW27 rotor. The supernatant material was carefully decanted, and residual sucrose solution was drained from the nuclear pellet by inverting the tube. The pellet was resuspended in solution B (25% (v/v) glycerol, 5 mM magnesium acetate, 50 mM Hepes - KOH pH 7.6, 5 mM dithiothreitol) at 0.5 - 2.0 x 10^8 nuclei/ml.

2.1.2. Preparation of crude nuclear preparations

A modification of the previous procedure avoiding the sedimentation of nuclei through 2M sucrose (Sarma, 1976) was used for many experiments on RNA synthesis *in vitro*. The homogenate in HM solution was centrifuged at 800 g for 10 minutes and the nuclear pellet was resuspended in solution B as described above.

2.1.3. Preparation of total cell homogenates

A further modification of the procedure described above was to homogenise cells directly in solution B containing, in some cases, 0.1% (v/v) Triton X-100 detergent, at a density of $\sim 10^8$ cells/ml. The homogenate was then used directly in incubation experiments as described for isolated nuclei. For comparison of isolated nuclei with the total cell homogenate, nuclei were centrifuged from this homogenate at 800 g for 10 minutes, and resuspended in solution B.

2.2. Incubation of nuclei

2.2.1. Nuclei from cells radioactively prelabelled in vivo

Nuclei from cells prelabelled with 5,6-^{3}H uridine for 10 minutes were resuspended in solution B at a density of 0.5 - 2.0 x 10^{8}/ml, at 4°C. The in vitro incubation was started by adding an equal volume of incubation salt solution (IM, 150 mM KCl, 5 mM magnesium acetate), mixing by brief agitation on a vortex mixer, and placing in a 25°C water bath. The incubation medium had a final composition of 12.5% v/v glycerol, 75 mM KCl, 5 mM magnesium acetate, 2.5 mM dithiothreitol and 25 mM Hepes - KOH pH 7.6. Other materials added to the incubation were either made up as concentrated solutions in distilled water, or dissolved in the incubation buffer. These were added to the incubation salt solution before starting the incubation. During the incubation, the nuclear suspension was shaken occasionally by agitation on a vortex mixer.

The amount and distribution of radioactivity during the incubation was assayed as follows:

The acid precipitable radioactivity in the total suspension was determined by removal of small aliquots (10 µl), after vigorous shaking of the suspension, and applied to Whatman 3MM discs. These were then placed in ice-cold 5% (w/v) trichloroacetic acid and processed as described in section 6.1.

The radioactivity in nuclei and supernatant material was found by centrifugation of a known volume (500 µl) of incubation mixture, removed at stated time intervals. This was centrifuged at 800 g for 10 minutes at 4°C. The supernatant was decanted and small aliquots (100 µl) spotted on Whatman 3MM cellulose paper discs.

The nuclear pellet was dissolved in the initial volume removed, of 1% (w/v) sodium dodecyl sulphate (SDS). Small aliquots of this solution were then applied to Whatman 3MM paper discs and processed for the determination of acid precipitable radioactivity as described in section 7.1.

2.2.2. RNA synthesis in isolated nuclei

Unlabelled HeLa cell nuclei prepared by the methods described above (section 2.1) were resuspended in solution B at a density of $0.5 - 2 \times 10^8$ nuclei/ml. An equal volume of IM solution containing unlabelled ribonucleoside triphosphate and tritiated UTP was prepared by adding 0.02 volumes of a stock solution of 40 mM ATP, 40 mM GTP, 40 mM CTP and 5 mM UTP in water, neutralised with KOH (stored -70°C), and 0.02 volumes of [5,6-^{3}H] uridine 5' triphosphate, ammonium salt (41 Ci/mmol, 1 mCi/ml) in water, prepared by lyophilising a sample of the same concentration in 50% ethanol solution, and redissolving in an equal volume of sterile distilled water.

To start the reaction, the nuclear suspension in solution B was mixed with an equal volume of IM containing the unlabelled nucleoside triphosphate and tritiated UTP, to give a final concentration of 0.4 mM each of ATP, GTP and CTP, 0.05M UTP and 10 μCi/ml [5,6-^{3}H]uridine 5' triphosphate at a final specific activity of 20 mCi/mmol. The incubation mixture was heated at 25°C in a water bath.

To measure incorporation of labelled UTP into acid precipitable material, small aliquots (10 μl) were removed from the

incubation mixture at indicated times and spotted on Whatman 3MM cellulose paper discs. These discs were placed in a beaker containing ice-cold 5% (w/v) trichloroacetic acid and 0.1M sodium pyrophosphate. The discs were then processed as described in section 6.1.

2.3. Preparation of cytosol and other cell fractions for incubation with nuclei

Cytosol was prepared as described for incubation with isolated HeLa cell nuclei (Sarma, 1976). The method is based on the preparation of an *in vitro* translation system from HeLa cells (Weber *et al*, 1975).

A HeLa cell pellet was resuspended in 2 volumes of homogenisation buffer (10 mM KCl, 1.5 mM magnesium acetate, 20 mM Hepes - KOH pH 7.4, 0.5 mM DTT), and the cells were allowed to swell for 10 minutes on ice before homogenisation in a tight fitting stainless steel Dounce homogeniser. The homogenate was centrifuged at 27,000 g for 4 minutes at 4°C (MSE 18), sedimenting the nuclei and mitochondria. The post mitochondrial supernatant material (PMS) was centrifuged in a Beckman 50 Ti rotor at 16,500 g(av) (R(av) 5.9 cm), for 1 hour at 4°C to give a pellet of polysomal material, and a supernatant solution termed "cytosol".

For incubation with nuclei, the solute concentrations in the PMS and cytosol fractions were adjusted to the levels in the nuclear incubation medium (75 mM KCl, 5 mM magnesium acetate, 5 mM DTT, 12.5% (v/v) glycerol and 50 mM Hepes - KOH pH 7.6), by addition of concentrated stock solutions of these solutes.

3. RNA

3.1. Extraction of RNA

3.1.1. Extraction of RNA from nuclei

A modification of the hot phenol-SDS method (Scherrer and Darnell, 1962) was used (Penman, 1966).

Nuclei were sedimented from the incubation mixture by centrifugation at 800 g for 10 minutes at 4°C (2,000 rpm) and resuspended in high salt buffer (HSB, 0.5M NaCl, 0.05M $MgCl_2$, 0.01M Tris-HCl pH 7.4) at approximately 5 x 10^7 nuclei/ml. The resultant viscous solution was stirred vigorously on a vortex mixer to disperse the clumped chromatin before addition of a solution of DNase I to a final concentration of 20 µg/ml. Electrophoretically purified DNase I (Worthington) was stored as a 1 mg/ml stock solution in 1 mM $MgCl_2$ containing 5 mM N-ethylmaleimide to inhibit contaminating ribonuclease activity. The chromatin was digested at 37°C for about 2 minutes, with gentle agitation, until the viscosity of the solution was much reduced. At this point the nuclei could be fractionated into crude nucleolar and nucleoplasmic fractions by sedimenting the nucleoli at 8,000 g for 5 minutes (Penman, 1969).

EDTA was then added to a final concentration of 30 mM, followed by addition of sodium dodecyl sulphate to a final concentration of 0.5% (w/v). An equal volume of phenol solution (80% w/v) was added and the contents were heated to 60°C (Perry, 1972) by placing in a water bath for 1 minute. After vigorous shaking, the tube was heated for another minute, reshaken, and a volume of chloroform (Perry, 1972) equal to the volume of phenol was added. The tube was again warmed to 60°C, shaken, and centrifuged at 1,500 g for 5

minutes at 20°C, to separate the organic and aqueous phases. At this stage a large white fluffy interphase of protein and SDS was found between the lower organic phase and the aqueous phase. The lower organic phase was removed by pasteur pipette, and an equal volume of chloroform was added to the remaining aqueous phase and interphase. These were again extracted at 60°C and centrifuged to separate the phases. This process was repeated until the interphase was reduced to a thin layer of denatured protein on the surface of the chloroform layer. The aqueous phase was then removed and the RNA precipitated by addition of 2 volumes of absolute ethanol, and storage at -20°C overnight.

3.1.2. Extraction of RNA from the supernatant fraction of isolated nuclei incubation buffer

To avoid the presence of K^+ ions in the phenol extraction (since K^+ ions form an insoluble precipitate with SDS; Palmiter, 1974)), the supernatant fraction was precipitated with two volumes of absolute ethanol at -20°C and collected by centrifugation at 12,000 g for 20 minutes at -10°C in the MSE 18 centrifuge. The pellet was resuspended in high salt buffer (0.5M NaCl, 50 mM $MgCl_2$, 10 mM Tris HCl pH 7.4) to which EDTA and SDS were added to final concentrations of 30 mM and 0.5% (w/v) respectively. This solution was then extracted once with phenol and chloroform and once with chloroform alone at 60°C, as described for nuclei lysed in HSB (Materials and Methods Section 3.1.1).

3.1.3. RNA extraction for assay of message activity in wheat germ extracts

To further purify RNA for translation studies *in vitro*, the

precipitate obtained from ethanol precipitation of phenol/chloroform extracted RNA was re-extracted by resuspending in 0.1M sodium acetate (pH 5.1) solution containing 0.5% (w/v) SDS (Palmiter, 1974). This solution was then extracted with an equal volume of aqueous phenol (80% w/v) at 60°C, to which was added the same volume of chloroform as described above. The process was repeated with chloroform alone, and the aqueous layer again ethanol precipitated in 2 volumes of ethanol at -20°C. The RNA was washed once by centrifugation, resuspension in 0.1M sodium acetate pH 5.1, and ethanol precipitation.

3.1.4. Extraction of radioactively labelled cytoplasmic RNA for sedimentation markers

HeLa cells were labelled with [5,6-^{3}H]uridine for 3 hours at 10 μCi/ml before harvesting as described in section 1.3. Cells were resuspended in reticulocyte standard buffer (RSB - 0.01M NaCl, 0.0015M $MgCl_2$, 0.01M Tris HCl pH 7.4), at a density of 5 x 10^7/ml, and swollen for 10 minutes on ice. The cells were broken in a stainless steel Dounce homogeniser (~ 10 strokes to attain 90% cell breakage). Nuclei and large cell debris were separated by centrifugation at 800 g for 10 minutes at 4°C, and the cytoplasmic fraction was decanted. The solution was made up to a final concentration of 30 mM EDTA and 0.5% (w/v) SDS, and the total cytoplasmic RNA was extracted by addition of an equal volume of 80% (w/v) phenol solution, shaking for 5 minutes at room temperature, followed by addition of an equal volume of chloroform and more shaking. The organic and aqueous phases were separated by centrifugation, followed by re-extraction of the aqueous phase with chloroform alone. The

resultant aqueous layer was added to two volumes of ethanol and the RNA was precipitated at -20°C.

3.2. Analysis of RNA

3.2.1. Sedimentation in sucrose gradients in LETS solution

36 ml 15-30% (w/v) sucrose gradients in LETS (0.1M LiCl, 0.01M EDTA, 0.1M Tris HCl pH 7.4, 0.2% (w/v) SDS) were formed in a cellulose nitrate tube using a linear gradient pourer. Ethanol precipitated RNA was resuspended in 2 ml LETS solution and layered over the gradient. The gradient was centrifuged at 30,000 g(av) (Rav 11.8 cm) for 17 hours at 20°C in a Beckman SW27 rotor. In some cases, samples were denatured by resuspending in 200 µl DMSO, 25 µl formamide, 25 µl LETS, heated to 45°C for 3 minutes and then made up to 2 ml with LETS (Derman et al, 1976).

The contents were fractionated by sucking the gradient from the bottom of the tube with a peristaltic pump and collecting timed fractions (approximately 2 ml). Fractions or aliquots of fractions were made 5% (w/v) with respect to trichloroacetic acid, and the acid insoluble material was collected on cellulose nitrate filters (Selectron - pore size 0.45 µm), after standing on ice for 10 minutes, and processed as described in section 6.1.

3.2.2. Denaturing gradients in 98% (v/v) formamide

For separation of RNA by size in denaturing conditions, the detailed description of formamide gradients by Ross (1976) was followed.

8-20% (w/v) sucrose gradients containing 98% (v/v)

formamide in 0.002M EDTA, 0.01M Tris HCl pH 7.5 were prepared in a Beckman SW56 rotor polyallomer tube. Solutions of 8, 12, 16 and 20% (w/v) sucrose were prepared in 0.002M EDTA, 0.01M Tris HCl pH 7.5 containing 98% (v/v) formamide (Fluka), and volumes of 0.7 ml 20% sucrose, 1.0 ml 16% sucrose, 1.0 ml 12% sucrose and 0.75 ml 8% sucrose were layered successively in the tube. The gradient was allowed to form by diffusion at room temperature for three hours.

Samples prepared by phenol extraction were precipitated from 67% (v/v) ethanol at -20°C and collected by centrifugation at 12,000 g(av) for 20 minutes at -10°C in the MSE 18 centrifuge. The RNA pellet was dried in a stream of air and dissolved in 0.075 ml 0.002M EDTA, 0.01M Tris HCl pH 7.5 solution, followed by 0.15 ml of 85% (v/v) formamide in the same solution. The sample was denatured by heating to 45°C for 2-5 minutes, and then layered over the gradients. Centrifugation was performed in a prewarmed Beckman SW56 rotor at 246,000 g(av) (Rav 8.78 cm) for 16 hours at 30°C.

Fractions of approximately 0.2 ml were collected by piercing the bottom of the tube with a syringe needle and collecting 4 drop fractions from the needle.

The acid precipitable radioactivity in each fraction was measured by adding 1 ml of 6% (w/v) trichloroacetic acid and precipitating on ice before filtration through Selectron filters (cellulose nitrate, 0.45 μm pore size).

3.3. Poly(U)Sepharose Chromatography

The method described by Jelinek *et al* (1973) for the selection of large hnRNA molecules on the basis of poly(A) content

was used. However, using the technique as described, only ~20% of polyadenylated hnRNA binds to the poly(U)Sepharose (Molloy et al, 1974). Therefore, the RNA was heated to 65°C and rapidly cooled before application to the column to denature the molecules (Nakazato and Edmonds, 1974).

0.25 g of poly(U)Sepharose (Pharmacia) was swollen in 10 ml of 0.1M NETS buffer (0.1M NaCl, 0.01M EDTA, 0.01M Tris HCl pH 7.4, 0.2% (w/v) SDS) at 4°C, then poured into a small column (2.0 x 0.6 cm) made by blocking a 5 ml pipette with a small glass bead. All glassware was siliconised (Repelcote, Hopkins and Williams), to prevent binding of RNA to the glass. The gel was washed with 10 volumes of 0.1M NETS buffer followed by 10 volumes of 90% formamide (Fluka) in ETS buffer (0.01M EDTA, 0.01M Tris HCl pH 7.4, 0.2% (w/v) SDS), and rewashed with 0.1M NETS before equilibration with 0.4M NETS (0.4M NaCl in ETS buffer).

A pellet of ethanol precipitated RNA was dried in air and resuspended in a small volume (0.1 or 0.2 ml) of 0.4M NETS, this volume being smaller than the excluded volume of the column. The sample was heated to 65°C for 3 minutes, cooled rapidly in ice/water mixture, and immediately applied to the top of the column. The sample was eluted with 0.4M NETS at a slow flow rate, at room temperature (20°C), such that the sample took approximately 10 minutes to pass through the column. The small eluted fraction was reapplied to the column and eluted at the same rate.

The bulk of the unbound material eluted in the first 1 ml, after which the column was washed extensively (at least 10 column volumes) with 0.4M NETS at room temperature.

For analytical purposes, the bound material was eluted in a stepwise fashion with increasing concentrations of formamide in ETS buffer from 0-50% (v/v) formamide in 5% steps. 1 ml of each concentration was applied to the column and eluted until the column bed was just covered, at which point the next concentration was applied. As shown in the Results section, material was eluted mainly at 5-10% (v/v) formamide and 35-45% (v/v) formamide concentrations. Therefore, for preparative purposes, the two classes of bound material were separated by eluting the column extensively with 10% (v/v) formamide in ETS, followed by elution with 90% (v/v) formamide in ETS.

3.4. Poly(A)Sepharose

Poly(A)Sepharose (Pharmacia) can be used to isolate RNA molecules containing regions rich in uridine residues (Molloy et al, 1974). The method used is exactly the same as for poly(U)Sepharose chromatography.

3.5. Wheat germ cell free translation system

RNA samples were tested for messenger activity in a wheat germ extract (Roberts and Paterson, 1973). Such extracts require optimisation of extract concentration, pH, Mg^{2+}, K^{+} and polyamine concentration for efficient translation of particular mRNA's (Marcu and Dudock, 1974). The optimum conditions for HeLa cell cytoplasmic mRNA translation have been worked out in this department (McGrath, 1978) and are 75 mM K^{+}, 1.5 mM Mg^{2+}, 0.2 mM spermidine.

3.5.1. Wheat germ extract

2 g of raw wheat germ (Bar-Rav Mill, Tel Aviv, Israel)

were ground with an equal weight of powdered glass (crushed pasteur pipettes) for 60 seconds in a cooled mortar. 4 ml of extraction buffer (20 mM Hepes KOH pH 7.6, 100 mM KCl, 1 mM magnesium acetate, 2 mM $CaCl_2$, 1 mM DTT) was added and the mixture was gently swirled for 15-30 seconds. The resultant thick paste was scraped into a glass tube and centrifuged at 16,000 rpm for 12 minutes at 4°C in a Sorvall SS34 rotor. The supernatant aqueous layer was withdrawn from between the pellet and the upper fatty layer with a pasteur pipette, and 1 ml was applied to a Sephadex G-25 (medium) column (21 x 1 cm) equilibrated with elution buffer (20 mM Hepes KOH pH 7.6, 120 mM KCl, 5 mM magnesium acetate, 1 mM DTT). The column was eluted at a flow rate of about 3 ml/minute with elution buffer. 0.5 ml fractions were collected, aliquots of which were assayed for absorbance at 260 nm. All fractions with optical density higher than 90 A_{260} units were pooled and centrifuged for 20 minutes at 4°C in the Sorvall SS34 rotor at 16,000 rpm. Small aliquots of the supernatant extract were stored at -70°C.

3.5.2. Protein synthesis assay

The assay mix, of final volume 50 µl, was prepared by mixing in order: 10 µl of energy mix (0.1M Hepes KOH pH 7.6, 0.09M KCl, 5 mM magnesium acetate, 12 mM DTT, 0.1 mg/ml creatine kinase, 3 mg/ml ATP, 0.05 mg/ml GTP, 10 mg/ml creatine phosphate, 1 mM spermidine trichloride), 2.5 µl L-[4,5-^{3}H]leucine (48 Ci/mmol, 1 mCi/ml), 15 µl wheat germ extract, and 22.5 µl RNA sample in distilled water.

Work in this department has shown that the wheat germ extract contains sufficient endogenous amino acids for the assay.

Creatine kinase is used at $^{1}/_{10}$ the published concentration (Marcu and Dudock, 1974), since the enzyme has been shown to contain contaminating ribonuclease activity (Singer, 1978).

Assays were incubated for 90 minutes at 25°C. 10 µl aliquots were taken at the end of the assay and applied to Whatman 3 MM paper discs for assay of incorporation of radioactivity into material insoluble in acid at 90°C (see Section 6.1).

4. Nuclear ribonucleoprotein particles

4.1.1. Extraction

Nuclear ribonucleoprotein particles containing heterogeneous nuclear RNA (hnRNP particles) were extracted from nuclei by sonication (Pederson, 1974) in isotonic salt buffer (ISB, 0.15M NaCl, 0.0015M $MgCl_2$, 0.01M Tris HCl pH 7.4) (Kish and Pederson, 1978).

Nuclei were resuspended in ISB at a density of 5 x 10^7/ml (Bhorjee and Pederson, 1973), and disrupted by sonication for 5-8 seconds at 1-3 amps with a $^{3}/_{8}$" diameter probe of the MSE 150 Watt Ultrasonic Disintegrator. This was found to be sufficient to break more than 95% of the nuclei. Under phase contrast microscopy free nucleoli and diffuse clumps of chromatin were visible. The density of chromatin clumps corresponded roughly to the original density of nuclei, and it was assumed that the brief sonication caused breakage of nuclei without much fragmentation of the chromatin, which did occur with longer sonication times.

To remove nucleoli, up to 10 ml of the sonicate was layered over 25-35 ml of 30% (w/v) sucrose in ISB buffer in cellulose nitrate

tubes and centrifuged at 3,300 g(av) (Rav 11.8 cm) for 15 minutes at 4°C in a Beckman SW27 rotor (Bhorjee and Pederson, 1973). The layer above the 30% (w/v) sucrose was decanted and labelled the post-nucleolar supernatant fraction (PNS).

4.1.2. Purification and analysis on sucrose density gradients

1-3 ml of PNS was layered over a 36 ml linear 15-30% (w/v) sucrose gradient in ISB, and centrifuged in the Beckman SW27 rotor for 17 hours at 4°C at 30,000 g(av) (Rav 11.8 cm). The gradient was fractionated by suction from the bottom with a peristaltic pump, and timed fractions of approximately 2 ml were collected.

For analysis of gradients, 50% (w/v) trichloroacetic acid was added to each tube to a final concentration of 5% (w/v), and acid precipitable material was collected on cellulose nitrate filters (Selectron, 0.45 μm pore size), and processed for liquid scintillation counting as described. The gradient pellet was resuspended in 1 ml of 0.5% (w/v) SDS solution and aliquots were assayed for acid precipitable radioactivity as for the gradient fractions.

For preparative purposes, small aliquots of each fraction were precipitated in 5% (w/v) trichloroacetic acid and the radioactivity collected on Selectron filters was assayed. Appropriate pools of gradient could then be made.

In order to compare the ribonucleoprotein material released from isolated nuclei during incubation, the supernatant fraction of the incubation medium was diluted with an equal volume of ISB to reduce the glycerol concentration, and was then layered on identical 15-30% (w/v) sucrose gradients in ISB to those described for hnRNP particles, and centrifuged under the same conditions.

4.2. Caesium chloride density gradient centrifugation

In order to measure the buoyant density of ribonucleoprotein particles, a method utilising the rapid fixation of the particles with glutaraldehyde and the short time required for centrifugation in preformed caesium chloride density gradients was followed (Baltimore and Huang, 1968). Pooled fractions from 15-30% sucrose gradients in ISB containing RNP particles were concentrated to volumes less than 1 ml and also the sucrose concentration was reduced by 'vacuum dialysis' against ISB. The apparatus consisted of a dialysis bag open to the atmosphere via a funnel, suspended in an evacuated conical flask containing the dialysis buffer. Thus, the processes of dialysis and ultrafiltration were in operation at the same time.

The sample was then fixed by addition of 25% (v/v) glutaraldehyde, which was neutralised to pH 7 with 1M $NaHCO_3$ just prior to use, to a final concentration of 6% (v/v). Samples were left for 4 hours on ice before layering onto preformed caesium chloride gradients.

4 ml linear CsCl gradients were formed in 2 x $\frac{1}{2}$" polyallomer tubes by mixing 2 ml 1.2 g/cm^3 CsCl solution with 2 ml 1.7 g/cm^3 CsCl solution in a gradient former. Caesium chloride solutions were dissolved in RSB containing 0.8% "Brij-58" detergent. 0.5 ml of fixed sample was layered over the gradient and centrifuged at 150,000g(av)(Rav 8.35 cm) in the Beckman SW50-1 rotor at $4^{\circ}C$ for 17 hours.

The gradient was fractionated by collecting drops from the

bottom of the tube punctured with a syringe needle.

The refractive index of every fifth fraction was measured to calculate the caesium chloride concentration and hence the buoyant density at that position in the gradient. Acid insoluble radioactivity was assayed by addition of an equal volume of 10% (w/v) trichloroacetic acid to each fraction and collection of the precipitate on cellulose nitrate filters (see section 6.1).

4.3. Oligo(dT)cellulose chromatography of RNP particles

The technique of binding a class of cytoplasmic RNP particles to oligo(dT)cellulose was first described by Lindberg and Sundqvist (1974). It was shown that whereas poly(A)$^+$ mRNA bound to oligo(dT)can be eluted by lowering the salt concentration, mRNP elution requires formamide and salt. It was therefore suggested that binding of mRNP to oligo dT involves both RNA and protein.

0.25 g of oligo(dT)cellulose (type T_2 - Collaborative Research) was packed in a siliconised disposable pipette to make a column 2.5 x 0.6 cm, and equilibrated with binding buffer (0.25M NET:- 0.25M NaCl, 10 mM EDTA, 10 mM Tris HCl pH 7.4) (Irwin et al, 1975).

Samples from sucrose gradient fractions were concentrated and dialysed by vacuum dialysis (see section 4.2) against 0.25M NET. Concentrated samples in 0.2 ml 0.25M NET were applied to the column at 4^{o}C and eluted at a flow rate of 0.1 ml/minute with 0.25M NET. The eluted fraction (0.5 ml) was repassed through the column and re-eluted with 0.25M NET. Fractions of 0.5 ml were collected.

After washing with 10 column volumes of 0.25M NET, the bound material was eluted with 10 volumes 25% (v/v) formamide in

0.25M NET, followed by 10 volumes of 50% formamide in 0.25M NET. Small aliquots for scintillation counting were applied to Whatman 3MM paper discs and assayed for acid insoluble radioactivity as described in section 6.1.

4.4. SDS polyacrylamide gel electrophoresis of proteins

The method described by LeStourgeon and Beyer (1978), based on the discontinuous polyacrylamide gel electrophoresis system of Laemmli (1970) was used.

4.4.1. Polyacrylamide gel preparation

A 10% (w/v) acrylamide resolving gel was prepared by mixing 13.3 ml of stock acrylamide solution (30% (w/v) acrylamide, 0.8% N,N'bisacrylamide), 10 ml of resolving gel buffer (1.5M Tris HCl pH 8.8, 0.4% SDS), and 16.5 ml distilled water. The mixture was "degassed" under vacuum in a round bottomed flask. To polymerise the gel, 0.15 ml of freshly prepared 10% (w/v) ammonium persulphate was added, followed by 0.01 ml N,N,N',N' tetramethylethylenediamine (TEMED).

A small slab gel was cast between two 8 x 8 cm glass plates, separated at the sides by 3 mm spacers and sealed at the bottom with waterproof tape, to a height of $6\frac{1}{2}$ cm. The gel was carefully overlaid with 0.1% (w/v) SDS solution.

After polymerisation (about 30 minutes), the overlay solution was poured off and a 3% (w/v) acrylamide stacking gel was cast over the resolving gel. The stacking gel was prepared by combining 6.5 ml H_2O, 2.5 ml stacking gel buffer (0.5M Tris HCl pH 6.8, 0.4% SDS), 1.0 ml stock acrylamide solution (30% w/v

acrylamide, 0.8% N,N'bisacrylamide) and 0.03 ml 10% (w/v) ammonium persulphate. The polymerisation was catalysed by 0.01 ml TEMED. A 14 space perspex comb was placed in the upper gel such that 1 cm deep wells were formed 0.5 cm above the resolving gel.

After polymerisation, the comb and the tape at the bottom of the gel were removed, the top of the stacking gel was rinsed with electrophoresis buffer, and the slab was placed in a "Uniscil" electrophoresis tank (Uniscience).

The upper and lower reservoirs contained electrophoresis buffer (0.025M Tris, 0.19M glycine, 0.1% (w/v) SDS). Up to 25 µl of sample was carefully poured in each well, displacing the electrophoresis buffer due to the higher density of the sample buffer.

Electrophoresis was carried out with a current of 15 mA until the marker dye had travelled 1 mm into the resolving gel, when the current was increased to 30 mA.

When the marker dye was about 0.5 cm from the bottom of the gel (about 3 hours running time), the current was switched off and the gel was removed, fixed and stained for 2 hours with 0.25% (w/v) Coomassie Brilliant Blue R250 in 9% (v/v) acetic acid, 45% (v/v) methanol. The gel was destained with repeated washes of 5% (v/v) methanol, 7.5% (v/v) acetic acid solution.

4.4.2. Preparation of samples for SDS polyacrylamide gel electrophoresis

1. Ethanol precipitation of RNP particles

Pooled fractions from sucrose density gradients or oligo(dT)

cellulose chromatography were precipitated with 67% (v/v) ethanol at -20°C for 4 hours. Precipitates were collected by centrifugation at 12,000 g for 20 minutes at -10°C, dried in a stream of air, and resuspended in sample buffer (0.01M sodium phosphate pH 7.4, 0.25M sucrose, 0.1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol) to a concentration of about 1 mg protein/ml). The sample was heated in a boiling water bath for 2 minutes, allowed to cool, and about 20 µg was layered into a well in the stacking gel.

2. Phenol extraction of proteins

In some cases, proteins were phenol extracted from samples (Le Stourgeon and Beyer, 1978), to remove nucleic acids from the sample. Samples were made up in 1 ml of protein extraction solution (2% (w/v) SDS, 20 mM EDTA, 20 mM Tris HCl pH 8.2, 1% (v/v) 2-mercapto-ethanol) and heated in a boiling water bath for 3 minutes. After cooling, 1 ml of aqueous phenol, saturated with protein extraction buffer, was added, and the solution was mixed for 5 minutes on a vortex mixer, at room temperature. The phases were separated by centrifugation at 3,000 rpm for 5 minutes, and the phenol phase was removed by pasteur pipette and dialysed overnight against 2 x 200 volumes of protein sample buffer (0.01M sodium phosphate, 0.1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, 0.25M sucrose). The non-diffusable material was concentrated by placing the dialysis bag in a water absorbent material such as "Sephadex" (Pharmacia) or "Carbowax" (Gurr).

Standard marker proteins were dissolved in sample buffer at a concentration of 1 mg/ml each. Proteins used were: bovine serum albumin (m.w. 68,000), glutamate dehydrogenase (m.w. 53,000) aldolase (40,000), lactate dehydrogenase (m.w. 36,000) and cytochrome c (m.w. 11,700).

4.4.3. Estimation of protein concentration

The dye binding method of Bradford (1976) for estimation of protein concentration was used.

The reagent was prepared by dissolving 100 mg of Coomassie Brilliant Blue G250 in 50 ml 95% (v/v) ethanol, adding 100 ml 85% (w/v) phosphoric acid, and diluting to 1 litre with distilled water, to give a solution of 0.01% (w/v) Coomassie Brilliant Blue, 4.7% (v/v) ethanol 8.5% (w/v) phosphoric acid.

0.1 ml of each sample was mixed with 5 ml of reagent and the absorbance at 595 nm was read after 2 minutes. Bovine serum albumin standards were prepared in the same buffer as the sample.

Samples in the sample buffer for SDS polyacrylamide gel electrophoresis (Section 4.4.2) were diluted $^{1}/_{10}$ with distilled water, and 0.1 ml was then analysed as before (Rubin and Warren, 1977).

5. Analysis of DNA in isolated nuclei

5.1. Sedimentation of DNA in neutral sucrose gradients

Nuclei to be analysed were resuspended in SSC buffer (0.15M NaCl, 0.015M Na citrate) at a density of 10^6 nuclei/ml. This suspension was made 0.5% (w/v) with respect to SDS, and Pronase (predigested for 3 hours at 37°C) was added to a final concentration of 500 µg/ml. The lysate was mixed by very gentle swirling of the tube and was incubated at 37°C for 1 hour. The SDS concentration was adjusted to give a final concentration of 1% (w/v) SDS. The lysate was then poured very gently, directly from the incubation tube, onto a 30 ml 15-30% (w/v) sucrose gradient in 0.1M NaCl, 0.001M EDTA, 0.01M

Tris HCl (pH 7.4), 0.5% (w/v) SDS, formed over a 6 ml 70% (w/v) sucrose cushion (Cheevers et al, 1972). The gradients were centrifuged at 64,000 g(av)(Rav 11.8 cm) for 14 hours at 25°C in the Beckman SW27 rotor. Gradients were collected by upward displacement.

5.2. Sedimentation of DNA in alkaline sucrose gradients

Cells or nuclei were resuspended in SSC (0.15M NaCl, 0.015M Na citrate) at a density of 4×10^6 nuclei/ml.

4 ml 15-30% (w/v) sucrose gradients in 0.5M NaCl, 0.25M NaOH, 0.001M EDTA, 0.01% SDS were formed over a 0.5 ml 70% (w/v) sucrose cushion (Cheevers et al, 1972). 0.25 ml of a solution containing 0.3M NaOH, 0.001M EDTA and 0.5% (w/v) SDS was layered over the sucrose gradient. 0.025 ml of the nuclear suspension in SSC, corresponding to 10^5 nuclei, was pipetted slowly into the overlay and incubated at 25°C for 16 hours. The gradients were centrifuged at 152,000g(av)(Rav 8.35 cm) at 25°C for 1 hour in the Beckman SW50.1 rotor. Gradients were harvested by upward displacement to avoid contamination of the sucrose gradient fractions with material collected on the 70% (w/v) sucrose cushion.

6. Miscellaneous Techniques

6.1. Detection of radioactivity by scintillation counting

6.1.1. Acid precipitable material collected on cellulose nitrate discs

Material to be assayed for radioactivity, typically density gradient fractions, was adjusted to 5% (w/v) trichloroacetic

acid and left on ice for at least 10 minutes. To the 0.2 ml fractions from 98% (v/v) formamide gradients, 1 ml of 6% (w/v) trichloroacetic acid was added to dilute the formamide. The material was filtered through a 0.45 μm pore size cellulose nitrate disc (Selectron-Filter), by suction, and washed with 10 ml of cold 5% (w/v) trichloroacetic acid. The discs were placed in plastic scintillation vials, dried in a 60°C oven for 1 hour, and covered with 5 ml of scintillation fluid (0.5% (w/v) diphenoxazole (PPO) in toluene).

6.1.2. Acid precipitable material collected on cellulose paper discs

Where stated, small aliquots of labelled material were applied to Whatman 3MM paper discs. Batches of discs were then plunged into ice-cold 5% (w/v) trichloroacetic acid (10 ml/disc) and washed successively with 5% (w/v) trichloroacetic acid (2 changes) ethanol (2 changes) and diethyl ether (2 changes). The discs were dried in a stream of air.

This procedure was modified in situation where radioactively labelled acid-soluble labelled material could bind directly to the discs or to acid insoluble material on the discs (Kammen et al, 1961).

(a) In nuclear incubations, labelled nucleoside triphosphates were washed off the filters with 5% (w/v) trichloroacetic acid containing 0.1M sodium pyrophosphate, before washing with 5% (w/v) trichloroacetic acid alone.

(b) In wheat germ translation assays, discs were placed in 10% (w/v) trichloroacetic acid containing 10^{-5}M L-leucine to dilute the labelled leucine in the assay.

The discs were then washed with 5% (w/v) trichloroacetic acid at 4°C, and then with 5% (w/v) trichloroacetic acid at 90°C for 10 minutes to solubilise charged tRNA's. The discs were then dried with ethanol and ether, as described above.

The discs were dried by evaporating ether in a stream of air, and were placed in plastic scintillation vials. 0.5 ml of 1M hyamine hydroxide in methanol was added to the vial and the labelled material was dissolved by heating in a 60°C oven for 30 minutes. 5 ml of 0.5% (w/v) PPO in toluene was added, and the vial was shaken vigorously before counting. Counting efficiency was 20% as determined by the "H-number" method in a Beckman LS8000 scintillation counter.

6.2. Precautions against ribonuclease contamination

Stringent precautions were taken to prevent ribonuclease contamination of samples containing RNA.

All glassware was sterilised by heating at 180°C overnight. Solutions were autoclaved at 15 p.s.i. for 30 minutes, except for solutions containing sucrose which were autoclaved at 5 p.s.i. for 50 minutes to avoid caramelisation. Buffers were stored in small aliquots at 4°C and used once only. Non-autoclavable solutions (e.g. DTT, nucleoside triphosphates) were prepared by dissolving the substance in sterile water or buffer solution.

Non-sterilisable equipment was washed with hot 2% (w/v) SDS containing, where applicable, 0.1% (v/v) diethyl pyrocarbonate (Mendelsohn and Young, 1978). Unless otherwise stated, all procedures were carried out at 4°C, and gloves were worn throughout to prevent contamination of samples with nucleases from human skin (Holley *et al*, 1961).

RESULTS

1. RNA synthesis in isolated nuclei

1.1 The size of RNA labelled in nuclei isolated by different procedures

1.1.1 The size of RNA labelled in purified nuclei

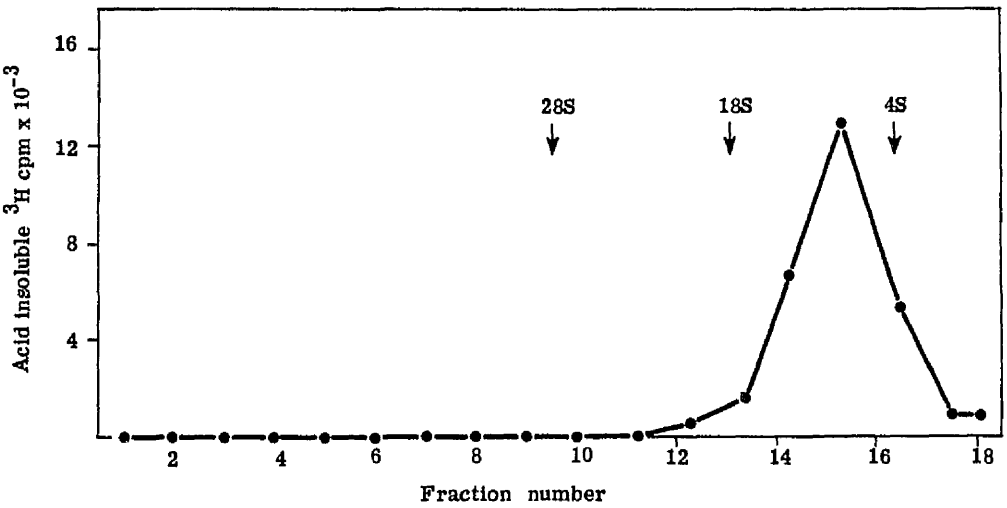
It has been known for some years that nuclei isolated from cells retain the ability to incorporate nucleoside triphosphates into RNA when incubated under suitable conditions. Many recent studies have been based on the conditions described by Marzluff *et al* (1973), who were able to prolong RNA synthesis in isolated mouse myeloma cell nuclei for over 2 hours by incubating at low temperature (25°C). In particular, a modification of these conditions was described (Sarma *et al*, 1976) in which nucleoside triphosphate incorporation into RNA in isolated HeLa cell nuclei was optimised by adjusting the temperature, pH and concentration of solutes in the incubation medium. These authors (Sarma *et al*, 1976) reported that 10% of the synthesised RNA was released into the incubation medium and had electrophoretic mobilities in acrylamide gels corresponding to ribosomal 5S RNA and 4.5S precursor transfer RNA. The rest of the synthesised RNA remained in the nucleus and was found to have an apparent high molecular weight (> 18SrRNA) as judged by acrylamide gel electrophoresis under non-denaturing conditions. Since this material was not seen to be processed to a smaller sized RNA during incubation, an impetus for examining this system was the possibility that this apparently high molecular weight material represented primary transcription products which were not processed in these isolated nuclei. Thus, it might be possible in such a system both to synthesise and characterise primary transcripts, and to study the

processing of these transcripts by addition of other factors to the system.

To examine this possibility, HeLa cells were pretreated with actinomycin D (0.04 μg/ml) to depress ribosomal RNA synthesis (Perry, 1963; Penman et al, 1968). HeLa cell nuclei were isolated by the method of Sarma et al (1976), by homogenisation in isotonic sucrose buffer (HM), centrifugation through 2M sucrose, and resuspension of the purified nuclear pellet in the incubation medium. The nuclear suspension was incubated for 10 minutes in the presence of [5,6 - ^{3}H]UTP and the four unlabelled ribonucleoside triphosphates. The nuclei were then centrifuged from the incubation medium, and the RNA was extracted from the nuclear pellet by the method of Penman (1969). The RNA radioactively labelled in vitro and extracted from these nuclei was analysed by sedimentation through an 8-20% (w/v) sucrose gradient under denaturing conditions (98% v/v formamide, 30°C) (Ross, 1976). The sedimentation behaviour was compared with radioactively labelled cytoplasmic RNA run in a parallel gradient to give an approximate estimate of molecular weight distribution. This estimate is an approximation since under these conditions, although most inter- and intra-molecular hydrogen bonded regions in hnRNA are eliminated (Federoff et al, 1977), the nuclear RNA may not be completely denatured (Spohr et al, 1976), and artefacts may also arise from partial renaturation of the marker ribosomal RNA (Boedtker and Lehrach, 1976).

The size distribution of the RNA synthesised by the isolated HeLa cell nuclei determined by this method was found to be in the range 4-18S (Figure 1). This is smaller than poly(A)$^{+}$ mRNA isolated directly from HeLa cells, which has an average sedimentation coefficient

Figure 1. Sedimentation in denaturing gradients of RNA labelled in purified nuclei.



HeLa cells were pretreated with actinomycin D (0.04 µg/ml) for 30 minutes. Nuclei were prepared by homogenisation of the cells in HM (0.3M sucrose, 2 mM magnesium acetate, 3 mM $CaCl_2$, 10 mM Hepes-KOH, pH 7.6, 0.5 mM DTT, 0.1% (v/v) Triton X-100) and purified by sedimentation through 2M sucrose (Sarma et al, 1976). Purified nuclei were resuspended and incubated at a density of ~ 200 x 10^6 nuclei in 2 ml of incubation medium (1 ml solution B + 1 ml IM) containing 0.4 mM each of ATP, GTP and CTP, 0.05 mM UTP and 20 µCi [5,6-^{3}H]UTP (41 Ci/mmol) at 25°C for 10 minutes. RNA was extracted from the nuclei and sedimented through 8-20% sucrose gradients in 98% (v/v) formamide at 157,000 g(av) (Rav 8.75 cm) for 17 hours at 30°C in a Beckman SW56 rotor.

Radioactively labelled cytoplasmic RNA was sedimented in a parallel gradient to provide sedimentation markers.

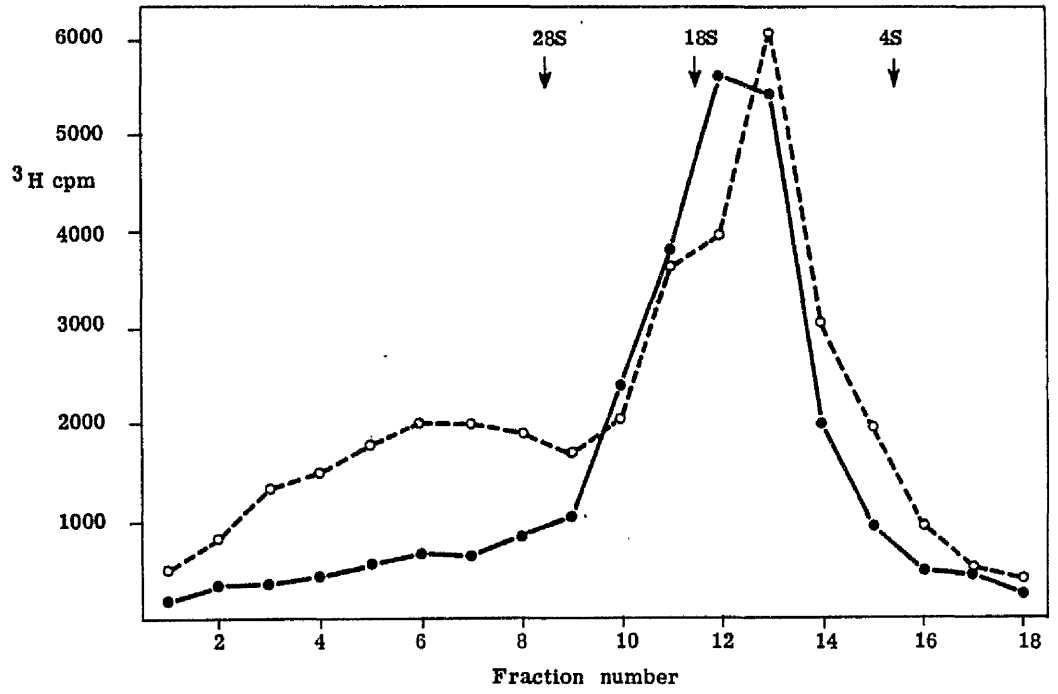
of 15S (Darnell, 1976), and is certainly smaller than expected from the polyacrylamide gel data of Sarma et al (1976). Their method, however, involves the centrifugation of nuclei through 2M sucrose, and it was initially thought that damage could occur to the nuclei during this step. Therefore, a modified procedure was devised, in which cells were homogenised either in the homogenisation medium (Methods section 2.1.2) or in solution B containing 0.1% (v/v) Triton X-100 (Methods section 2.1.3). The nuclei were then isolated, by low speed centrifugation, to give a crude nuclear preparation which was then suspended in the incubation medium.

1.1.2. The size of RNA labelled in crude nuclear preparations

When RNA, labelled in nuclei prepared by simply homogenising in solution B, was analysed on a denaturing gradient (Figure 2) the labelled material from actinomycin D pretreated cells was found to sediment mainly between 5-25S. Although some higher molecular weight material was found in this particular gradient, this result was not always reproducible. A large proportion of the material has sedimentation coefficients between 10S and 18S. Very little radioactively labelled small RNA (4-5S) was found remaining inside the nucleus, but was found in the incubation medium after removal of the nuclei by centrifugation (see Section 3).

The RNA synthesised in these crude nuclear preparations was larger than obtained when the nuclei were purified through 2M sucrose according to the initial protocol. Whilst still not of the large size expected from the original report of Sarma et al (1976), it seemed unlikely that any further modifications of this type (reducing mechanical damage to nuclei, and reducing the time of

Figure 2. Effect of actinomycin D pretreatment on the size of RNA synthesised <u>in vitro</u> by crude nuclei



Nuclei were prepared from control and actinomycin D treated (0.04 μg/ml, 30 minutes) cells by homogenisation in solution B containing 0.1% (v/v) Triton X-100 and isolated by low speed centrifugation (800 g, 10 minutes). The crude nuclear pellet was resuspended and incubated at a density of ~ 200 x 10^6 nuclei in 2 ml incubation medium containing ATP, GTP, CTP (0.4 mM), UTP (0.05 mM) and [5,6-^{3}H]UTP (20 μCi) for 30 minutes at 25°C. RNA was extracted from the nuclei and sedimented through 8-20% sucrose gradients in 98% formamide, 246,000 g(av) (Rav 8.78 cm) for 17 hours at 30°C.

o- - - -o- - - -o nuclei from control cells

●———●———● nuclei from cells pretreated with 0.04 μg/ml actinomycin D for 30 minutes.

preparation) could have much further effect. Therefore, the crude nuclei prepared by low speed centrifugation were further characterised in terms of RNA synthesising ability.

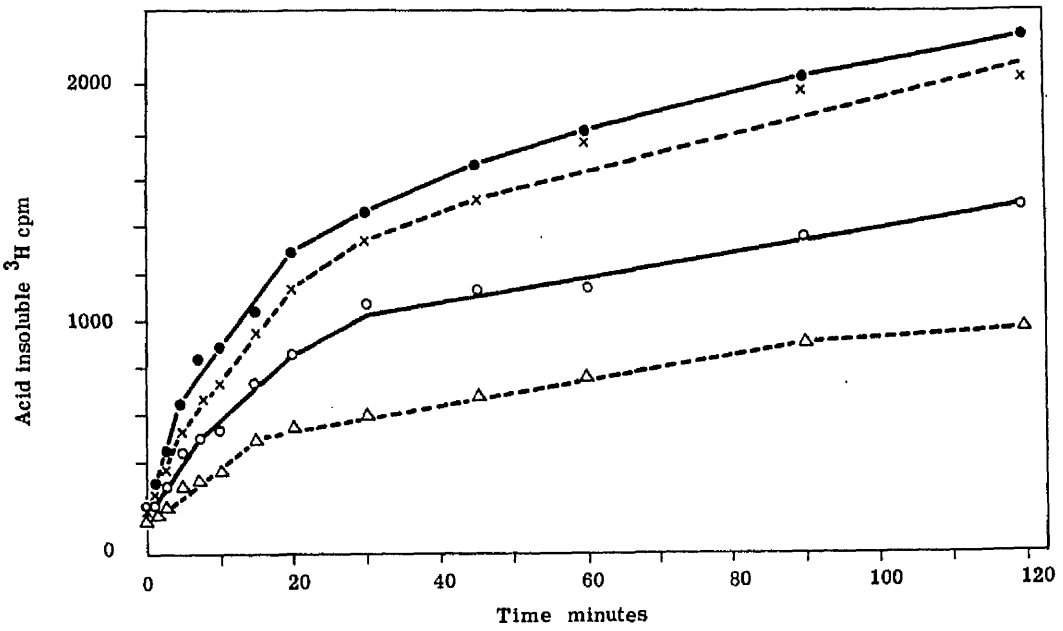
1.2. Characterisation of RNA synthesis in crude nuclear preparations

1.2.1 The effect of inhibitors on the incorporation of labelled precursors into RNA

It was found that the crude nuclei from untreated cells incorporated radioactive UTP into acid insoluble material for more than 2 hours at 25°C (Figure 3) whereas the incorporation into purified nuclei stopped after 10 minutes (results not shown). The incorporation in this case was bi-phasic, with a rapid rise in incorporation over the first 10-20 minutes followed by a steady increase for the next 2 hours. This pattern of incorporation has been found in isolated nuclei from a number of cell types (Ernest et al, 1976; McNamara et al, 1975), including HeLa cell nuclei (Sarma et al, 1976). In myeloma nuclei the incorporation is sometimes biphasic and sometimes linear (Marzluff, 1978). In Figure 7, for instance, it can be seen that the incorporation is linear at 25°C for 30 minutes indicating that this variability in incorporation is also found in HeLa nuclei. The reasons for this commonly observed biphasic incorporation are not yet clear.

Pretreatment of cells with actinomycin D (0.04 μg/ml) reduced the level of incorporation, but the nuclei were still active after 2 hours. This concentration of actinomycin D inhibits ribosomal RNA synthesis in HeLa cells in vivo (Penman et al, 1968) and it will be shown that this pretreatment of cells also inhibits nucleolar RNA synthesis in vitro.

Figure 3. Effect of actinomycin D pretreatment of cells and incubation with α-amanitin on ^{3}H-UTP incorporation by isolated HeLa nuclei.



Crude HeLa cell nuclei were prepared by homogenisation in solution B containing 0.1% (v/v) Triton X-100 and centrifugation at 800 g for 10 minutes. Nuclei were resuspended and incubated at a density of ~ 150 x 10^{6}/2 ml incubation medium, containing nucleoside triphosphates (0.5 mM ATP, GTP and CTP, 0.05 mM UTP) and [5,6-^{3}H]UTP (0.0125 mCi/2 ml). At the indicated times 50 µl aliquots were spotted onto Whatman 3MM paper discs and processed for counting of acid precipitable radioactivity.

●——●——● Nuclei from untreated cells.

×- - -×- - -× Nuclei from cells pretreated with 0.04 µg/ml actinomycin D for 30 minutes.

○——○——○ Nuclei from untreated cells incubated in the presence of 1 µg/ml α-amanitin

△- - -△- - -△ Nuclei from cells pretreated with 0.04 µg/ml actinomycin D incubated in the presence of 1 µg/ml α-amanitin.

Addition of α-amanitin (1 μg/ml) to the nuclear incubation buffer reduced incorporation in nuclei from both untreated and actinomycin D treated cells. It has been shown that α-amanitin at this concentration specifically inhibits purified RNA polymerase II (Lindell et al, 1970; Kedinger et al, 1970) and has the same effect in isolated HeLa cell nuclei (Zylber and Penman, 1971; Weil and Blatti, 1976). The contribution of RNA polymerase II to total incorporation of radioactivity into acid insoluble material can therefore be calculated from this data. Assuming that actinomycin D pretreatment preferentially affects RNA polymerase I activity in isolated nuclei, the RNA polymerase I contribution to total incorporation can also be calculated, as can the residual incorporation when both these RNA polymerase classes are inhibited, which is probably due to RNA polymerase III activity.

The relative contribution of each RNA polymerase to total acid insoluble radioactivity incorporation was found to vary slightly between different experiments, possibly due to differences in the growth state of cells used at different times (Johnson et al, 1974).

1.2.2 The effect of actinomycin D pretreatment on the size of RNA labelled in vitro

In Figure 2 the sedimentation of RNA synthesised in nuclei from actinomycin D treated cells is compared with that from untreated cells. In untreated cell nuclei, RNA sedimenting at > 28S was found in addition to the main peak of material of 15-18S sedimentation coefficient. Although 0.04 μg/ml actinomycin D selectively inhibits the synthesis of ribosomal RNA in intact HeLa cells (Penman et al,

1968), it also inhibits up to 30% of the hnRNA synthesis (Penman et al, 1968). It was therefore possible that the actinomycin D sensitive large RNA was hnRNA, and that the synthesis of RNA smaller than expected in pretreated nuclei was somehow related to the effects of actinomycin D.

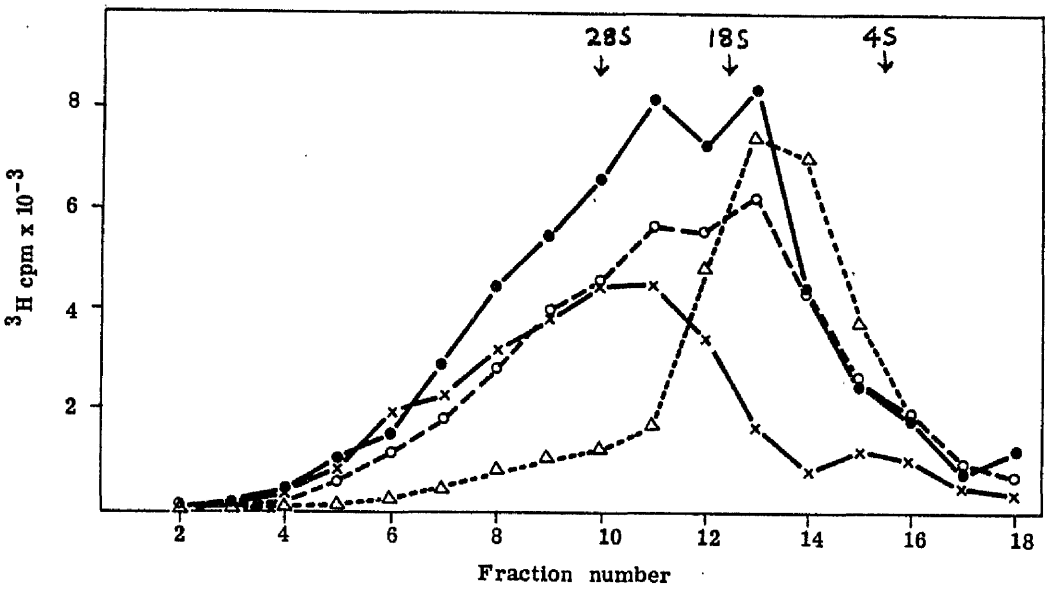
1.2.3 The effect of α-amanitin on the size of RNA labelled in vitro

Addition of α-amanitin to nuclei from untreated cells at concentrations which selectively inhibit RNA polymerase II activity was found to inhibit the synthesis of material with a peak at 15-18S, whereas synthesis of RNA larger than this was insensitive to α-amanitin (Figure 4). Therefore, it is probable that the large (>28S) RNA is synthesised by RNA polymerase I whilst the smaller α-amanitin-sensitive material is synthesised by RNA polymerase II.

1.2.4 The effect of incubation with Actinomycin D on the size of RNA labelled in vitro

The effect of incubating the isolated nuclei in the presence of 0.04 μg/ml actinomycin D is shown in Figure 4. The effect is dramatically different from that of pretreatment of cells in vivo with the same concentration of the drug, in that there is some inhibition of synthesis of all size classes of RNA rather than a complete inhibition of large RNA synthesis. This is in agreement with the results of Lindell (1976), which showed that RNA polymerase I activity in isolated nucleoli from rat liver was insensitive to low concentrations of actinomycin D. In these studies RNA polymerase II had a biphasic response to the drug, with ~ 20% of activity inhibited in the concentration range 1-100 μg/ml actinomycin D and the remaining 80% of activity inhibited by 1-100 μg/ml. It has been proposed (Lindell

Figure 4. Effect of actinomycin D treatment in vivo and in vitro on the size of RNA labelled in isolated nuclei



Nuclei were prepared by homogenisation in HM and centrifugation at 800 g for 10 minutes. The crude nuclear pellet was resuspended such that ~ 100 x 10^6 nuclei were incubated in 2 ml of incubation medium for 30 minutes at 25°C. RNA was extracted from the nuclei and sedimented through 8-20% sucrose denaturing gradients at 246,000 g(av) (Rav 8.78 cm) for 12 hours at 30°C.

●——●——● Nuclei from untreated cells.

△- - - -△- - - -△ Nuclei from cells pretreated with 0.04 µg/ml actinomycin D for 30 minutes

○- - - -○- - - -○ Nuclei from untreated cells incubated with 0.04 µg/ml actinomycin D

×——×——× Nuclei from untreated cells incubated with 1 µg/ml α-amanitin.

et al, 1978), that the observed inhibition of ribosomal RNA synthesis in rat liver by low levels of actinomycin D in vivo is caused by inhibition of the synthesis of mRNA(s) involved in the control of ribosomal RNA transcription, rather than direct inhibition of nucleolar transcription.

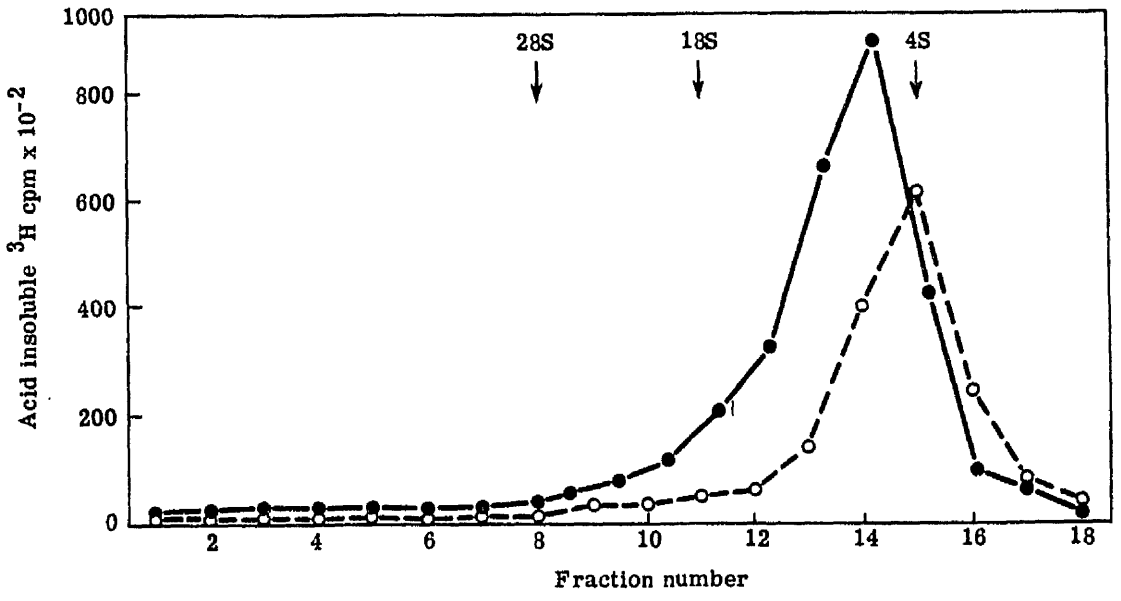
1.2.5 The nuclear location of RNA synthesis in isolated nuclei

Figure 5 shows the size of RNA labelled for 30 minutes in isolated nuclei and detectable in nucleolar and nucleoplasmic subfractions prepared by the method of Penman (1969). After incubation, nuclei were lysed with high salt and digested with DNase I. The material remaining in the supernatant fraction after centrifugation at 10,000 rpm for 20 minutes was termed "nucleoplasmic" whilst the pellet was designated "nucleolar". In Figure 5a, the "nucleoplasmic" RNA synthesised in the absence of α-amanitin sediments between 4S and 18S, but synthesis in the presence of the inhibitor results in a peak of material of $\sim$ 4S. The synthesis of "nucleoplasmic" RNA is insensitive to actinomycin D pretreatment of cells. On this basis, it is concluded that the α-amanitin-insensitive RNA is synthesised by RNA polymerase III and is of the expected size for the major products of this polymerase class (5S rRNA and 4.5S pre tRNA). RNA polymerase II synthesises "nucleoplasmic" material smaller than 18S in this experiment.

In Figure 5b a large proportion of in vitro synthesised RNA recovered in the "nucleolar" pellet sediments as a broad peak between 18S and 45S. The synthesis of this material is not inhibited by α-amanitin (1 μg/ml), but is inhibited by actinomycin D pretreatment of cells. On the other hand, the peak of low molecular weight

Figure 5a. Effect of actinomycin D pretreatment of cells and α-amanitin on the size of RNA synthesised in vitro.

(a) Nucleoplasmic fraction



1 ml aliquots of the 2 ml incubations shown in Figure 3 were removed after 30 minutes and the nuclei were sedimented and resuspended in 2 ml HSB (0.5M NaCl, 0.05M $MgCl_2$, 0.01M Tris-HCl pH 7.4) containing 20 μg/ml DNaseI. The viscous solution was incubated at 37°C until the viscosity was much reduced (~ 2 minutes). The solution was centrifuged at 8,000 g(av) for 5 minutes.

The nucleoplasmic supernatant fraction was phenol extracted. The RNA was analysed on formamide gradients, as described in Figure 2, at 246,000 g(av) (Rav 8.78 cm) for 17 hours at 30°C in the Beckman SW56 rotor.

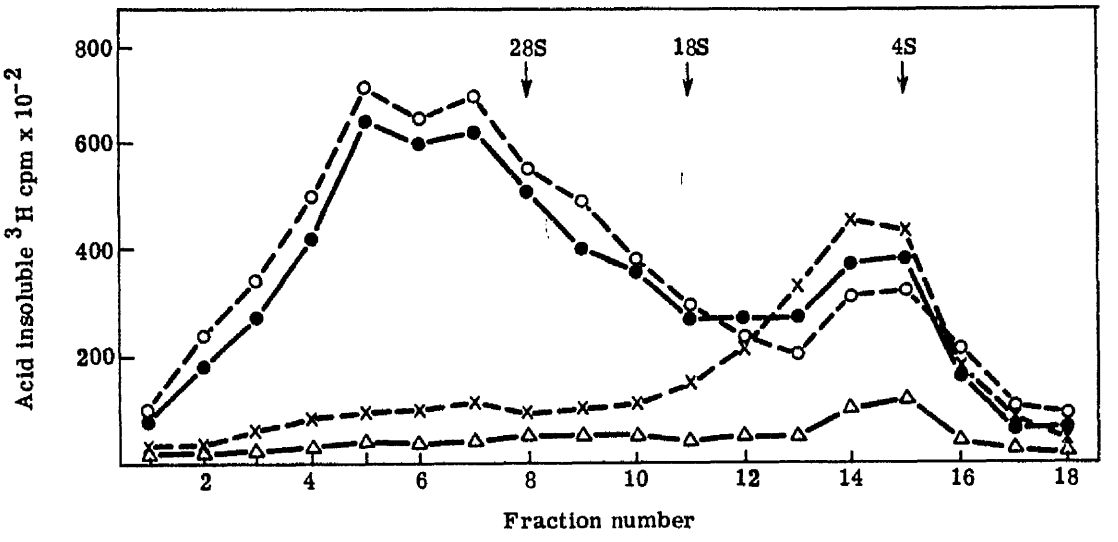
●——●——● Nuclei from untreated cells.

○- - -○- - -○ Nuclei from untreated cells incubated with 1 μg/ml α-amanitin.

Actinomycin D pretreatment of cells had no effect on the sedimentation of nucleoplasmic RNA from control and α-amanitin treated nuclei (results not shown).

Figure 5b. Effect of actinomycin D pretreatment of cells + α-amanitin on the size of RNA synthesised in vitro

(b) Nucleolar fraction



The nucleolar pellet from Figure 5a was resuspended in HSB, DNase digested, and extracted with phenol.

The RNA was analysed on formamide gradients as described in Figure 2 at 246,000 g(av) (Rav 8.78 cm) for 17 hours at 30°C in the Beckman SW56 rotor.

●——●——● Nuclei from untreated cells

o----o----o Nuclei from untreated cells incubated with 1 μg/ml α-amanitin

×----×----× Nuclei from cells pretreated with actinomycin D (0.04 μg/ml)

△——△——△ Nuclei from cells pretreated with actinomycin D (0.04 μg/ml) incubated with α-amanitin (1 μg/ml).

material is not sensitive to actinomycin D treatment of cells, but is inhibited by α-amanitin.

The conclusion from these experiments is that by the criteria of size and location, transcription by RNA polymerases I and III is occurring normally in the isolated nuclei. Thus, RNA polymerase I synthesises a product of up to 45S size found exclusively in the nucleolar fraction, whereas the only product insensitive to both actinomycin D pretreatment and α-amanitin is ~4S in size, and found mainly in the nucleoplasmic fraction.

The product attributable to RNA polymerase II activity is found in both the nucleolar and nucleoplasmic fractions. It is likely that in this crude fractionation procedure large fragments of chromatin are present in the nucleolar pellet, and that nascent RNA is still associated with the chromatin. The size of product however in both fractions is much smaller than would be expected from the size range of pulse-labelled hnRNA, the major RNA polymerase II product *in vivo*.

2. Why is the RNA synthesised by RNA polymerase II in isolated nuclei smaller than hnRNA?

Many of the following experiments have been designed to analyse the discrepancy between the size of RNA synthesised by RNA polymerase II in isolated nuclei and the size of RNA synthesised by the same enzyme class *in vivo*. A major incentive for doing this work was that a number of reports have shown a similar discrepancy using isolated nuclei from different cell types (e.g. Ernest *et al*, 1976; Schafer, 1977; Land and Schafer, 1977; Marzluff, 1978;

Ganguly, 1978), yet, in some of these systems (Panyim et al, 1978; Smith et al, 1976; Orkin et al, 1978; Nguyen-Huu et al, 1978) the correct de novo transcription of specific message sequences has been demonstrated, and the concentration of the synthesised sequence shown to be several orders of magnitude greater than expected if the genome were randomly transcribed.

Moreover, the results shown above indicate that RNA synthesis by RNA polymerases I and III in vitro is comparable to that in vivo in terms of the size of product, which would imply a specific defect in RNA polymerase II activity or the products of this activity, rather than a more general defect in the isolated nuclei system.

2.1. Is the small size of RNA labelled in isolated nuclei due to a slow elongation rate in vitro?

One possible reason for the small size of RNA synthesised in actinomycin D pretreated nuclei is that the elongation rate in vitro is roughly 100 times slower than in vivo, in rat liver nuclei (Coupar and Chesterton, 1977) and chick oviduct nuclei (Cox, 1976), and therefore the size distribution seen in gradients represents a "nascent transcript profile" (Derman and Darnell, 1976). This would occur if the time of incubation resulted in addition of a relatively small number of nucleotides to each nascent chain, making no significant increase in the size of the chain. In this case the size distribution of labelled chains will represent the size distribution of nascent transcripts.

In most isolated nuclei systems it has been difficult to demonstrate reinitiation in vitro by RNA polymerase II (Gilboa et al, 1977) and it has been shown that the majority (90%) of RNA polymerase

II activity in isolated myeloma nuclei involves elongation of existing chains, even when initiation by RNA polymerase II can be detected (Smith et al, 1978).

2.1.1 RNA synthesis at 25°C does not increase the length of nascent RNA chains

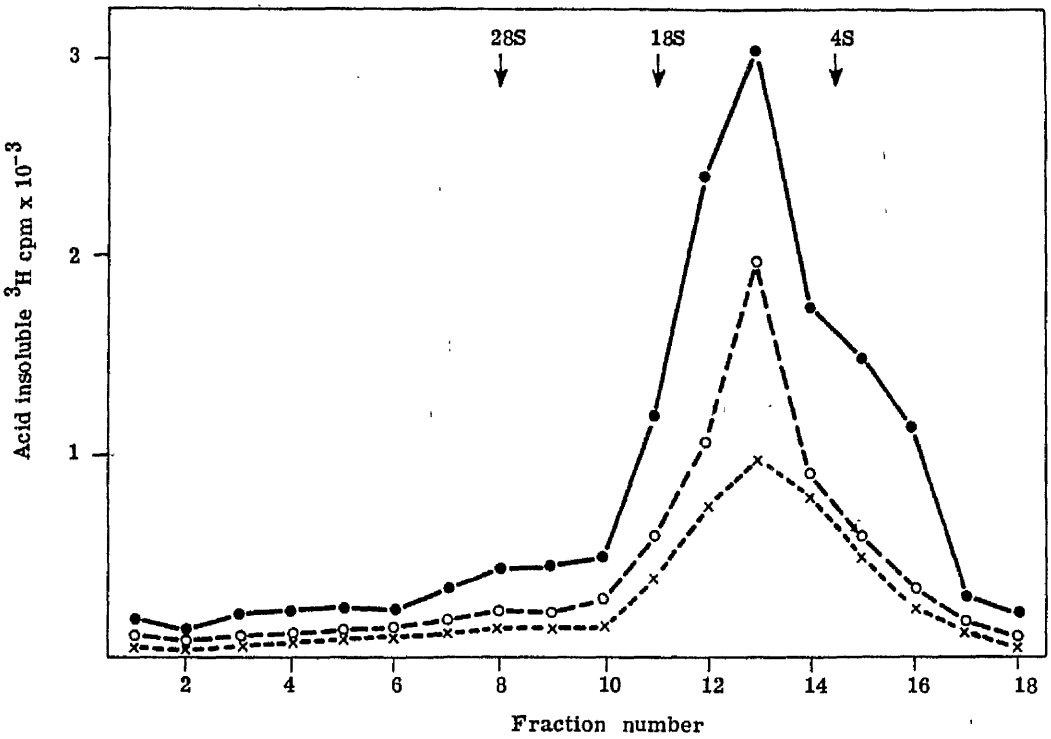
Figure 6 shows the size of RNA synthesised at 25°C for 10 and 30 minutes. Although there is an increase in the amount of radioactivity from 10 to 30 minutes, the RNA labelled for these times sediments at the same position suggesting that little observable growth in chain length has taken place between 10 and 30 minutes. Analysis of the size of RNA synthesised for times from 5 minutes up to 2 hours in separate experiments did not reveal any appreciable increase in size.

Since it appeared from this experiment that the chain elongation rate is not sufficient at 25°C to support discernible growth of RNA chains, it was of interest to study RNA synthesis at higher temperatures to see if a faster elongation rate could be achieved.

2.1.2 The size of RNA synthesised in vitro at 37°C

Figure 7 shows the incorporation of labelled precursor into acid insoluble material in isolated nuclei incubated at 25°C and 37°C. The incorporation at 25°C is linear for 30 minutes, but at 37°C incorporation stops after 20 minutes. This has been reported in a number of isolated nuclei systems (Sarma et al, 1976; Marzluff et al, 1973; Ernest et al, 1976; Udvardy and Seifart, 1976), perhaps indicating a common mechanism in these different cell types. It has

Figure 6. Effect of incubation time at 25°C on the size of RNA labelled in isolated nuclei.



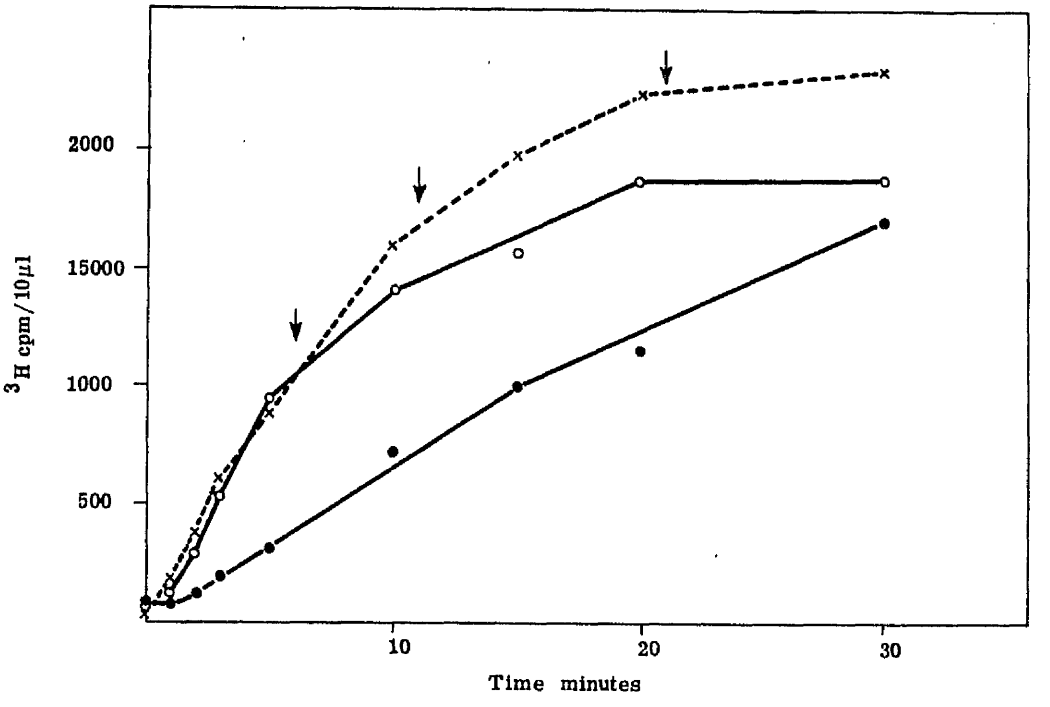
Crude nuclei from actinomycin D (0.04 μg/ml) treated cells were prepared by homogenisation in HM, centrifugation at 800 g for 10 minutes and resuspension in solution B. Nuclei were incubated at 25°C for the indicated times at a density of 100×10^6 nuclei in 2 ml incubation medium containing nucleoside triphosphates and labelled UTP. RNA was extracted from nuclei and analysed as in Figure 2.

●——●——● Nuclei incubated for 30 minutes at 25°C.

○---○---○ Nuclei incubated for 10 minutes at 25°C.

×----×----× Nuclei incubated for 10 minutes at 25°C, centrifuged and resuspended in incubation medium without nucleoside triphosphates, and incubated for a further 20 minutes at 25°C.

Figure 7. Incorporation of [5,6-^{3}H]UTP into acid insoluble material in nuclei incubated at 25°C and 37°C.



Crude nuclei from actinomycin D (0.04 µg/ml) treated HeLa cells were prepared by low speed centrifugation after homogenisation in HM. Nuclei were resuspended in solution B to which an equal volume of solution IM containing nucleoside triphosphates was added to give a final concentration of 0.4 mM ATP, GTP, CTP, 0.05 mM UTP and 20 µCi of [5,6-^{3}H]UTP (41 Ci/mmol) in a volume of 2 ml containing 100×10^6 nuclei. Duplicate 10 µl aliquots were taken at each time and spotted on Whatman 3MM discs.

●——●——● Nuclei incubated at 25°C.

○——○——○ Nuclei incubated at 37°C.

×- - -×- - -× Nuclei incubated at 37°C. Arrows denote addition of nucleoside triphosphates and ^{3}H UTP. Each addition was of an amount equal to the original amount of triphosphates present in the incubation medium.

been suggested that the cessation of incorporation at 37°C could be partly due to hydrolysis of the nucleoside triphosphates (Busiello and Girolamo, 1975), but addition of triphosphates at the indicated time intervals did not affect the cessation of incorporation.

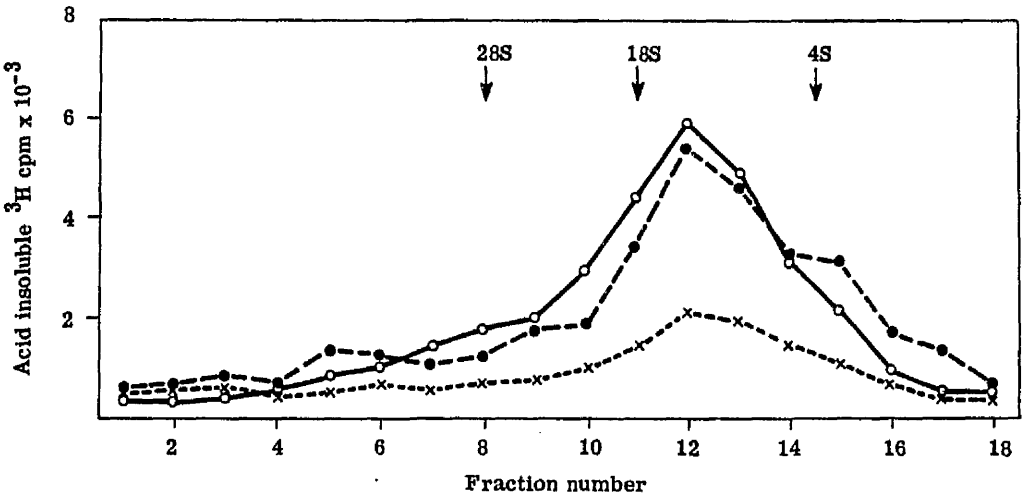
The size of RNA synthesised at 37°C in cell homogenates is shown in Figure 8. RNA synthesised for 5 and 30 minutes at this temperature was compared with RNA synthesised for 5 minutes at 25°C. The amount of labelled RNA is very similar after 5 and 30 minutes incubation, in agreement with the time course of incorporation (Figure 7). The size is also the same at the two times, indicating a stability of this nascent RNA at 37°C. Despite the increase in incorporation at 37°C, the size of product is the same as that synthesised for 5 minutes at 25°C.

2.1.3 Increase in RNA size during incubation of nuclei at 37°C

When the sedimentation of RNA labelled at 37°C for shorter times was examined, an apparent growth in RNA chain length was observed (Figure 9). The material labelled after 1 minute had an average sedimentation coefficient of roughly 4S, compared to a peak between 4 and 18S after 5 minutes. (The nature of the secondary peak between 18 and 28S is unknown, but was not reproducible, as seen in the previous figure.)

There are a number of possible explanations for this observation. Marzluff and Cooper (1978) have reported that the RNA labelled in the first minute of incubation of nuclei isolated from myeloma cells untreated with actinomycin D is mainly small (4-5S) RNA and large RNA (probably precursor ribosomal RNA). This may represent preferential completion of new completed chains. Therefore, in this

Figure 8. Effect of temperature on the size of RNA labelled in total cell homogenates.



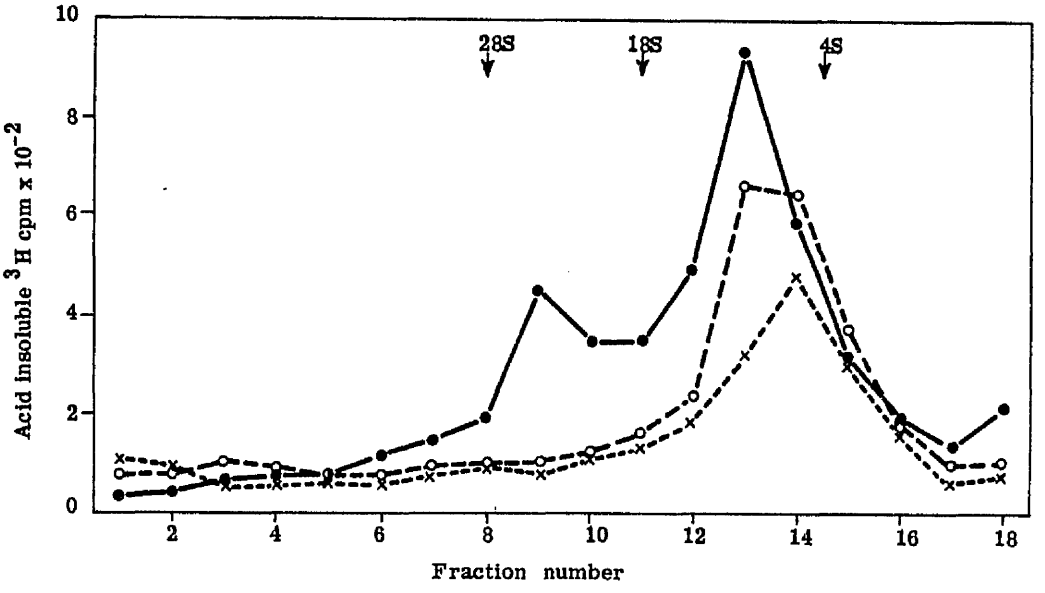
HeLa cells were treated with actinomycin D (0.04 μg/ml) for 30 minutes before harvesting. Cell homogenates were prepared by homogenisation of cells in solution B containing 0.10% (v/v) Triton X-100. Each incubation was prepared by mixing 1 ml of homogenate with 1 ml of IM (150 mM KCl, 5 mM magnesium acetate) containing nucleoside triphosphates and labelled UTP to give a final concentration of ~ 50 x 10^{6} cells/2 ml incubation. RNA was extracted from nuclei isolated from the homogenate, after incubation, by centrifugation and analysed on denaturing gradients as described in Figure 2.

o——o——o Nuclear RNA from homogenate incubated at 37°C for 30 minutes.

●– – –●– – –● Nuclear RNA from cell homogenate incubated at 37°C for 5 minutes.

×----×----× Nuclear RNA from cell homogenate incubated at 25°C for 5 minutes.

Figure 9. The size of RNA labelled in cell homogenates incubated for short times at 37°C.



Homogenates of actinomycin D treated cells were prepared in solution B containing 0.1% Triton X-100 as in Figure 8. Homogenates were incubated at 37°C, and nuclear RNA was extracted and analysed as described in Figure 2.

×----×----× Nuclear RNA from cell homogenates incubated at 37°C for 1 minute.

o— —o— —o Nuclear RNA from cell homogenates incubated at 37°C for 2 minutes.

●——●——● Nuclear RNA from cell homogenates incubated at 37°C for 5 minutes.

case, the synthesis after 1 minute at 37°C could be mainly completion of existing chains on the short tRNA and 5S rRNA transcription units by RNA polymerase III, followed by RNA polymerase II activity at the later times, resulting in apparent growth.

Another explanation could be that at 37°C elongation is relatively rapid in the first few minutes, such that only after 1 minute is a "nascent transcript profile" seen, whereas at later times the majority of chains reach completion (which would also explain the cessation of incorporation). Following the analysis of Dernan and Darnell (1976) this would imply that in the isolated nuclei the effective transcription units transcribed by RNA polymerase II are shorter than *in vivo*. Possible reasons for this will be discussed later.

2.2. The stability of RNA labelled in vitro

Another possible explanation for the finding that the product of RNA polymerase II catalysed synthesis *in vitro* is smaller than hnRNA found *in vivo*, is that the RNA is degraded either during the incubation or in the subsequent RNA extraction procedures. This would provide a simple answer to the observed facts since hnRNA is notoriously labile compared, for instance, to ribosomal RNA, both metabolically (Penman *et al*, 1968; Herman and Penman, 1977) and in terms of sensitivity to contaminating nucleases during isolation procedures.

2.2.1 The stability of RNA labelled in vitro in nuclei incubated at 25°C

To determine whether the nascent RNA was being degraded during the incubation, the fate of RNA labelled for 10 minutes at 25°C

in isolated nuclei was followed by separating the nuclei from the incubation medium by centrifugation, and resuspending in fresh medium without nucleoside triphosphates, for 20 minutes at 25°C (Figure 6). The RNA was found to remain the same size during the post-label incubation.

2.2.2 The stability of RNA labelled in vitro in nuclei incubated at 37°C

Returning to Figure 8, it was shown that labelled RNA remained stable at 37°C during the period after the cessation of synthesis (i.e. between 5' and 30'). In this case, a total cell homogenate was used, rather than crude nuclei, which means that even in the presence of possible cytoplasmic nucleases the nascent nuclear RNA was not degraded at 37°C.

The results discussed in section 2.1.3 also show that the size of RNA produced is not the limit digest size of a rapid processing reaction, since RNA synthesised for very short times is smaller, rather than larger than the final product. These results cannot, however, rule out a processing reaction which cleaves RNA during transcription. This will be discussed later in terms of recent observations on the processing of hnRNA *in vivo*.

2.2.3 The effect of proflavin on the size of RNA labelled in vitro

A further attempt to clarify this point was to analyse synthesis in the presence of proflavin. Proflavin has been reported to inhibit the processing of pre-rRNA and hnRNA (Brinker *et al*, 1973), and processing of adenovirus specific nuclear RNA late in infection (Madore and Bello, 1978). The suggested mode of action is by inter-

calation into double stranded regions of RNA, which could be sites for processing or recognition by processing nucleases (Ryskov et al, 1976; Naora and Whitelam, 1975). Proflavin has also been shown to inhibit the processing in isolated rat liver nuclei of pre-labelled RNA, primarily 45S precursor rRNA (Yannarell et al, 1977).

The effect of proflavin on RNA synthesis in vitro was a slight increase in peak sedimentation coefficient, but there was no increase in the amount of RNA sedimenting faster than 18S (Figure 14). When proflavin was added after 10 minutes of incubation, no size increase was observed. The interpretation of these results is that although the proflavin may stabilise the small RNA to some extent, it is either ineffective in stabilising larger RNA, or that little RNA of size $>$ 18S is being synthesised.

It is also possible that the isolation and extraction procedures cause degradation of the RNA. However, Figure 26 demonstrates that RNA labelled with [5,6 - ^{3}H]uridine for 10 minutes in vivo in the presence of actinomycin D and extracted from purified isolated nuclei is much larger than that synthesised in vitro, and is comparable in size to HeLa hnRNA extracted by other methods and sedimented in denaturing gradients (Derman and Darnell, 1976).

Thus, a general conclusion from these experiments is that the nascent RNA labelled in vitro by RNA polymerase II activity is remarkably stable. It will be shown later that this material is not transported from the isolated nuclei. These results are actually in accord with those of Sarma et al (1976) despite the apparent difference in size of the RNA polymerase II products described by them, and shown here.

2.3. Does the size of RNA labelled in vitro reflect the state of the chromatin template?

A different approach to the problem of the small size of putative hnRNA being synthesised in vitro was to examine conditions which might favourably alter the chromatin template of RNA synthesis. In particular, a change in the state of transcriptionally active chromatin to an inactive state during incubation could result in a decrease in the elongation rate, or complete termination of transcription.

2.3.1 The effect of ionic strength on the size of RNA labelled in vitro

Isolated RNA polymerase II has an optimum activity on a naked DNA template in high concentrations of $(NH_4)_2SO_4$ (Widnell and Taka, 1966; Roeder and Rutter, 1969). It is also possible that increasing the ionic strength of the incubation medium could alter the conformation of chromatin.

Therefore, the effect of increasing the KCl concentration in the incubation medium from 75 mM to 400 mM was tested. It was observed that lysis of some of the nuclei occurred in this medium. However, although RNA synthesis still occurred in this medium, the size of product was found to be the same as RNA labelled in control (75 mM KCl) incubation medium. Nuclei appeared to remain intact under phase contrast microscopy when incubated in 0.35M KCl, but again the labelled RNA size was the same as in control nuclei (results not shown).

Using $(NH_4)_2SO_4$ in the incubation medium instead of KCl

resulted in an increase in the size of RNA labelled *in vitro*. At an $(NH_4)_2SO_4$ concentration of 100 mM, some lysis of nuclei occurred. Therefore, the effect of incubating nuclei in 90 mM $(NH_4)_2SO_4$ (in which lysis was not observed by phase contrast microscopy) on the size of RNA labelled *in vitro* was determined. The results shown in Figure 10 demonstrate the sedimentation of RNA labelled in equal numbers of nuclei incubated either in 90 mM $(NH_4)_2SO_4$ or 75 mM KCl. It was found that, whilst the incorporation of label into RNA in nuclei incubated in 90 mM $(NH_4)_2SO_4$ was no greater than in control nuclei, the RNA was reproducibly larger.

2.3.2 The effect of temperature on the size of RNA labelled in vitro

It might be expected that temperature could affect the conformation of the chromatin or its interaction with factors involved in transcription and consequently have some effect on transcription. However, it has already been demonstrated (Figure 8) that the size of RNA synthesised for five minutes at 37°C is the same as that synthesised at 25°C.

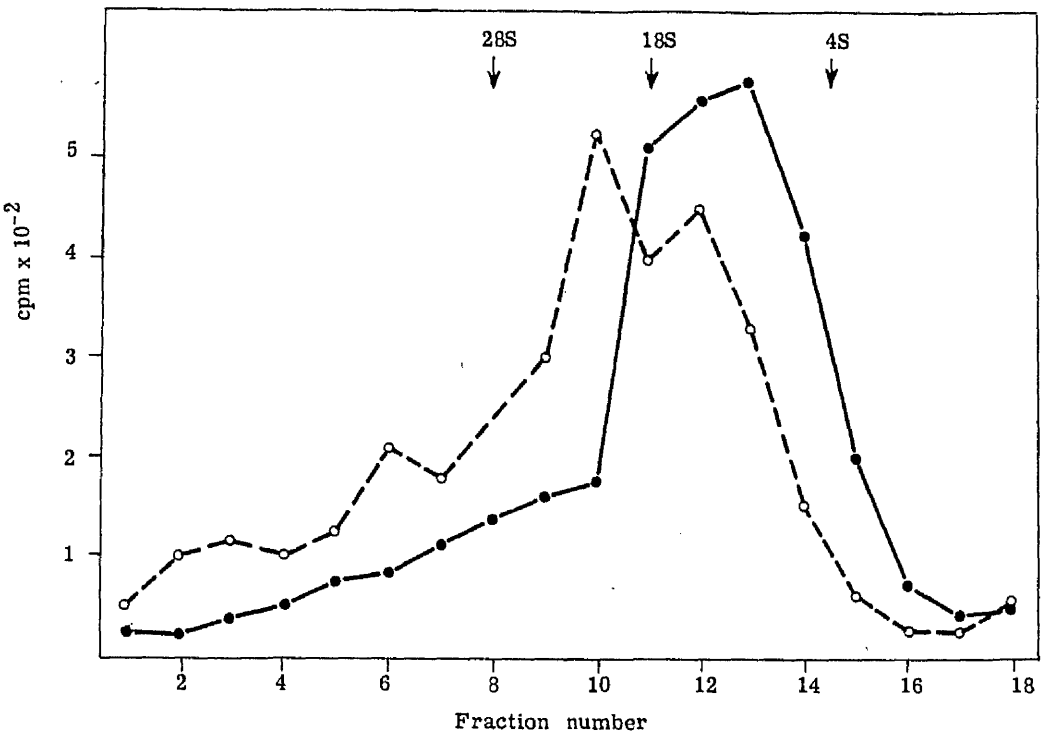
2.3.3 The effect of histone modification on the size of RNA labelled in vitro

Other factors which could alter the template activity of chromatin are the various post-translational modifications of histones, which include acetylation, methylation, phosphorylation and ADP-ribosylation (see Introduction, section

2.3.3.1 Acetylation

Acetylation of histones has been implicated with a role in transcription. Indirectly, increased transcriptional activity has

Figure 10. The effect of ionic strength on the size of RNA labelled in isolated nuclei.



Crude nuclear preparations, isolated by low speed centrifugation from a HeLa cell homogenate in HM, were incubated as indicated.

●——●——● Control: 100 x 10^6 nuclei in 1 ml solution B + 1 ml IM (150 mM KCl, 5 mM magnesium acetate) i.e. Final [KCl] of 75 mM.

○- - -○- - -○ High ionic strength: 100 x 10^6 nuclei in 1 ml solution B + 1 ml 180 mM $(NH_4)_2SO_4$, 5 mM magnesium acetate, i.e. Final $[(NH_4)_2SO_4]$ of 90 mM.

RNA was extracted and analysed as in Figure 2.

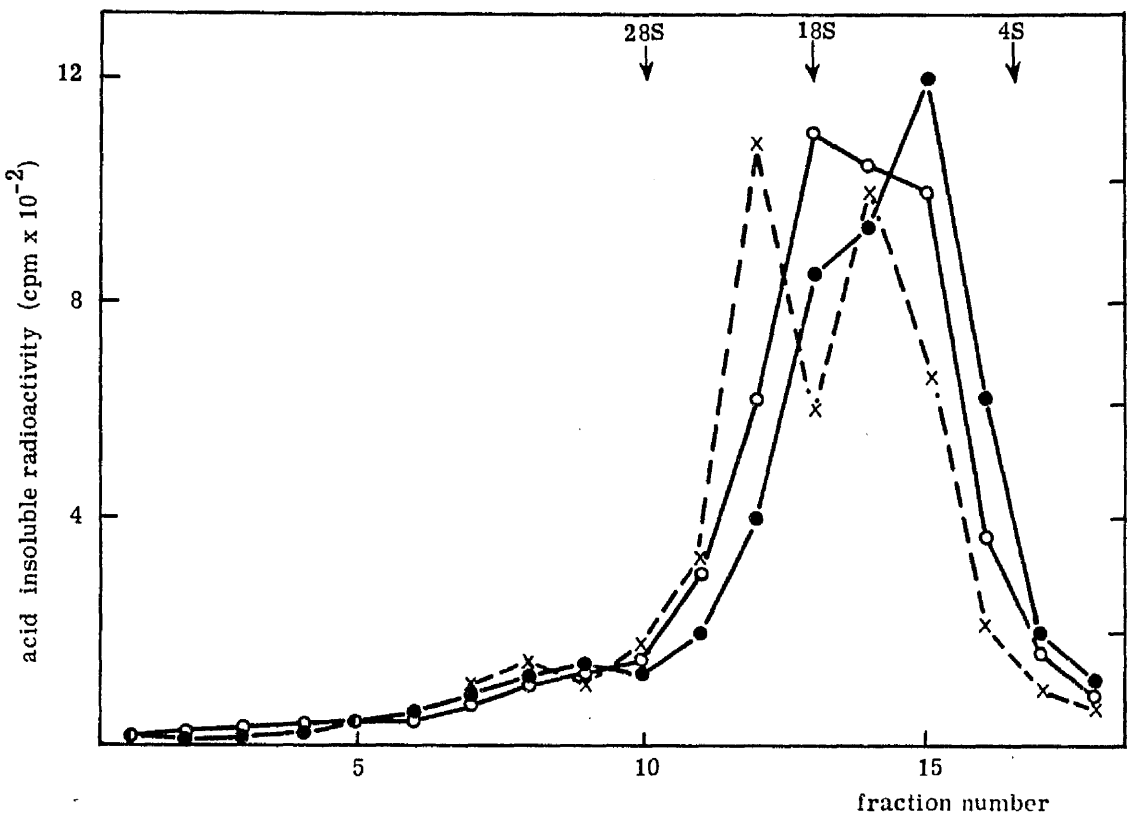
been associated with increased acetylation (Ruiz-Carrillo et al, 1975). More recently, acetylated histones have been shown to be preferentially associated with template-active chromatin (Davie and Candido, 1978) and to be preferentially released from chromatin under conditions which selectively digest transcriptionally active chromatin (Simpson, 1978b; Vidali et al, 1978).

Two approaches to studying the effect of acetylation on RNA synthesis in vitro were used. It is possible to acetylate histones in vitro in isolated nuclei by incubation with acetyl CoA (e.g. Vidali et al, 1968). Therefore, the effect of adding acetyl CoA to the isolated nuclei incubation medium on RNA synthesis was determined. Addition of acetyl CoA to the incubation resulted in an increase in incorporation of label into acid insoluble material of, on average, 50%. Figure 11a shows the sedimentation in denaturing gradients of RNA synthesised in the presence of acetyl CoA (1 mM). Material synthesised in the presence of acetyl CoA was found to be larger; in the size range 5-25S compared to the control range of 4-22S.

The other approach to studying the acetylation of histones is by using sodium butyrate. Addition of butyrate to HeLa cells has been shown to cause the hyperacetylation of histones, mainly H_3 and H_4 (Riggs et al, 1977). This is due to the inhibition of deacetylation by butyrate (Candido et al, 1978), which leads to hyperacetylation because the rapid turnover of acetyl groups on these histones is prevented (Ruiz-Carrillo et al, 1975).

Pretreatment of cells with butyrate at a concentration known to cause gross hyperacetylation in HeLa cells (Riggs et al, 1977)

Figure 11a. The effect of butyrate pretreatment on the size of RNA labelled in isolated nuclei.



Crude nuclei were prepared by low speed centrifugation from homogenates of actinomycin D treated HeLa cells in HM solution. Butyrate treated cells were grown in the presence of 6 mM sodium butyrate for 12 hours, and nuclei from these cells were also incubated in the presence of 6 mM butyrate. All nuclei were incubated at 25°C for 30 minutes (~ 100 x 10^6 nuclei in 2 ml incubation medium).

RNA was extracted and analysed on denaturing sucrose gradients as in Figure 2.

●——●——● Nuclei incubated in incubation medium.

×– – –×– – –× Nuclei incubated in incubation medium containing 1 mM acetyl CoA.

○——○——○ Nuclei incubated in incubation medium containing 6 mM sodium butyrate.

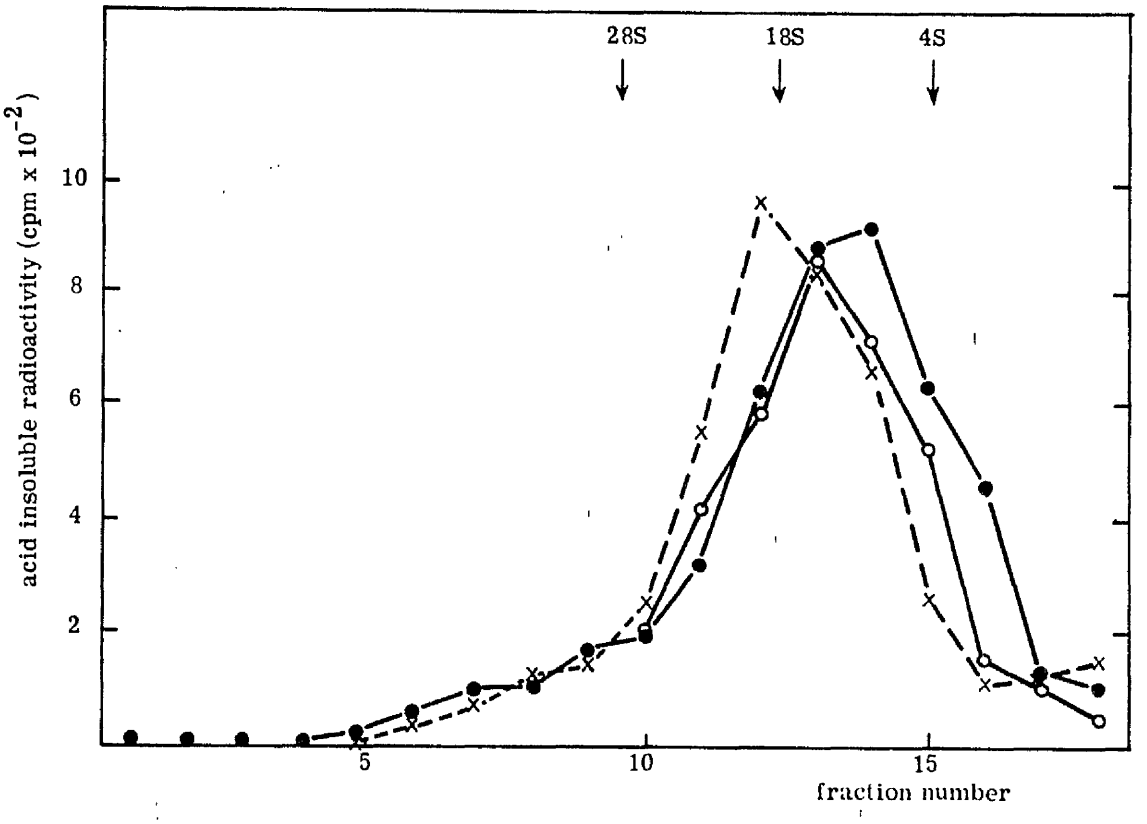
These nuclei were prepared from cells pretreated with 6 mM sodium butyrate for 12 hours.

effected only a small increase in the size of RNA labelled *in vitro*. In this experiment, butyrate was also present in the incubation medium to prevent deacetylation of the hyperacetylated histones (Candido *et al*, 1978). The size increase was not as great as that caused by acetyl CoA, even though it might be expected that more acetyl groups would be present in butyrate treated cell nuclei than in the *in vitro* acetylated nuclei.

In another experiment (Figure 11b), an attempt to increase the effects of acetylation was made by pre-incubating nuclei for 5 minutes with acetyl CoA and butyrate before starting RNA synthesis. It was expected that the preincubation might increase the level of acetylation before the start of RNA synthesis, and that butyrate would inhibit any deacetylation. However, the size increase was only moderate. Addition of butyrate alone at the start of synthesis had very little effect. Thus, although the effect of *in vitro* acetylation is to increase both incorporation of label and the size of RNA, the effect of butyrate is less than would be predicted from its dramatic effect on acetylation of histones H_3 and H_4.

In order to characterise the effect of acetyl CoA on RNA synthesis, the incorporation of [1 - ^{14}C]acetyl CoA into proteins in isolated nuclei was examined. Figure 11c shows a time course of incorporation of ^{14}C acetyl groups into hot acid precipitable material in isolated nuclei. More than 90% of these ^{14}C acetyl groups were incorporated into chromatin proteins soluble in 0.2M H_2SO_4 (results not shown). Solubility in 0.2M H_2SO_4 is a characteristic property of histones, making it probable that a large proportion of the proteins acetylated *in vitro* were histones.

Figure 11b. The effect of incubation with butyrate and acetyl CoA on the size of RNA labelled in isolated nuclei



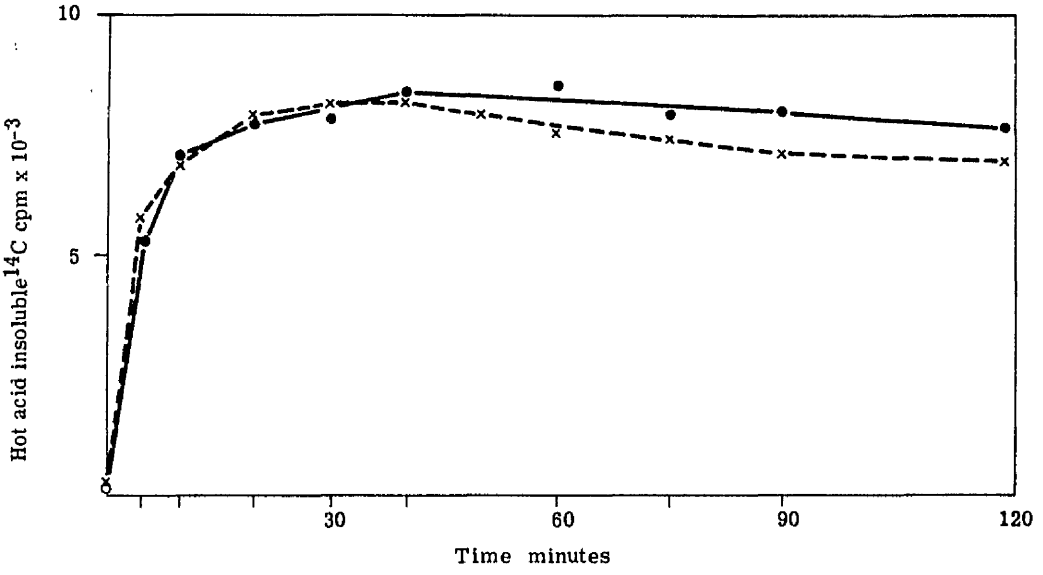
Nuclei were prepared and incubated as in Figure 11a. RNA was extracted and analysed on denaturing gradients as in Figure 2.

●——●——● Nuclei incubated for 30 minutes at 25°C.

o——o——o Nuclei incubated for 30 minutes at 25°C in 6 mM butyrate.

x----x----x Nuclei pre-incubated for 5 minutes at 25°C with 5 mM acetyl CoA and 6 mM butyrate in the absence of nucleoside triphosphates, then for 30 minutes with 2.5 mM acetyl CoA, 6 mM butyrate and nucleoside triphosphates.

Figure 11c. Time course of acetylation of proteins in isolated nuclei



Isolated nuclei were prepared from HeLa cells by homogenisation of cells in homogenisation buffer (HM), and low speed centrifugation. The crude nuclear preparation was resuspended in incubation medium (equal volumes of solution B and IM) containing [1-^{14}C]acetyl coenzyme A (58.7 mCi/mmol) at a final concentration of 0.1 μCi/ml. Nuclei were incubated at 25°C, and the reaction was stopped at the indicated times by addition of excess 15% (w/v) trichloroacetic acid containing 0.1M sodium pyrophosphate. Incubation tubes were then heated at 60°C for 1 hour for determination of hot acid insoluble radioactivity.

●——●——● Nuclei incubated at 25°C in incubation medium.

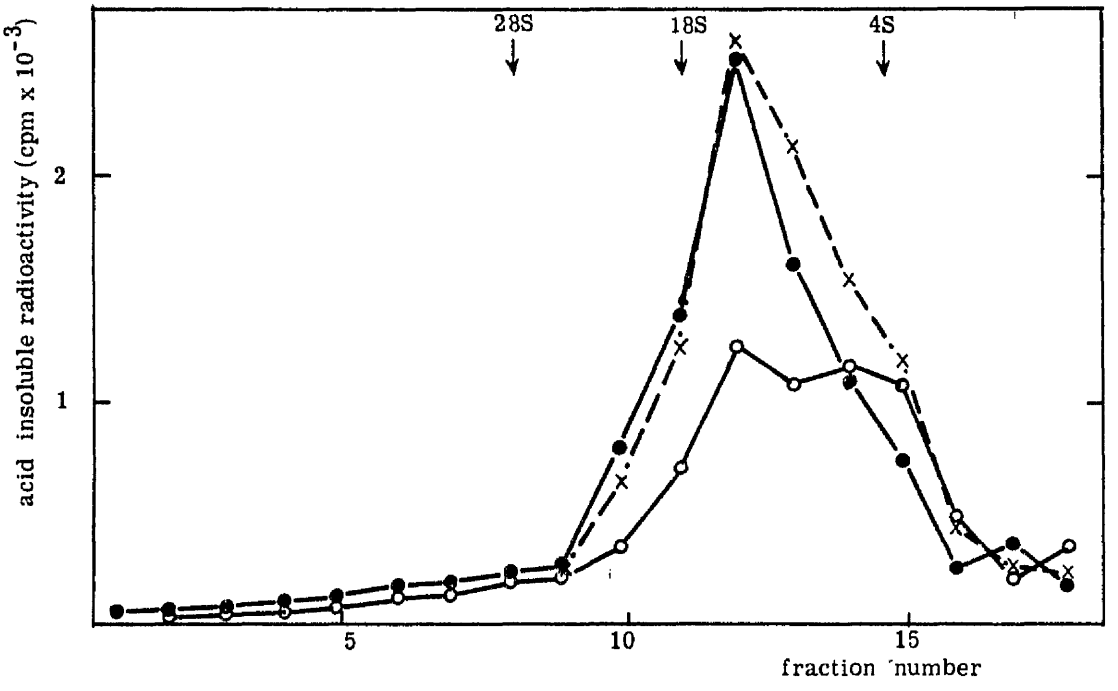
×----×----× Nuclei incubated at 25°C in incubation medium containing the four ribonucleoside triphosphates at the usual concentrations for RNA synthesis (0.4 mM each ATP, GTP, CTP, 0.05 mM UTP).

It was found that the incorporation of acetyl groups into protein was rapid, reaching a plateau within 10 minutes. The addition of more labelled acetyl CoA after 20 minutes did not increase this incorporation, suggesting that the plateau was not simply caused by depletion of acetyl CoA. It was observed that the acetylation of protein in nuclei incubated in the presence of ribonucleoside triphosphates (at the usual concentration for RNA synthesis), reached the same plateau level during the early stages of incubation. However, this level decreased slightly (but reproducibly) more rapidly than the level of acetylation in nuclei incubated without NTPs. This implies that although the extent of acetylation is not appreciably altered in the presence of RNA synthesis, the turnover of acetyl groups may be greater.

2.3.3.2. Methylation

A similar approach was adopted in the study of the effect of methylation on the size of RNA synthesised in vitro. Figure 12 is a denaturing gradient analysis of RNA synthesised in isolated nuclei after preincubation for 30 minutes with 1 mM S-adenosyl methionine (Ado-Met). This time of preincubation was chosen since, unlike acetylation which reaches a steady state level within 10 minutes, the methylation of nuclear protein was found to be linear up to at least 30 minutes (results not shown). Again, a large proportion of methyl groups were incorporated into proteins soluble in 0.2M H_2SO_4, probably histones. In this, and other, experiments, there was a decrease in the size of RNA labelled in nuclei incubated in the presence of Ado-Met. The effect of Ado-Met was found to be antagonistic to that of acetyl CoA, such that incubation in the presence of both at the particular concentrations indicated resulted in RNA of a size very similar to the control.

Figure 12. The effect of acetyl CoA and S-adenosyl methionine on the size of RNA labelled in isolated nuclei.



Nuclei were prepared as described in Figure 11 and incubated for 30 minutes before addition of RNA precursors and then incubated at 25°C for a further 30 minutes.

●——●——● Nuclei pre-incubated for 30 minutes, incubated with ribonucleoside triphosphates for 30 minutes.

o——o——o Nuclei pre-incubated for 30 minutes with Ado-Met (1 mM), incubated with ribonucleoside triphosphates and Ado-Met for 30 minutes.

x- - -x- - -x Nuclei pre-incubated with Ado-Met (1 mM) and acetyl CoA (2.5 mM), then incubated in the same medium with ribonucleoside triphosphates for 30 minutes.

RNA was extracted from nuclei and analysed as in Figure 2.

To determine whether the effect of Ado-Met was at the level of transcription or was a post-transcriptional event (for instance by stimulating processing of the RNA by acting as a substrate for methylation of the RNA as demonstrated in isolated nuclei (Winicov and Perry, 1976; Groner and Hurwitz, 1975)), Ado-Met was added to the "pulse-chase" type experiment described earlier (Figure 13). RNA was labelled for 10 minutes *in vitro*, after which the nuclei were centrifuged from the labelling medium and resuspended in fresh medium without triphosphates. The presence of Ado-Met in the fresh medium made no difference to the size of product recovered from the nuclei after the "post-label" incubation. The effect of Ado-Met would therefore appear to be at the level of transcription.

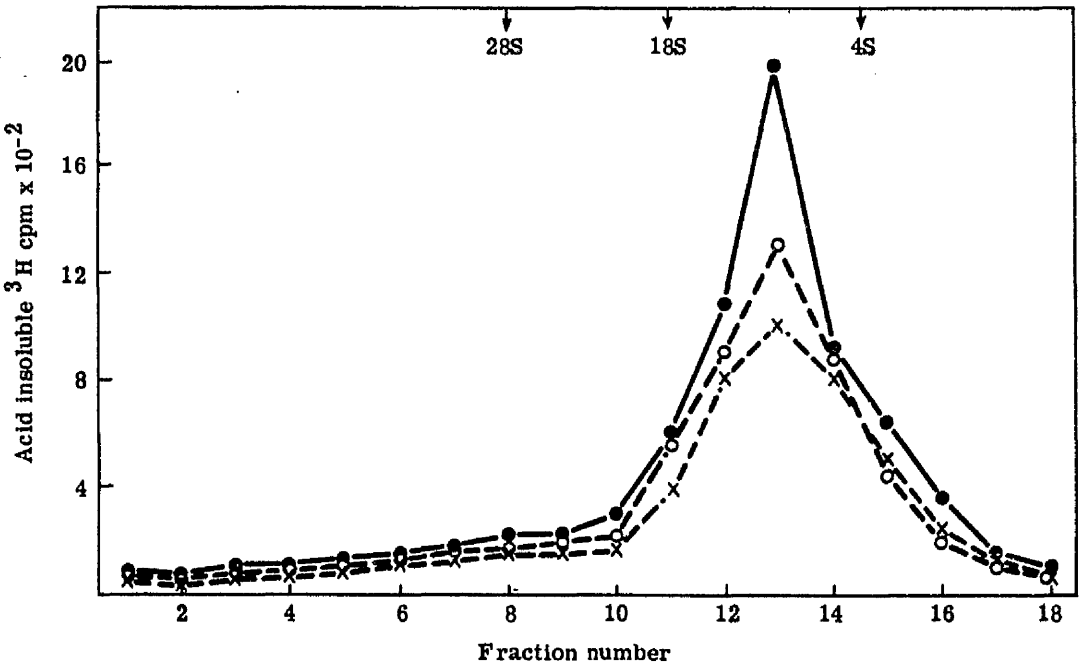
2.3.3.3. Phosphorylation

Following the reasoning applied above, the effects of phosphorylation were examined by incubating nuclei with a final concentration of ATP of 1 mM, with or without cyclic AMP. Only a very small increase in the size of RNA labelled *in vitro* was observed.

2.3.3.4. ADP-ribosylation

Similarly, incubating nuclei with concentrations of NAD^+ known to support ADP-ribosylation in isolated nuclei (Hayashi and Ueda, 1977) had no effect on the RNA size. Apart from effects due to chromatin modification, NAD^+ has been reported to inhibit RNA polymerase I activity in isolated nuclei by directly modifying RNA polymerase I, but to stimulate RNA polymerase II by an unknown mechanism (Furneaux and Pearson, 1978). Another report mentioned that by inhibiting DNase activity, NAD^+ was capable of increasing the

Figure 13. The effect of S-adenosyl methionine on the stability of RNA labelled in isolated nuclei.



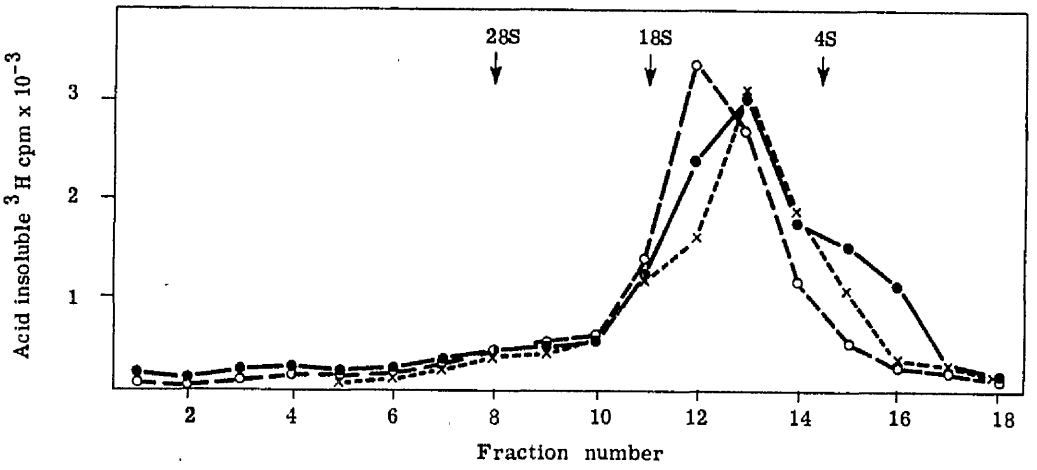
Nuclei were prepared and incubated as in Figure 6. After 10 minutes incubation at 25°C, nuclei were centrifuged and resuspended in fresh incubation medium without nucleoside triphosphates. These were incubated for a further 20 minutes, with or without S-adenosyl methionine. The RNA was analysed on denaturing gradients, and compared with RNA from nuclei labelled for 10 minutes.

●——●——● Nuclei incubated at 25°C for 10 minutes.

×– – –×– – –× Nuclei labelled at 25°C for 10 minutes, then incubated at 25°C for 20 minutes in the absence of NTP's.

o– – –o– – –o Nuclei labelled at 25°C for 10 minutes, then incubated at 25°C for 20 minutes in the presence of Ado-Met, and the absence of NTP's.

Figure 14. The effect of proflavin on the size of RNA labelled in isolated nuclei.



Crude isolated nuclei prepared by centrifugation at low speed from a HeLa cell homogenate in HM were incubated at a density of ~100 x 10^6 nuclei/2 ml incubation medium.

RNA was analysed on denaturing gradients as in Figure 2.

●——●——● Nuclei incubated at 25°C for 30 minutes.

o——o——o Nuclei incubated with 100 μM proflavin at 25°C for 30 minutes.

x----x----x Nuclei incubated at 25°C for 10 minutes, then a further 20 minutes in the presence of proflavin (100 μM).

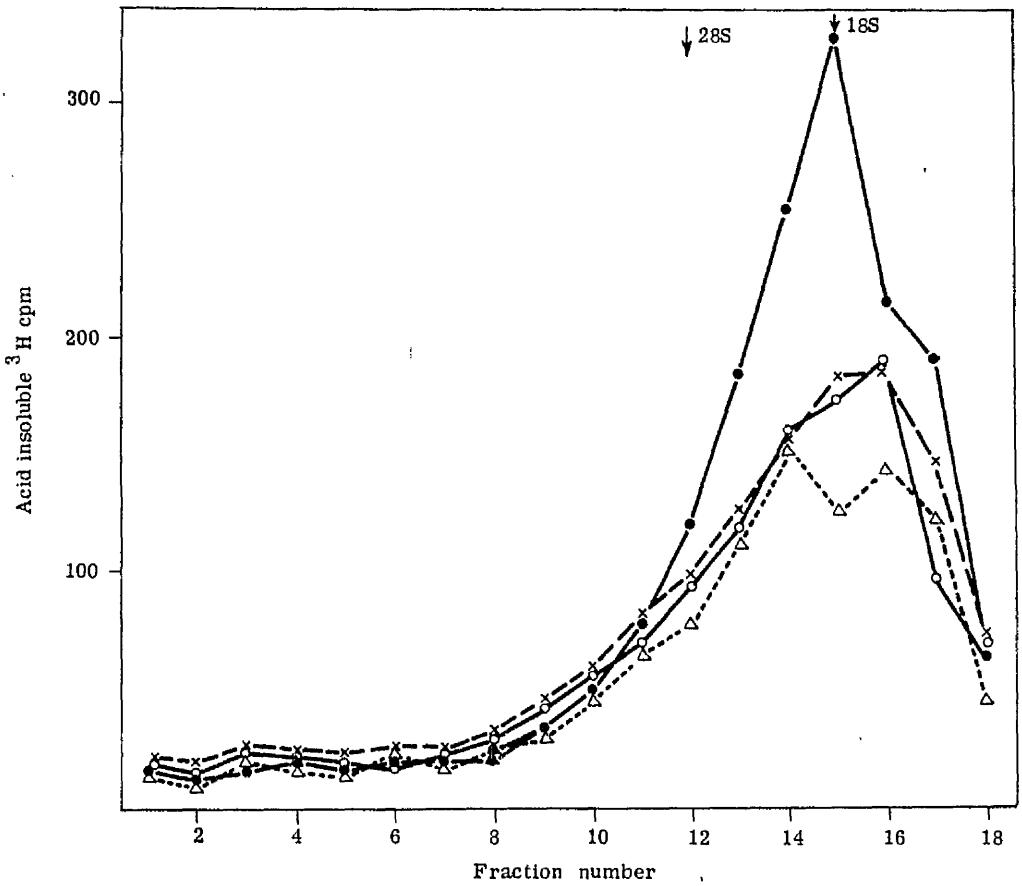
size of RNA synthesised in isolated rat liver nuclei (Lindell, 1977), yet in the present case, no effect of this kind was observed.

2.4 Other factors which might influence RNA synthesis in vitro

2.4.1 The effect of cytoplasmic factors on the size of RNA synthesised in vitro

It has been observed in a number of isolated nuclei systems that the addition of cytosol or cytoplasm enhances RNA synthesis (Wu and Zubay, 1974; McNamara *et al*, 1975; Mory and Gefter, 1977; Bastian, 1977; Icekson, 1979), including the isolated HeLa nuclei system used in this study (Sarma, 1976). The most direct way of testing the effect of cytoplasm, or leaked nuclear components contained in the cytoplasmic fraction, was to homogenise the cells directly into solution B, and use this homogenate in the RNA synthesis medium in place of nuclei resuspended in solution B. It can be seen in Figure 16 that the size of α-amanitin sensitive labelled RNA from homogenates is comparable to that synthsised in crude nuclei isolated from the homogenate. The non-ionic detergent Triton X-100 was added to solution B to solubilise cellular membranes during homogenisation. When non-homogenised cells were treated with Triton X-100 and used in the RNA synthesis medium they were found capable of synthesising RNA, but again only of a peak size of roughly 15S. It is presumed that the Triton-treated cells were lysed due to solubilisation of cellular membranes, leaving a nucleus embedded in the cytoskeletal structure described by Lenk *et al* (1977). Thus, this would tend to eliminate damage done to the nuclei or removal of the cytoskeleton as reasons for abnormal RNA synthesis in isolated nuclei.

Figure 15. The effect of detergent on the size of RNA labelled in HeLa cell homogenates and isolated nuclei.



HeLa cells treated with actinomycin D (0.04 μg/ml) were homogenised in solution B with or without 0.1% (v/v) Triton X-100. The homogenates, and corresponding crude nuclei prepared by low speed centrifugation (800 g for 10 minutes) and resuspension in solution B, were incubated for 15 minutes at 25°C. The nuclear RNA was analysed by denaturation in 80% DMSO, 10% formamide at 50°C for 5 minutes and centrifuged on 15-30% sucrose gradients in LETS buffer at 30,000 g(av) (Rav 11.8 cm) for 17 hours at 20°C in the Beckman SW27 rotor.

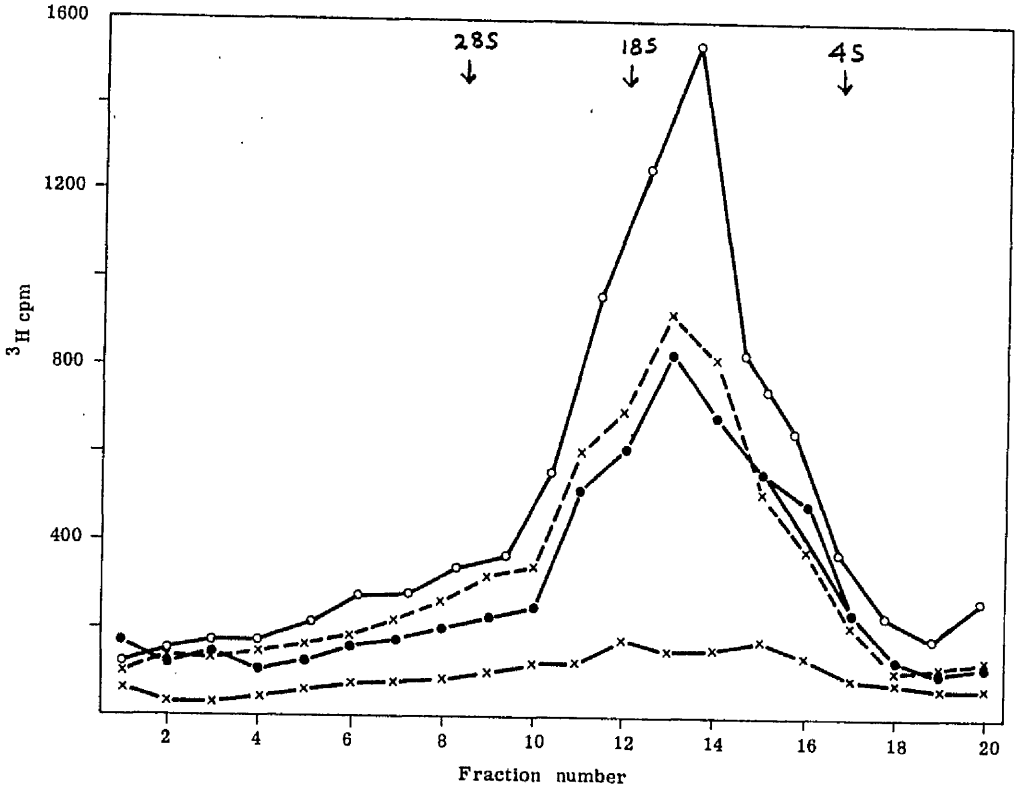
●——●——● Nuclei isolated from cell homogenate prepared in the presence of 0.1% (v/v) Triton X-100.

o——o——o Nuclei isolated from cell homogenate prepared in the absence of Triton X-100.

△- - -△- - -△ Cell homogenate prepared and incubated in the presence of Triton X-100 (0.1%).

×- - -×- - -× Cell homogenate prepared and incubated in the absence of Triton X-100.

Figure 16. The effect of cytoplasmic factors on the size of RNA labelled in isolated nuclei.



HeLa cells treated for 30 minutes were suspended in solution B containing 0.1% (v/v) Triton X-100. Part of the suspension was homogenised, and nuclei were isolated from part of this homogenate by low speed centrifugation, and resuspended in solution B. Equivalent amounts of cell suspension, homogenate and nuclei in solution B were assayed by addition of an equal volume of IM containing nucleoside triphosphates and incubated for 5 minutes at 25°C. The RNA was extracted from nuclei and analysed as in Figure 2.

o——o——o Nuclear RNA from detergent treated cell suspension incubated at 25°C for 30 minutes.

x - - -x - - -x Nuclear RNA from cell homogenate incubated at 25°C for 30 minutes.

●——●——● Nuclear RNA from isolated nuclei incubated at 25°C for 30 minutes.

x——x——x Nuclear RNA from isolated nuclei incubated with α-amanitin (1 µg/ml) at 25°C for 30 minutes.

2.4.2. The effect of removal of nuclear membranes on the size of RNA labelled in vitro

To test whether the Triton X-100 was having an effect on RNA synthesis either directly, or by removal of both nuclear membranes (Aaronson and Blobel, 1974; Scheer *et al*, 1976), cell homogenates were prepared in solution B without Triton X-100 and these and the crude nuclei isolated from them were compared with Triton X-100 prepared homogenates and nuclei. Again, no difference in sedimentation in denaturing gradients of the labelled products was found (Figure 15). Thus, the presence or absence of nuclear membranes would appear not to be important for *in vitro* RNA synthesis.

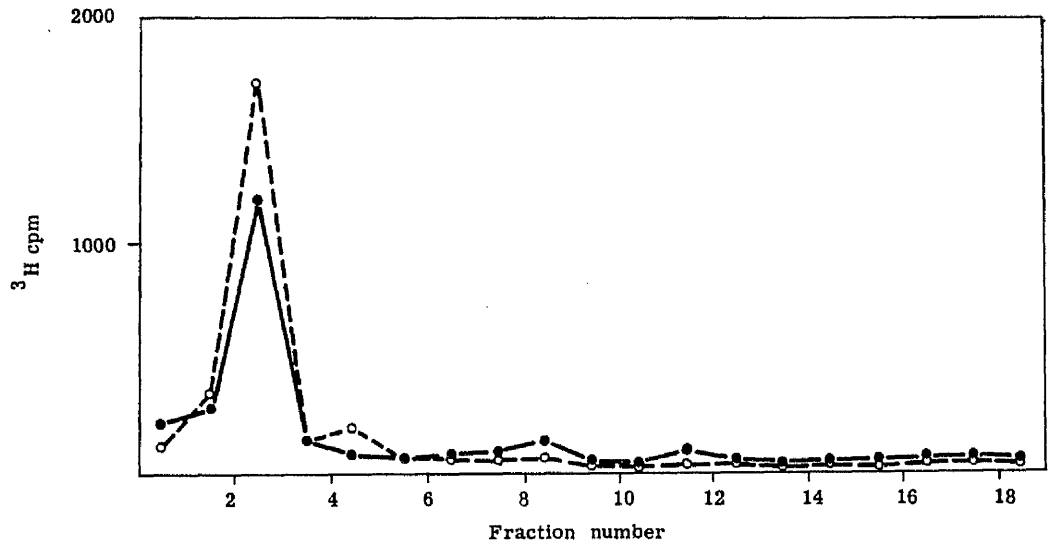
2.5. The size of DNA template in isolated nuclei

2.5.1. The size of DNA in isolated nuclei as determined by neutral sucrose gradient sedimentation

Another approach to the problem of small RNA synthesis was to look at the size of DNA in the isolated nuclei, since enzymatic cleavage or mechanical shearing could reduce the DNA to a size too small to support complete transcription of lengthy transcription units. To do this without introducing artefacts of shearing during the preparation of DNA, cells or nuclei were lysed on top of either neutral or alkaline sucrose gradients according to the method of Cheevers *et al* (1972).

Under the conditions used, the labelled DNA from incubated nuclei was still found to sediment onto the cushion in neutral SDS-sucrose gradients even after pronase treatment (Figure 17). Thus, there appeared to be no degradation of double stranded DNA during incubation of nuclei.

Figure 17. Sedimentation in neutral SDS-sucrose gradients of DNA from isolated nuclei.



HeLa cells were labelled for 48 hours with 3.3 μCi/ml [6-^{3}H] thymidine (27 Ci/mmol). Crude nuclei were prepared by homogenisation in HM and low speed centrifugation (800 g, 10 minutes). Nuclei were resuspended in solution B, to which an equal volume of IM was added, to give a final concentration of roughly 2.5 x 10^6 nuclei/ml. Nuclei were resuspended in SSC at a density of 10^6 nuclei/ml and made 0.5% (w/v) w.r.t. SDS. Pronase was added to a final concentration of 500 μg/ml and the lysate was incubated at 37°C for 1 hour. 10% SDS was added to give a final concentration of 1% (w/v) SDS. The lysate was poured very gently onto a 30 ml 15-30% (w/v) sucrose gradient in 0.1M NaCl, 0.001M EDTA, 0.01M Tris-HCl (pH 7.4) 0.5% SDS formed over a 6 ml 70% (w/v) sucrose cushion. The gradients were centrifuged at 64,000 g(av) (Rav 11.8 cm) for 14 hours at 25°C in the Beckman SW27 rotor.

●——●——● Non-incubated nuclei.

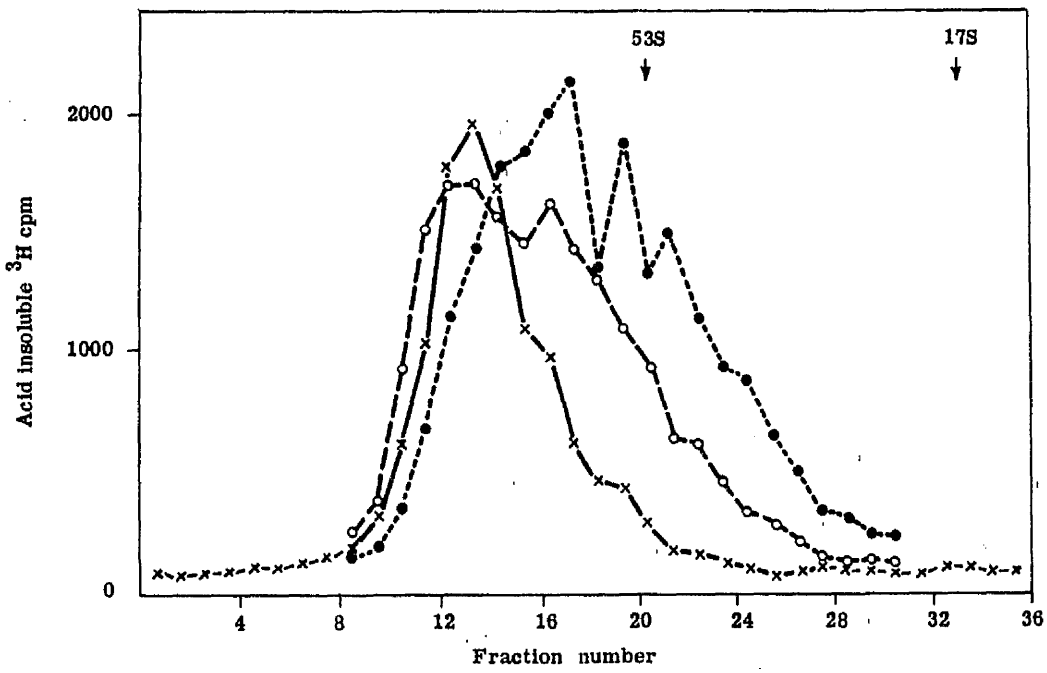
o- - -o- - -o Nuclei incubated at 25°C for 30 minutes before lysis.

2.5.2. The size of nuclear DNA as determined by sedimentation in alkaline sucrose gradients

In contrast to neutral gradients, when alkaline gradients were run, cellular DNA sedimented near to, but not on, the dense sucrose cushion (Figure 18). Similar sedimentation behaviour has been shown for DNA from mouse embryo cells (Cheevers et al, 1972) and Chinese hamster cells (Cleaver, 1974). When whole cells and isolated nuclei were compared on alkaline gradients, the labelled DNA from isolated nuclei was found to have a slower sedimentation behaviour. Furthermore, the labelled DNA from nuclei incubated at 25°C for 30 minutes was found to be smaller than DNA from non-incubated nuclei. This difference in the single strand length of DNA from intact cells, isolated nuclei and incubated nuclei, was found reproducibly, although the extent of size difference varied between experiments. It was also noticed that the pattern of degradation was reproducible, in that the single strand length of cellular DNA is fairly homogeneous, but is degraded to a more heterogeneous population of lengths upon isolation and incubation of nuclei.

Sedimentation markers were run in a parallel gradient. These were ^{14}C-labelled form I and II SV40 DNA (Materials section), with sedimentation coefficients of 53S and 17S respectively. From these it was determined that the average sedimentation coefficient of cellular DNA is $\sim$ 100S whilst the average sedimentation coefficient of DNA from nuclei incubated at 25°C for 30 minutes was $\sim$ 70S. Using the formula of Studier (1965) which relates the sedimentation coefficient of DNA in alkaline gradients to molecular weight, an average single-strand chain length for cellular DNA of 6×10^5 nucleo-

Figure 18. Alkaline sucrose-gradient centrifugation of DNA from cells and nuclei.



HeLa cells were labelled at 48 and 24 hours before harvesting with 2.5 μCi/ml [6-^{3}H]thymidine (27 Ci/mmol). Crude nuclei were prepared by homogenisation in HM and low speed centrifugation (800 *g* for 10 minutes). Nuclei were resuspended in SSC at a density of roughly 4 x 10^6 nuclei/ml. 0.25 ml of alkaline lysis solution (0.3N NaOH, 0.001M EDTA, 0.5% (w/v) SDS) was layered over a 4 ml 15-30% (w/v) sucrose gradient in 0.5M NaCl, 0.25N NaOH, 0.001M EDTA, 0.01% SDS, formed on a 0.5 ml 70% (w/v) sucrose cushion. 0.025 ml of the nuclear suspension in SSC, corresponding to 10^5 nuclei, was carefully pipetted into the overlay and lysed at 25°C for 16 hours. The gradients were centrifuged at 152,000 g(av) (Rav 8.35 cm) at 25°C for 1 hour in the Beckman SW50.1 rotor.

×———×———× Cells applied directly to alkaline gradients.

o– – –o– – –o Isolated nuclei applied directly to alkaline gradients.

●-----●-----● Isolated nuclei incubated at 25°C for 30 minutes in normal incubation medium before layering on alkaline gradients.

tides was estimated, compared to a value of 2 x 10^5 nucleotides for DNA in incubated nuclei. One important thing to notice from this figure is that the length of single strands of DNA in incubated nuclei is still very large (~ 200 kilobases) compared to the size of transcription units in HeLa cells (number average:- 5 kilobases) (Derman and Darnell, 1976; Goldberg et al, 1977a).

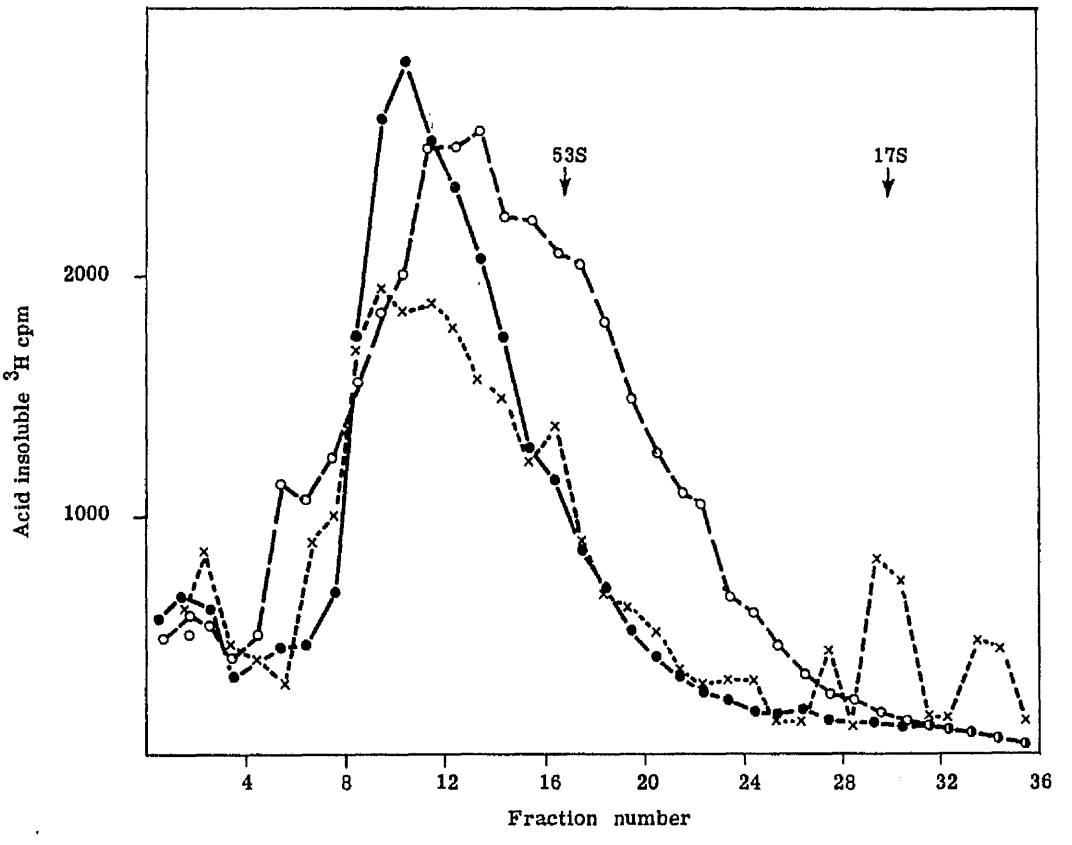
2.5.3. The effect of factors shown to alter the size of RNA labelled in vitro on the size of DNA in isolated nuclei

Although the DNA in incubated nuclei is still relatively large, it is possible that the difference in size between DNA in intact cells and DNA in incubated nuclei is caused by nicking of the DNA during isolation and incubation of nuclei. It is known that the transcriptionally active regions of chromatin are more susceptible to nuclease action than the bulk of chromatin (see Introduction 1.1.5), and therefore, the nicking of DNA in isolated nuclei could occur preferentially in transcriptionally active DNA, causing the small size of RNA labelled in vitro. To test this possibility, the effect of various factors, which had been shown to affect the RNA labelled in isolated nuclei, on single strand DNA length in incubated nuclei was determined.

2.5.3.1. The effect of incubation temperature on the size of DNA in incubated nuclei

Figure 19a shows the sedimentation behaviour of DNA in alkaline gradients, prepared from nuclei incubated at different temperatures. It can be seen that the DNA from nuclei incubated at 37°C for 5 minutes is of the same size as DNA from nuclei incubated at 25°C for 30 minutes. On the other hand, incubation of nuclei at

Figure 19a. Alkaline sucrose gradient centrifugation of DNA from incubated nuclei - The effect of incubation temperature.



HeLa cells were labelled and harvested as in the previous Figure 18. Crude nuclei were prepared and incubated at a density of $\sim$ 2 x 10^6 nuclei/ml in incubation buffer. $\sim$ 0.2 x 10^6 nuclei from each incubation were lysed on top of alkaline gradients as described in Figure 18 and centrifuged at 152,000 g(av) (Rav 8.35 cm) at 25°C for 1½ hours in the Beckman SW50.1 rotor.

●——●——● Nuclei incubated at 25°C for 30 minutes.

X----X----X Nuclei incubated at 37°C for 5 minutes.

O--O--O Nuclei incubated at 37°C for 30 minutes.

37°C for 30 minutes results in a reduction in size of the single strand length of DNA compared to incubation at 25°C. This size reduction corresponds to a reduction in average length from ~ 200 kilobases to ~ 100 kilobases. Referring back to section 2.1.2, it was found that RNA synthesised at 37°C for 5 minutes was the same size as RNA synthesised at 25°C for 5 or 30 minutes. However, RNA synthesis at 37°C was found to stop completely within 20 minutes. Therefore, this cessation of RNA synthesis could possibly be related to the reduction in size of DNA during incubation at 37°C.

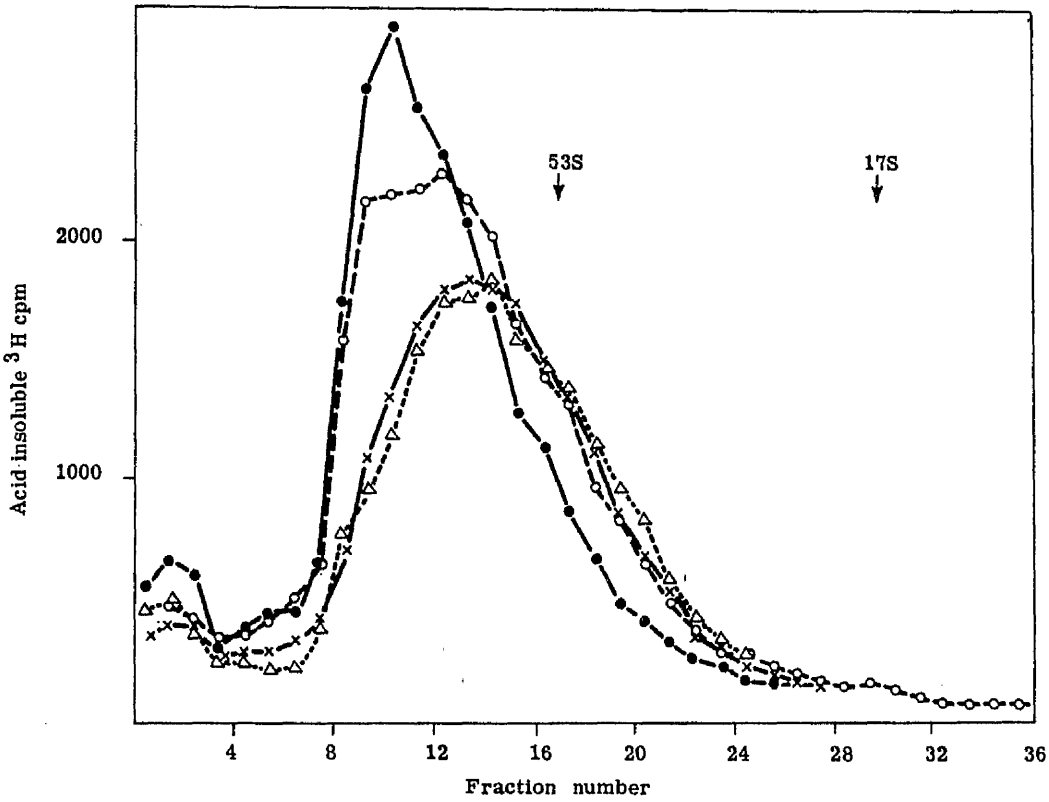
2.5.3.2. The effect of RNA synthesis on the size of DNA in isolated nuclei

The previous results on DNA size in incubated nuclei have all concerned nuclei incubated in the absence of added ribonucleoside triphosphates. When ribonucleoside triphosphates were added to the incubation medium at the normal concentrations used for RNA synthesis, a slight reduction in DNA size was observed (Figure 19b). Thus, this result is consistent with the proposal that DNA in isolated nuclei is preferentially nicked in transcriptionally active regions, and that increasing the RNA synthetic activity by adding ribonucleoside triphosphates might increase the proportion of nuclease sensitive sites in the DNA.

2.5.3.3. The effect of acetyl CoA on the size of DNA in isolated nuclei

The addition of acetyl CoA to the incubation medium resulted in a decrease in the single strand length of DNA in incubated nuclei (Figure 19b). This size decrease was more pronounced than that caused

Figure 19b. Alkaline sucrose gradient centrifugation of DNA from incubated nuclei - The effect of nucleoside triphosphates and acetyl CoA.



Experiment as in Figure 19a.

●——●——● Nuclei incubated at 25°C for 30 minutes

o- - -o- - -o Nuclei incubated at 25°C for 30 minutes with ribonucleoside triphosphates.

×——×——× Nuclei incubated at 25°C for 30 minutes with acetyl CoA (1 mM).

△- - - -△- - - -△ Nuclei incubated at 25°C for 30 minutes with acetyl CoA (1 mM) and ribonucleoside triphosphates.

by incubation with ribonucleoside triphosphates, and incubation of nuclei with both acetyl CoA and triphosphates had no more effect than acetyl CoA alone. It is known that regions of chromatin containing acetylated histones are more susceptible to nuclease attack (Introduction 1.2.1), and indeed, this is one reason why acetylation of histones has been correlated with transcriptional activity. Therefore, these results are explicable at the level of nicking of DNA in incubated nuclei. On the other hand, these results are quite contrary to the proposal that the synthesis of small RNA is due to nicking of the DNA template, since acetyl CoA increases the size of RNA labelled in vitro (section 2.3.3.1), yet decreases the size of DNA template. The DNA in nuclei incubated with acetyl CoA has an average single strand length of $\sim$ 100 kilobases, still an order of magnitude higher than the longer HeLa cell transcription units.

2.5.3.4. Factors with no effect on the size of DNA in incubated nuclei

The effect of adding various other factors to incubating nuclei on DNA size was tested. It was found that incubating with Ado-Met had no effect on the single strand length of DNA compared to DNA from control incubated nuclei. In section 2.3.3.2, it was shown that addition of Ado-Met to incubating nuclei resulted in a decrease in the size of RNA labelled in vitro. The lack of effect of Ado-Met on DNA size would suggest that this effect on RNA size is not caused by reduction in the size of the DNA template.

Another way of determining the effect of nicking of DNA on RNA transcript size was to look for factors which might inhibit

nicking, or repair it. It has been reported that the polyamines, spermine and spermidine, inhibit a Ca^{2+}/Mg^{2+} dependent endonuclease in rat liver nuclei which is active during isolation and incubation of nuclei (Keichline et al, 1976). Therefore, these polyamines were included in the homogenisation and incubation buffers at the reported concentrations (0.15 mM spermine, 0.5 mM spermidine). However, this had no effect on the single strand length of DNA compared to DNA in nuclei isolated and incubated without polyamines.

It has also been suggested that some DNase II may be associated with HeLa cell nuclei (Slor, 1973) and it is known that this enzyme is inhibited by (SO_4^{2-}) ions (Oshima and Price, 1974). Given the finding that the RNA synthesised in nuclei incubated in 90 mM (NH_4^+)SO_4 was larger than that synthesised in 75 mM KCl (Figure 10), it was of interest to determine the effect of $(NH_4)_2SO_4$ on single strand DNA length in incubated nuclei. No difference in the single strand DNA length between nuclei incubated in 90 mM $(NH_4)_2SO_4$ and 75 mM KCl was observed. Furthermore, incubation of nuclei in 90 mM $(NH_4)_2SO_4$ in the presence of acetyl CoA resulted in further degradation of the DNA compared to nuclei incubated in the presence of 90 mM $(NH_4)_2SO_4$ alone (results not shown). Therefore, the effect of $(NH_4)_2SO_4$ on the size of RNA synthesised in isolated nuclei does not appear to be due to an inhibition of DNA degradation by SO_4^{2-} ions.

3. Transport of RNA labelled in vitro from isolated nuclei

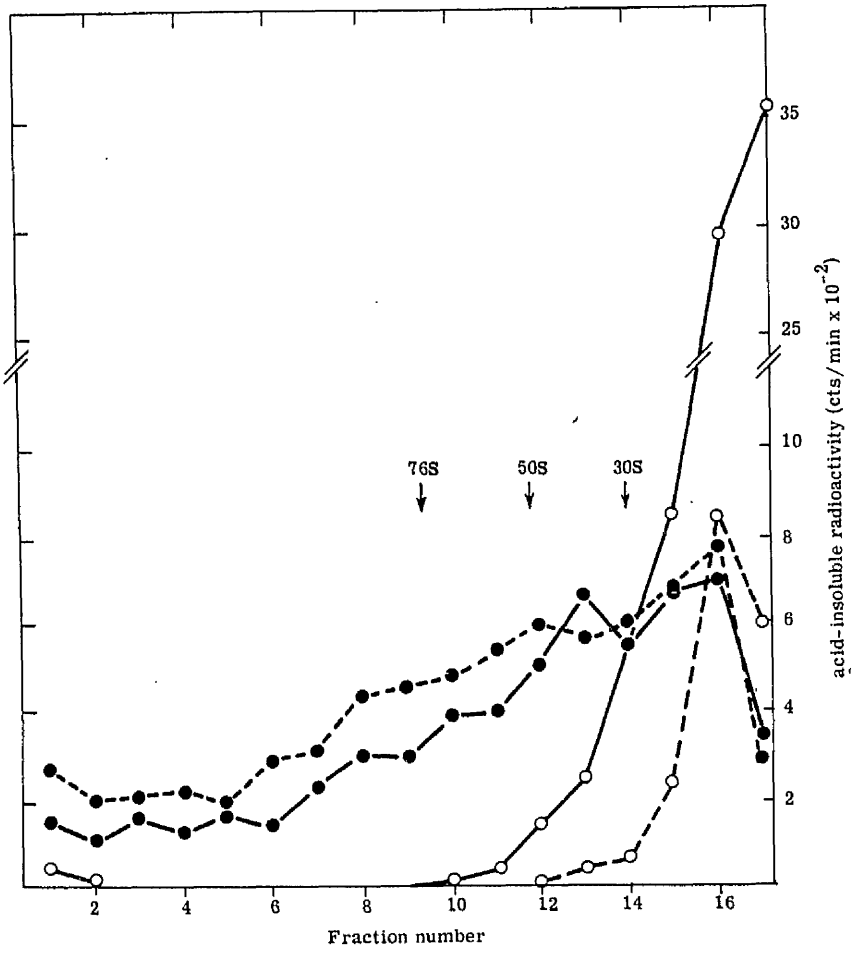
3.1. Some RNA labelled in vitro is incorporated into nucleoplasmic hnRNP particles

The evidence presented in Figures 6 and 8 indicated that

the size of RNA labelled *in vitro* was stable. This could either be due to the fact that the processing and degradative enzymes in the isolated nucleus are absent, or that the labelled chains are not being completed, and remaining in a nascent "non-processable" state.

As discussed in the introduction, electron microscopical evidence has shown that rapidly labelled RNA is synthesised at the periphery of the chromatin, incorporated into perichromatin fibrils, and then migrates into the interchromatin space (Puvion and Moyne, 1978). These interchromatin fibrils have been suggested (Devillier, 1977) to be identical to the hnRNP particles which can be released from nuclei by extraction (Samarina *et al*, 1968; Beyer *et al*, 1977) or disruption (Stevenin *et al*, 1975; Pederson, 1974). Results which will be presented later indicated that sonication of nuclei released a constant proportion of the rapidly labelled RNA in the form of heterogeneously sedimenting hnRNP particles, and that the procedure described by Pederson (1974) could therefore be used to fractionate the rapidly labelled RNA into "chromatin-bound" and "free" hnRNP particle fractions. When free hnRNP particles were extracted in this way from nuclei labelled for 10 minutes *in vitro* and sedimented through sucrose gradients, 13% of the total nuclear acid insoluble radioactivity was recovered in the "free hnRNP" fraction (Figure 22). In other cases, the number of counts in the gradient was so small that it was not possible to determine the figure. On the other hand, of the total nuclear radioactivity incorporated during a 10 minute pre-label of cells *in vivo*, about 50% is found in the heterogeneously sedimenting "free" hnRNP fraction.

Figure 22 Sedimentation of nuclear RNA particles labelled in isolated nuclei



Purified nuclei from HeLa cells were prepared by homogenisation in HM solution and sedimentation through 2M sucrose. Nuclei were resuspended in solution B and incubated with an equal volume of solution IM containing the four nucleoside triphosphates and labelled UTP at 25°C for 10 minutes. After incubation, nuclei were resuspended in ISB solution and sonicated briefly. The sonicate was fractionated into "nucleolar" and "post-nucleolar supernatant" fractions as described. The post-nucleolar supernatant material was analysed by sedimentation through 15-30% sucrose gradients in ISB at 300,000 g(av) (Rav 11.8 cm) for 17 hours at 4°C in the SW27 rotor.

●——●——● Nuclei were labelled for 10 minutes at 25°C

●- - -●- - -● Nuclei were labelled for 10 minutes at 25°C, then centrifuged, resuspended in incubation medium without nucleoside triphosphates, and incubated for a further 30 minutes

○——○——○ Supernatant incubation medium after centrifugation of nuclei in ●——●——●

○- - -○- - -○ Supernatant incubation medium after centrifugation of nuclei in ●- - -●- - -●

Furthermore, although particles of size > 80S are found here, the average size is smaller than the size of hnRNP particles labelled in vivo (compare, for instance, with Figure 28). The implication is that most of the material labelled in vitro remains bound to the chromatin fraction. Any labelled particles which can be released by sonication sediment slower than those labelled in vivo, which probably reflects the small size of the RNA labelled in vitro.

3.2. Release of RNA labelled in vitro from isolated nuclei

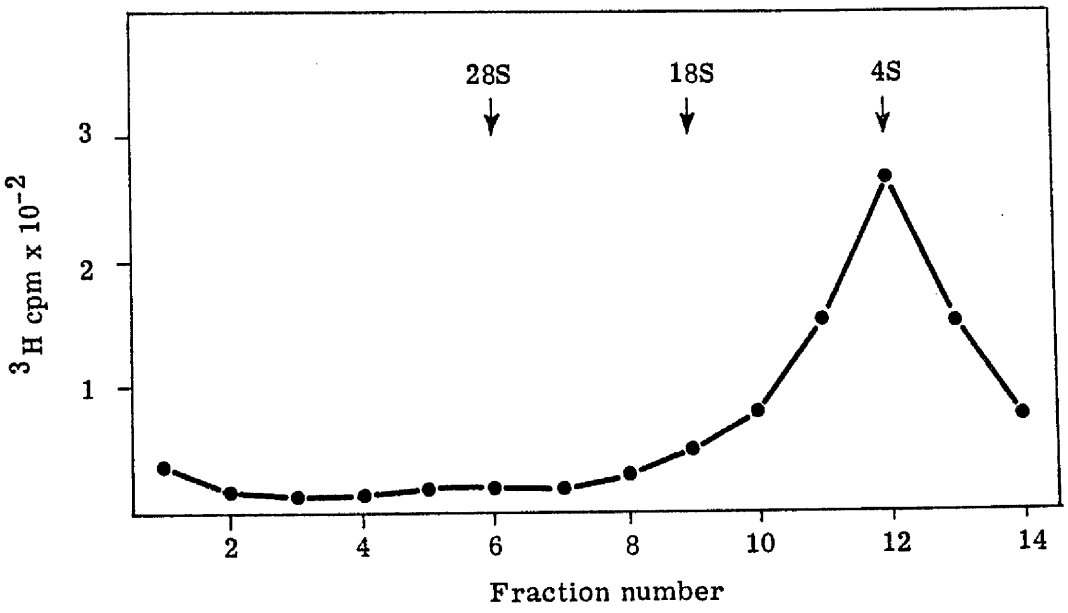
3.2.1. Release of small RNA labelled in vitro

The size of RNA labelled in vitro which was released into the incubation medium was determined by phenol extraction of the supernatant fraction of the incubation medium, obtained by low speed centrifugation, of nuclei and analysis on denaturing sucrose gradients (Figure 23a). Most of the label is found in RNA sedimenting at 4S, although some larger RNA may also be present in the released material. This is in agreement with the results of Sarma et al (1976) who found that much of the released RNA electrophoresed in gels at the positions of marker 5SrRNA and 4.5Spre-tRNA. The conclusion is that most of the released RNA is probably 5SrRNA and 4.5Spre-tRNA synthesised by RNA polymerase III and released rapidly from the nucleus. The larger RNA polymerase II products are either not released, or are released in small amounts.

3.2.2. Release of large RNA labelled in vitro

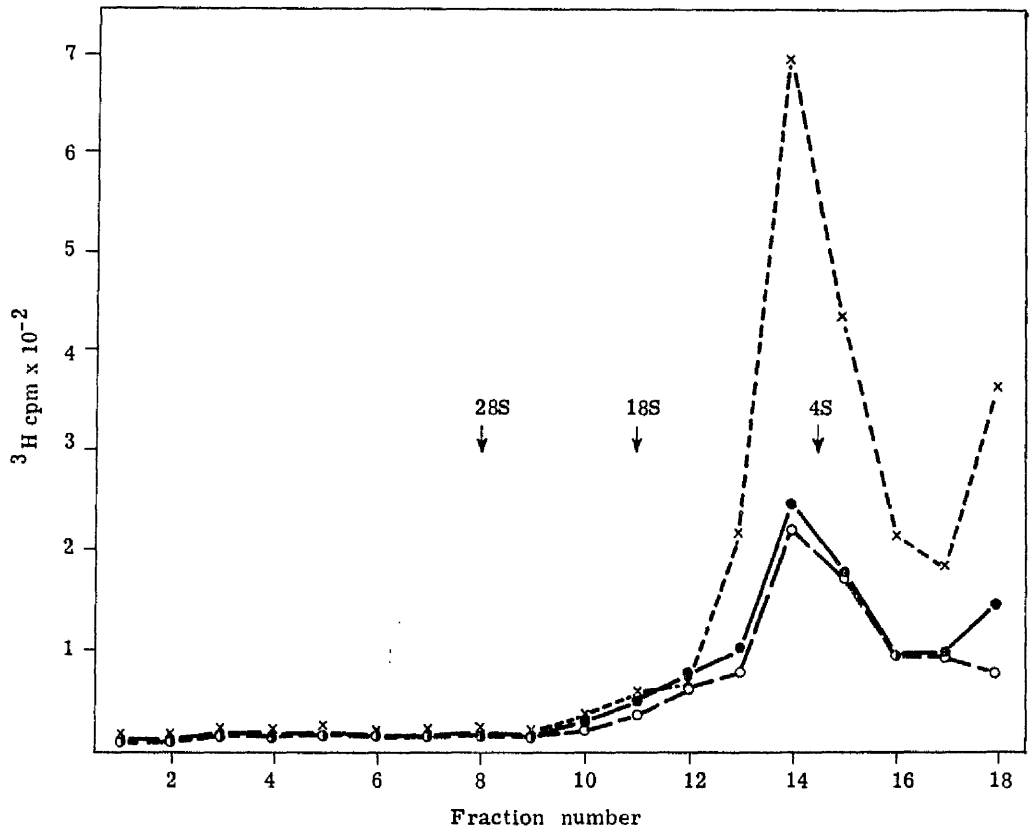
Although little RNA sedimenting faster than 4-5S RNA was released from isolated nuclei at 25°C, it is possible that under different conditions, more large RNA would be transported. In Figure

Figure 23a Size of RNA labelled in isolated nuclei and released into the incubation medium



Crude nuclei, prepared by homogenisation of actinomycin D treated HeLa cells in HM solution and low speed centrifugation, were incubated for 30 minutes at 25°C under normal RNA synthesising conditions. Nuclei were separated from the incubation medium by centrifugation at 3,000 rpm for 5 minutes, and 2 volumes of ethanol were added to the supernatant fraction. The precipitate obtained by centrifuging the supernatant material, after precipitation overnight at -20°C, was phenol extracted, and the RNA was sedimented through 8-20% sucrose gradients in 98% (v/v) formamide as in Figure 2.

Figure 23b The labelled RNA recovered in the supernatant fraction of homogenates and nuclei after incubation at 37°C



Cell homogenates in solution B containing 0.1% (v/v) Triton X-100 were prepared from cells with or without actinomycin D treatment (0.04 µg/ml for 30 minutes). Homogenates, and crude nuclei isolated from the homogenates by low speed centrifugation, were incubated at 37°C for 30 minutes in the standard RNA synthesising system. Nuclei were sedimented from the incubation medium by centrifugation at 3,000 rpm for 5 minutes. RNA recovered in the supernatant fraction was extracted and analysed as in Figure 23a.

●——●——● Cells untreated. Total cell homogenate incubated at 37°C for 30 minutes.

o- - -o- - -o Cells treated with actinomycin D (0.04 µg/ml). Total cell homogenate incubated at 37°C for 30 minutes.

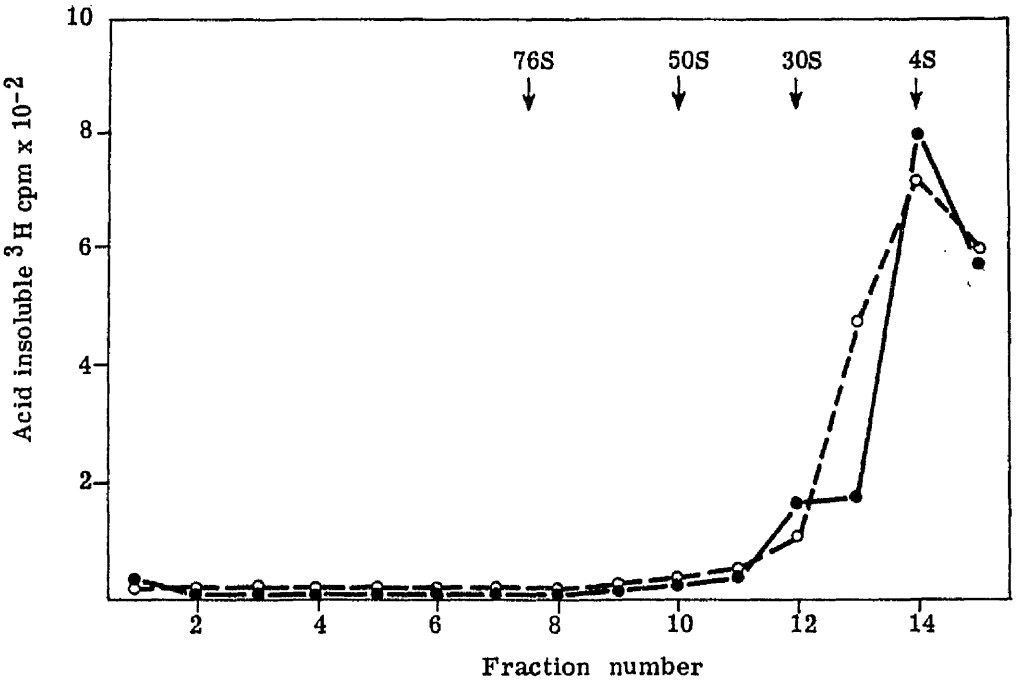
×- - -×- - -× Cells untreated. Crude nuclei prepared from cell homogenate and incubated at 37°C for 30 minutes.

23b the effect on RNA release from nuclei of incubating both isolated nuclei and total cell homogenates at 25°C and 37°C was examined. Although a change in temperature from 25°C to 37°C has been reported to increase the energy-dependent release of pre-labelled RNA from isolated nuclei (Clawson and Smuckler, 1978), incubation at 37°C in the present case had no effect on the size of released RNA. The addition of cytoplasmic factors has also been thought to control the release of prelabelled mRNA-like RNA from isolated nuclei (Yannarell et al, 1977), and of RNA labelled in vitro (Sarma, 1976). However, a comparison of material released from isolated nuclei and nuclei in total cell homogenates did not reveal any difference in the amount of large RNA released, although the release of 4-5S RNA from nuclei in homogenates was inhibited.

When homogenates from control cells were compared with those from 0.04 μg/ml actinomycin D treated cells, mainly 4-5S material was released in both cases. This is an important result since it demonstrates the lack of effect of Actinomycin D treatment on the release of in vitro labelled RNA. This point is mentioned because high concentrations of actinomycin D have been shown to inhibit transport of messenger RNA in vivo (Eckert et al, 1975), possibly by inhibiting the postulated function of the nucleolus in mRNA transport (Sidebottom and Harris, 1969).

Factors which stimulated the synthesis of larger RNA were also investigated for their ability to stimulate release of RNA larger than 4-5S RNA. In this particular experiment (Figure 24), the released material was sedimented through aqueous sucrose gradients in order to detect rapidly sedimenting ribonucleoprotein material. As

Figure 24. Sedimentation of released material labelled in isolated nuclei:- Effect of agents which increase the size of labelled nuclear RNA



Crude nuclei were incubated under normal RNA synthesising conditions at 25°C for 30 minutes. The supernatant fraction obtained after sedimentation of nuclei from the incubation mixture was diluted with an equal volume of ISB buffer and sedimented on 15-30% (w/v) sucrose gradients in ISB at 300,000 g(av) (Rav 11.8 cm) for 17 hours at 4°C in the Beckman SW27 rotor.

●——●——● Nuclei incubated at 25°C for 30 minutes.

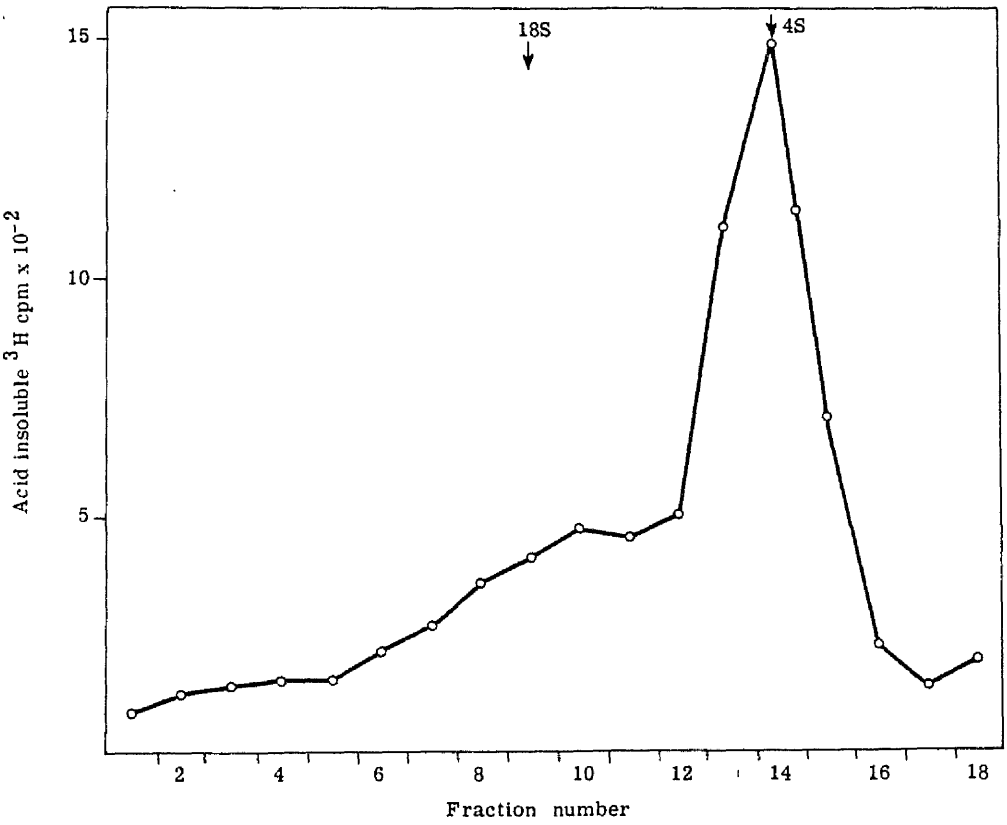
o- - -o- - -o Nuclei incubated at 25°C for 30 minutes with acetyl CoA (1 mM).

will be shown later, prelabelled RNA is released from nuclei in the form of RNP particles sedimenting at 40S. However, little RNA labelled *in vitro* was observed to be released in such particles. Moreover, material labelled *in vitro* in the presence of acetyl CoA, which has been shown to be larger than the control (Figure 11), is still not released in this fashion.

On the other hand, when the RNA labelled in nuclei incubated in 90 mM $(NH_4)_2SO_4$ and released into the incubation medium was analysed, a shoulder of material sedimenting faster than 4S was seen (Figure 25a). This could be due either to lysis of some nuclei at this ionic strength, or reflect transport of transcripts which appear to be full length when synthesised at this salt concentration, and might therefore be processed correctly.

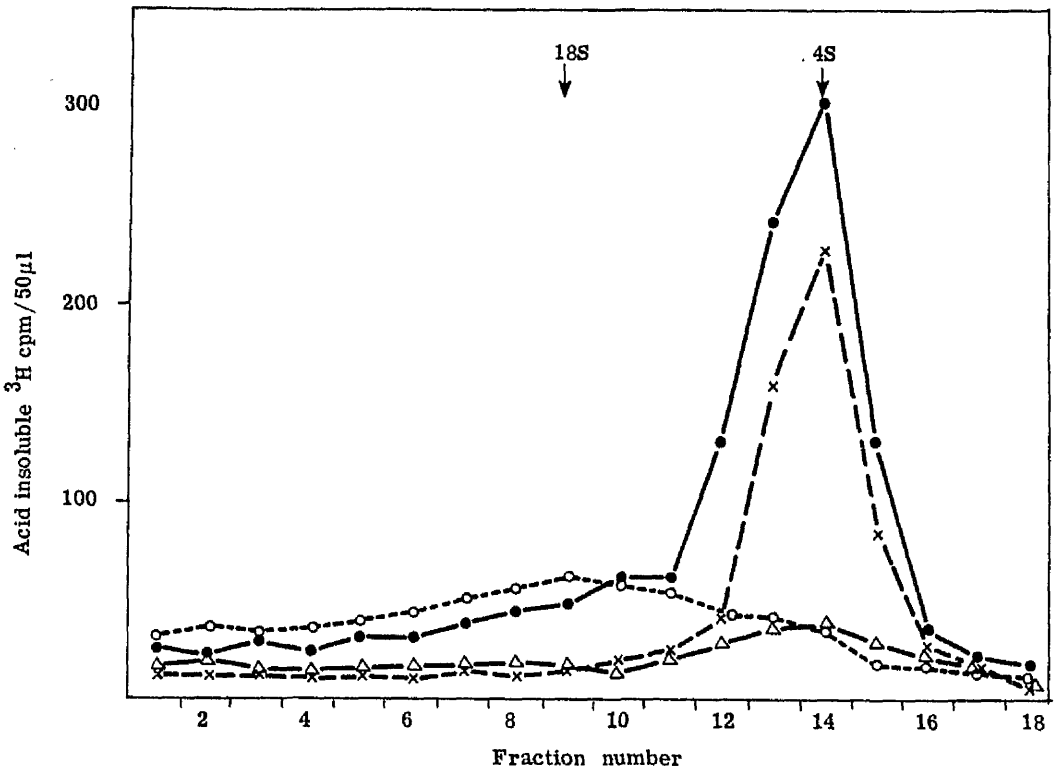
To reduce the former possibility, RNA was labelled in nuclei incubated in a lower concentration of $(NH_4)_2SO_4$ (80 mM), and the RNA released was analysed (Figure 25b). It can be seen that the proportion of released material sedimenting faster than 4S is reduced, compared to the previous figure, suggesting that lysis of nuclei might be responsible for some of this released material. The nuclear RNA labelled under these conditions was still as large as at 90 mM $(NH_4)_2SO_4$ and the bulk of this material was found to be α-amanitin sensitive (results not shown). Thus, although cells were not pre-treated with actinomycin D, very little ribosomal RNA synthesis occurs at this salt concentration, as found previously (Zylber and Penman, 1971). The small shoulder of rapidly sedimenting RNA released from these nuclei was also α-amanitin sensitive (Figure 25b). Moreover, it was found that the material released from nuclei which

Figure 25a. Sedimentation of released RNA labelled *in vitro* in nuclei incubated in 90 mM $(NH_4)_2SO_4$



Untreated HeLa cells were homogenised in HM buffer, and nuclei were prepared by low speed centrifugation and resuspended in solution B. An equal volume of a solution containing 180 mM $(NH_4)_2SO_4$, 5 mM $Mg(OAc)_2$, ribonucleoside triphosphates and ^{3}H UTP was added to give an incubation medium in which all the constituents were at the usual concentrations, except that KCl (75 mM) was replaced by $(NH_4)_2SO_4$ (90 mM). Nuclei were incubated at 25°C for 30 minutes, following which a supernatant fraction was obtained by centrifugation of the nuclei from the incubation medium. The RNA released into the supernatant fraction was extracted and analysed as described in Figure 23a.

Figure 25b. Sedimentation of released RNA labelled *in vitro* in nuclei incubated in 80 mM $(NH_4)_2SO_4$



RNA synthesis *in vitro* was performed as described in Figure except that the final concentration of $(NH_4)_2SO_4$ was 80 mM as opposed to 90 mM. The released RNA was extracted and analysed as in Figure 23a.

(a) ●——●——● RNA released from nuclei incubated for 30 minutes in incubation medium containing 80 mM $(NH_4)_2SO_4$, ribonucleoside triphosphates and ^{3}H UTP.

(b) ×——×——× RNA released from nuclei incubated as (a), with α-amanitin (1 µg/ml).

(c) o - - -o - - -o Nuclei incubated as (a), centrifuged, resuspended and incubated in medium without ribonucleoside triphosphates and ^{3}H UTP for 60 minutes. RNA released during the 60 minute "post-label" incubation was analysed.

(d) △——△——△ Nuclei incubated as (b), then resuspended in medium without NTPs and ^{3}H UTP for 60 minutes. RNA released during the 60 minute "post-label" incubation was analysed.

were labelled for 30 minutes and then incubated for 60 minutes in incubation medium lacking ribonucleoside triphosphates consisted mainly of RNA sedimenting more rapidly than 4S RNA (Figure 25b).

A large proportion of mRNA appears in the cytoplasm *in vivo* between 30 and 90 minutes after synthesis (Herman and Penman, 1977). Therefore, it is possible that the release of RNA synthesised by RNA polymerase II *in vitro* between 30 and 90 minutes of incubation represents the specific transport of mRNA sequences transcribed *in vitro*. This possibility is examined in the following section.

3.2.3. Poly(U) Sepharose affinity chromatography of RNA labelled in vitro

One indirect way of distinguishing the hnRNA and mRNA population is by their characteristic binding to poly(U) Sepharose (see section 5.2.1). In particular, a proportion of hnRNA molecules contain an oligo(A) tract of 20-30 nucleotides which is not present in mRNA (Venkatesan *et al*, 1979).

Also, while only ~ 20% of pulse-labelled hnRNA has a poly(A) tail of ~ 200 nucleotides (Herman and Penman, 1977) about 70% of mRNA is polyadenylated (Milcarek *et al*, 1974). Oligo(A) and poly(A) containing molecules can be distinguished since they are eluted from poly(U) Sepharose at different formamide concentrations, whilst molecules without either type of adenylate tract do not bind to poly(U) Sepharose (See Figure 34).

Table 1a shows the binding characteristics of various RNA samples with respect to the proportion of total labelled RNA not bound to poly(U) Sepharose (defined as $(A)^-$), the proportion eluted from

(b). As in (a), except that RNA was synthesised in nuclei incubated in an incubation medium containing 90 mM $(NH_4)_2SO_4$.

(c). Nuclei were incubated as described in Figure 25b, i.e. RNA was synthesised for 30 minutes in incubation medium containing 80 mM $(NH_4)_2SO_4$. The nuclei were then incubated for a further 60 minutes in 80 mM $(NH_4)_2SO_4$ medium without ribonucleoside triphosphates or ^3H UTP. The nuclear RNA was then extracted and analysed as for (a).

(d). The RNA released into the supernatant fraction of both the 30' labelling incubation and the 60' post-label incubation in (c) above was extracted and run on denaturing gradients (Figure 25b, samples (a) and (c)). Material sedimenting faster than ~8S in both gradients was pooled, precipitated with 2 volumes of ethanol, and resuspended in poly(U) Sepharose binding buffer.

Table 1a. Poly(U) Sepharose affinity chromatography of RNA labelled in vitro

Sample	RNA non-bound to poly(U) Sepharose $(A)^-$. % of total c.p.m.	RNA eluted from poly(U) Sepharose with 0-10% formamide (oligo(A)) % of total c.p.m.	RNA eluted from poly(U) Sepharose with 20-90% formamide $(poly(A)^+)$ % of total c.p.m.
(a) Nuclear RNA labelled in nuclei incubated in 75 mM KCl	76	19	5
(b) Nuclear RNA labelled in nuclei incubated in 90 mM $(NH_4)_2SO_4$	68	25	7
(c) Nuclear RNA labelled in nuclei incubated in 80 mM $(NH_4)_2SO_4$	63	29	8
(d) Released RNA labelled in nuclei incubated in 80 mM $(NH_4)_2SO_4$	60	27	13

(a). Cells were pretreated with actinomycin D (0.04 μg/ml) for 30 minutes. Cells were homogenised and nuclei prepared as in Figure 23. Nuclei were incubated for 30 minutes at 25°C in the usual incubation medium (i.e. 75 mM KCl). Nuclear RNA was extracted as described in Figure 2 and resuspended in poly(U) Sepharose binding buffer (0.4M NETS). RNA was applied to poly(U) Sepharose and eluted with increasing concentrations of formamide as described in Table 1b.

poly(U) Sepharose with formamide concentrations between 0-10% (v/v) (defined as oligo $(A)^+$), and the proportion eluted with formamide concentrations between 20-90% (v/v) (defined as poly$(A)^+$).

A number of important points emerge from this study. The first two samples are nuclear RNA labelled *in vitro* in nuclei incubated either in the normal incubation medium (75 mM KCl) or in 90 mM $(NH_4)_2SO_4$. It can be seen that in both cases $\sim$ 20% of labelled RNA elutes in the "oligo$(A)^+$" fraction. It will be shown later (section 5.2.2), that a similar proportion of hnRNA labelled for 10 minutes *in vivo* is found in this fraction, indicating that in terms of transcription of oligo(A) sequences, the transcription *in vitro* is the same as that *in vivo*.

It can also be seen that in both cases, some of the RNA labelled *in vitro* appears in the poly$(A)^+$ class. This would imply that in both incubation media, a similar proportion of RNA transcripts are terminated and polyadenylated, despite the difference in size of the transcripts labelled in the normal and 90 mM $(NH_4)_2SO_4$ incubation media.

From the experiment described in Figure 25b, RNA sedimenting faster than the major peak of 4-5S RNA, released both during the 30 minute labelling incubation and the 60 minute post-labelling incubation, was pooled and characterised in terms of poly(U) Sepharose binding. This was compared with the RNA retained in these nuclei after 30 minutes labelling incubation and 60 minutes post-labelling incubation. It can be seen (Table 1a) that the pattern of poly(U) Sepharose binding of the released RNA sample is very definitely "hnRNA-like" rather than "mRNA-like" in terms of both the presence of a substantial

"oligo(A)$^+$" fraction, and the low proportion of labelled RNA in the "poly(A)$^+$" fraction indicating that specific transport of mRNA does not occur, even during 90 minutes of incubation. The significance of the slightly higher proportion of labelled RNA present in the poly(A)$^+$ fraction in released RNA than in retained RNA is not clear (particularly when the small number of counts in the released RNA sample is taken into account). In this particular experiment, ~ 2% of the total radioactivity incorporated into RNA *in vitro* was released as RNA sedimenting faster than the major peak of 4-5S RNA during the 30 minutes labelling incubation, and a further ~ 4% was released during the 60 minute "post-label" incubation. The "hnRNA-like" poly(U) Sepharose binding of this released RNA tends to indicate that the release is mainly due to lysis of nuclei, or non-specific leakage, rather than the specific transport of mRNA sequences.

4. Studies on RNA, prelabelled in vivo, in isolated nuclei

4.1. Possible processing of RNA in isolated nuclei

4.1.1. Decrease in size of prelabelled RNA in isolated nuclei

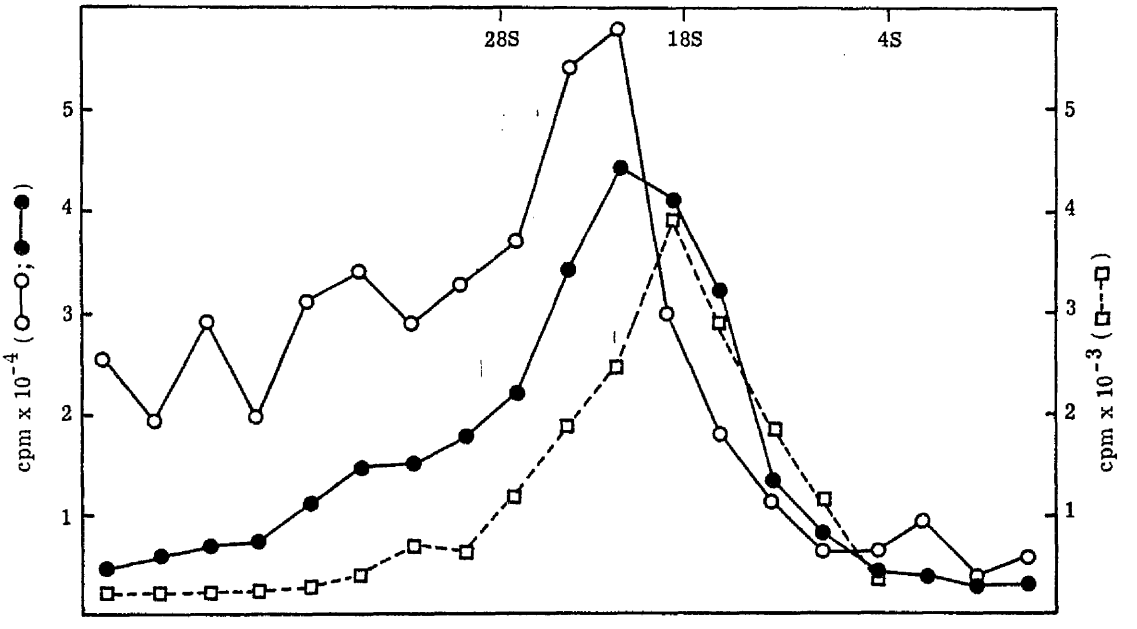
The results described in the previous section indicated that hnRNA labelled *in vitro* in isolated nuclei is stable and is not released from nuclei during incubation. This could either be due to the abnormally small size of the RNA, or to a defect in the processing "machinery" of the isolated nuclei. Therefore, the following experiments were performed to investigate the ability of nuclei to process hnRNA prelabelled *in vivo*. It was also hoped that data from these experiments might throw light on the relatively unexplored areas associated with the processing of hnRNP particles and their movement from chromatin through the nucleoplasm and transport to the cytoplasm.

For experiments in processing and transport, purified nuclei were made by the original method of Sarma et al (1976). Although it has been shown in the first half of this section that nuclei prepared by this method synthesised RNA smaller than crude nuclei, it was more important for these studies that contamination by cytoplasmic nucleases, and by cytoplasmic mRNA, was reduced to a minimum.

When cells were prelabelled for 10 minutes in vivo in the presence of actinomycin D (0.04 μg/ml), the RNA extracted from nuclei prepared by the original method of Sarma et al (1976) had a heterogeneous sedimentation distribution with a peak around 20-25S, but containing material $>$ 45S (Figure 26). The shape of the sedimentation profile was reproducible (see also Figure 27), and differed from that of hnRNA from HeLa cells prepared in this laboratory by more rapid procedures (Strachan, 1979) which had a smoother profile with a peak of $\sim$ 28S. It is possible that some of the large hnRNA is degraded during the preparation of these isolated nuclei.

Incubation of prelabelled isolated nuclei caused a decrease in the size and amount of extractable nuclear RNA. The nuclear RNA was reduced to a size range with average sedimentation coefficient of 20S in denaturing conditions. Comparison with cytoplasmic poly(A)$^+$ RNA from HeLa cells (figure not shown) shows that the population of labelled RNA molecules retained in the nucleus after incubation is slightly larger on average than the mRNA population, and contains very few labelled molecules $<$ 10S in size. This limited decrease in size could possibly reflect specific processing of hnRNA molecules, rather than gross degradation by non-specific nucleases.

Figure 26. The effect of incubation on the size of nuclear RNA labelled in vivo



HeLa cells were treated with actinomycin D (0.04 μg/ml) for 20 minutes, and then labelled with [5,6-^{3}H]uridine (0.2 mCi/50 ml, 45 Ci/mmol) for 15 minutes. Purified nuclei were prepared by homogenising cells in HM solution and sedimentation through 2M sucrose. Nuclei were incubated in incubation medium (solution B/IM) at 25°C for 30 minutes. RNA was extracted from nonincubated nuclei, incubated nuclei, and the supernatant incubation medium. RNA was analysed on denaturing gradients at 250,000 g(av) (Rav 8.78 cm) for 16 hours at 30°C in the Beckman SW60 rotor.

○——○——○ RNA from non-incubated nuclei

●——●——● RNA from nuclei incubated at 25°C for 30 minutes

□---□---□ RNA from the supernatant incubation medium obtained by centrifugation of nuclei from the medium after incubation at 25°C for 30 minutes.

(N.B. different scale.)

4.1.2. The effect of proflavin on possible processing in vitro

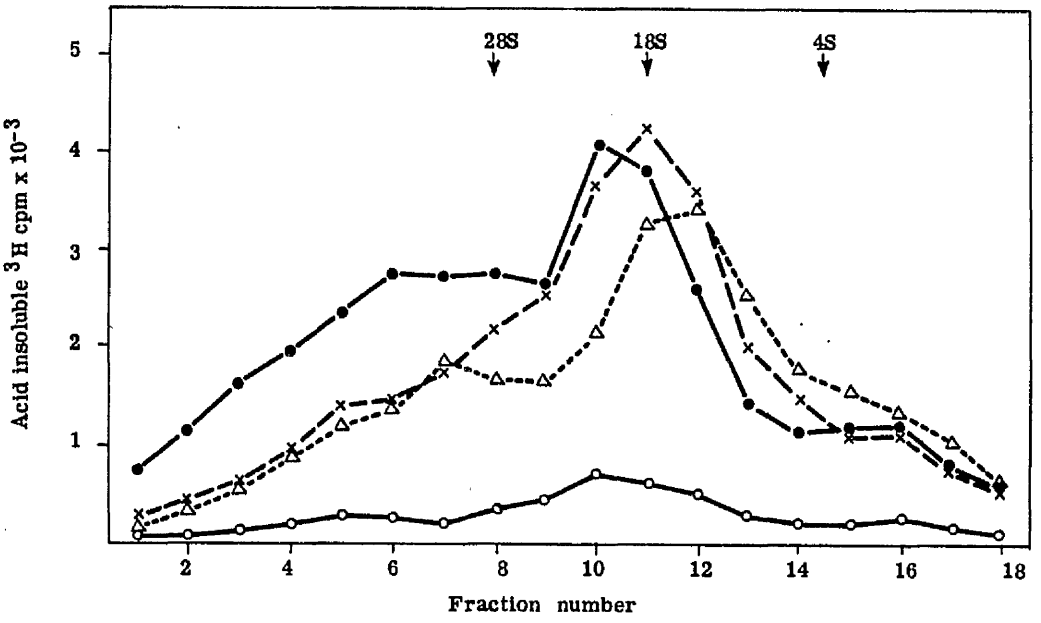
In order to investigate further the processing of pre-labelled RNA in isolated nuclei, the effect of proflavin was examined. It has been shown that proflavin inhibits the processing of adenovirus precursor mRNA, apparently by intercalating in the double stranded regions of the RNA (Madore and Bello, 1978). It has also been reported that incubation of isolated rat liver nuclei with proflavin inhibits the degradation of large RNA, mainly ribosomal precursor RNA (Yannarell et al, 1977). The effect of proflavin on the size reduction described here is shown in Figure 27. In contrast to the reports mentioned, proflavin did not inhibit size reduction. If anything, the RNA from nuclei incubated with proflavin was smaller than the control. The implication is that this degradation does not involve double stranded regions of the RNA or is not inhibited by intercalation in these regions. However, this does not necessarily mean that the size reduction does not represent specific processing, since it has not been proved that processing events involve double-stranded regions in eukaryotic systems. Although these regions have been implicated in the formation of mRNA (Naora and Whitelam, 1975; Ryskov et al, 1976) by their sequence homology with mRNA, sequence analysis of regions flanking the junctions of intervening sequences in non-contiguous genes known to be transcribed into hnRNA have not revealed any striking regions of complementarity (Breathnach et al, 1978; Catterall et al, 1978).

4.2. Possible processing of hnRNP particles in isolated nuclei

4.2.1. Isolation of nucleoplasmic hnRNP particles

There is now a lot of evidence that nascent hnRNA is

Figure 27. The effect of proflavin on the size of labelled RNA in incubated nuclei



Purified nuclei from actinomycin D treated cells were incubated as described in Figure 26, and the RNA analysed on denaturing gradients (246,000 g(av) (Rav 8.78 cm) 17 hours 30°C, SW56 rotor).

●——●——● RNA from non-incubated nuclei

×---×---× RNA from nuclei incubated at 25°C for 30 minutes

△----△----△ RNA from nuclei incubated at 25°C for 30 minutes with proflavin (100 μM)

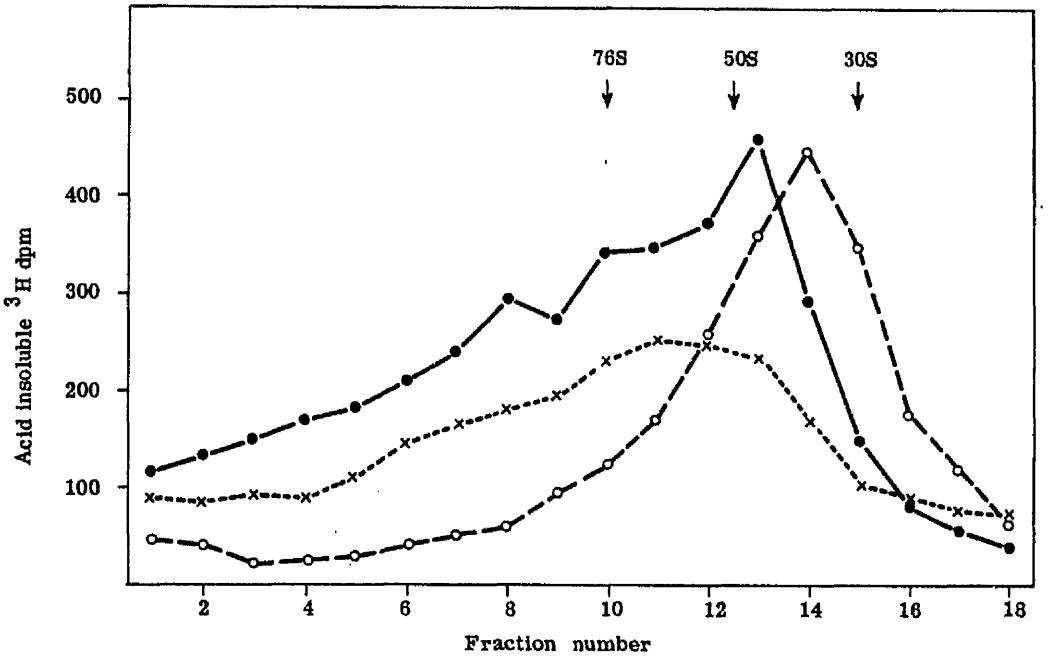
○——○——○ RNA from non-incubated nuclei prepared from cells prelabelled *in vivo* in the presence of proflavin (100 μM).

associated with proteins and some of this RNA can be extracted in the form of hnRNP particles after release from the chromatin. However, it is not yet clear whether the pool of free extractable hnRNP particles constitutes messenger RNA precursors being processed and destined for transport to the cytoplasm, or whether they constitute a fraction of the hnRNA which is retained in the nucleus.

Using the method described by Pederson (1974), it was possible to extract labelled hnRNP particles from nuclei by sonication. After purification by centrifuging nucleoli from the sonicate through 30% (w/v) sucrose, the remaining "post-nucleolar supernatant" fraction was sedimented through a sucrose gradient. The non-incubated nuclear hnRNP profile in Figure 28 shows a typical size distribution of "free" particles from nuclei. It was found that the time of sonication under the conditions described was critical for achieving a balance between complete nuclear breakage, and damage to the released particles, resulting in 40S "monomer" particles (see Introduction). Between 5 to 10 seconds sonication (at 1-3 amps) was necessary for breakage of 95-100% of nuclei, whereas 15 seconds sonication generated slower sedimenting particles. Monitoring by phase contrast microscopy showed that the shorter sonication time disrupted nuclei to form large chromatin fragments and nucleoli. Further sonication reduced the size of the chromatin fragments.

Because of the large size of "chromatin" fragment generated by this procedure, the subsequent purification step designed to sediment only nucleoli through 30% (w/v) sucrose (Pederson, 1974) also sedimented 50% of the rapidly labelled RNA in the sonicate of

Figure 28. Sedimentation of nuclear and released RNP particles from incubated nuclei



Purified nuclei were prepared from HeLa cells treated for 30 minutes with actinomycin D (0.04 μg/ml) and labelled for 10 minutes with [5,6-^{3}H]uridine (0.2 mCi/50 ml, 41 Ci/mmol). Nuclei were resuspended in incubation medium (solution B + IM) and incubated as shown. After incubation nuclei were sedimented by centrifugation at 3,000 rpm for 5 minutes and resuspended in ISB. Nuclear RNP particles were released by sonication and fractionation into nucleolar and "post-nucleolar supernatant" fractions. The "post-nucleolar supernatant" material was sedimented through 15-30% (w/v) sucrose gradients in ISB at 300,000 g(av) (Rav 11.8 cm) for 17 hours at 4°C. The supernatant fraction of the incubation medium was diluted with an equal volume of ISB and sedimented on a parallel gradient.

●——●——● RNP particles from nonincubated nuclei.

×----×----× RNP particles from nuclei incubated at 25°C for 30 minutes.

o--o—o RNP particles released from nuclei incubated at 25°C for 30 minutes into the supernatant fraction of the incubation medium.

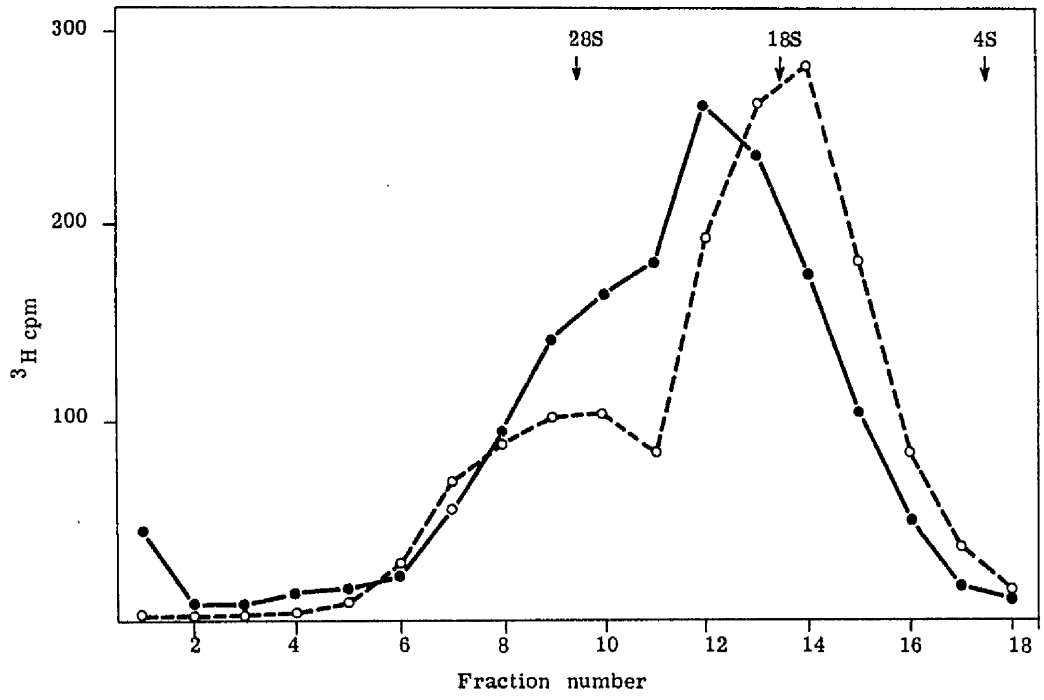
nuclei prelabelled in the presence of actinomycin D. Only a small proportion of the labelled supernatant material formed a pellet in the subsequent sucrose gradient sedimentation step. This pellet was described as the "chromatin" fraction by Pederson (1974). However, in this case, the fact that the bulk of the chromatin was removed in an earlier fractionation step than expected proved beneficial by lowering the risk of contamination of the "free" hnRNP fraction by chromatin bound material, and allowing the possibility of extracting RNA directly from the "post-nucleolar supernatant" fraction.

4.2.2. hnRNP particles from isolated nuclei incubated at 25°C

The effect of nuclear incubation on this free hnRNP pool was determined. Figure 28 compares the sedimentation in sucrose gradients of hnRNP particles from nonincubated and incubated nuclei. The amount of radioactivity in the extractable hnRNP pool decreased during incubation, yet the size distribution remained constant. That is, loss of material occurred in all size classes of particles. When RNA was extracted from the "post-nucleolar supernatant" fraction containing 90% of the radioactivity in soluble particles, RNA with a peak sedimentation coefficient of 20S on denaturing gradients was recovered (Figure 29). After incubation of nuclei there was a decrease in the sedimentation coefficient, indicating degradation of the RNA. Thus, the possible processing of RNA occurs in the hnRNP fraction as well as in total nuclear RNA.

Mild RNase treatment of native hnRNP particles results in cleavage to a homogeneous monomeric size of 30-50S (Samarina et al, 1968; Pederson, 1974), yet the cleavage of hnRNP RNA in isolated

Figure 29. Size of RNA in RNP particles from prelabelled nuclei



RNA was extracted from the "post-nucleolar supernatant" fractions of purified nuclei, obtained as described in Figure 28. The RNA was sedimented on 8-20% (w/v) sucrose gradients in 98% (v/v) formamide at 246,000 g(av) (Rav 8.78 cm) for 17 hours at 30°C in the Beckman SW56 rotor.

●——●——● RNA from RNP particles extracted from non-incubated nuclei.

o - - -o- - -o RNA from RNP particles extracted from nuclei incubated at 25°C for 30 minutes

nuclei does not affect the size of the particles. Therefore the cleavage apparently occurs in positions which do not affect the polyparticle structure of the particles, possibly within the monomeric 40S structures.

4.3. Release of prelabelled RNA from incubated nuclei

4.3.1. Sedimentation of prelabelled RNP particles released from incubated nuclei

The same figure shows the sedimentation of rapidly labelled particles released into the incubation medium during the incubation. A homogeneous peak of material sedimenting consistently at 40S as compared with the sedimentation of EDTA dissociated ribosomal subunits (30S and 50S), was released. Much of the further work was designed to characterise this transported material and compare it with the nuclear restricted material. The basis of this comparison was the working hypothesis that the released material might be selectively transported from the nuclei during incubation and that, although originating from the pool of extractable hnRNP particles, the released particles are a specific subclass of this population.

4.3.2. Sedimentation of released RNA in denaturing gradients

Returning to Figure 26, the sedimentation in denaturing gradients of RNA released from incubated nuclei is compared with that of nuclear RNA before and after incubation. The released RNA is characterised by a relatively narrow size distribution with an average sedimentation coefficient of about 18S, indicating very little leakage of large hnRNA during incubation, and no observable release of small RNA. Very little small RNA (4-5S) is found either in the released

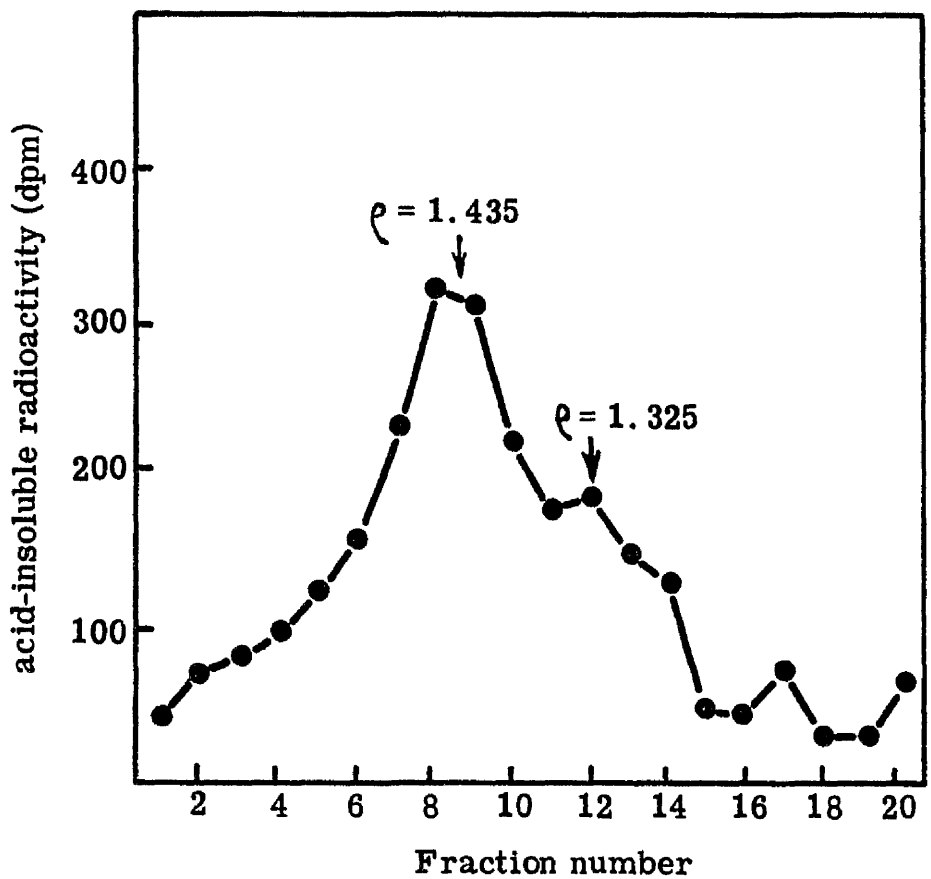
fraction, or restricted to the nucleus. 5S ribosomal RNA and 4S tRNA appear to be rapidly transported to the cytoplasm, and therefore only a small pool of labelled small RNA in the nucleus would be expected (Burdon, 1975).

Only a small proportion of total labelled nuclear RNA was released from the nuclei during incubation. Quantitation of this amount showed that a consistent 8-12% of the acid precipitable labelled material remaining in the incubation mixture after incubation at 25°C for 30 minutes was recovered in the supernatant fraction.

4.3.3. The buoyant density of RNP particles released from incubated nuclei

A major diagnostic feature of nuclear and cytoplasmic messenger RNP particles is the high protein/RNA ratio which results in a characteristic buoyant density in CsCl of approximately 1.4 g/cm^{-3} (see Introduction). Analysis of the released 40S particles after fixation revealed a disperse density distribution with a peak corresponding to a density of 1.43 g/cm^3 (Figure 30a). Some apparently less dense material was also seen but this could be due to degradation of particles, resulting in their not reaching the equilibrium position at the end of centrifugation (Greenberg, 1976). However, little material with a density of roughly 1.55 g/cm^3 was found, indicating the absence of labelled ribosomal particles, nor was any free RNA observed at the bottom of the gradient. hnRNP particles extracted from nuclei by sonication had a similar buoyant density (Figure 30b).

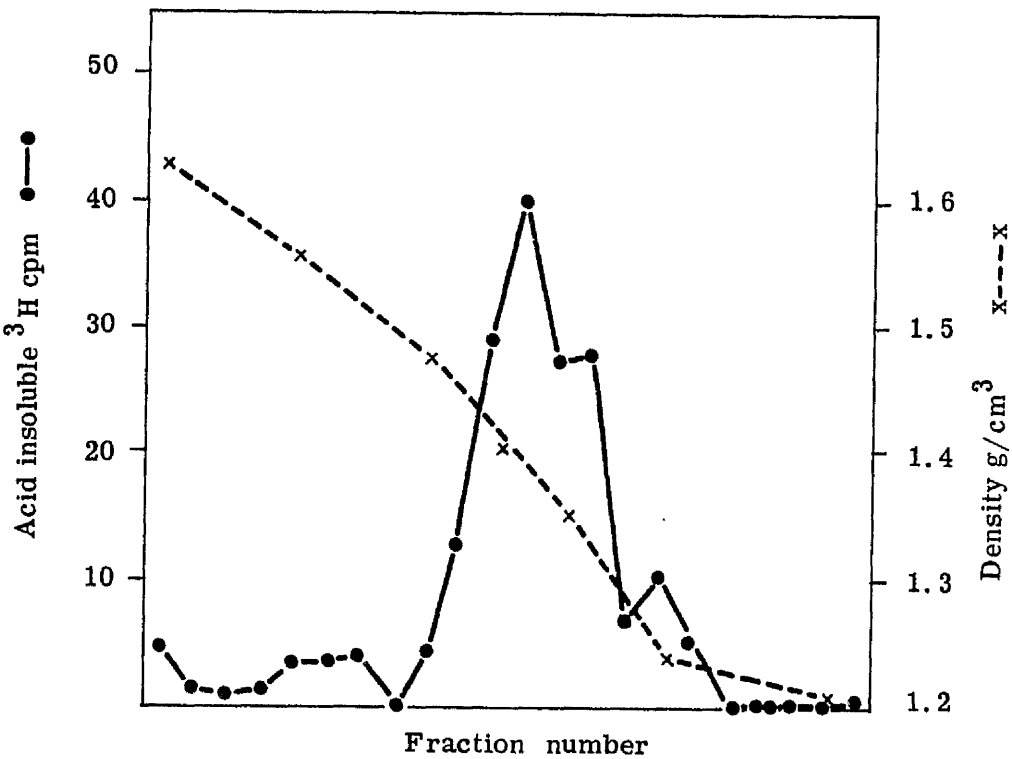
Figure 30a. Buoyant density of labelled RNP particles released from incubated nuclei



HeLa cells were pretreated for 20 minutes with actinomycin D (0.04 μg/ml) and labelled for 10 minutes with [5,6-^{3}H]uridine. Released RNP particles from incubated nuclei were sedimented through 15-30% (w/v) sucrose gradients in ISB, as described in Figure 28. Fractions containing the labelled 40S particles were pooled and dialysed against ISB in a vacuum dialysis apparatus. The concentrated particles were fixed for 4 hours on ice with 6% glutaraldehyde before layering on preformed 20-60% (w/w) CsCl gradients and centrifuging at 149,000 g(av) (Rav 8.35 cm) for 15 hours at 4°C in the Beckman SW50.1 rotor.

●——●——● Acid insoluble ^{3}H cpm in released RNP.

Figure 30b. Buoyant density of hnRNP particles extracted by sonication from purified nuclei



HnRNP particles were extracted from purified nuclei by sonication, and sedimented through 15-30% (w/v) sucrose gradients in ISB as described in Figure 28. Nuclei were prepared from cells labelled for 10 minutes with [5,6-^{3}H] uridine after 20 minutes actinomycin D treatment. Labelled hnRNP particles sedimenting between 60-120S were pooled and dialysed against ISB in a vacuum dialysis apparatus. Concentrated particles were fixed with 6% glutaraldehyde and sedimented through preformed CsCl gradients as described in Figure 30a.

5. Comparison of RNP particles released during incubation, with RNP particles retained during incubation

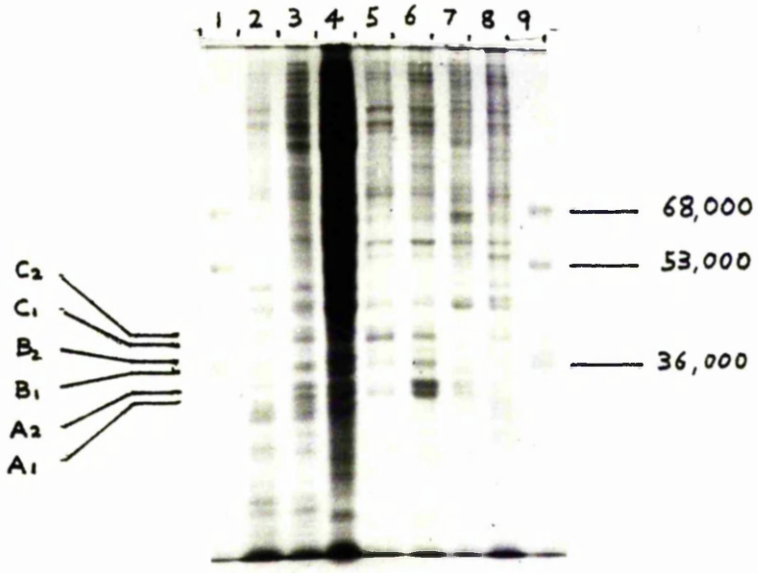
5.1. Comparison of proteins associated with released RNP particles with nuclear hnRNP particles

5.1.1. Proteins cosedimenting with labelled RNA

Having observed a difference in the size of particles released from or retained in nuclei after incubation, it was of interest to determine whether the released RNP particles differed in other ways from the RNP particles remaining in the nucleus. From a size point of view, at least, the released particles are similar to the HeLa messenger RNP particles described by Spohr et al (1970). The proteins contained in these HeLa cell free mRNP particles and polysomal mRNP particles have been reported to differ from each other (Liautard et al, 1976) and from hnRNP particles extracted by sonication from HeLa nuclei (Kumar and Pederson, 1975; Liautard et al, 1976). Therefore, it is possible that nuclear particles selected for transport might contain some messenger-specific proteins, although none of these have been detected in the nuclear RNP particle pool except for the poly(A)specific protein of 72-78K molecular weight (Kish and Pederson, 1975, 1976; Schwartz and Darnell, 1976).

In order to compare the protein composition of the RNP particles retained and released from nuclei in these studies, proteins extracted from isotonic salt sucrose gradient fractions were analysed by SDS polyacrylamide gel electrophoresis (Figure 31). The protein composition of rapidly sedimenting nuclear particles extracted by sonication from incubated nuclei (tracks 5 and 6) is very heterogeneous,

Figure 31. SDS polyacrylamide gel electrophoresis of nuclear and released particles from incubated nuclei



RNP particles released from incubated nuclei and extracted by sonication were sedimented through sucrose gradients as shown in Figure 28. Pooled fractions were precipitated with two volumes of ethanol and resuspended in SDS gel sample buffer (0.01M sodium phosphate pH 7.4, 0.1% SDS, 1% 2-mercaptoethanol, 0.25M sucrose). Samples were placed in boiling water for 2 minutes, cooled, and layered on the gel. Samples were electrophoresed at 30 mA for 3 hours through a 10% polyacrylamide gel.

Track 1 and 9	Molecular weight markers
2	50-150S released material
3	30-50S released material
4	0-30S released material
5	50-150S retained material
6	30-50S retained material
7	0-30S retained material
8	pellet nuclear material

with at least 30 discernible bands. The six major proteins in the molecular weight range 30-40K resemble the 3 pairs of proteins described by Beyer et al (1977) in particles extracted by diffusion from HeLa cell nuclei, and suggested by them to play a "packaging" role in the structural organisation of hnRNA in the nucleus. The nomenclature of Beyer et al (1977) defines a major doublet, A_1 and A_2 (32 and 34K molecular weight), and two less prominent doublets, B_1 and B_2 (36 and 37K) and C_1 and C_2 (42 and 44K). These proteins are probably highly resolved components of what was previously thought to be a single major hnRNP protein with a molecular weight of 40K (Pederson, 1974) (see Introduction, section 4.1.4.2).

These major proteins are depleted in material pooled from the top of the sucrose gradient (track 7), whereas many of the other proteins are found on particles with a wide range of sedimentation behaviour. The only other major bands which only cosediment with labelled RNP particles are bands of 110K, 100K and 84K molecular weight. However, cosedimentation is obviously a necessary but not sufficient condition for assignment of genuine hnRNP particle proteins.

The proteins found associated with the released 40S RNP particles (track 3) are very similar to those found in the corresponding region (30-50S) of the nuclear restricted RNP particle gradient (track 6). In particular, the three major pairs of bands (A, B and C) are present in the released fraction. These proteins, however, can also be readily seen in the released material at the top of the gradient (0-30S) (track 4). Most of the higher molecular weight proteins sedimenting at 40S are also found in the material between 0-30S. However, some labelled released material sedimenting faster

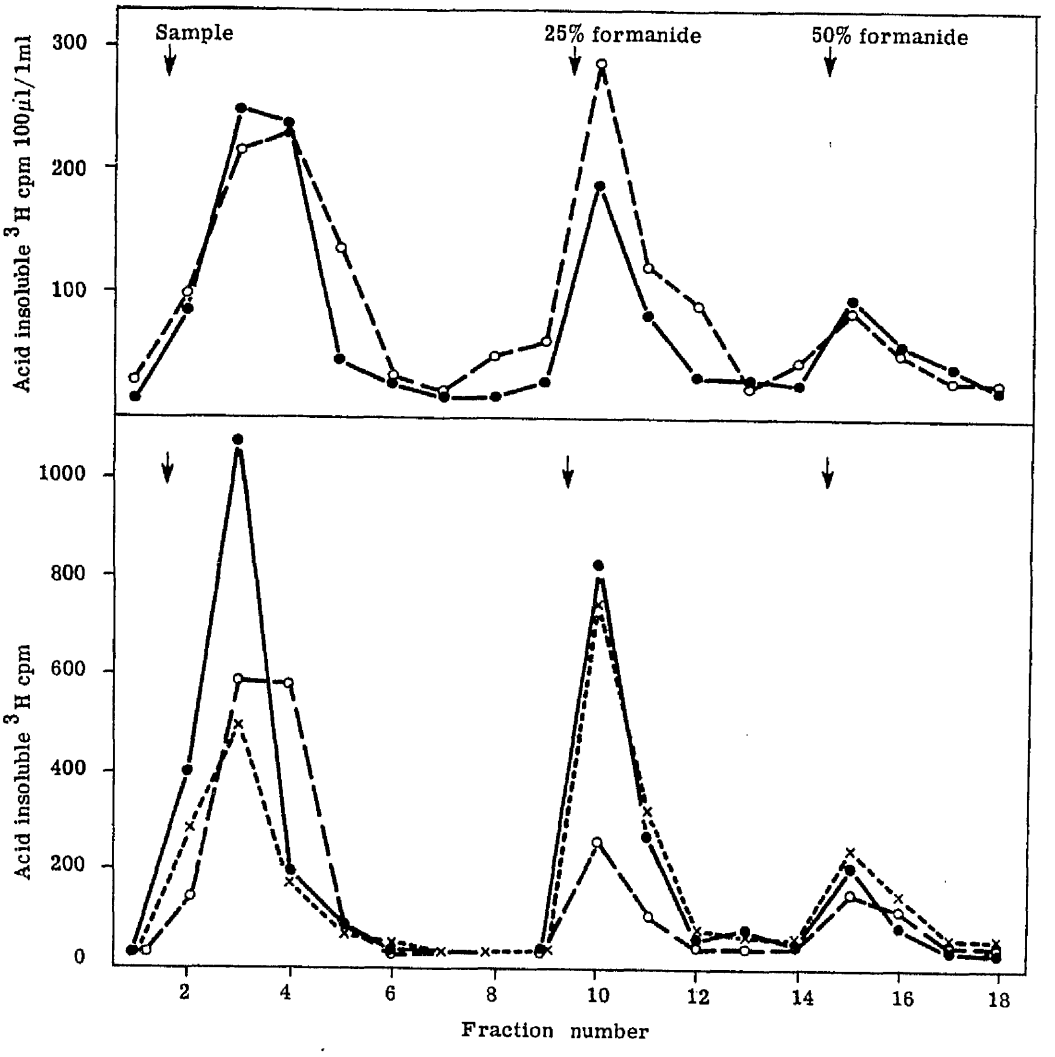
than 40S was also found (track 2) and contained much less of these high molecular weight proteins.

One major difference between the released and retained particles is the presence in the released fractions sedimenting at 40S and above of low molecular weight proteins running faster in the gel than the major A_1 and A_2 pair of proteins. From the molecular weights of these proteins it is suggested that they are ribosomal, since the pattern of banding is similar to that described by Beyer et al (1977) and Karn et al (1977) in the same gel system. The diffuse band of $\sim$ 30,000 daltons could be histone H_1 (Beyer et al, 1977; Karn et al, 1977). These low molecular weight proteins are not found in the material sedimenting between 0-30S and could therefore be attached to co-sedimenting pre-ribosomal particles released during the incubation. Although these cells were labelled in the presence of low concentrations of actinomycin D to inhibit ribosomal RNA synthesis, 28S rRNA sequences remain in the nucleus for $\sim$ 60 minutes before the mature 28S RNA is transported to the cytoplasm. The transport of ribosomal RNA is discussed latter.

5.1.2. Purification of RNP particles by oligo(dT) affinity chromatography

In order to purify further the released and retained particles, oligo (dT) cellulose affinity chromatography was employed. This method was first used by Lindberg and Sundquist (1974) to separate messenger ribonucleoprotein particles from ribosomal particles and other contaminants and has been used to isolate a fraction of HeLa hnRNP particles (Kumar and Pederson, 1975). About 40% of labelled hnRNPs bound to columns of oligo(dT) cellulose, and could be released

Figure 32. Oligo(dT) cellulose affinity chromatography of nuclear and released particles from incubated nuclei



Sucrose gradient fractions of retained and released particles (as in Figure 28), corresponding to material sedimenting between 30-60S, were pooled and dialysed against oligo(dT) binding buffer in vacuum dialysis apparatus. Samples were applied to oligo(dT) cellulose columns and eluted with 25% (v/v) and 50% (v/v) formamide.

Graph (a).

●——●——● 30-60S material released from nuclei incubated at 25°C for 30 minutes.

o - - -o - - -o 30-60S material released from nuclei incubated at 25°C for 30 minutes in the presence of dialysed cytosol.

Graph (b).

●——●——● 30-60S material retained in non-incubated nuclei.

o - - -o - - -o 30-60S material retained in nuclei incubated at 25°C for 30 minutes.

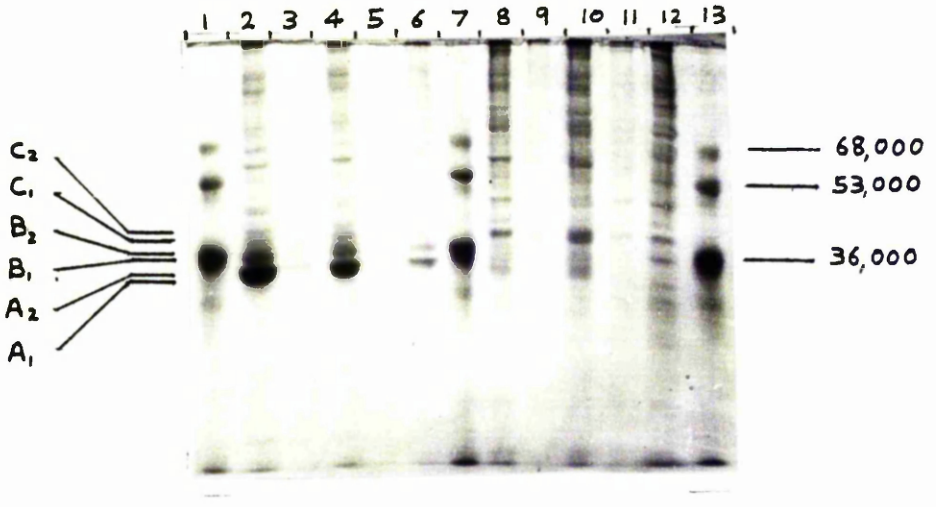
× - - - - × - - - - × 30-60S material retained in nuclei incubated at 25°C for 30 minutes in the presence of dialysed cytosol.

following sequential elution with 25% (v/v) and 50% (v/v) formamide (Figure 32). The binding of both retained and released particles showed no significant deviation from this percentage, which would tend to indicate a similarity between these particles, at least in terms of binding to oligo(dT). However, the nature of the affinity for oligo(dT) of these particles is complex and involves an interaction of protein as well as poly(A) regions in the RNA with the oligo(dT) (Lindberg and Sunquist, 1972; Sundquist et al, 1977b). Thus comparison of binding may not correlate with any well defined characteristic of the particles. A greater percentage of nuclear and released particles from nuclei incubated with cytosol bound to the column. The significance of this is difficult to interpret in the light of results obtained in the next section.

5.1.3. Proteins associated with oligo(dT) cellulose fractionated RNP particles

Figure 33 shows in tracks 2-6 the pooled material eluted from oligo(dT) columns with 25 and 50% (v/v) formamide. The recovery of retained and released RNP particles (tracks 3 and 5) was low following this two step purification procedure. However, in the same experiment, nuclei were incubated in medium containing cytosol, and the retained and released RNP particles were also fractionated on oligo(dT) cellulose. The recovery in this case was better, possibly due to a "carrier" effect of cytosol materials. There is no observable difference between the protein constituents of hnRNPs extracted from nonincubated nuclei and those of the retained RNP particles remaining in nuclei incubated with cytosol (tracks 2 and 4). Moreover, the main bands in these fractions can also be seen in the

Figure 33. SDS polyacrylamide gel electrophoresis of proteins associated with particles purified by oligo(dT) cellulose affinity chromatography.



Retained and released particles from incubated nuclei were fractionated on oligo(dT) cellulose asin Figure 32. Non-bound and bound material (pooled material eluted with 25% and 50% formamide) was precipitated with two volumes of ethanol and resuspended in SDS gel sample buffer. Electrophoresis was carried out as in Figure 31.

Tracks 1, 7 and 13			Molecular weight standards	
2	material bound to oligo(dT) cellulose	8	material not bound to oligo(dT) cellulose	Non-incubated nuclear particles
3		9		Incubated nuclear particles
4		10		Incubated + cytosol nuclear particles
5		11		Released particles
6		12		Released + cytosol particles

bound fraction of particles released into medium containing cytosol (track 6), particularly the major proteins in the molecular weight range 30-40,000. The proteins in the non-bound fraction of each particle sample were also run on the gel. The pattern of bands is quite different from that of the bound fractions. Analysis of the major proteins in the 30-45,000 molecular weight range shows that the major A_1 + A_2 doublet of the Beyer et al nomenclature (poorly resolved in the gel), is found exclusively in the bound fraction of nuclear hnRNP particles, along with four other proteins with mobilities corresponding to B_1, B_2, C_1 and C_2 of the Beyer et al (1977) nomenclature. A single, or possibly two, proteins with mobility equivalent to the C group proteins were also found in the non-bound fractions.

Comparing the bound and non-bound fractions of material released during incubation (tracks 6 and 12) reveals the presence of proteins of molecular weight lower than 30,000 in the non-bound fraction which are not present in the bound fraction. This would indicate that even in the presence of cytosol, the protein composition of the bound released particles is very similar to that of nuclear RNP particles prepared by sonication. It has been shown that the main proteins associated with HeLa cell free cytoplasmic mRNP particles are small with molecular weights ranging from 10-30,000 (Liautard et al, 1976) and are quite distinct from the hnRNP proteins. Thus, the low molecular weight proteins co-sedimenting with the released particles (Figure 31) could be informosomal. Moreover, it might be expected that incubation in the presence of cytosol might lead to a "changeover" of particle proteins from hnRNP type to informosomal,

since it has been suggested that informosomes are formed by the interaction of cytosolic RNA binding proteins with transported RNA (Preobrazensky and Spirin, 1978; Egly and Stevenin, 1977). However, since these low molecular weight proteins are not apparent in the oligo(dT) bound fraction of released particles, and informosomes are known to bind to oligo(dT) (Jeffery, 1977), it is probable that these proteins are ribosomal rather than informosomal.

5.1.4. Is the release of RNP particles "specific transport" or "passive diffusion"?

The similarity of the protein composition of released and retained RNP particles would at first sight tend to support the conclusion that the release of particles is a random leakage during incubation, analogous to the "extraction" method for preparing "40S" hnRNP particles from intact nuclei by diffusion into pH 8.0 buffer (Samarina et al, 1968). However, comparison of the protein composition of particles prepared by disruption of nuclei (e.g. sonication) and diffusion from intact nuclei has revealed large differences between the two. In general, the extraction method, which relies on endogenous ribonuclease activity to cleave particles to 40S monomer size, results in a much simpler protein composition, containing only the major protein(s) of 30-40,000 molecular weight (Pederson, 1974; Beyer et al, 1977). However, in this case, the released particles, although mainly of monomer size (40S), contain many more proteins, even after purification by sucrose gradients and oligo(dT) cellulose. Therefore, in a contradictory way, the similarity of released and restricted particle proteins might actually be

indicative of a more physiological type of transport from the isolated nuclei than the leakage of degraded nuclear particles.

To explore this possibility, various characteristics of the released and restricted nuclear RNA were examined.

5.2. Comparison of released and retained pre-labelled RNA in incubated nuclei

5.2.1. Affinity chromatography of RNA on poly(U) Sepharose

Approximately 20% of pulse-labelled HeLa cell hnRNA has been found to be polyadenylated (Herman and Penman, 1977), whereas 70% of cytoplasmic mRNA in the same cells has a poly(A) tail (Mikarek et al, 1974). Therefore, the labelled RNA released during incubation in these studies can be analysed to determine whether this fraction is enriched in polyadenylated molecules compared to the total nuclear RNA. This was performed by measuring the binding of ^{3}H -uridine labelled RNA to poly(U) Sepharose columns (Molloy et al, 1974). Poly(U) Sepharose was used in preference to oligo(dT) cellulose, since the length of the poly(U) ($\sim$100 nucleotides) makes it possible to differentiate polyadenylate tracts of different lengths. In particular, it has been shown that HeLa cell hnRNA contains transcribed oligo(A) sequences of 20-30 nucleotides long which are found only in non-polyadenylated (poly(A)$^-$) hnRNA molecules (Venkatesan et al, 1979). It is possible to distinguish molecules containing oligo(A) and poly(A) tracts by elution from poly(U) Sepharose with different formamide concentrations (Molloy et al, 1974).

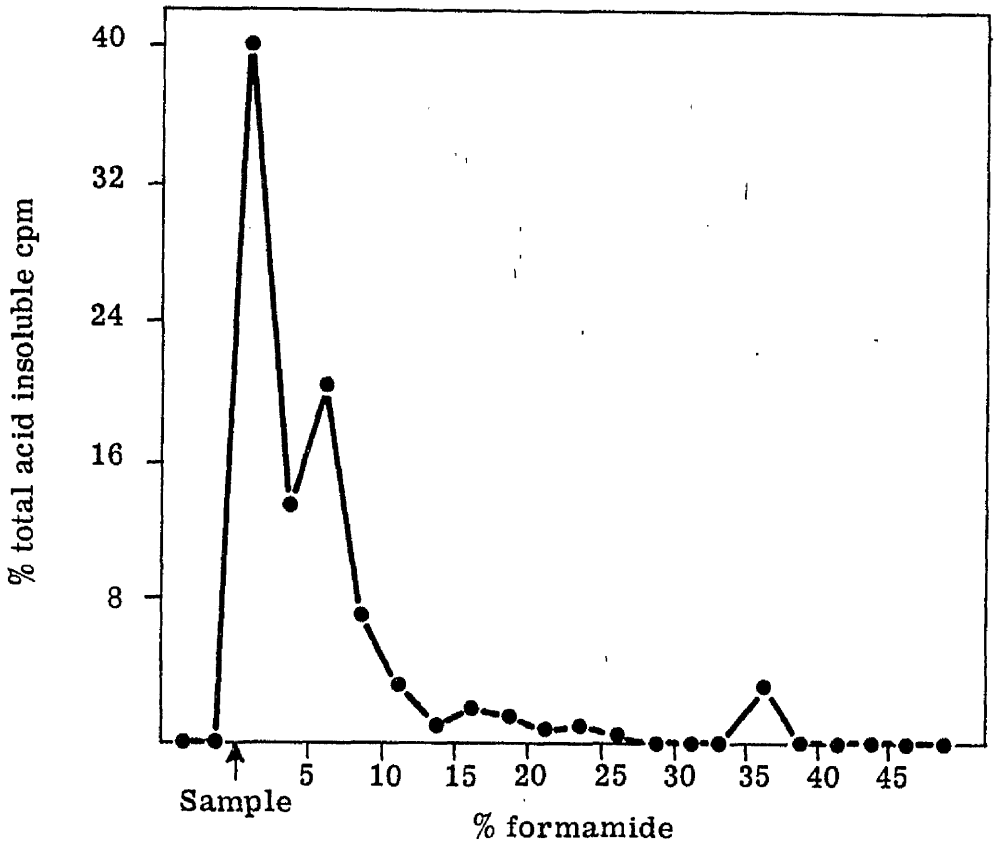
It was found that both retained and released RNA contain labelled material which is eluted from poly(U) Sepharose with 5%

formamide and 35% formamide, but very little which is eluted with intermediate concentrations of formamide (Figure 34). It has been shown that material eluted at these formamide concentrations contains oligo(A) stretches of 20-30 nucleotides, and poly(A) tracts of about 200 nucleotides respectively (Molloy et al, 1974). On this basis, material which did not bind to poly(U) Sepharose was termed $(A)^-$ and presumed to contain molecules containing only tracts of (A) residues less than $\sim$ 10 nucleotides. Material which was eluted with 10% (v/v) formamide was termed oligo$(A)^+$ and presumed to contain mainly molecules containing a transcribed oligo(A) tract of 20-30 nucleotides, whilst material which was eluted by 90% (v/v) formamide was assumed to be polyadenylated RNA and termed poly$(A)^+$ RNA.

5.2.2. Comparison of released and retained RNA in terms of binding to poly(U) Sepharose

Table 1 shows the results of two estimations of poly(U) Sepharose binding of total nuclear, retained and released pre-labelled RNA. The released RNA contains a significant proportion of molecules which behave like oligo(A) containing hnRNA on poly(U) Sepharose, yet normal cellular HeLa mRNA does not appear to contain any oligo(A) sequences (Venkatesan et al, 1979). Therefore, by this criterion, the released RNA is "hnRNA-like" rather than "mRNA-like". However, Edmond's group have proposed that oligo(A) in hnRNA may serve as a primer for post-transcriptional polyadenylation to explain the absence of oligo(A) in either polyadenylated hnRNA or cytoplasmic mRNA (Venkatesan et al, 1979). The sequence complexity of oligo(A)-adjacent sequences in 30S hnRNP particles extracted by diffusion into pH 8.0 buffer from intact nuclei has been determined (Kinniburgh and

Figure 34. Poly(U) Sepharose affinity chromatography of RNA released from incubated nuclei.



RNA was extracted from the supernatant fraction of a nuclear incubation mixture, after incubation of purified nuclei isolated from HeLa cells labelled for 10 minutes with [5,6-^{3}H]uridine after 20 minutes actinomycin D treatment. RNA was resuspended in Poly(U) Sepharose binding buffer and applied to the column. The sample was eluted by stepwise elution with increasing concentrations of formamide, from 5-50% (v/v) formamide.

Martin, 1976b). The abundant class of oligo(A)-adjacent sequences was also found in the abundant class of cytoplasmic poly(A)$^+$ mRNA. Therefore, the fact that a larger proportion of released RNA is found in the oligo(A) class than of the nuclear RNA could indicate a preferential release of messenger sequences.

The proportion of pulse labelled hnRNA in the poly(A) containing fraction is lower than described elsewhere (Molloy et al, 1974; Herman and Penman, 1977), and this could be due to the low efficiency of binding of large hnRNA to poly(U) Sepharose (Molloy et al, 1974). On the other hand, the samples were partially denatured by heating to 60°C and rapidly cooled immediately before application to the column, which would overcome aggregation of poly(A) with U-rich sequences and general secondary structure which might prevent binding to the column (Herman and Penman, 1977). It is possible that the poly(A) sequences are selectively degraded during preparation of the nuclei. Extraction of 30S RNP particles from nuclei in pH 8.0 buffer taking advantage of endogenous nuclease activity, results in release of 15S particles containing only poly(A) (Quinlan et al, 1974) and thus the region joining the poly(A)-protein complex to the remainder of the hnRNP polypeptide may be susceptible to mild nuclease activity.

Comparison of the proportion of labelled RNA in each class (Table Ib) shows a very low proportion of labelled RNA retained in nuclei after incubation in either of the (A)$^+$ classes compared to the original nuclear RNA or the released RNA. This would imply a selective release of (A)$^+$ molecules (both oligo(A) and poly(A)). Although the proportion of released label in the poly(A)$^+$ class is

precipitable counts were determined in each fraction, and expressed as a percentage of the total counts eluted from the column.

Table (a). Total nuclear RNA from non-incubated and incubated nuclei, and RNA released from incubated nuclei were characterised in terms of binding to poly(U) Sepharose.

Table (b). RNA from non-incubated and incubated nuclei, and RNA released from incubated nuclei was passed through a poly(A) Sepharose column. The non-bound material (defined as $(u)^-$ - see Table 2) was then characterised in terms of binding to poly(U)-Sepharose.

Table 1b Binding of nuclear and released RNA to Poly(U) Sepharose

		Labelled RNA not bound to poly(U) Sepharose $[(A)^-]$	Labelled RNA eluted from poly(U) Sepharose with 10% formamide (oligo$(A)^+$)	Labelled RNA eluted from poly(U) Sepharose with 90% formamide (poly$(A)^+$)
Sample		% total c.p.m.	% total c.p.m.	%total c.p.m.
(a) Nuclear RNA from non-incubated nuclei	Expt 1	65	25	10
	Expt 2	80	15	5
RNA retained in incubated nuclei	Expt 1	88	10	2
	Expt 2	89	9	2
Released RNA from incubated nuclei	Expt 1	64	30	6
	Expt 2	72	25	3
(b) $(u)^-$non-incubated nuclear RNA.		84	12	4
$(u)^-$retained RNA.		88	9.5	2.5
$(u)^-$released RNA.		73	20	7

HeLa cells were treated with actinomycin D (0.04 µg/ml) for 30 minutes before labelling for 10 minutes with $[5,6\text{-}^3H]$ uridine. Purified nuclei were prepared and incubated as described. RNA was extracted from the nuclei and the supernatant fraction of the incubation medium and resuspended in 0.4M NETS. Each sample was passed twice through the column after heating to 60°C for 3 minutes followed by rapid cooling. Bound RNA was eluted with 10% (v/v) formamide (oligo$(A)^+$ fraction) and 90% (v/v) formamide (poly$(A)^+$ fraction). The acid

lower than in the original hnRNA, the size of this RNA is significantly smaller than the original hnRNA (Figure 26). Therefore, since a random leakage of small molecules after their formation by degradation of large poly(A)$^+$ molecules would result in a much smaller proportion of released label in the poly(A)$^+$ class than of the original RNA, this result is still consistent with a preferential release of (A)$^+$ RNA.

5.2.3. Poly(A) Sepharose affinity chromatography of RNA

HeLa cell hnRNA contains (U)-rich tracts of 30-40 nucleotides in length (Molloy et al, 1974). It has been estimated that large polyadenylated hnRNA contains on average 2-3 (U)-rich tracts per molecule whereas small poly(A)$^+$ hnRNA contains on average 0.25 (U)-rich tracts/molecule (Molloy et al, 1974). HeLa poly(A)$^+$ mRNA contains on average 0.20 (U)-rich tracts/molecule (Edmonds et al, 1976) (i.e. 20% of poly(A)$^+$ mRNA molecules have one (U)-rich segment unless the (U)-rich segments are concentrated in a smaller number of molecules).

Poly(A)-Sepharose affinity chromatography was used to define a non-bound (U)$^-$ class and a bound (U)$^+$ (U-rich) class which was eluted from the column with 90% (v/v) formamide. It was found that a similar proportion of (U)$^+$ RNA was released or restricted after nuclear incubation (Table 2). During extraction by diffusion into pH 8.0 buffer of 30S monomer particles from intact ascites nuclei, a large proportion of nuclear poly(A) is released in a separate 15S particle which also contains (U)-rich sequences (Quinlan et al, 1977), whereas both poly(A) and oligo(A) remain associated with the polypeptides released from HeLa nuclei by sonication (Kish and Pederson,

Table 2 Binding of nuclear and released RNA to Poly(A) Sepharose

Sample		Labelled RNA not bound to poly(A) Sepharose $[(u)^-]$ % total c.p.m.	Labelled RNA eluted from poly(A) Sepharose with 90% formamide $[(u)^+]$ % total c.p.m.
(a)			
Non-incubated nuclear RNA.		83.5	16.5
Retained RNA from incubated nuclei.		90.5	9.5
Released RNA from incubated nuclei.	Expt 1	89	11
	Expt 2	87.5	12.5
(b)			
Nuclear $(A)^-$ RNA		90	10
Retained $(A)^-$ RNA		89	11
Released $(A)^-$ RNA		80	20

The experiment was carried out as described in Table 1b. RNA samples were passed through poly(A) Sepharose columns and divided into non-bound (u^-) and bound (u^+) (eluted with 90% (v/v) formamide) fractions. Acid precipitable counts in each fraction were determined and expressed as a percentage of the total counts eluted from the column.

Table (a). Nuclear RNA from non-incubated nuclei, RNA retained in incubated nuclei, and RNA released from incubated nuclei were characterised in terms of poly(A) Sepharose binding.

Table (b). RNA samples as above were first passed through poly(U) Sepharose columns, and the non-bound RNA $(A)^-$ was then characterised in terms of poly(A) Sepharose binding.

1975) and are apparently base paired with oligo(U) sequences (Kish and Pederson, 1977). Therefore, if the particles released during incubation are analogous to particles extracted from nuclei in pH 8.0 buffer by the action of endogenous nucleases, it might be expected that the released RNA would be depleted in poly(A) and $(U)^+$ sequences compared to the nuclear RNA. However, since this is not the case, it is possible that the release reaction studied here is more analogous to specific transport of mRNA than to extraction of hnRNP monomer particles.

It was possible to apply the material which did not bind to poly(U) Sepharose directly to a poly(A) Sepharose column, and vice versa. In this way, it was possible to characterise the $(A)^-$ and $(U)^-$ classes of RNA. In general, the binding to poly(U) Sepharose of $(U)^-$ RNA reflects the binding of total RNA, as does the binding of $(A)^-$ RNA to poly(A) Sepharose.

5.3. Factors affecting the release of prelabelled RNA from isolated nuclei

5.3.1. The effect of ATP on the release of RNA from isolated nuclei

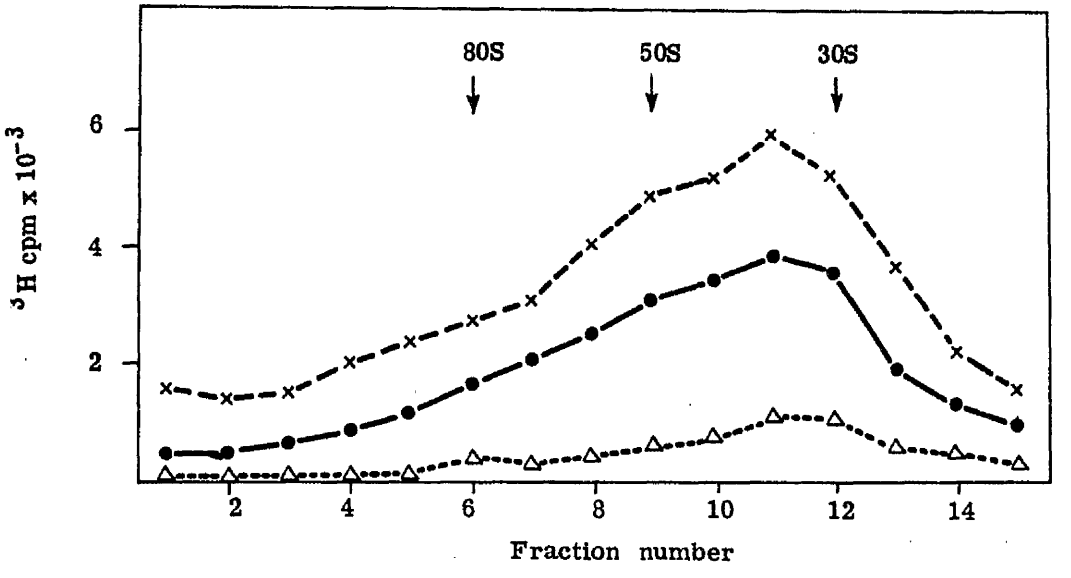
It has been shown by a number of groups that release of rapidly labelled RNP material from isolated nuclei is stimulated by ATP, although explanations for this differ. An energy role for ATP has been suggested by Webb's group (Schumm and Webb, 1972), and recently supported by Clawson *et al* (1978). A site specific binding role of ATP causing a conformation change in the transport machinery has also been proposed (Ishikawa, 1978). On the other hand, a chelating role has been proposed by other groups, since other chelating agents

can mimic the effect of ATP, and a high Mg^{2+} ion concentration inhibits release (Chatterjee and Weissbach, 1973; Raskas and Rho, 1976). This may be due to leakage caused by chelation of Mg^{2+} ions necessary for the integrity of the nuclear envelope (Stuart et al, 1975).

Using the optimum concentration of 7.5 mM ATP (Clawson and Smuckler, 1978; Chatterjee and Weissbach, 1973) a larger proportion of labelled RNA was released during incubation than from the control nuclei. When this concentration was "compensated" for chelating activity by adding Mg^{2+} ions to a final concentration of 7.5 mM (Cornish-Bowden, 1976), this stimulation was inhibited. Moreover, increasing the Mg^{2+} concentration in the normal incubation medium from 5 mM to 10 mM inhibited the normal release. These results are more consistent with a chelating function of ATP rather than a specific role in transport. 7.5 mM EDTA also stimulated release which could be compensated by addition of 7.5 mM Mg^{2+}. Quercitin had no effect on release, although it has been shown that this is an inhibitor of a nuclear envelope nucleoside triphosphatase which has been implicated in the energy dependent transport of RNA from isolated pig liver nuclei (Agutter et al, 1976).

When material released under these conditions was sedimented through sucrose gradients, no difference in the size of material released was observed (Figure 35). The size of RNA released was also the same whether released into control, high Mg^{2+} or ATP containing medium (results not shown). The effect of ATP and Mg^{2+} on the free hnRNP pool was much less pronounced. HnRNPs were extracted by sonication of nuclei after incubation with ATP, or high Mg^{2+} concen-

Figure 35. The effect of ATP on sedimentation of particles released from isolated nuclei



HeLa cells were labelled with [5,6-^{3}H] uridine for 10 minutes after 20 minutes treatment with actinomycin D (0.04 µg/ml). Purified nuclei were prepared and incubated at 25°C for 30 minutes as described in Figure 26. Nuclei were sedimented after incubation, and the supernatant fraction was diluted with an equal volume of ISB and sedimented through 15-30% (w/v) sucrose gradients as described in Figure 28. The effect of incubation in the presence of 7.5 mM ATP, and 10 mM Mg^{2+} was compared to the control incubation medium of 5 mM Mg^{2+}.

●——●——● Nuclei incubated in normal incubation medium (5 mM Mg^{2+})

×– – –×– · –× Nuclei incubated in 5 mM Mg^{2+} + 7.5 mM ATP

△– – –△– – –△ Nuclei incubated in 10 mM Mg^{2+}

tration (Figure 36). The RNP distribution profiles are the same as the control, and contain the same amount of label. The RNA size in these nuclei was unaffected by these incubation conditions.

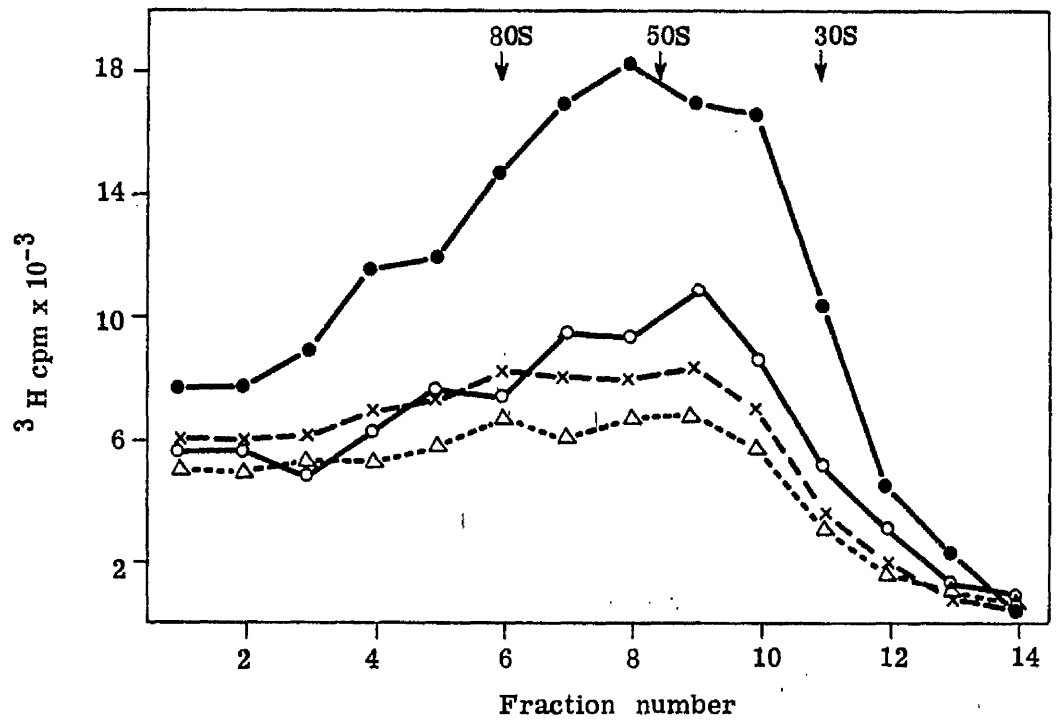
5.3.2. The effect of cytosol on the release of labelled RNA

It has been suggested, particularly by Webb and coworkers (Schummet al, 1973; Yannarell et al, 1976), that release of labelled RNA from isolated rat liver nuclei is controlled by cytoplasmic factors present in dialysed cytosol. However, in HeLa nuclei, dialysed cytosol had no effect on the amount of released RNA. Studies on the size of released RNP particles, their binding to oligo(dT) (Figure 34), and their protein composition (Figure 35), all indicated no discernible effect of cytosol on the released particles. The size of RNA in the released particles was also unaffected by cytosol.

5.3.3. The effect of nuclear membranes on the release of RNA from isolated nuclei

Another factor which could influence the release of RNP particles from these nuclei is the presence or absence of nuclear membranes. It has been shown that the non-ionic detergent, Triton X-100, will remove both the outer and inner membranes of nuclei (Aaronson and Blobel, 1974; Scheer et al, 1976), leaving an intact nuclear structure held together by the underlying protein lamina (Aaronson and Blobel, 1975) and/or an internal protein matrix (Berezney and Coffey, 1974). The presence or absence of membrane has no effect on the release of RNA from isolated myeloma cell nuclei (Stuart et al, 1977). The amount of RNA released from these HeLa nuclei was independent of whether the nuclei were prepared by homogenisation in

Figure 36 The effect of ATP on sedimentation of particles extracted from incubated nuclei.



The incubated nuclei from the experiment described in Figure 36 were sonicated and the total sonicate was sedimented through 15-30% (w/v) sucrose gradients as described in Figure 28.

●——●——● Sonicate of non-incubated nuclei

o——o——o Sonicate of nuclei incubated at 25°C for 30 minutes in 5 mM Mg^{2+}

×---×---× Sonicate of nuclei incubated in 5 mM Mg^{2+} and 7.5 mM ATP

△----△----△ Sonicate of nuclei incubated in 10 mM Mg^{2+}

medium with or without Triton X-100 (results not shown). It is generally assumed, although with little evidence (Stevens and Swift, 1966; Monneron and Bernard, 1969) that RNA is transported through the lumen of nuclear pores. These remain embedded in the protein lamina of the nucleus after removal of the membranes (Aaronson and Blobel, 1975), and therefore an absence of effect in this experiment is not unexpected, although a role for nuclear membranes in the control of transport through pores has been postulated (Herlan and Wunderlich, 1979).

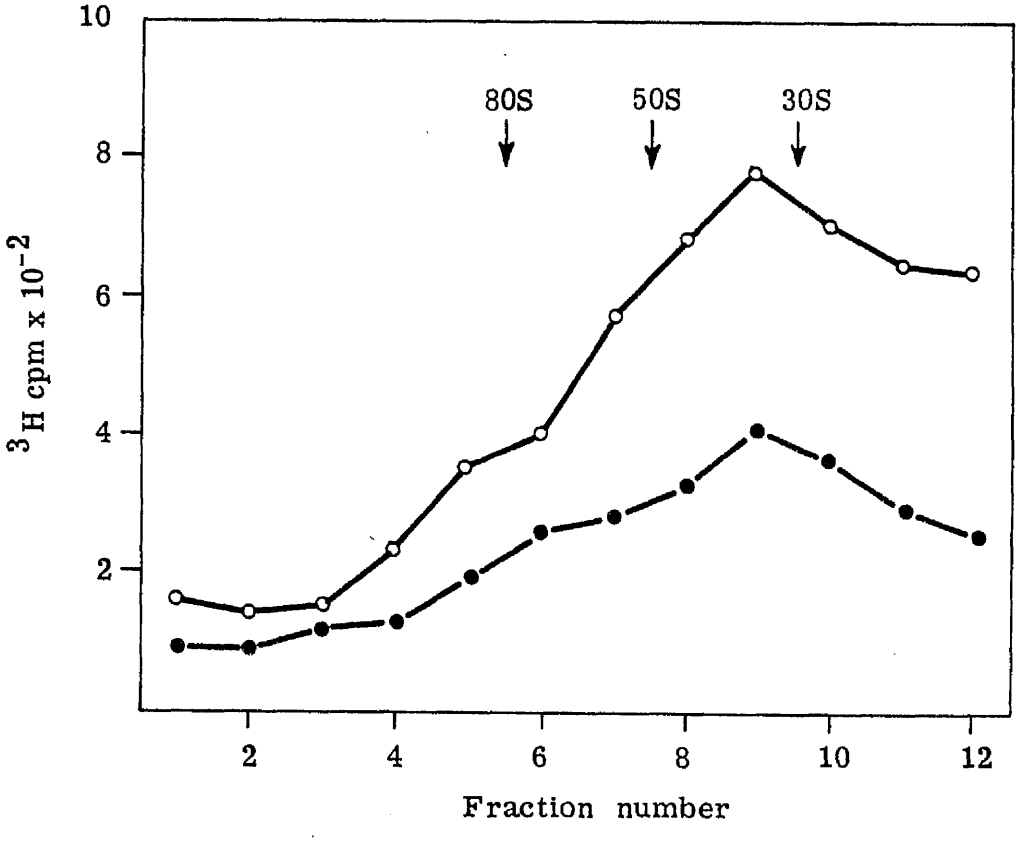
5.4. The effect of actinomycin D pretreatment of cells on release of labelled RNA from isolated nuclei

5.4.1. The effect of actinomycin D pretreatment on the size of released material

In most of the studies on transport of rapidly labelled RNA outlined above, cells were treated with low levels (0.04 μg/ml) of actinomycin D to inhibit labelling of ribosomal RNA. However, a role for the nucleolus in mRNA transport was proposed a number of years ago (Sidebottom and Harris, 1969) based on the effects of u.v. microbeam inactivation of nucleoli *in vivo* on RNA transport. More recently, it has been suggested that mRNP particles are transported from the nucleus in polysome-like structures (Goidl *et al*, 1975). It was therefore of interest to determine the effect of actinomycin D pretreatment on the release of labelled RNA, and at the same time examine the release of labelled ribosomal RNA from isolated nuclei.

Figure 37 shows the sedimentation profiles of RNP particles released from nuclei labelled *in vivo* for 10 minutes with or without

Figure 37. The effect of actinomycin D treatment of cells on release of labelled RNP particles from isolated nuclei.



HeLa cells were labelled for 10 minutes with [5,6-^{3}H]uridine (0.25 mCi/50 ml). Purified nuclei were prepared and incubated at 25°C for 30 minutes. The material released was analysed on 15-30% sucrose gradients in ISB as described in Figure 26. The effect of treating cells with actinomycin D (0.04 µg/ml) for 20 minutes before labelling was compared with a non-treated control.

o——o——o Non-treated cells

●——●——● Cells treated for 20 minutes with actinomycin D (0.04 µg/ml)

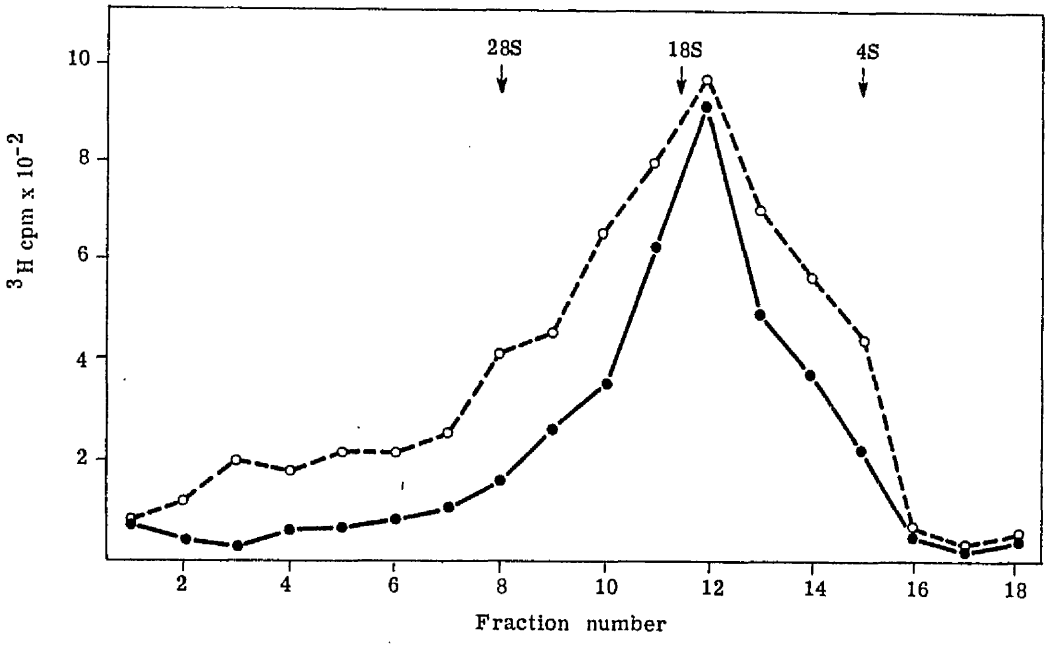
actinomycin D pretreatment. The profiles are similar, with a peak of material sedimenting at 40S. Analysis of released RNA on denaturing gradients showed that although the peak of labelled RNA has a sedimentation coefficient of approximately 18S, the profile of RNA from untreated cell nuclei is broader, with a possible "shoulder" of material sedimenting at 28S (Figure 38). There is also more small (4-10S) material present. The results are consistent with the release of labelled ribosomal RNA occurring during incubation, since the release of mature ribosomal RNA would be mainly 18S after the short labelling time. The results do not indicate any large effect on the release of hnRNP particles of actinomycin D pretreatment.

5.4.2. The release of ribosomal RNP particles

When cells were labelled for 12 hours rather than 10' to achieve steady state labelling of long-lived RNA, it was found that material smaller than 40S was also released. When untreated cells were labelled for this time, an extra peak of 50-70S material was seen in the sedimentation profile of released material (Figure 39). A 55S RNP particle containing 32S precursor ribosomal RNA has been identified in HeLa cells (Warner and Soeiro, 1967) and mature 28S rRNA is released in a 60S particle. RNA in the peak was heterogeneous in denaturing gradients, much of it being $>$ 28S in size. This is a similar size to material transported from rat liver nuclei in the presence of cytosol and tRNA (Sato et al, 1977a), and is probably partially processed ribosomal RNA.

If this is the case, similar unlabelled partially processed ribosomal RNA particles are probably still released into the incubation medium from nuclei pretreated for 30 minutes with actinomycin D, since

Figure 38. The effect of actinomycin D treatment of cells on the release of labelled RNA from isolated nuclei

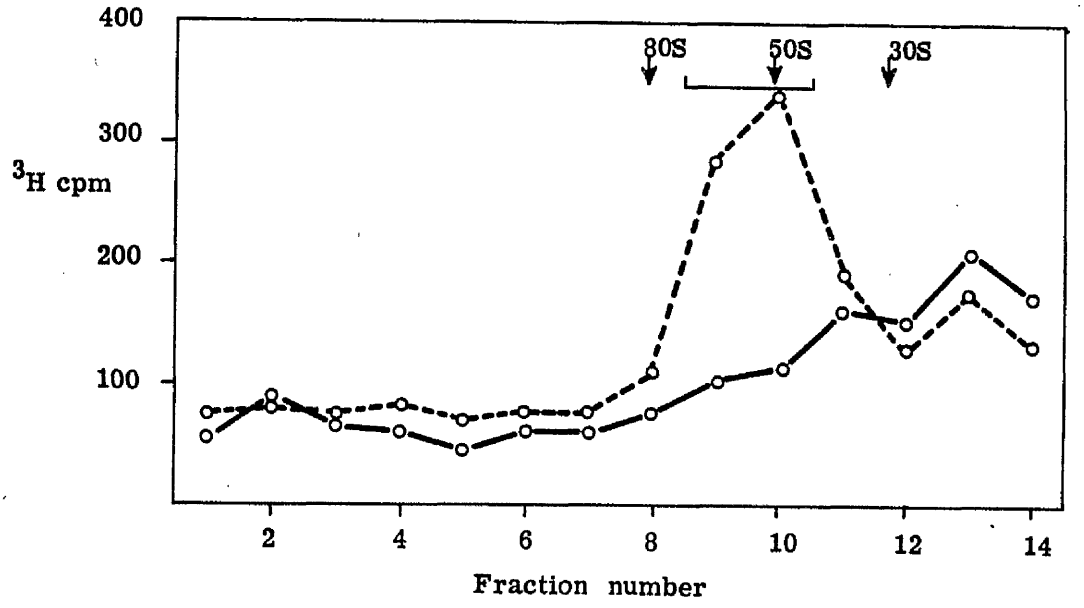


RNA was extracted from the incubation medium supernatant fraction of the previous experiment (Figure 37) and analysed on denaturing gradients as described in Figure 2.

o - - - o - - - o Untreated cells

● ——— ● ——— ● Cells treated for 20 minutes with actinomycin D (0.04 μg/ml)

Figure 39. The effect of actinomycin D on release of material from incubated nuclei prepared from cells labelled for 12 hours.



HeLa cells were labelled for 12 hours with 5,6-^{3}H uridine in the presence or absence of actinomycin D (0.04 µg/ml). Purified nuclei were prepared and incubated at 25°C for 30 minutes. The material released into the supernatant fraction of the incubation medium was analysed on 15-30% sucrose gradients in ISB as described in Figure 26.

o- - -o- - -o Cells labelled in the absence of actinomycin D

o ——— o ——— o Cells labelled in the presence of actinomycin D (0.04 µg/ml).

28S rRNA processing takes 1 hour between synthesis and transport (Penman, 1969). It was observed that fractions from sucrose gradients of released material sedimenting at 40S and above contained a number of low molecular weight proteins not found in the nucleoplasmic hnRNP particles or the nucleosol (Section 5.1.1).

Although it is possible that these proteins were informosomal (Liautard et al, 1976) the fact that they were not selectively bound to oligo(dT) cellulose would tend to confirm their ribosomal nature. It has been reported that addition of poly(U) to isolated HeLa cell nuclei releases polyribosomes (Goidl et al, 1975) which could be involved in nuclear protein synthesis, or transport of mRNA. However, it was found that addition of poly(U) at the same concentration as used by Goidl et al (1975) to isolated HeLa cell nuclei labelled in vivo for 10 minutes with or without actinomycin D treatment, actually inhibited release of labelled particles. Moreover, it was not possible to extract any labelled hnRNP particles by sonication, due to sedimentation of these particles to the bottom of the 15-30% sucrose gradient. It is suggested that poly(U) aggregates the nuclear particles and this prevents release of particles.

5.5. Is the RNA released from isolated nuclei mRNA?

5.5.1. Correlation of the release of labelled RNA with the mRNA activity of total released RNA

To determine whether the total RNA population released during incubation was messenger-like, its ability to stimulate incorporation of labelled amino acid into acid precipitable material in a wheat germ protein synthesis system was assayed.

Table 3 shows two separate experiments in which different dilutions of released RNA were tested in the wheat germ system. The activity of RNA released from the same number of nuclei incubated in normal, high Mg^{2+} and +ATP medium was compared with the supernatant RNA from non-incubated nuclei. The purpose of this was to correlate the relative changes in message activity with the ability of the different incubation conditions to release labelled RNA (see Section 5.3.1), and thus reduce the real problem of residual contamination by cytoplasmic messenger RNA.

The supernatant material from non-incubated nuclei gives a small stimulation, particularly at its highest concentration. This is most likely to be due to contaminating mRNA, despite the purification of nuclei by homogenising in Triton X-100 containing medium, and centrifuging through 2M sucrose. Alternatively, some unalebelled message-like RNA may leak out of the nuclei immediately on resuspension in the incubation medium.

At the highest concentration, RNA released during 30 minutes incubation stimulates incorporation up to 5 times that of the endogenous message activity, and more than twice that of the non-incubated control RNA. The material released in the presence of 10 mM Mg^{2+} has much less activity, only slightly more than control.

The RNA released by ATP was found to inhibit the endogenous activity of the system at its highest concentration. This was probably due to the large precipitate obtained after ethanol precipitation of this sample, which could be ATP or its derivatives. Repeated washes and ethanol precipitations from pH 5.0 and pH 9.0 buffers did not completely remove this precipitate. However, at

Table 3 Messenger activity of RNA released from isolated nuclei in a wheat germ cell-free translation system

RNA dilution	0 dilution		¼ dilution		1/5 dilution		1/10 dilution		1/12.5 dilution	
Sample	^{3}H cpm	stimu-lation	^{3}H cpm	stimu-lation	^{3}H cpm	stimu-lation	^{3}H cpm	stimu-lation	^{3}H cpm	stimu-lation
no RNA	990		990		900		990		900	
0.3 µg "0" RNA	2990	3.0	1714	1.7	1459	1.6	1364	1.4	1160	1.3
0.7 µg "30" RNA	5624	5.7	2322	2.3	1932	2.1	1632	1.6	1366	1.5
1.5 µg "ATP" RNA	297		2511	2.5	2508	2.7	2704	2.7	2692	3.0
0.5 µg "10 mM Mg^{2+}" RNA	3408	3.4	1895	1.9	1458	1.6	1552	1.6	1136	1.3

HeLa cells were treated with actinomycin D (0.04 µg/ml) for 30 minutes. Purified nuclei were prepared and incubated under the conditions described in Figure 37. Equal numbers of nuclei were used in each incubation. RNA was extracted from the supernatant fraction of the incubation and washed by repeated ethanol precipitation and resuspension. The activity of RNA from equal numbers of nuclei was determined by its ability to stimulate incorporation of L[4,5-^{3}H]leucine into acid insoluble material in a wheat germ cell free protein synthesising system Different dilutions of added RNA were compared with the endogenous activity of the wheat germ system.

lower concentrations of this sample, a marked stimulation was observed compared to the normal incubated sample. Therefore, there appears to be a correlation between the amount of labelled RNA and the messenger activity released from incubated nuclei under various conditions, indicating that some of the messenger activity is actually released from the nuclei, and not residual mRNA contamination.

5.5.2. Comparison of the messenger activity of RNP and deproteinised RNA released from isolated nuclei

In Table 4 the message activity of released RNP particles was compared with an identical sample from which the RNA was phenol extracted. A stimulation by the released RNA was again observed, but the corresponding RNP sample had no activity in the assay. This could either be due to the proteins bound to the RNA, or to other cosedimenting inhibitory factors. Most experiments comparing the message activity of polysomal mRNP with the deproteinised mRNA have detected no difference (Ernst and Arnstein, 1975; Chen et al, 1976) whereas assay of "free" cytoplasmic globin mRNPs has shown an inhibitory activity associated with the particles although the constituent mRNA has message activity (Civelli et al, 1976). The implication of these results is that the proteins associated with released RNP particles also inhibit translation, whereas the RNA contained in the particles does have messenger activity.

The same experiment was performed on different size classes of retained nuclear hnRNP. The native hnRNP did not have any activity, but some activity was detected in the deproteinised RNA. A number of experiments have attempted to show message activity in

Table 4 Messenger activity of RNP particles released from isolated nuclei

	0.5 µg RNA		0.1 µg RNA	
	^{3}H cpm	stimu-lation	^{3}H cpm	stimu-lation
no RNA	852		900	
30' inc supt RNP RNA	4340	5.1	1900	2.1
> 80S hnRNP RNA	2700	3.3	2017	2.2
20-80S hnRNP RNA	1785	2.1	-	-
30' inc supt RNP	630	-	796	-
> 80S hnRNP	452	-	663	-
20-80S hnRNP	340	-	-	-

Purified nuclei were prepared and incubated as previously described, and RNP particles from the released supernatant fraction, and extracted by sonication, were sedimented through 15-30% (w/v) sucrose gradients as described in Figure 28. Fractions corresponding to sedimentation coefficients of 20-80S and 80-120S were pooled, diluted with an equal volume of ISB, and sedimented at 254,300 g(av) (Rav 6.3 cm) for 5 hours at 4°C in the Beckman 60 Ti rotor. Pools were divided equally between two centrifuge tubes. 1 pellet was resuspended in distilled water, while the corresponding pellet was extracted with phenol/chloroform. The wheat germ assay was performed as in the previous Table.

hnRNA, although it is extremely difficult to rule out mRNA contamination in this type of experiment (Ruiz-Carillo et al, 1973; Williamson et al, 1973). The finding of message activity in the nuclear restricted hnRNP particle as well as in the released RNA could indicate that the released RNA is not necessarily a specific class of messenger-like RNA, although the results suggest that the released fraction is enriched in messenger-like RNA compared to the nuclear RNA from an equivalent number of nuclei.

DISCUSSION

1. Isolated nuclei as a tool for the study of eukaryote transcription

In the Introduction (section 2.2), a brief outline of a number of techniques which have been used to study transcription *in vitro* was given. One of these, the technique of incubating isolated nuclei such that chromatin is transcribed by endogenous RNA polymerases, has been used in the present study. A number of experiments were therefore performed in order to characterise the ability of isolated HeLa cell nuclei to synthesise RNA *in vitro* and also to process and transport this RNA.

1.1. Transcription of genes for small RNAs by RNA polymerase III

The most successful investigations of specific gene transcription in isolated nuclei have been the study of small RNA transcription (i.e. 5SrRNA and 4-5S precursor tRNA) by RNA polymerase III. These investigations have been helped by the small size of transcription unit, and the homogeneity of product. In particular, it has been relatively simple to determine the fidelity of 5S RNA synthesis in isolated nuclei to the extent that it is known that the genes are copied and terminated correctly in isolated HeLa cell nuclei (Yamamoto and Siefart, 1977a; Hamada *et al*, 1979) and that reinitiation of transcription occurs with fidelity *in vitro* (Smith *et al*, 1978). From these studies it has also been possible to isolate a putative primary transcription product which is longer than the mature 5S RNA, but has not previously been detected in mammalian cells *in vivo* due to its rapid processing (Hamada *et al*, 1979).

The transcription of tRNA genes also appears to be faithful

in isolated HeLa cell nuclei by the criteria of the size of transcripts ($\sim$ 4.5S) which can be converted to mature 4S tRNA size by cytoplasmic extracts (McReynolds and Penman, 1974; Weil et al, 1976). In the present studies, RNA sedimenting between 4-5S was synthesised in vitro by a polymerase activity in the nucleoplasm insensitive both to low levels of α-amanitin, and pretreatment of cells with low levels of actinomycin D (Figure 5b). Most of this material was recovered in the post-nuclear supernatant fraction of the incubation medium. This rapid release of small RNA from isolated nuclei has been reported elsewhere (McReynolds and Penman, 1974; Marzluff et al, 1974). By the criteria of size alone, it would appear that the products of RNA polymerase III transcription are normal in the isolated HeLa nuclei used in the present study. Indeed, the reported fidelity of transcription of these genes in vitro coupled with a reduction in the rate of processing and the rapid release of these transcription products into the incubation medium, makes this system a potentially valuable method for isolating highly labelled precursor molecules in high concentrations. This technique could be further extended to isolate reinitiated molecules by incorporating [γ-S]-NTP into the initiated 5' terminal of these molecules (Smith et al, 1978).

1.2. The transcription of ribosomal RNA by RNA polymerase I in vitro

Analysis of ribosomal RNA transcription in vitro has also benefited from the homogeneity of transcription product, and the possibility of inhibiting RNA polymerase II and III transcription with high levels of α-amanitin (Weinmann and Roeder, 1974). The use of physically isolated nucleoli has been another way of analysing

transcription by RNA polymerase I alone (e.g. Saiga and Higashinakawa, 1979). In terms of the size and processing of transcription products (Buisello and Girolamo, 1975; Udvardy and Siefert, 1976) and the complementarity of labelled sequences (Reeder and Roeder, 1972), ribosomal RNA appears to be elongated (and terminated) with fidelity *in vitro*. However, the level of reinitiation *in vitro* is low or undetectable (Udvardy and Siefert, 1976; Gilboa and Aviv, 1977; Smith *et al*, 1978). Ribosomal RNA synthesis has been demonstrated in isolated HeLa nuclei (Busiello and Girolamo, 1975; Udvardy and Siefert, 1976), and the results obtained in the present case are in accord with this. In particular, a large RNA product of similar size distribution to these previous studies was labelled in the nucleolar fraction of the isolated nuclei by an RNA polymerase activity insensitive to low levels of α-amanitin, but inhibited by pretreatment of cells with low levels of actinomycin D (Figure 5a). On this basis, it is possible that ribosomal RNA transcription is occurring normally *in vitro*, and the level of initiation may simply be undetectable due to the large size of transcription unit. Again, if this is the case, the possibility exists of using this technique further to gain insight into some aspects of ribosomal RNA transcription and processing that are not readily amenable to study *in vivo*. For example, the role of ribosomal RNA methylation in processing might be determined in a system in which non-methylated primary transcripts were produced. Another aspect of rRNA metabolism about which little is yet known is the formation of pre-ribosomal ribonucleoprotein particles, and the finding that ribosomal proteins are selectively taken up by isolated nuclei synthesising ribosomal RNA (Bolla *et al*, 1977) might be a useful starting place for such a study.

Another aspect of ribosomal RNA synthesis confirmed in this study is the lack of inhibition of synthesis *in vitro* by low concentrations of actinomycin D (Figure 4). It has been suggested that the effect of actinomycin D on ribosomal RNA synthesis *in vivo* is by inhibition of synthesis of an mRNA coding for a protein involved in ribosomal RNA synthesis (Lindell, 1976; Lindell *et al*, 1978). It would be useful to examine this further by looking at rRNA synthesis in heterologous incubations of nuclei and cytosol from normal and actinomycin D treated cells. This type of assay might then be useful in order to identify the proteins necessary for rRNA synthesis.

1.3. Transcription of hnRNA in isolated nuclei by RNA polymerase II

Both the size and sequence complexity of RNA polymerase II products has prevented the study of transcriptional fidelity *in vitro* by this enzyme. Although molecular hybridisation techniques are available for determining the sequence specificity of transcription of total or particular genes, most of these require separation of RNA synthesised *in vitro* from pre-existing RNA. This is possible using mercurated nucleotides, which, when incorporated into RNA *in vitro* enable this newly synthesised RNA to be separated from bulk nuclear RNA on sulphydryl columns (Dale and Ward, 1975). Using this technique, it has been reported that the transcription of particular mRNA sequences in isolated nuclei occurs at a much higher level than expected for random transcription of the genome. For example, the specific transcription of well characterised genes (see Introduction), such as globin (Fodor and Daly, 1977; Orkin and Swerdlow, 1977, 1978), ovalbumin (Roop *et al*, 1978; Nguyen-Huu *et al*, 1978) and immunoglobulin

K light chain (Smith et al, 1976) has been demonstrated using this technique. In the case of ovalbumin, it was demonstrated that intervening sequences in the ovalbumin gene are also specifically transcribed. Other less well characterised gene products also appear to be specifically transcribed using this technique (e.g. vitellogenin - Panyim et al, 1978; α-2-globulin - Chan et al, 1978). In other cases of sequences produced in very high concentration, specialised techniques have been used to demonstrate specific transcription without the use of mercurated nucleotides (e.g. silk fibroin genes - Suzuki and Giza, 1976; histone genes in sea urchin embryos - Shutt and Kedes, 1974; Levy et al, 1978).

In one case, the transcription of histone genes by RNA polymerase II in isolated HeLa cell nuclei incubated under the conditions used in the present study was shown to be at a much higher level than predicted for random transcription of the genome (Detke et al, 1978). This would tend to indicate that the system used in the present study may retain the potential for faithful transcription of genes by RNA polymerase II.

However, there is still some doubt as to whether the transcription of mRNA sequences by RNA polymerase II in isolated nuclei is simply the elongation of existing RNA chains, or whether correct initiation of these transcription units also takes place in vitro. There have been a number of reports indicating that re-initiation of RNA transcription in vitro is mainly or totally by RNA polymerase III (Gilboa et al, 1977; Udvardy and Seifert, 1976).

On the other hand, using a novel technique of γ-S purine triphosphate incorporation, followed by isolation of chains initiated

in vitro on mercury columns, it was found that half the incorporation into chains initiated *in vitro* was by RNA polymerase II but that only 5-10% of incorporation by RNA polymerase II was into chains initiated *in vitro* (Smith *et al*, 1978).

Other recent techniques have also been used to demonstrate initiation by RNA polymerase II *in vitro*. Thus, the density of some RNA molecules synthesised in vitro with mercurated nucleotides was consistent with incorporation of the mercurated nucleotides into the full length of the molecule (Mory and Gefter, 1978). The specific initiation of the adenovirus major late transcription unit has also been detected in nuclei from adenovirus infected cells (Manley *et al*, 1979). Therefore, it is possible that initiation by RNA polymerase II also occurs in the present system, although this was not tested.

However, one thing which is apparent from many reports of RNA synthesis in isolated nuclei is that the products of transcription by RNA polymerase II are smaller than expected from the average transcription unit size determined by other methods (see Introduction) (Ernest *et al*, 1976; Schafer, 1977; Land and Schafer, 1977; Smith *et al*, 1978; Ganguly, 1978). This small size of RNA polymerase II product labelled *in vitro* makes it uncertain as to whether termination by this polymerase class occurs correctly *in vitro* or whether some defect in the transcriptional apparatus prevents full length transcription to the termination signal at the end of the transcription unit.

Whilst the problem of initiation by RNA polymerase II has been examined by a number of groups, the termination reaction has not really been considered. Indeed, it has been implied by many groups that the synthesis of mRNA-sized molecules by RNA polymerase II is a satisfactory length of transcription product. In very few cases has

the RNA labelled by RNA polymerase II *in vitro* been shown to have a size comparable to hnRNA labelled *in vivo* (Mory and Gefter, 1977). This problem of the small size of RNA polymerase II transcripts obtained in the present study and the common finding by other workers of the same phenomenon, might indicate a fundamental problem preventing full length transcription by RNA polymerase II *in vitro*.

2. The problem of short RNA polymerase II transcripts

2.1. Prevention of full length transcription at the level of chromatin

A number of experiments performed in this study suggest that the size of RNA synthesised *in vitro* by RNA polymerase II is related to the conformation of the chromatin template. In particular, increasing the ionic strength (Figure 10) and presumptive modification of histones (Figures 11b and 12) altered the size of RNA labelled *in vitro*. It is therefore necessary to determine whether the effects of high ionic strength, acetyl CoA, and Ado-Met on RNA synthesis are consistent with their known effects on chromatin structure or whether other effects of these compounds could be responsible for the change in RNA size.

2.1.1. Is the effect of salt concentration on transcription related to its effects on chromatin structure?

These studies have shown that the substitution of 90 mM $(NH_4)_2SO_4$ for 75 mM KCl in the incubation medium resulted in an increase in the size of RNA labelled *in vitro* by RNA polymerase II (Figure 10). There are a number of possible reasons for this increase in size. It is known that purified RNA polymerase II from

a number of different species is stimulated by high ionic strength when assayed on a naked DNA template (Roeder, 1976; Beebee, 1977). The concentration of $(NH_4)_2SO_4$ used is also sufficient to remove a number of loosely bound non-histone proteins from isolated chromatin (Spelsberg and Hnilica, 1971), and histone H_1 in some tissues (Long et al, 1979; Hnilica, 1975). Thus, the ionic strength could affect transcription by altering the chromatin conformation either by the removal of inhibitory non-histone proteins, or possible decondensation by removal of histone H_1, given the possible role of histone H_1 in chromatin condensation (see Introduction, section 1.1.4).

It has been reported that Xenopus erythrocyte nuclei swell on transfer from 100 to 200 mM ionic strength and become much less refractile, reflecting some decondensation of chromatin. It was found that RNA synthesis in these isolated Xenopus erythrocyte nuclei was activated under these conditions (Hilder and MacLean, 1974). The swelling of nuclei and concomitant decondensation of chromatin have been observed in many cases of nuclear reactivation (e.g. Gurdon, 1974; Harris, 1974) and swelling appears to be a prerequisite for activation of DNA synthesis (de Roeper et al, 1977). However, the nuclear swelling in these latter cases is apparently caused by lowering the cytoplasmic cation concentration (Barry and Merriam, 1972) which also decondenses chromatin (Leake et al, 1972) by dissociating cations from the chromatin (Jacobs et al, 1976). However, the swelling induced by low ionic strength in isolated Xenopus erythrocyte nuclei did not activate RNA synthesis, suggesting that nuclear swelling and decondensation of chromatin alone are not sufficient to increase RNA synthesis in isolated nuclei (Hilder and MacLean, 1974).

The effect of ionic strength on transcription may therefore not be connected with gross decondensation, but may also affect the conformation of chromatin at a more subtle level. Evidence for this has come from the study of transcription of SV40 "minichromosomes" *in vitro*. It has been observed that salt concentrations from 100-600 mM NaCl increasingly stimulate the elongation of transcription by *E. coli* RNA polymerase of reconstituted SV40 "minichromosomes" (Wasylyk *et al*, 1978). The same effect is also observed for added eukaryotic RNA polymerases I and II (Wasylyk *et al*, 1979) and for isolated native SV40 transcriptional complexes (Gariglio *et al*, 1979). Since it was shown that the nucleosomes did not dissociate or "slide" along the DNA at the salt concentration used, and that the polymerases elongated RNA chains across regions of DNA occupied by nucleosomes, a local loosening of histone-DNA interactions was postulated to explain this result, as opposed to a mechanism of nucleosome unfolding (which would be favoured at low salt) nucleosome dissociation, or nucleosome sliding (Wasylyk *et al*, 1978).

It is not clear whether the high ionic strength used here is within the range of physiological ionic strength likely to exist within the nucleus *in vivo*. The nucleus appears to be freely permeable to ions (Siebert, 1972), yet there is a steep sodium ion concentration gradient across the nuclear membrane, such that Na^+ ions are 11 times more concentrated in the nucleus than in the cytoplasm, and K^+ ions are also slightly more concentrated in the nucleus. It is probable that many of these cations are bound to protein and DNA in the nucleus and do not contribute to the ionic strength of the nucleus, which is probably close to that of the whole cell (Siebert, 1978). However, changes in the cell ionic strength,

or the concentration of particular ions, will not only affect the ionic activity in the nucleus, but also the binding of cations to the chromatin. The finding that small changes in divalent cation concentration can dramatically change the binding of divalent cations to chromatin, and alter the condensation of the chromatin makes it possible that the ionic environment of the nucleus may control certain functions, and that the ionic composition of the incubation medium used for isolated nuclei might be critical for correct transcription.

Most studies of RNA polymerase II transcription using isolated nuclei have either used a "physiological" buffer containing between 75-150 mM KCl, Mg^{2+} ions, glycerol, thiol reagents, etc (based on Marzluff et al, 1973), or have based the ionic composition on the ionic requirements of purified RNA polymerase II (0.1M $(NH_4)_2SO_4$, Mn^{2+} ions). The low ionic strength buffer usually allows prolonged synthesis for a number of hours, although often only at temperatures below 30°C (e.g. Marzluff et al, 1973; Sarma et al, 1976). In the high ionic strength buffer, initiation is inhibited (Cox, 1976; Coupar and Chesterton, 1977) and prolonged synthesis is often not possible (Zylber and Penman, 1971), yet, as found in this case, larger transcripts than in the "physiological" buffer are generated (e.g. Zylber and Penman, 1971). Only in the case of adenovirus infected cell nuclei (Weber et al, 1977; Evans et al, 1978), and isolated adenovirus (Wilhelm et al, 1976) transcription complexes has the specific transcription of expressed sequences been demonstrated in an "$(NH_4)_2SO_4/Mn^{2+}$" system. However, specific initiation of adenovirus transcription has only been demonstrated in a "K^+/Mg^{2+}"

type system in isolated nuclei (Manley et al, 1979), and in a reconstituted system (Weil et al, 1979b), and is unlikely to be possible in high concentrations of $(NH_4)_2SO_4$. In the latter study, only short transcripts were generated. It may therefore be that the optimal salt conditions for correct initiation, full length elongation, and termination will be a compromise between the two types of system described, and require careful optimisation of all the constituent ions.

2.1.2. The role of histone acetylation in chromatin transcription

The acetylation of histones in the nucleus has been correlated with transcriptional activity (Allfrey et al, 1977). Acetylated histones are found preferentially in transcriptionally active chromatin fractions (Levy-Wilson et al, 1977; Davie and Candido, 1978), and hyperacetylated chromatin has a number of the characteristics of transcriptionally active chromatin (Vidali et al, 1978; Simpson, 1978).

In the present study, acetyl groups were rapidly incorporated into histones in isolated nuclei (Figure 11c). Acetyl CoA was also found to stimulate the incorporation of triphosphates into RNA in isolated nuclei, resulting in a larger RNA product (Figures 11a and 11b). These experiments would suggest that the acetylation of histones is responsible for this enhanced transcriptional activity.

The role of acetylation in transcriptionally active chromatin is not clear. Histones H_3 and H_4 are acetylated on specific lysine residues in the NH_2-terminal region of the molecules (Isenberg, 1979) which are probably bound to the DNA chain. The effect of reducing the positive charge of this region will presumably

loosen the binding of histone to DNA, facilitating transcription. Thus, acetylation may be required to maintain regions of chromatin in an "active" conformation. The simplest explanation for the results obtained here is that deacetylation of histones, which is known to occur during extraction and incubation of nuclei (Perry, 1979) partially "deactivates" the transcribed regions of chromatin resulting in the small size of RNA product observed. Acetylation in vitro partially alleviates this block, resulting in an increased size of RNA product. Further evidence in favour of this idea is the fact that incubation of nuclei with acetyl CoA causes a reduction in the size of the nuclear DNA, suggesting a transition of some chromatin to an "active" conformation (Figure 19b) more susceptible to endogenous nucleases.

However, other results obtained in this study are not consistent with this hypothesis. In particular, pretreatment of cells with sodium butyrate at concentrations known to cause massive hyperacetylation of histones (Riggs et al, 1977) caused only a small increase in the size of RNA labelled in nuclei isolated from these cells (Figure 11a). However, the effect of butyrate on transcription in vivo is also small compared to its effect on acetylation. Thus it has been reported recently that butyrate induces new gene expression in Friend cells (Reeves et al, 1979a). However, only 38% more new RNA sequences appear to be transcribed, and the effect of butyrate may be related to its ability to induce erythroid differentiation in these cells (Leder and Leder, 1975), rather than hyperacetylation of histones, since DMSO, which also induces differentiation, has no effect on histone acetylation (Riggs et al, 1977). It has also been found that in HTC cells no new gene expression is

observed after butyrate treatment (Cousens et al, 1979). In the latter paper it was observed that different regions in chromatin differed in their accessibility to histone acetylase, such that normally acetylated histones were preferentially multiacetylated. Therefore, the small effect of butyrate on RNA synthesis in vitro might be due to the fact that only chromatin already in an active conformation is further acetylated when butyrate inhibits the deacetylase. Alternatively, it is probable that butyrate has a number of other effects which might indirectly affect transcription in an antagonistic fashion (Prasad and Sinha, 1976).

An alternative explanation might be that transcription activity is related to the rapid turnover of acetyl groups observed in vivo (Jackson et al, 1975) rather than the presence of acetyl groups per se. Thus, it is found that there is an increased turnover of acetyl groups on SV40 minichromosomes in infected cells compared to bulk cellular chromatin (Chestier and Yaniv, 1979). In the present work, there was a slight increase in acetyl group turnover in nuclei synthesising RNA compared to inactive nuclei (Figure 11c).

Further work in this system is required to determine whether histones are acetylated randomly in vitro, or whether active regions are preferentially acetylated. The work on DNA size suggests that a number of nuclease resistant sites become susceptible to degradation following acetylation (Figure 19b) suggesting the former possibility. Thus, it would be of interest to determine whether new sequences are transcribed in vitro following acetylation, and also to use this system to study the effect of acetylation on the trans-

cription of specific gene sequences. It is also possible that the full effect of acetylation *in vitro* is masked by the concomitant enhanced degradation of DNA by endogenous nucleases. This endogenous nuclease does not appear to be DNase II since it is not inhibited by sulphate which is known to inhibit DNase II (Oshima and Price, 1974) nor does it closely resemble the Ca^{2+}/Mg^{2+} dependent nuclease found in rat liver, since it is not appreciably inhibited by polyamines (Keichline *et al*, 1976). In principle however, an ability to inhibit this enzyme might enable a full length product to be synthesised in the presence of acetyl CoA, or alternatively, might show whether nicking of DNA is actually an integral part of the transcription process, and directly related to the ability of acetyl CoA to enhance transcription.

2.2. The DNA template in isolated nuclei

2.2.1. Is nicking of DNA in isolated nuclei responsible for the small size of RNA transcripts?

As suggested in the last section, the state of the DNA template in isolated nuclei may be critical for the success of RNA synthesis experiments. Extensive degradation of the template will lead to a decrease in the size of RNA likely to be transcribed, and in some tissue nuclei this has been found to be a problem. For instance, in rat liver nuclei, nicking of the DNA by the Ca^{2+}/Mg^{2+} degraded endogenous nuclease resulted in a decrease in RNA size (which could be prevented by inhibition of the enzyme with NAD^{+}) (Lindell, 1977). In HeLa cell nuclei there is very little degradation of the DNA template such that single stranded fragments many times larger than the larger HeLa cell transcription units are still

present after incubation (Figure 18). However, it is possible that since transcriptionally active regions of chromatin are preferentially sensitive to various nucleases (Weintraub and Groudine, 1976; Gottesfeld et al, 1974; Bloom and Anderson, 1978), nicking occurs selectively in the active regions of the genome. That this is the case is supported by the slight increase of DNA nicking in the presence of NTPs, i.e. RNA synthesis (Figure 19b).

It has been proposed that the different conformation of transcriptionally active chromatin detected by preferential nuclease sensitivity is not a result of the presence of transcribing RNA polymerases or nascent RNA. Thus, genes with a history of, or a potential for transcriptional activity, appear to be maintained in this "active" conformation (see Introduction). The small increase in DNA digestion during RNA synthesis in isolated nuclei would appear to be contrary to this. However, it has been suggested that the chromatin in isolated nuclei may be "deactivated", possibly due to deacetylation of histones (section 2.1.2) as an explanation for the small size of RNA transcription in vitro. It is possible that transcription of this partially "deactivated" chromatin causes a localised change in conformation to the "active" state selectively digested by nucleases. The implication of this would be that any elongating polymerase would be selectively terminated by nuclease activity!

The contradictory effects of acetyl CoA on RNA synthesis and DNA degradation are difficult to explain. It has already been suggested that acetylation of chromatin might result in its transition from an inactive to a transcribable state (section 2.1.2).

This would increase the length of RNA transcribed but might also increase the degradation of the DNA in the acetylated chromatin. However, it might then be thought that the increased nicking of DNA would decrease the size of RNA transcripts obtained. One explanation for this would be that acetyl CoA acetylates chromatin not actually being transcribed *in vitro*, since addition of acetyl CoA to incubated nuclei results in more DNA nicking than addition of NTPs (Figure 19b). Thus, the actual nicking of transcribed DNA after acetylation might be less than in control nuclei. Alternatively, the positive effects of acetylation on transcription may simply be greater than the negative effects on DNA degradation.

The effects of acetyl CoA and $(NH_4)_2SO_4$ on DNA degradation were quite different, since DNA degradation in nuclei incubated in 90 mM $(NH_4)_2SO_4$ was not increased (results not shown), despite the similar effects of acetyl CoA and $(NH_4)_2SO_4$ on the size of RNA transcribed *in vitro*. In a previous section, the possible effect of increasing the ionic strength on chromatin conformation was discussed (section 2.1.1.). It was proposed that a local loosening of histone-DNA interaction by increased ionic strength would allow the passage of RNA polymerase across regions of DNA occupied by nucleosomes. Acetylation of histones will also cause a loosening of histone-DNA interactions, and this has been proposed as a mechanism for maintaining the conformation of transcriptionally active chromatin (section 2.1.2.). It is therefore possible that the "open" chromatin conformations induced by high ionic strength and acetylation are different with respect to their sensitivity to endogenous nucleases, or that the effect of $(NH_4)_2SO_4$ on transcription *in vitro* does not involve chromatin structure.

Other possible effects of $(NH_4)_2SO_4$ on transcription cannot include inhibition of DNA degradation, which might be expected if the degrading nuclease were DNase II (Oshima and Price, 1974). Thus, addition of acetyl CoA to nuclei incubated in 90 mM $(NH_4)_2SO_4$ resulted in degradation of the nuclear DNA (results not shown).

2.2.2. Is nicking of DNA an integral part of the transcription process?

An alternative explanation for the effect of acetyl CoA on RNA synthesis and DNA degradation is that nicking is an integral part of the transcription process. It is known that RNA polymerase II initiates at nicks and single stranded regions on a naked DNA template *in vitro* (Beebee, 1977). Regions of single stranded DNA have been found in the genome and appear to be enriched in transcribed sequences (Hannania *et al*, 1977; Leibovitch *et al*, 1979). It has been suggested that these regions arise by selective nicks produced by a single strand specific nuclease or DNA unwinding enzyme. It has also been reported that nucleosomes can form on single stranded DNA, and proposed that during transcription the nucleosomes may segregate onto one strand (Palter *et al*, 1979). Moreover, it is possible that this segregation would be more favourable at high salt concentration (Wasylyk *et al*, 1979; Gariglio *et al*, 1979), and in acetylated chromatin (Yamamoto and Alberts, 1976). Therefore, nicking might be involved in the generation of single stranded regions of DNA with the potential for transcriptional activity. Alternatively it is possible that nicking by an unwindase or gyrase enzyme is part of a process involved in the supercoiling of DNA. It is known

that RNA polymerase II transcribes naked supercoiled SV40 DNA more efficiently than linear DNA (Mandel and Chambon, 1974a). Eukaryotic DNA is found to be organised in supercoiled loops following removal of histones (Cook and Brazell, 1975; Benyajati and Worcel, 1976). It has been proposed that further generation of negative supercoils in a supercoiled "domain" in the eukaryotic nucleus would result in a more "open" conformation, effectively activating this region of chromatin for transcription (Colman and Cook, 1977).

Thus, it might be that the increased DNA nicking observed during RNA synthesis and acetylation reflects an increase in supercoiling activity. It might be argued conversely that in this model, nicking would tend to unwind supercoils, deactivating regions of chromatin, and could therefore explain the small size of RNA synthesised *in vitro*, generated by a small number of nicks.

2.3. Premature termination sites in eukaryotic transcription units

There is evidence in prokaryotes that the expression of some operons may be controlled by premature termination of transcription (attenuation) (Adhya and Gottesman, 1979) (e.g. the trp operon (Bertrand *et al*, 1975)). There is now evidence for premature termination of transcription of the major late adenovirus transcription unit (Evans *et al*, 1979). As many as70-80% of initiated chains fail to continuepast 2,000 nucleotides, such that a peak of 18S material complementary to the 5' terminal sequence is found in very briefly labelled cells. The same phenomenon occurs *in vitro* in isolated adenovirus infected HeLa cell nuclei (Weber *et al*, 1977). The drug DRB (5,6 dichloro-1-β-D ribofuranosyl-benzimidazole) appears to

enhance this premature termination (Fraser et al, 1978). It has been possible to define multiple discrete sites of termination in the adenovirus major late transcription unit which are enhanced by DRB to the extent that no full length transcription occurs (Fraser et al, 1979).

The effect of DRB on HeLa cellular RNA synthesis is to inhibit the synthesis of 70% of hnRNA synthesis, but 100% of mRNA synthesis (Sehgal et al, 1976). The pulse labelled DRB-resistant RNA in HeLa cells has a prominent peak at ~ 18S (Sehgal et al, 1976). The DRB-resistant RNA synthesised by RNA polymerase II in isolated HeLa cell nuclei is mainly 140-740 nucleotides long (Tamm, 1977). Therefore, it is possible that cellular transcription units also contain specific premature termination signals which can be enhanced by DRB. A more recent paper has shown that these short chains are also made in control isolated HeLa cell nuclei, in apparent molar excess but that their synthesis is increased after DRB treatment (Tamm and Kikuchi, 1979). In these studies, nuclei were incubated in 100 mM $(NH_4)_2SO_4$ (Tamm, 1977).

It is possible that premature termination is even more predominant under the relatively low salt conditions of incubation of nuclei used in the present study. For example, premature termination of RNA polymerase II transcription on naked supercoiled SV40 DNA was observed at low salt concentrations, but did not occur at higher salt concentrations (Mandel and Chambon, 1974b). In general, RNA synthesised under the relatively low salt conditions of Marzluff et al (1973) tends to be shorter than that in higher salt conditions (e.g. Zylber and Penman). In particular, premature termination of

adenovirus transcription by RNA polymerase II in low salt conditions was observed in a reconstituted system (Weil et al, 1979b), whereas long transcripts are generally obtained at higher salt conditions in isolated nuclei (e.g. Weber et al, 1977; Yang and Flint, 1979). It has been concluded that the increase in RNA polymerase II transcripts in 90 mM $(NH_4)_2SO_4$ in the present study may not involve the same mechanism as acetylation, i.e. by altering the conformation of chromatin (section 2.1.2). It is therefore possible that the effect of $(NH_4)_2SO_4$ is to decrease the amount of premature termination at particular sites in the DNA, rather than an effect on chromatin structure.

A recent paper, in agreement with this, demonstrates that $(NH_4)_2SO_4$ in concentrations between 80-160 mM suppresses the correct termination of ribosomal RNA transcription in isolated Tetrahymena nucleoli (Leer et al, 1979). It is suggested that high salt removes a termination factor which is required for correct termination in vitro in low salt, but that the RNA polymerase I reads through the termination signal when the factor is dissociated in high salt. It would appear that the salt concentration of the incubation medium of isolated nuclei may require careful optimisation to suppress premature termination, but allow correct termination.

It is not clear whether premature termination also involves such dissociable factors, or whether the effect of salt is on the 2^o structure of RNA, which appears to be involved in the mechanism of premature termination in prokaryotes (Oxender et al, 1979).

2.4. Preferential transcription of small transcription units

2.4.1. Small hnRNA

The range of transcription unit sizes in HeLa cells is 2-20 kilobases (Derman et al, 1976; Goldberg et al, 1977b). It has been suggested that a large proportion of rapidly labelled hnRNA of all size classes are primary transcripts (Goldberg et al, 1977b; Giorno and Sauerbier, 1976). Thus, the preferential transcription of the smaller transcription units would generate RNA molecules of ~ 2 kilobases (i.e. ~ 18S). Evidence for a discrete subclass of small hnRNA has been obtained by salt fractionation of nuclei (ShnRNA) (Price et al, 1974), and has some characteristics of a separate kinetic class, having a short half life (Herman and Penman, 1977). Although originally proposed as a class of mRNA precursors (Price et al, 1974), only 40% of poly(A)-adjacent sequences in ShnRNA are mRNA sequences, compared to 65% in the bulk of hnRNA (Herman and Penman, 1976; Herman, 1979). Thus, ShnRNA differs from the small hnRNA which constitutes the bulk of steady state hnRNA (Sphor et al, 1974) which are enriched in mRNA sequences, consistent with their being products of the processing of larger mRNA precursors (Sippel et al, 1977; Hames and Perry, 1977). ShnRNA may therefore be transcribed from small transcription units, which might have other characteristics differing from the bulk of hnRNA transcription units which would lead to their preferential transcription.

It has been reported that not all the RNA polymerase molecules active *in vivo* are active *in vitro*. Only about a hundred of the few thousand transcription units transcribed by RNA polymerase III *in vivo* appear to be active *in vitro* (Gilboa and Aviv, 1977). Also,

the predominant labelled RNA products in the first minutes of myeloma nuclei incubation are small and very large molecules which appear to be completed transcription products (Marzluff et al, 1978b). In contrast, the material labelled between 10 and 11 minutes of incubation is predominantly 18S, with neither small nor large molecules apparent. Thus, it appears that the early transcription is due to rapid preferential completion of RNA chains near the 3' terminal of the transcription unit, leading to an over-representation of small RNAs and 45S RNA. Later in the reaction, the labelled product is RNA in the process of elongation. This might explain the typical biphasic response observed in a number of systems, including myeloma cell nuclei (Marzluff et al, 1973) and HeLa cell nuclei (Figure 3). The only indirect evidence against this proposal is the finding that the RNA labelled in vitro does not appear to be processed and is not released from the nuclei, as might be expected if complete transcripts of small hnRNA were preferentially synthesised in vitro. It will be argued in section 4.4 that this lack of processing and transport is due to the lack of full length transcription by RNA polymerase II.

3. Processing of RNA in isolated nuclei

3.1. Turnover of prelabelled hnRNA

The RNA labelled in vitro by RNA polymerase II was found to be stable when incubated further in the absence of nucleoside triphosphates (Figure 6). In general, RNA labelled in vitro in isolated HeLa cell nuclei is stable, in that no degradation to acid soluble material is observed (Sarma et al, 1976; Busiello and Girolamo, 1975; Udvardy and Siefart, 1976). It therefore seems unlikely that

degradation by contaminating nucleases causes the small size of RNA transcribed *in vitro*, by RNA polymerase II, particularly in view of the stability at 37°C (Figure 8), and the large size of RNA polymerase I transcripts (Figure 5a).

This stability contrasts with the decrease in size and amount of prelabelled hnRNA in incubated nuclei (Figure 26). In view of the absence of contaminating nucleases proposed above, it is possible that this degradation represents normal processing events which occur *in vivo*. Thus, it is known that the bulk of hnRNA is not transported to the cytoplasm, but turns over in the nucleus. The most recent attempts to measure the kinetics of this turnover in HeLa cells have shown that the decay of total hnRNA follows first order kinetics for at least 3 hours, with a half-life of 70 minutes (Herman and Penman, 1977). Multiple decay rates were estimated for subfractions of the hnRNA. This rate of decay is quite consistent with the extent of hnRNA degradation observed in isolated nuclei incubated at 25°C for 30 minutes (Figure 26). Furthermore, the extent of decrease in size of prelabelled hnRNA in these isolated nuclei (Figure 26) is consistent with observations *in vivo*. For example, rapidly labelled hnRNA in HeLa cells is 5-30 kilobases in size (Spohr *et al*, 1976), and may consist mainly of primary transcripts (Goldberg *et al*, 1977b). This large hnRNA turns over with a half-life of 15-20 minutes, whilst smaller hnRNA of 2-6 kilobases is considerably more stable, with a half life of 3-5 hours (Spohr *et al*, 1974; Scherrer *et al*, 1979). Small hnRNA is enriched in mRNA sequences (Hames and Perry, 1977; Sippel *et al*, 1977). It is therefore possible that these small stable hnRNA molecules are the products of large precursor mRNA molecules. The stages of processing of

particular mRNA precursors are in accord with this general scheme, in that the primary transcripts of mRNA genes appear to be rapidly processed in a stepwise manner down to a nuclear RNA the size of the mature mRNA (e.g. adenovirus late mRNAs (Nevins, 1979), globin mRNA (Kinniburgh and Ross, 1978)). These processing steps involve both endonucleolytic cleavage (e.g. adenovirus late mRNA (Nevins and Darnell, 1978a) and splicing out of intervening sequences in an apparently ordered manner (e.g. Nevins, 1979; Kinniburgh and Ross, 1979).

Since these steps occur rapidly in vivo, it would be useful to study them in vitro, in which they might be slower, or possibly controllable. For instance, the recent discovery in isolated HeLa nuclei of a precursor of 5S rRNA which is rapidly processed in vivo makes it possible to define the 5S rRNA transcription unit and study the processing of this precursor (Hamada et al, 1979). In a sense, this type of system is analogous to the use of mutants defective in tRNA processing in yeast to isolate precursor tRNAs and study their processing in vitro (e.g. Knapp et al, 1978; Peebles et al, 1979; Knapp et al, 1979).

The processing events involved in mRNA production have not yet been well characterised in isolated nuclei. Only in the case of adenovirus mRNAs has the availability of specific probes for these processing events been utilised. Thus, an early report showed that adenovirus "late" RNA labelled in infected cells was cleaved during incubation of isolated nuclei (Brunner and Raskas, 1972).

More recently these experiments were extended by probing with restriction fragments of the adenovirus genome from the region

coding for 22S fibre mRNA (Zimmer et al, 1978). Non-incubated nuclei contained specific RNA species of 35S, 28S and 22S. Following incubation of isolated nuclei, the 35S RNA disappeared, and a new 25S RNA appeared.

Splicing of a prelabelled adenovirus "early" RNA in isolated nuclei has been reported (Blanchard et al, 1978). A 5 kilobase nuclear poly(A) RNA containing intervening sequences was processed to give a 2 kilobase product of identical size to the mature mRNA, from which the intervening sequences had been deleted. Although this splicing only occurred in the presence of a cytoplasmic extract, it is probable that the enzymes responsible leaked out of the nuclei under the hypotonic isolation conditions used in this experiment. Recent results suggest that "late" adenovirus RNA labelled in isolated nuclei in vitro can be spliced correctly in the absence of any additional factors (Yang and Flint, 1979). Apart from adenovirus infected cells, the processing of RNA in isolated nuclei has not been studied at the level of specific mRNA precursors.

It is possible that cleavage and splicing reactions require that the RNA is held in a specific conformation by RNA-RNA or RNA-protein interactions. The sequences at the junctions between coding and non-coding regions of the genome, although conserved (Catterall et al, 1978; Dawid and Wahli, 1979) are not consistent with a model in which double stranded regions could form between the ends of an intervening sequence. However, the regions to be spliced could be held in juxtaposition by the tertiary folding of the RNA, governed by sequences distant from the splicing points (Khoury et al, 1979). Alternatively, these regions could be recognised and positioned by

virtue of their interaction with other RNAs, (e.g. small "hnRNAs" Davidson and Britten, 1979; small nuclear RNAs, Murray, 1979; Roberts, 1980), or with proteins, in RNP particles (Calvet and Pederson, 1978). It may therefore turn out that some processing reactions are only possible when the RNA is in its "native" state, i.e. in isolated nuclei or isolated RNP particles.

Since the degradation of prelabelled RNA in the nuclei used in this system is consistent with the occurrence of normal processing events, this type of system might be useful in the study of these processing events. However, it would be more useful if it were possible to process primary transcripts synthesised in vitro. In this regard, it is possible that since processing may occur in these nuclei, that the RNA synthesised in vitro is rapidly processed during transcription, resulting in its small size in the present study. There is evidence from ... studies of transcriptional activity that the transcripts of some genes are cleaved during transcription (Laird et al, 1976; McKnight and Miller, 1976; Old et al, 1977). The E.M. evidence of McKnight and Miller (1976), on the silk fibroin gene confirms the biochemical evidence for the cleavage of fibroin mRNA transcripts during synthesis (Lizardi, 1976). Furthermore, in the adenovirus major late transcription unit, cleavage and polyadenylation at one of five sites during transcription determines the particular mRNA expressed (Nevins and Darnell, 1978a). Moreover, the discrete sizes of adenovirus RNA transcribed in vitro correspond to the sizes of adenovirus RNA prelabelled in vivo and processed in vitro (Zimmer et al, 1978).

The conjunction of this rapid processing with a lack of full

length transcription might also explain the stability of this RNA, since full length transcription of the adenovirus major late transcription unit is still required, after cleavage of the transcript, for successful processing of the cleaved transcript (Goldberg et al, 1978). However, the effects of acetyl CoA and $(NH_4)_2SO_4$ on RNA transcript size are not readily compatible with this theory since it might not be expected that acetylation and high ionic strength would directly inhibit processing.

4. Release of RNA from isolated nuclei

4.1. Is the release of RNA from isolated nuclei the specific transport of mRNA?

A number of groups have studied the release of RNA pre-labelled in vivo from isolated nuclei. In many cases this has been claimed to represent a model system for the transport of mRNA from the nucleus to the cytoplasm. However, the experiments which have been used to support these claims have mainly been tests which might distinguish hnRNA from ribosomal RNA and tRNA, but could not distinguish specific mRNA transport from leakage of hnRNA.

It was found in the present study that when cells were pre-labelled in the presence of actinomycin D, the labelled material released from isolated nuclei had a peak size of 40S (Figure 28) and a peak density of 1.43 g/cm^3 (Figure 30a). These figures are consistent with the labelled material being either HeLa cell mRNP particles (Spohr et al, 1970) or hnRNP "monomer" particles similar to those released from HeLa nuclei at 20°C in an hnRNP pH 8.0 extraction buffer (Pederson, 1974; Beyer et al, 1977). The released RNA had the same

size distribution (Figure 26) as HeLa cell mRNA (Darnell, 1976). However, this RNA could also be degraded hnRNA, since the prelabelled RNA retained in the nuclei was also reduced in size during incubation (Figure 26).

One way in which hnRNA differs from mRNA is the presence in hnRNA of unusual base sequences which appear not to be present in mRNA. For example, oligo(A) sequences of 20-30 nucleotides in length are found in HeLa cell hnRNA but have not been detected in HeLa cell cytoplasmic mRNA (Venkatesan et al, 1979). Oligo(A) containing molecules can be separated from poly(A)$^+$ molecules and (A)$^-$ molecules on the basis of affinity for poly(U) Sepharose (Molloy et al, 1974) (Figure 34). Furthermore, the same method can be used to determine the proportion of polyadenylated molecules, which also distinguishes hnRNA and mRNA populations since $\sim$ 70% of mRNA molecules in HeLa cells are polyadenylated (Milcarek et al, 1974), whilst only $\sim$ 20% of HeLa cell hnRNA contains poly(A) (Molloy et al, 1974).

It was found that a comparable proportion of both released and retained RNA was eluted from poly(U) Sepharose with low concentrations of formamide, a characteristic of oligo(A) containing molecules (Molloy et al, 1974 (Table 1b). Also, both released and retained RNA contained only a small proportion of polyadenylated molecules. The results therefore strongly indicate a release of "hnRNA-like" molecules, rather than a specific transport of "mRNA-like" RNA.

Another test which was used to distinguish hnRNA from mRNA was that of in vitro translation. This type of experiment suffers from the major problem of mRNA contamination of hnRNA. That this

can occur is demonstrated by the fact that large hnRNA could be translated into globin sized proteins (e.g. Ruiz-Carrillo et al, 1973), despite the presence of inserts in globin precursor mRNA (Tilghman et al, 1978b; Kinniburgh et al, 1978).

In the present case, it was observed that material in the post-nuclear supernatant fraction from non-incubated nuclei had significant messenger activity (Table 3), despite the fact that little labelled RNA was released from nuclei into this fraction (Figure 35). The RNA released during incubation of nuclei significantly increased the message activity of the supernatant fraction. In order to correlate this apparent release of message activity with the release of labelled RNA during incubation, the effects of ATP and high Mg^{2+} on the release of message activity was observed. In this way a correlation between the amount of labelled RNA released (Figure 35) and the release of messenger activity was obtained, suggesting that the amount of message activity released during incubation was related to the release of labelled RNA, which might not be expected from contamination by cytoplasmic mRNA.

However, it was also observed that the RNA retained in the nucleus had some message activity (Table 4). This could be due to mRNA contamination. Alternatively, this could be due to processed nuclear mRNA precursors, since in all the system studied so far, complete processing of mRNA precursors to native mRNA occurs in the nucleus, such that precursor molecules the size of the native mRNA can be found in the nucleus (e.g. adenovirus late mRNA precursors, Nevins, 1979; globin mRNA precursors, Ross and Kinniburgh, 1978), immunoglobin light chain mRNA precursor (Gilmore-Herbert et al, 1978a).

Although the released RNA had a higher specific activity than retained RNA, the difference is not large enough to support an argument in favour of specific transport, particularly in view of the high background activity of the released RNA of non-incubated nuclei.

Another approach used to analyse the material released from isolated nuclei was to characterise the proteins cosedimenting with the labelled RNA released during incubation. The major proteins associated with HeLa cell nuclear and cytoplasmic RNP particles are different (Kumar and Pederson, 1975; Liautard et al, 1976). In particular, the major 30-40K molecular weight proteins of hnRNP particles cannot be detected in SDS polyacrylamide gels of informosomes or polysomal mRNPs (Kumar and Pederson, 1975; Liautard et al, 1976), nor can they be detected immunologically in the cytoplasm (Lukanidin et al, 1972; Martin et al, 1979a).

The results obtained showed that the pattern of proteins released was similar to that of hnRNPs retained in nuclei and extracted by sonication, particularly with respect to the major proteins of 30-40K molecular weight (Figure 31). Moreover, the particles containing these proteins were preferentially bound to oligo(dT) cellulose (Figure 33), whilst the low molecular weight proteins also found cosedimenting with labelled RNA in the released fraction did not bind to oligo(dT). This would tend to indicate that these low molecular weight proteins are not informosomal although of comparable size (Liautard et al, 1976), but more probably ribosomal (Figure 31). Moreover, no "changeover" of these hnRNP proteins to mRNP proteins was observed when the nuclei were incubated with cytosol (Figure 33). In general, therefore, the release of

particles from isolated nuclei resembles the diffusion of hnRNP particles rather than the specific transport of mRNP particles. The absence of mRNP particles from the released fraction was also suggested by the lack of messenger activity in the released particles (Table 4). Thus, taken together, the results from the poly(U) binding of released RNA and the protein composition of released particles do not provide any evidence for specific mRNA transport, although they cannot completely rule out this possibility, whilst the results from the wheat germ cell free system protein synthesis assays are somewhat ambiguous. It must be borne in mind that these three separate experiments are examining three distinct populations of released material. The poly(U) Sepharose studies characterise only the RNA prelabelled *in vivo* whilst the protein synthesis assay detects message activity within the total RNA population. The protein characterisation studies show the major proteins which cosediment with labelled RNA, and copurify on oligo(dT) cellulose.

However, the results from poly(U) binding show that the release of labelled RNA is probably leakage, and this is in accord with the observation that within 30 minutes of incubation at 25°C, ~ 10% of the labelled RNA was released. This does not correspond to the figure of 3-6% of labelled hnRNA transported to the cytoplasm over a period of 2 hours in HeLa cells, *in vivo* (Herman and Penman, 1977). The release of messenger activity in the total released RNA population might reflect the specific transport of mRNA, but it seems likely from the results with labelled RNA that the bulk of this release is leakage of hnRNA. Since the major released proteins copurifying with labelled RNA are those found associated with

hnRNP particles retained in the nuclei, it again seems likely that the bulk of RNA release from these nuclei is a result of hnRNA leakage.

4.2. Is the release of RNA from isolated nuclei energy dependent?

It has been stressed that little evidence of specific mRNA transport from isolated nuclei has been reported. However, a number of groups have presented evidence for the energy-dependent release of RNA from isolated nuclei, which has been presumed to be specific mRNA transport. Much of this evidence has come from the effect of ATP on the release of RNA from rat liver nuclei incubated at 25-37°C. It seems likely that under these conditions much of the nuclear RNA will be degraded and released as 40S hnRNP monomer particles. Thus, in some cases, the proportion of labelled nuclear RNA released is much higher than the 3-6% expected from kinetic studies of mRNA transport *in vivo* (Herman and Penman, 1977). Furthermore, it has been shown that RNP particles bound to rat liver chromatin can be released either with ATP or the pH 8.0 extraction conditions for hnRNP particles from intact rat liver nuclei (Ishikawa *et al*, 1974), suggesting that these processes are analogous. The RNA release from isolated HeLa cell nuclei in the present study was stimulated by high levels of ATP (Figure). However, the addition of Mg^{2+} ions to compensate for the chelating ability of ATP prevented this increase. The addition of this further Mg^{2+} alone inhibited release, whilst EDTA stimulated release. Similar results were found previously with isolated HeLa cell nuclei (Chatterjee and Weissbach, 1973), and suggest that the role of ATP in stimulation is by chelating Mg^{2+} ions which inhibit RNA release. However, conflicting results obtained in other systems have led to other theories.

ATP was found to lyse myeloma cell nuclei, resulting in an increase in release of RNA (Stuart et al, 1975). No lysis was observed in the present study, and ATP stimulated release only of small hnRNP particles (Figure 35) whilst lysis might be expected to release large particles the size of those remaining in the nuclei (Figure 36).

However, other groups have not been able to demonstrate a stimulatory effect of other chelators such as EDTA or sodium pyrophosphate (Ishikawa et al, 1969; Raskas and Rho, 1973; Agutter et al, 1976). The inhibitory effect of magnesium itself has not been examined, however. The theory generated has then depended on the effects found for other nucleotides, ATP analogues, and inhibitors. In particular, in adenovirus infected KB cells (Raskas and Rho, 1973) and rat liver (Ishikawa et al, 1978) the analogues AMP-PCP and AMPCP-P were both as effective as ATP, leading to the suggestion that the specific binding of ATP to the nucleus without hydrolysis is required for transport. This theory is supported by the effects of ADP and pyrophosphate which are independently half as effective as ATP (Raskas and Rho, 1973) but together have the same effect as ATP (Ishikawa et al, 1978). Other groups have found no stimulation with ADP or ATP analogues (Agutter et al, 1976, 1978b) and used this to support a theory of energy dependent transport, whilst other groups finding half the effect of ATP with ADP or either α-β or β-γ methylene analogue have suggested that the hydrolysis of both phosphodiester bonds in ATP can be coupled to RNA transport (Clawson et al, 1979) (such that the transport is proportional to the energy charge). Clawson (1979) has also determined the activation energy of ATP dependent RNA release and suggested that this is different from that expected for passive diffusion.

However, in most of these cases, the extent of ATP stimulation of release results in a much larger proportion of labelled RNA released than expected for specific mRNA transport. Thus, whatever the effect of ATP on release, it does not appear to be a specific effect on mRNA transport. It will be of interest to reexamine the effects of ATP when a system for the specific transport of mRNA has been established.

4.3. Release of hnRNP particles from isolated nuclei

Although the released particles had a protein composition similar to that of hnRNP particles retained in the nucleus (Figure 31), they differ in this respect from hnRNP monomer particles extracted in pH 8.0 buffer at 0°C by the method of Samarina et al (1968). It is generally observed that hnRNP monomer particles extracted by this method have a simple protein composition consisting solely of the major 30-40K molecular weight proteins (Kinniburgh et al, 1976c). When the method has been applied to HeLa cells a raised temperature of 20 or 37°C is required, possibly due to the low levels of endogenous RNase in these cells. The particles obtained by this method from HeLa cells are similar in size and density to those observed in this study (Pederson, 1974; Beyer et al, 1977). However, the extracted particles had a simple protein composition consisting of the major 30-40K proteins (Beyer et al, 1977) and not containing the higher molecular weight proteins found associated with hnRNP particles extracted by sonication (Pederson, 1974) and the particles released from nuclei in this study (Figure 31).

The problem of artefacts occurring due to aggregation of extensively digested hnRNP particles has been raised by Stevenin et al

(1979). It was proposed that the association of the minor higher molecular weight proteins with hnRNP particles is RNase sensitive and that they are therefore released from monoparticles during the extensive degradation of RNA involved in the method of Samarina et al (1968). The major 30-40K proteins may then rearrange to form 40-45S aggregates associated with degraded RNA. The results obtained in this study are consistent with this model. The RNA in the 40S particles released in this study is ~ 2,000 nucleotides long (Figure 26), whilst many reports of RNA size in pH 8.0 extracted 40S monoparticles suggest only a few hundred nucleotides (Beyer et al, 1977; Maundrell et al, 1979). The protein composition of the particles in this study is similar to the heterogenous composition of nuclear hnRNP polyparticles, whilst pH 8.0 extracted monoparticles of similar size containing much degraded RNA have a much simpler composition. Thus, the particles released in this study may have a structure closer to that of native hnRNP particles than the particles normally extracted by the method of Samarina et al (1968). It has been assumed that the extraction of hnRNP particles from intact nuclei is the result of diffusion of nucleoplasmic particles (Billings and Martin, 1978). The results obtained by many groups show that principally 40S monoparticles are extracted following RNase action. Although early reports have suggested that larger particles can be extracted in the presence of RNase inhibitor, this does not agree with the necessity to raise the temperature of extraction for cells low in RNase activity (Pederson, 1974; Beyer et al, 1977). It is possible, therefore, that the diffusion channel has a diameter of equivalent size to the monoparticle diameter, such that larger polyparticles cannot pass through this channel. It is known that the

nuclear pore is selective to the passive diffusion of molecules in terms only of size, such that a patent pore channel radius of ~40Å has been defined *in vivo* (Paine *et al*, 1975a and b). This is smaller than the size of monoparticles (100-300Å) (Stevenin *et al*, 1976).

An alternative explanation for the apparent selective release of 40S sized particles in this study (Figure 28) is that hnRNA is held in the nucleus and is not free to diffuse unless degraded by RNase action, or is released by disruption of nuclei.

Growing RNA chains are linked to DNA by the transcribing RNA polymerase, as observed in chromatin spreads (e.g. Foe *et al*, 1976). This nascent RNA is incorporated into RNP particles during elongation (Malcolm and Somerville, 1974; Laird *et al*, 1976). Biochemical studies have also shown that rapidly labelled RNA is associated with chromatin (Monahan and Hall, 1975). However, much of this RNA can be released from the chromatin of rat liver by incubating with chelating agents (Ishikawa *et al*, 1974), or by a pH 8.0 extraction procedure as used on intact nuclei (Northemann *et al*, 1978). In myeloma cells, 80% of the labelled hnRNP remains bound to chromatin and can be released by a pH 8.0 extraction procedure (Kimmel *et al*, 1976). It therefore seems likely that the extractable chromatin-bound hnRNP particles are not growing RNA chains, but are mature RNA molecules still bound to chromatin (Bachellerie *et al*, 1975; Augenlicht and Lipkin, 1976; Kimmel *et al*, 1976).

There is also evidence that hnRNA is associated with other structures within the nucleus. Thus, a large network of RNP fibrils was isolated using gentle methods for disruption of nuclei, suggesting that normal extraction methods release RNP particles by shearing

this network during sonication, or by nuclease digestion during incubation of intact nuclei (Faiferman et al, 1975). This network is connected to the nucleoli, the inner nuclear membrane and the nuclear pores (Miller et al, 1978). It has been proposed (Herman et al, 1978) that the bulk of hnRNA is in fact associated with the nuclear pore-lamina complex (Aaronson and Blobel, 1975) or the nuclear protein matrix (Berezney and Coffey, 1977). It appears that poly(A) and double stranded RNA sequences may be the specific points of attachment of hnRNA to these structures (Herman et al, 1978). It may therefore be significant that the released RNA contained a very low proportion of poly(A) molecules, yet a high proportion of oligo(A)$^+$ molecules, since oligo(A) is not apparently associated with the nuclear matrix (Table 1b).

The hnRNP particles retained in incubated nuclei were the same size as those in non-incubated nuclei (Figure 28), despite the fact that the RNA decreased in size during incubation (Figure 26). This would tend to support the idea of a network of RNP fibrils connected in part by protein-protein interactions, such that sonication of both control and incubated nuclei would generate the same size distribution of particles by random shearing.

4.4. Release of RNA labelled in vitro from isolated nuclei

Although some of the hnRNA prelabelled in vivo was released as 18S RNA in 40S RNP particles, the majority of RNA labelled in vitro which was released from nuclei was small RNA of 4-5S labelled by RNA polymerase III (Figure 23a). It is assumed that this released RNA is predominantly 5S rRNA and tRNA. It would be of great interest to determine whether this released RNA was also processed in

the isolated nuclei, or represented a purified population of small RNA precursor molecules.

It was occasionally observed that the released RNA labelled in vitro contained a shoulder of higher molecular weight RNA (Figure 23a). A number of different types of modification were tried in order to increase the amount of larger RNA released. A number of important negative results were obtained. Thus, no effect of cytoplasmic factors on the releaseof RNA synthesised in vitro was observed. Cytosol has been reported to stimulate the release of prelabelled RNA from rat liver nuclei (Schumm and Webb, 1972; Yannarell et al, 1976). Although these authors suggested the presence of stimulatory proteins in cytosol (Schumm et al, 1973), it has more recently been found that cytosol stimulates the release of ribosomal RNA (Sato et al, 1977a) and that the stimulatory factor is RNA, or any other polyanion such as poly(U) (Sato et al, 1977b).

This latter result may also explain the observation that polyanions release ribosomes from isolated nuclei, apparently as polyribosomes (Goidl et al, 1975). This observation was taken as evidence for nuclear polysomes, either involved in nuclear protein synthesis, or involved in the transport of mRNA (Goidl, 1975). This latter theory is supported by ultrastructural evidence of nuclear polysomes (Bachellerie et al, 1975) and earlier observations that a functional nucleolus is required for mRNA transport (Sidebottom and Harris, 1969). This might also tie in the inhibitory effect of high concentrations of actinomycin D on mRNA transport (Eckert et al, 1975).

However, pretreatment of the cells used in this study with

low levels of actinomycin D had no apparent effect on the transport of either RNA prelabelled *in vivo* or RNA synthesised *in vitro* (Figure 23b). The effect of poly(U) on the transport of prelabelled RNA from isolated nuclei was also tested, but it was found that transport was inhibited, and that no hnRNP particles could be extracted from the isolated nuclei after incubation, by sonication, suggesting that poly(U) had caused aggregation of the nuclear particles (results not shown). Therefore, no effect of cytoplasm or the nucleolus on transport was observed.

One possible explanation for the lack of release of large RNA labelled *in vitro* by RNA polymerase II is related to the small size of this RNA. Thus, the nascent RNA might remain bound to chromatin via RNA polymerase II, or even if prematurely terminated, might not be sufficiently large to be processed and transported correctly. For example, full length transcription of the adenovirus major late transcription and is required for correct processing of the late mRNA, even though the primary transcript is cleaved before termination of transcription (Goldberg *et al*, 1978). To test this possibility, the effect on release of RNA larger than 5S of factors which increased the size of RNA synthesised *in vitro* was examined. It was found that acetyl CoA did not affect the size of *in vitro* labelled RNA released from the nuclei (Figure 24).

On the other hand, incubation of nuclei in 90 mM $(NH_4)_2SO_4$ did increase the amount of large RNA released after labelling *in vitro* (Figure 25a). Although this would correlate well with the observation that 90 mM $(NH_4)_2SO_4$ increased the size of RNA polymerase II transcripts *in vitro* (Figure 10), it seemed more

likely that the release was a result of the observed lysis of some nuclei at this ionic strength. Incubation in 80 mM $(NH_4)_2SO_4$, in which no lysis was observed whilst large RNA was still synthesised, reduced the amount of large RNA released between 0-30 minutes of incubation to roughly 2% of the total labelled nuclear RNA. A further 4% of the labelled nuclear RNA was released between 30-90 minutes incubation in the absence of RNA synthesis (Figure 25b). These figures correlate well with the observation that 3-6% of labelled nuclear RNA is transported to the cytoplasm *in vivo* (Herman and Penman, 1977). It would therefore be of interest to determine whether the large RNA lebelled in vitro in 80 mM $(NH_4)_2SO_4$ and released from nuclei was enriched in mRNA sequences.

In very few cases has the transport of RNA synthesised *in vitro* from isolated nuclei been reported, other than small RNA synthesised by RNA polymerase III. For example, the RNA released from isolated HeLa cell nuclei after synthesis *in vitro* is mainly 4-5S RNA (McReynolds and Penman, 1974; Sarma *et al*, 1976). The apparent lack of transport of RNA polymerase II transcripts found by a number of groups may be related to the small size of RNA polymerase II transcripts, found in these systems.

In only a few cases has the transport of RNA synthesised by RNA polymerase II been reported (Biswas *et al*, 1976; Mory and Gefter, 1977; Roy *et al*, 1979). Moreover, the possibility of leakage from these nuclei cannot be ruled out. For instance, the binding of released RNP particles to olgio(dT) cellulose has been used as a test for poly(A) containing RNA (presumed to be mRNA), (Biswas, 1978; Roy *et al*, 1979), yet as shown in this study, although

roughly 50% of the released RNP particles bound to oligo(dT) cellulose (Figure 32), a much smaller proportion of the RNA in these particles was actually polyadenylated (Table 1b). In other cases, the binding of oligo(dT) has been used to measure the proportion of polyadenylated molecules in the released RNA (Mory and Gefter, 1977; Biswas et al, 1976) yet the conditions used for binding may not discriminate molecules containing internal oligo(A) sequences from polyadenylated molecules (Cooper and Marzluff, 1978).

Furthermore, in two recent studies it has been claimed that the RNA labelled in vitro by RNA polymerase II, and released from isolated nuclei, is specifically associated with mRNP proteins (Roy et al, 1979) or hnRNP proteins (Chisick et al, 1979). However, the problem of separating new transcripts from endogenous RNA (which has plagued studies of transcription in vitro), has not been eliminated from these studies, nor has the problem of artefactual binding of protein to RNA. Thus, there is no clear indication in these studies that the major proteins observed on SDS polyacrylamide gels are actually the proteins associated with the labelled RNA.

It has been suggested earlier (section 2.3), that the incubation conditions necessary for correct initiation, elongation, and termination by RNA polymerase II may need careful optimisation. It has also been shown that in no case has the specific transport from isolated nuclei of mRNA sequences been shown, whether these are prelabelled in vivo (section 4.1) or synthesised in vitro. However, the preliminary results presented here suggest that under

conditions which increase the length of RNA polymerase II transcripts *in vitro*, a small proportion of these transcripts are released in a manner consistent with specific mRNA transport. It may be that this transport is possible as a consequence of full length transcription of transcription units by RNA polymerase II under these conditions. Thus, the optimisation of an isolated nuclei system for full length transcription by RNA polymerase II might allow the study not only of this transcription, but also of the processing and transport of the transcription products *in vitro*.

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