"Possible RNA Processing Enzymes in HeLa Cell Nuclei"

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by

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Summary

1. HnRNP particles were isolated from monolayer cultures of HeLa cells and characterized with respect to their sedimentation in sucrose density gradients (s values from 0 to >250s), their buoyant density in CsCl density gradients following aldehyde fixation of the particles (1.390 $g.cm^{-3}$), their heterogeneous complement of polypeptides as analysed on SDS-polyacrylamide gels (species from 38,000 to >150,000 daltons) and the heterogeneous sedimentation of their rapidly labelled RNA component (s values from 0 to >40s).

2. Various potential RNA processing enzyme activities were investigated in relation to their possible association with HeLa hnRNP particles or related subnuclear fractions of chromatin and nucleosol.

(a) Exoribonuclease activity which was dependent on Mg^{2+} ions was found to be largely confined to a nucleosol fraction and, to a lesser extent, to the chromatin fraction. No exoribonuclease activity could be detected in association with HeLa hnRNP particles.

(b) Endoribonuclease activity of poor substrate specificity was detected in association with HeLa hnRNP particles, chromatin and nucleosol fractions. The hnRNP particle-associated activity was stimulated by Mg^{2+} but did not appear to be strictly dependent on the presence of Mg^{2+}. The same activity permitted degradation of HeLa hnRNA to molecular species of lower average sedimentation coefficient than present in mRNA but without any accompanying production of acid-soluble components.

(c) No double-strand RNA-specific RNase activity could be detected in association with HeLa hnRNP particles. Instead such activity appeared to be largely confined to the nucleosol fraction.

(d) RNA guanylyltransferase activity could not be detected in association with HeLa hnRNP particles. Again such activity appeared to be confined to the nucleosol fraction.

(e) RNA ligase activity similar to the bacteriophage T_4 activity could not be detected in any of the HeLa subnuclear fractions tested.

(f) No poly A synthetase activity could be detected in association with HeLa hnRNP particles. Rather, such activity was prominent in a HeLa nucleosol fraction and, to a lesser extent, in the corresponding chromatin fraction. The nucleosol-located activity was optimally stimulated in the presence of a poly A primer and appeared to be more dependent on the presence of exogenously provided RNA primer than the corresponding chromatin-associated activity.

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Abbreviations

AMP	-	Adenosine 5' monophosphate
ATP	-	Adenosine 5' triphosphate
BSS	-	Balanced salts solution (defined in Materials & Methods 2.1)
cDNA	-	Complementary deoxyribonucleic acid
CMP	-	Cytidine 5' monophosphate
CTP	-	Cytidine 5' triphosphate
DEAE-	-	Diethylaminoethyl-
DNase	-	Deoxyribonuclease
DTT	-	Dithiothreitol
EDTA	-	Ethylenediaminetetra-acetic acid
GMP	-	Guanosine 5' monophosphate
GTP	-	Guanosine 5' triphosphate
HEPES	-	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
hnRNA	-	Heterogeneous nuclear ribonucleic acid
hnRNP	-	Heterogeneous nuclear ribonucleoprotein
HSHMT	-	High salt-high Mg^{2+}-tris buffer (defined in Materials & Methods 2.1)
Kb	-	Kilobases
LETS	-	Lithium-EDTA-tris-SDS buffer (defined in Materials & Methods 2.1)
LSLMT	-	Low salt-low Mg^{2+}-tris buffer (defined in Materials & Methods 2.1)
mRNA	-	Messenger ribonucleic acid
mRNP	-	Messenger ribonucleoprotein
NEM	-	N-ethylmaleimide
poly A	-	Polyadenylic acid
poly C	-	Polycytidylic acid
poly G	-	Polyguanylic acid
poly U	-	Polyuridylic acid
P.N.S.	-	Post-nucleolar supernatant fraction (defined in Materials & Methods 3.2)
PPO	-	2,5-Diphenyloxazole
POPOP	-	1,4-bis[2(5-phenyloxazolyl)] benzene
rRNA	-	Ribosomal ribonucleic acid
RNase	-	Ribonuclease
s	-	Sedimentation coefficient (Svedberg unit)
SDS	-	Sodium dodecyl sulphate
SSC	-	Standard saline citrate (0.15M NaCl, 0.015M sodium citrate)

tRNA	-	Transfer ribonucleic acid
TEMED	-	N,N,N',N'-tetramethylethylenediamine
TCA	-	Trichloroacetic acid
Tris	-	Tris (hydroxymethyl) aminomethane
UMP	-	Uridine 5' monophosphate
UTP	-	Uridine 5' triphosphate

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INTRODUCTION

TABLE 1 Characteristic Features of Major RNA Classes in Eukaryotic Cells

Class	Abundance (% of total cellular RNA)	Sedimentation Coefficient	Base Composition	Characteristic Structural Elements	Kinetics of Radioactive Labelling	Subcellular Localization	Function
Ribosomal RNA (rRNA)	80	Four species: 17-18s, 25-28s, 5.8s, 5s	High GC content	High degree of secondary structure; large number of methylated nucleosides	Very slow incorporation; stable	Ribosomes	Structural template for protein synthesis
Transfer RNA (tRNA)	10-15	Large number of different species, all at about 4s	Relatively high GC content	High degree of secondary and tertiary structure; wide variety of modified nucleosides	Moderately rapid incorporation; stable	Cytosol	Liaison between amino acids and mRNA in protein synthesis
Heterogeneous nuclear RNA (hnRNA)	<1	Heterodisperse ~10s to >70s	Relatively low GC content	Large poly A tract at 3'end; capped structures at 5'end; oligo A sequences; oligo U sequences; double stranded regions; internal m^6Ap residues	Very rapid incorporation; rapid decay	Nucleus	Partly as precursors to mRNA; some may be structural, some may be regulatory
Messenger RNA (mRNA)	<1-3	Heterodisperse ~8s to >30s	Relatively low GC content	Large poly A tract at 3'end; capped structures at 5'end; oligo U sequences; internal m^6Ap residues	Moderately slow incorporation; moderately stable	Cytoplasmic	Functional template for protein synthesis

1. TRANSCRIPTION

Ribonucleic acid (RNA) exists in eukaryotic cells in a number of different molecular classes which can be distinguished on the basis of several properties including range of sedimentation coefficients, base composition, possession of characteristic structural elements, kinetics of radioactive labelling and subcellular localization (TABLE 1). In each case, however, the ultimate origin of the RNA species occurs at the chromosomal level: individual RNA molecules are synthesized from four ribonucleoside triphosphates (ATP, CTP, GTP, UTP) using the DNA of chromatin as a template. In this way the genetic information which codes for the development and functioning of organisms, and which is housed in the elaborate chromosomal structure, can be transferred to RNA molecules at certain specific regions of the DNA called genes. The mechanism whereby the transfer of genetic information occurs from DNA to RNA, in both prokaryotes and eukaryotes, is known as transcription and is catalysed by an enzyme designated as DNA-dependent RNA polymerase (E.C 2.7.7).

1.1 The enzyme, RNA polymerase

DNA-dependent RNA polymerase activity was first demonstrated in rat liver nuclei (Weiss & Gladstone, 1959; Weiss, 1960). However subsequent research revealed the considerable difficulty in solubilizing the chromatin-bound eukaryotic RNA polymerases in contrast to the situation in bacteria where the existence of comparatively large amounts of soluble enzyme was demonstrated. Consequently the most instructive information concerning the nature of the transcriptional process has been derived from prokaryotic systems.

1.1.1 Prokaryotic RNA polymerase and the mechanism of transcription

Bacterial RNA polymerases from different species appear to be closely related in subunit structure. The E.coli enzyme has four major subunits: α (40,000 daltons);

β(150,000 daltons); β'(160,000 daltons); and σ(86,000 daltons) (Chamberlin, 1976). The protomeric form has the structure $\alpha_2\beta\beta'\sigma$ (Travers & Burgess, 1969) called the holoenzyme which can be resolved by phosphocellulose chromatography into the "core" enzyme $\alpha_2\beta\beta'$ and the sigma factor, σ (Burgess, 1971).

The structural complexity of RNA polymerase is paralleled by the complexity of the transcription process it catalyses. Four component steps have been distinguished: (1) template site selection and activation; (2) RNA chain initiation; (3) RNA chain elongation; and (4) RNA chain termination and enzyme release (Anthony et al, 1966). Initially, after a series of unproductive random interactions with the DNA template the holoenzyme binds strongly to a specific promotor region. There follows a change in the conformational state of the DNA in the promotor region (probably strand separation of the DNA double helix over 4 to 8 base pairs - Saucier & Wang, 1972) after which the holoenzyme is ready to commence RNA synthesis but using only one of the strands as a template. RNA chain initiation proceeds with the enzyme catalysing the coupling of ATP or GTP with a second ribonucleoside triphosphate to generate, by elimination of inorganic pyrophosphate, a dinucleoside tetraphosphate pppPupX (Pu=purine, X=any nucleoside) which remains tightly bound to the enzyme-DNA complex. Elongation of the nascent RNA chain can then be accomplished by addition of successive nucleoside monophosphate residues, derived from various substrate nucleoside triphosphates, to the initial dinucleoside tetraphosphate at the 3'OH terminus. In this reaction the sequence of nucleotides in the DNA template dictates, by means of Watson-Crick base-pairing rules, the sequence of nucleotides incorporated into the growing RNA chain. Finally at a defined termination point in the DNA sequence the RNA polymerase detaches from its template along with the completed RNA chain or primary transcript as it is known.

The functions of individual subunits have been partially elucidated. Sigma factor appears to be required

for activating selective initiation of RNA synthesis and is thought to interact with β', the subunit believed to participate in template binding of RNA polymerase to DNA (Zillig et al, 1976). Subunit β participates in binding of the substrate and may also contribute to the interaction of core enzyme with σ, while α carries a binding site for β and is also involved in σ binding (Walter et al, 1968; Seifert et al, 1969). In addition, another factor ρ, has been implicated as a termination factor acting at certain termination sites.

1.1.2 Eukaryotic RNA polymerase

1.1.2.1 Nomenclature and structure

In eukaryotic systems the observed heterogeneity of RNA polymerase species, within a single cell type, affords a further level of complexity in the transcriptional apparatus. Typically, three major classes of RNA polymerase can be distinguished by their order of elution from DEAE-sephadex in response to an increasing salt gradient (numbered progressively I, II and III - Roeder & Rutter, 1969) or by their relative sensitivities to the toxin α-amanitin (A - relatively insensitive, B - very sensitive and C - moderately sensitive, Kedinger et al, 1971). Subspecies of each main category can be further distinguished on the basis of their elution from ion exchange resins and their α-amanitin sensitivity. In each case the various RNA polymerase species are macromolecular multi-subunit enzymes having molecular weights of ~500,000 with each enzyme consisting of two large (>100,000 daltons) subunits and several smaller subunits (Beebee & Butterworth, 1977).

1.1.2.2 Function

The first indication that RNA polymerases I, II and III have a nuclear residence and are probably involved in the transcription of nuclear genes was provided by the demonstration that highly purified sea urchin nuclei contain substantial amounts of each of these activities (Roeder & Rutter, 1969). Further, it was shown that following subfractionation of isolated nuclei, RNA

polymerase I was almost exclusively localized in the nucleolus while polymerases II and III were confined to the nucleoplasmic fraction (Roeder & Rutter, 1970).

A more direct approach to the examination of the functions of different RNA polymerase classes has utilized the observation that the α-amanitin-sensitivities of the endogenous RNA polymerase activities parallel those of the purified enzymes (e.g. Lindell et al 1970; Weinmann et al, 1974). Studies employing this approach have established that the nucleolar polymerase I transcribes rRNA genes, nucleoplasmic polymerase II transcribes genes coding for hnRNA and viral mRNA precursors, and polymerase III transcribes tRNA genes and 5s rRNA genes as well as those coding for low molecular weight viral RNAs. In addition it has recently been shown that a group of small nuclear RNA molecules are synthesized by a type I RNA polymerase from HeLa cells (Benecke & Penman, 1977).

1.1.2.3 Control

The control mechanisms which regulate transcription in eukaryotes are at present largely undefined. However, the existence of three distinct forms of RNA polymerase in eukaryotes with specificities for certain genes or classes of genes represents at least a coarse level of control. Additionally, RNA polymerases display micro-heterogeneity within each major class and are subject to modifications such as phosphorylation (Bell et al, 1976). Further, proteins have been isolated which stimulate the activity of the polymerases *in vitro* although considerable doubt is still attached to their roles *in vivo* (see Beebee & Butterworth, 1977). Studies to examine the specificity and regulation of transcription have largely been conducted using *in vitro* systems.

1.1.2.4 *In vitro* transcription

Initial studies employed DNA or chromatin isolated from eukaryotic nuclei as a template for exogenous RNA polymerase, usually the readily available, highly purified E.coli RNA polymerase. However, perhaps not surprisingly,

the fidelity of *in vivo* transcription could not be reproduced in these systems. More promising results could be obtained using homologous RNA polymerases. In particular RNA polymerase III has been shown to transcribe, selectively and accurately, the 5s RNA genes in oocyte chromatin (Parker & Roeder, 1977) while HeLa cell polymerase III also appears to synthesize specifically 5s RNA from isolated HeLa chromatin (Yamamoto et al, 1977). Although there is no unambiguous evidence that RNA polymerase I specifically and accurately transcribes deproteinized nucleolar DNA or chromatin, a considerable amount of specificity of transcription has been claimed for such systems (Matsui et al, 1977; Daubert et al, 1977). However RNA polymerase II does not appear to be able to transcribe isolated chromatin accurately (e.g. Wilson et al, 1975).

Similar studies have been extended to isolated nuclei which have the advantage of containing chromatin templates in a minimally damaged form. The genes for 5sRNA and for tRNA are transcribed selectively and accurately by RNA polymerase III added to isolated nuclei (Sklar & Roeder, 1977; Parker et al, 1978) while the addition of homologous polymerases I and II proved ineffective in stimulating the transcription of these genes.

RNA polymerase I transcripts synthesized in isolated nuclei have been shown in some cases to be as large as the 45s rRNA precursors formed *in vivo* and these may undergo a limited amount of processing to generate molecular species resembling mature rRNA molecules (Zylber & Penman, 1971; Udvardy & Seifart, 1976). In addition, rRNA synthesis in isolated Xenopus nuclei has been shown to occur by asymmetric transcription (Udvardy & Seifart, 1976).

Although the RNA species synthesized by RNA polymerase II in isolated nuclei are generally small in size, usually being about 10s-25s (e.g. Biswas et al, 1976), the transcript sizes *in vivo* and *in vitro* have been shown in one case to be identical when estimated under denaturing conditions (Mory & Gefter, 1977). Asymmetric transcription

of specific genes by polymerase II in isolated nuclei has also been reported (Orkin, 1978; Levy et al, 1978). However the synthesis achieved appears to be met very largely by elongation of pre-formed transcripts rather than by initiation (e.g. Levy et al, 1978).

Finally, the Xenopus oocyte affords a promising system for the study of *in vitro* transcription. Cloned Xenopus genes for 5s RNA and for the initiator tRNA have been faithfully transcribed in this system following the injection of the RNA into the oocyte nuclei (Brown & Gurdon, 1978; Kressmann et al, 1978). Further, extracts from Xenopus germinal vesicles have been shown to direct accurate transcription of cloned Xenopus 5s RNA genes and cloned tRNA genes from Drosophila and yeast (Birkenmeier et al, 1978; Schmidt et al, 1978).

1.2 The template, chromatin

1.2.1 General structure

Eukaryotic DNA is associated with various proteins to form a complex deoxyribonucleoprotein structure which, when isolated from interphase nuclei, is generally referred to as chromatin. The composition of chromatin is defined by four major elements: DNA; histones; non-histone proteins; and a small quantity of RNA. The DNA component is largely in the form of a double helix of 20Å diameter which combines with protein to give a fibre 30Å in diameter. Aggregation and condensation of the 30Å-diameter fibre generates structures of about 100Å in diameter which, when slightly extended, resemble beads on a string. The beads, or nucleosomes, represent basic repeat units. They consist of a well defined length (usually about 200 base pairs) of DNA complexed with an octamer of histones with two copies each of histones H2A, H2B, H3 and H4 (Hewish & Burgoyne, 1973; Noll, 1974; Kornberg, 1974). The nucleosome is comprised of a relatively nuclease-resistant core region containing 140 base pairs contiguous with a linker region of variable length (Axel, 1975; Sollner-Webb & Felsenfeld, 1975).

The structure of the nucleosome core has been defined by X-ray crystallography as a flat disc about 110Å in diameter, 57Å in height which is generated by supercoiling of the double helical DNA to give approximately $1\frac{3}{4}$ turns of a superhelix of 80Å diameter and 28Å pitch (Finch et al, 1977). A superhelical arrangement of this type constitutes the first higher order of structure responsible for maintaining the DNA in the extremely compact form found in the nucleus, and arrangement of the nucleosome cores in linear array defines chromatin fibres of 100Å in diameter. Larger chromatin fibres of 200Å-300Å in diameter have been considered to arise from coiling of the nucleosome chain to give a solenoidal structure 300Å in diameter (Finch & Klug, 1976) while others have considered the fibre to be discontinuous and made up of 200Å-"superbead" units with about 8 nucleosomes per superbead (e.g. Renz et al, 1978; Hozier et al, 1977).

The location of the fifth histone species H_1 is not known precisely but it is thought to be bound to chromatin, at least in part, through attachment to the DNA linker regions, and appears to modify or stabilize interactions between nucleosomes (see Felsenfeld, 1978). As yet virtually nothing is known about the location of the non-histone proteins bound to chromatin. However, studies on histone-depleted DNA have shown that about 20-30 species of non-histone protein maintain the DNA in a highly folded state. The proteins are arranged in a central core or scaffold which serves as an anchorage point for loops of DNA between 45,000 and 90,000 base pairs in length (Laemmli et al, 1978; Paulson & Laemmli, 1977). In addition the presence of some non-histone proteins in nucleosomes has been suggested by experiments performed by Defer et al (1978) and Bakayer et al (1978) while an acidic protein which binds histones and transfers them to DNA, thereby assembling nucleosomes, has been identified and purified from eggs of Xenopus laevis (Laskey et al, 1978).

1.2.2 Structure of transcriptionally active chromatin

Studies of the transcription of specific genes (e.g. globin genes) in isolated chromatin by exogenous RNA polymerases have shown that chromatin structure determines in part which genes are transcribed by the cell (Axel et al 1973; Gilmour & Paul, 1973). Distinction between transcriptionally active and inactive chromatin has been probed using specific nucleases, and although no distinction is evident with staphylococcal nuclease (Axel et al, 1975), pancreatic DNase I appears to digest the transcriptionally active regions preferentially (Weintraub & Groudine, 1976). However, the transcription complex itself does not seem to confer special sensitivity to DNase I since gene sequences that are infrequently represented in the mRNA population are nevertheless DNase-sensitive (Garel & Axel, 1977).

Electron microscopic visualization of transcriptionally active ribosomal RNA genes has shown that the underlying chromatin appears smooth and free of nucleosomes, while measurements of the ribosomal transcription units indicate that the DNA must be extended in the B form (Foe, 1978; McKnight et al, 1978; Franke et al, 1978). In the case of non-ribosomal transcription units nucleosome-like beads are visible on moderately actively transcribed regions, albeit with a reduced periodicity relative to inactive regions. However no beads are visible on the extensively active transcriptional units of lampbrush chromosomes (Franke et al, 1978; Scheer, 1978) and a correlation has been drawn between the degree of transcriptional activity and the frequency of nucleosomes for both nucleolar and non-nucleolar chromatin (Scheer, 1978).

1.2.3 Sequence organisation

In addition to gene sequences which are present in a single or limited number of copies per haploid genome there are sequence elements which are present in several hundred to hundreds of thousand copies per nucleus (Britten & Kohne, 1968). Hybridization studies have revealed that there are two major patterns of sequence organization.

In Xenopus and many other organisms 50% of the genome is accounted for by an alternating arrangement of unique sequence of about 1,000 nucleotides long and repetitive sequences 200-400 nucleotides long, 40% by interspersion of very long (several x 10^3 nucleotides) unique sequence and comparatively short repetitive sequences, and the remaining 5-10% by clustered highly repetitive sequences (e.g. Graham et al, 1974). However in Drosophila, in addition to the clustered highly repetitive sequences, there are interspersed unique and repetitive sequences covering a wide range with unique sequences from 2,500-40,000 base pairs and repetitive regions from 500-13,000 base pairs (Manning et al, 1975). The unique copy sequences adjacent to repetitive DNA have been shown to harbour 80-100% of the structural gene sequences (Davidson et al, 1975).

The topography of DNA sequences coding for specific RNA species has been investigated in a number of cases. In particular the reiterated genes are often found in clustered arrays of tandemly arranged repeat units with each repeat unit consisting of a coding component and a non-transcribed spacer component. They include the genes for 18s and 28s ribosomal RNA from several species (Wensink & Brown, 1971; Glover & Hogness, 1977; Philippsen et al, 1978), 5s rRNA from Xenopus (Brown et al, 1971), some tRNA genes from Drosophila (Yen et al, 1977) and Xenopus (Clarkson et al, 1978) and also the genes coding for histone mRNA in sea urchin (Cohn et al, 1976). Whereas the transcribed component of each repeat unit (i.e. coding region + transcribed non-coding spacer) remains constant there is generally considerable length and sequence heterogeneity of the non-transcribed spacer region.

A further level of complexity has become apparent following the very recent discovery that many genes have their coding sequences interrupted by one or more non-coding sequences. Although "split" genes include some representatives of the reiterated genes, including some rRNA genes from Drosophila (Glover & Hogness, 1977) and

some tRNA genes from yeast (Goodman et al, 1977; Valenzuela et al, 1978), they seem to be well represented in single-copy genes coding for various mRNA species. They include the genes for rabbit and mouse β-globin (Jeffreys & Flavell, 1977; Tilghman et al, 1978a), chicken ovalbumin (Garapin et al, 1978; Catterall et al, 1978; Weinstock et al, 1978) and the V regions of mouse immunoglobulin λ and κ light chains (Brack & Tonegawa, 1977; Rabbitts & Forster, 1978). In addition a number of viral mRNAs are composed of segments coded for by disparate regions of the genome as in the case of mRNAs specified by adenovirus (Berget et al, 1978; Broker et al, 1978), SV 40 (Aloni et al, 1978) and polyoma (Horowitz et al, 1978a).

2. PRODUCTS OF TRANSCRIPTION AND POST-TRANSCRIPTIONAL PROCESSING

The products of transcription in eukaryotic nuclei, the primary transcripts, are RNA species which in the vast majority of cases undergo a series of processing steps to generate the mature functional species. Four major classes of processing events can be distinguished:

(1) Cleavage - reduction in length of the primary transcription product or its derivatives by means of scission mechanisms catalysed by specific endoribonucleases.

(2) Modification - alteration of primary nucleotide sequences as a result of base or sugar modification e.g. methylation.

(3) Terminal Addition - addition of specific nucleotide sequences to the 5' or 3' end of the appropriate RNA species e.g. polyadenylation (see 5.1.1), "capping" (see 5.1.2) and addition of the CCA sequence to the 3' end of tRNA (see 2.1.1).

(4) Splicing - ligation of RNA sequences transcribed from disparate components of a transcription unit following excision of the transcribed intervening sequences (see 5.1.3).

In both prokaryotes and eukaryotes processing of RNA proceeds in a non-conservative fashion i.e. much of the sequence of the primary transcription products is not represented in the mature RNA species. The primary transcript is delineated by its transcription unit which can be defined as a contiguous segment of DNA whose components are transcribed co-ordinately proceeding from a single promoter site. Following cleavage of the precursor RNA molecules, discarded sequences are presumed to be hydrolysed by non-specific nucleases.

2.1 Processing of RNA in prokaryotes

2.1.1 Transfer RNA

RNA species which act as precursors to some E.coli tRNA and phage T_4-encoded tRNA molecules have in many cases been demonstrated to contain extra nucleotides at both the 5' and the 3' ends and the larger precursors have

ribonucleoside triphosphates at their 5' ends (Barrell et al, 1974; Chang & Carbon, 1975; Sakano & Shimura, 1978). In addition multimeric precursors containing up to 7 or 8 individual tRNA sequences separated by internal spacer-regions have been identified (Sakano & Shimura, 1978; Guthrie et al, 1973; Schedl et al, 1975; Carbon et al, 1975). Enzymes capable of processing precursors of these kinds have been identified in prokaryotic cells.

The most thoroughly characterized tRNA processing enzyme is RNase P (Robertson et al, 1972) which introduces a single cleavage at the 5' end of the precursor tRNA species thereby generating the 5' end of the mature tRNA species. That RNase P plays an essential role in tRNA biosynthesis is strongly suggested by the isolation of temperature-sensitive mutants of E.coli which are defective in the RNase P activity and in tRNA biosynthesis (Schedl & Primakoff, 1973).

In addition, an endoribonuclease activity which cleaves polymeric tRNA precursor molecules internally in the spacer segments has been partially purified and designated ribonuclease O (Sakano & Shimura, 1975) or P_2 (Schedl et al, 1976). The partially purified enzyme appears to be inactive on the majority of monomeric as well as dimeric tRNA precursors (Shimura et al, 1978).

The enzyme activity that processes the 3' end of the tRNA precursors has been suggested to be E.coli ribonuclease II (Schedl et al, 1976) but a nuclease P_3 which can cleave the 3' end of tRNA precursors *in vitro* does not copurify with RNase II (Bikoff et al, 1975). In addition Shimura et al (1978) have described two activities of this type designated Q and Y in E.coli while evidence has been presented that there may be at least two enzymic activities which remove the extra 3' nucleotides *in vivo* (Seidman et al, 1975a).

The processing of tRNA precursors probably involves an alternating pattern of attack by the various cleavage enzymes on multimeric precursors, the order of the steps being determined by the structure of the precursor (Seidman et al, 1975b). A sequential model for the processing of E.coli tRNA precursors has been advanced along those lines (Schedl et al, 1975), while studies

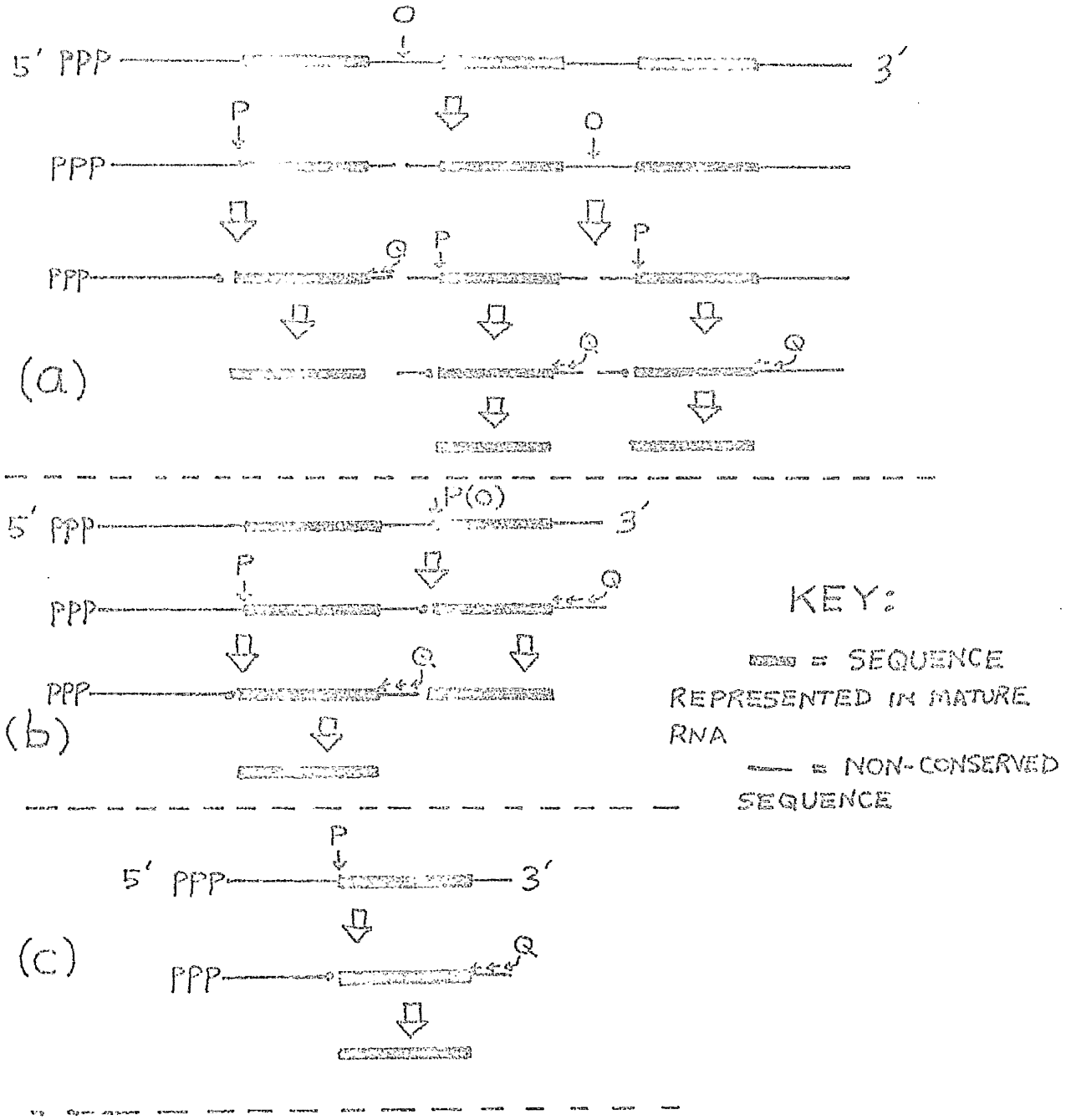


Fig 1 Modes of Cleavage Processing of E.coli tRNA Precursors

Sequential attack by RNases O,P and Q leads to formation of mature tRNA species from various precursors such as oligomers (a), dimer (b) and monomer (c). The intervening sequence between coding regions in a dimeric precursor may be cleaved by RNase O, or more usually by RNase P.

on the *in vitro* processing of tRNA precursors have demonstrated a sequential series of reactions involving at least three different enzymatic activities (Schedl et al, 1976; Sakano & Shimura, 1978). The latter authors have concluded that the multimeric precursors are usually processed first by RNase O and the resulting monomeric or dimeric precursors are then processed by RNase P. After the removal of the extra sequence at the 5' end, RNase Q trims 3' extra nucleotides off these small precursors (see Fig 1).

Nucleoside modifications are largely confined to the cleaved products (Altman & Smith, 1971) while the enzyme tRNA nucleotidyl transferase is required to add the CCA sequence to the 3' terminus of certain tRNA processing intermediates (Deutscher et al, 1975) but is not required for the processing of some tRNA species.

2.1.2 Ribosomal RNA

In E.coli there appears to be at least 7 rRNA transcription units located at 7 different chromosomal sites (for review, see Nomura et al, 1977). They appear to have the general structure: 5'-leader sequence-16s-$tRNA_{1 or 2}$-~~spacer, or 2~~-spacer-23s-spacer-5s-trailer sequence-3' where 16s, 23s, 5s refer to sequences in the DNA which code for 16s, 23s and 5s rRNA respectively. When a single tRNA-coding sequence is present it is that coding for $tRNA^{Glu}$ or when there are two sequences they are those coding for $tRNA^{Ile}$ and $tRNA^{Ala}$ in the transcription units analysed thus far. In addition, tRNA genes have occasionally been identified at the distal end of the transcription unit and there is strong evidence that the genes for $tRNA^{Trp}$ and $tRNA_1^{Asp}$ located at the distal end of the rrnC operon are co-transcribed with the genes in this operon (Morgan et al, 1978).

Normally in growing cells it is not possible to detect the primary transcript. However, in a mutant strain of E.coli which is deficient in the enzyme RNase III cleavage is partially blocked and a large 30s pre-rRNA can be readily detected which contains, *inter alia*, the

sequences for 16s rRNA and 23s rRNA (Pettijohn et al 1970). However kinetic analyses of radioactive label incorporated into rRNA species shows that the 30s RNA is probably not the major precursor to the 16s and 23s rRNAs synthesized in these mutants (Gegenheimer & Apirion, 1975).

A battery of different ribonuclease activities have been implicated in the processing of rRNA in prokaryotes (see Fig 2). They include: RNase III - cleaves the pre-rRNA at two sites to generate precursors to 16s and 23s rRNA (Apirion & Lassar, 1978; Gegenheimer et al, 1977); RNase E - generates the precursor to 5s rRNA (Gegenheimer et al, 1977; Apirion & Lassar, 1978); RNase P - required for the release of spacer tRNAs (Gegenheimer & Apirion, 1978); RNase M_5 - may process the 5' end of the precursor to 5s rRNA, at least in B.subtilis (Sogin & Pace, 1974); and RNase M_{16} - may process the precursor to 16s rRNA and probably includes two enzymic activities specific for the 5' end (Dahlberg et al, 1978) and the 3' end (Hayes & Vasseur, 1976).

Most of the cleavages probably occur when the rRNAs are associated with proteins in pre-ribosomal particles. If a supply of ribosomal proteins is lacking, the efficiency of the cleavage reactions is greatly reduced and pre-ribosomal particles accumulate (Chang & Irr, 1973; Dean & Sykes, 1974). In addition some cleavages, especially secondary ones, are catalysed by enzymes which specifically require their substrates to have a ribonucleoprotein organisation (Dahlberg et al, 1978; Hayes & Vasseur, 1976). In contrast, primary cleavages which occur during or very soon after transcription utilize, as substrates, RNA species which contain all the requisite signals for cleavage in their structure (Nikolaev et al, 1975a) and include those catalysed by RNase III (Nikolaev et al, 1973). However ribosomal proteins appear to be bound to precursor rRNA at a very early stage and a large precursor particle containing 30s pre-rRNA and the majority of the proteins found in mature ribosomes has been identified in RNase III$^-$

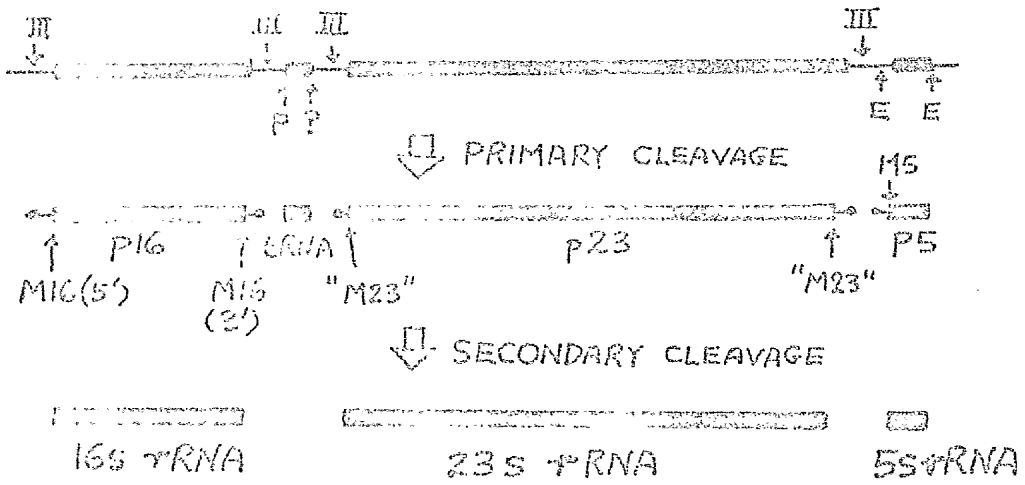


Fig 2 Mode of Cleavage Processing of rRNA Precursors in Prokaryotes

Primary cleavages are effected by RNases III, P and E and an unidentified nuclease (?) to generate p16, p23, p5 and mature tRNA. Secondary cleavages are effected by M16 probably as two different activities specific for 5' and 3' ends of p16, M5 and the hypothetical "M23" - see text.

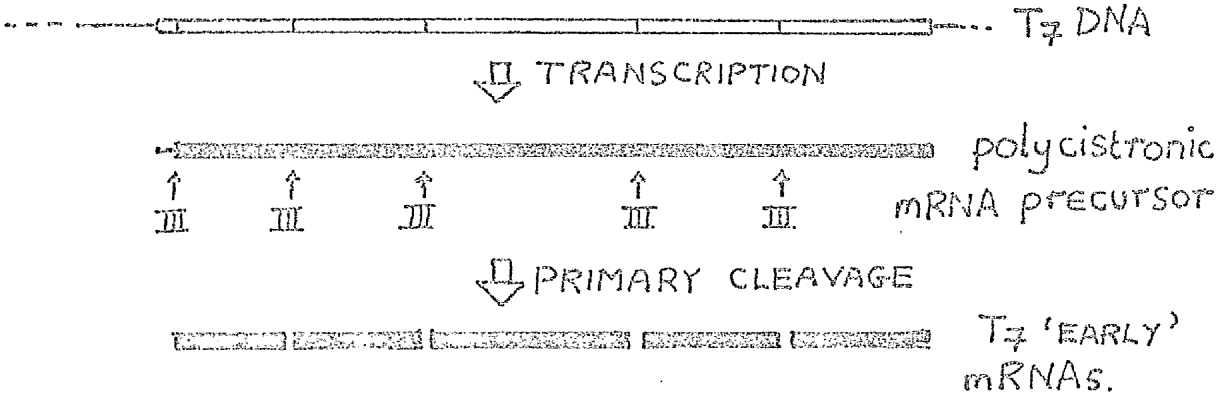


Fig 3 Post-transcriptional Processing of T_7 early mRNAs by RNase III

mutants of E.coli (Nikolaev et al, 1975b; Duncan & Gorini, 1975).

The majority of chemical modifications (methylation, pseudouridylation) of the primary transcript appear to occur shortly after transcription and generally are confined to sequences present in the mature rRNA (for review, see Hadjiolov & Nikolaev, 1976). The sequence of action and structural requirements for modifying enzymes remain, however, completely unknown.

2.1.3 mRNA

Although post-transcriptional processing has not been invoked for normal bacterial mRNAs it is clearly detectable in the production of "early" mRNAs specified by bacteriophage T_7. In RNase III$^-$ mutants of E.coli the early region of T_7 DNA is transcribed as a single unit to produce large molecules essentially identical to those transcribed *in vitro* by E.coli RNA polymerase. Molecules of both types are cleaved by purified RNase III *in vitro* to produce the mRNA species observed *in vivo* (Dunn & Studier, 1973a)- see Fig 3. In addition Majumder et al (1977) have implicated RNase III in the processing of high molecular weight T_3 RNA polymerase transcripts to form mature late T_3 mRNAs.

2.2 Processing of RNA in eukaryotes

2.2.1 tRNA

Although tRNA precursor molecules were first discovered in cultured mammalian cells (Burdon et al, 1967) further research has been considerably impeded by the difficulty in obtaining specific precursors in sufficiently pure quantities although in this regard 2-dimensional electrophoretic systems have enjoyed partial success (Garber et al, 1978). Studies on the size of the precursors under denaturing conditions have revealed that they are considerably larger than the mature species (Burdon & Clason, 1969). Also tRNA precursors from Bombyx mori have been shown to contain additional sequences at both 5' and 3' termini and some of the precursors may contain triphosphorylated nucleotides at their extreme

5' ends (Garber et al, 1978). However there is no unambiguous evidence that multimeric tRNA transcripts are present in eukaryotic organisms although tRNA precursors have been identified in yeast mutants which are large enough to contain two tandem tRNAs (Hopper et al, 1978).

Enzymes which are thought to process eukaryotic tRNA precursors include an activity designated RNase P.Hsa which has been partially purified from the cytoplasm of human KB cells, and is identical to E.coli RNase P (see 2.1.1) in many properties including substrate specificity and identity of specific cleavage products (Koski et al, 1976). An activity that removes extra nucleotides from the 3' end of tRNA precursors to generate 4s RNA fragments has also been reported in a variety of systems (Smillie & Burdon, 1970; Marzluff et al, 1974; Altman et al, 1975). Also it has been recently shown that wild type yeast strains contain an activity which specifically removes the intervening sequences in yeast $tRNA^{Tyr}$ and $tRNA^{Phe}$ precursors, transcribed from "split genes" (see 1.2.3), to generate mature-sized tRNA species (Knapp et al, 1978; O'Farrell et al, 1978).

The tRNA precursors isolated from mammalian cells contain methylated bases and pseudouridine but these modifications are incomplete compared with mature tRNA and base modification of tRNA occurs at various stages of processing (Munns & Katzman, 1973; see also Smith, 1976).

The location and sequence of processing events in eukaryotic tRNA biogenesis is poorly understood. Examination of the kinetics of radioactive labelling of tRNA suggests that it is very rapidly exported from the nucleus to the cytoplasm, and a cytoplasmic location for at least the final processing steps is indicated by the ability to isolate tRNA precursors from the cytoplasm but not apparently from the nucleus (Burdon et al, 1967). Also many tRNA-processing enzymes have a cytoplasmic residence including RNase P.Hsa (Koski et al, 1976), tRNA nucleotidyl transferase (Mukerji & Deutscher, 1972) and a 3'-specific cleavage enzyme (Altman et al, 1975).

In addition, although the bulk of the tRNA methylases has been claimed to reside in the nucleus (Kahle et al, 1971), in vivo labelling experiments have indicated that the methylation of tRNA occurs principally in the cytoplasm (Muramatsu, M. & Fujisawa, 1968). Finally, it is interesting to note that the yeast $tRNA^{Tyr}$ and $tRNA^{Phe}$ precursors which contain the transcribed intervening sequences also contain fully matured 5' and 3' termini which suggests that removal of the intervening RNA sequence is a relatively late event in processing (Knapp et al, 1978).

2.2.2 5s RNA

In higher eukaryotes 5s rRNA is transcribed from reiterated DNA sequences at chromosomal locations which are different from those representing the other rRNA species. Thus far no precursor to 5s RNA has been reported in mammals while the 5s RNA from many eukaryotes contains a 5' triphosphorylated nucleotide, thereby implying that the 5' terminus of the primary transcript is retained (Benbamou & Jordan, 1976). However, when Drosophila cells are exposed to elevated temperatures, a putative precursor ($5s^{+}$) accumulates, consisting of the complete nucleotide sequence of 5s supplemented on the 5' end by an extra 15 nucleotides (Rubin & Hogness, 1975). The $5s^{+}$ species has moreover been indicated to be a precursor to 5s RNA in Drosophila cells continuously cultured at 25°, by a kinetic analysis which also revealed an extremely rapid rate of processing of the $5s^{+}$ species (Levis, 1978).

2.2.3 rRNA

The ribosomal DNA of eukaryotes occurs as tandemly repeated units consisting of a transcribed component (transcription unit) and a non-transcribed spacer of variable length. Generally the transcription unit is organized as:

5' - 18s-Sp1' - 5.8s-Sp1" - 28s - Sp2 - 3'

where 18s, 28s and 5.8s refer to sequences coding for 18s, 28s and 5.8s rRNA species respectively, Sp1' and Sp1" are short internal transcribed spacers and Sp2

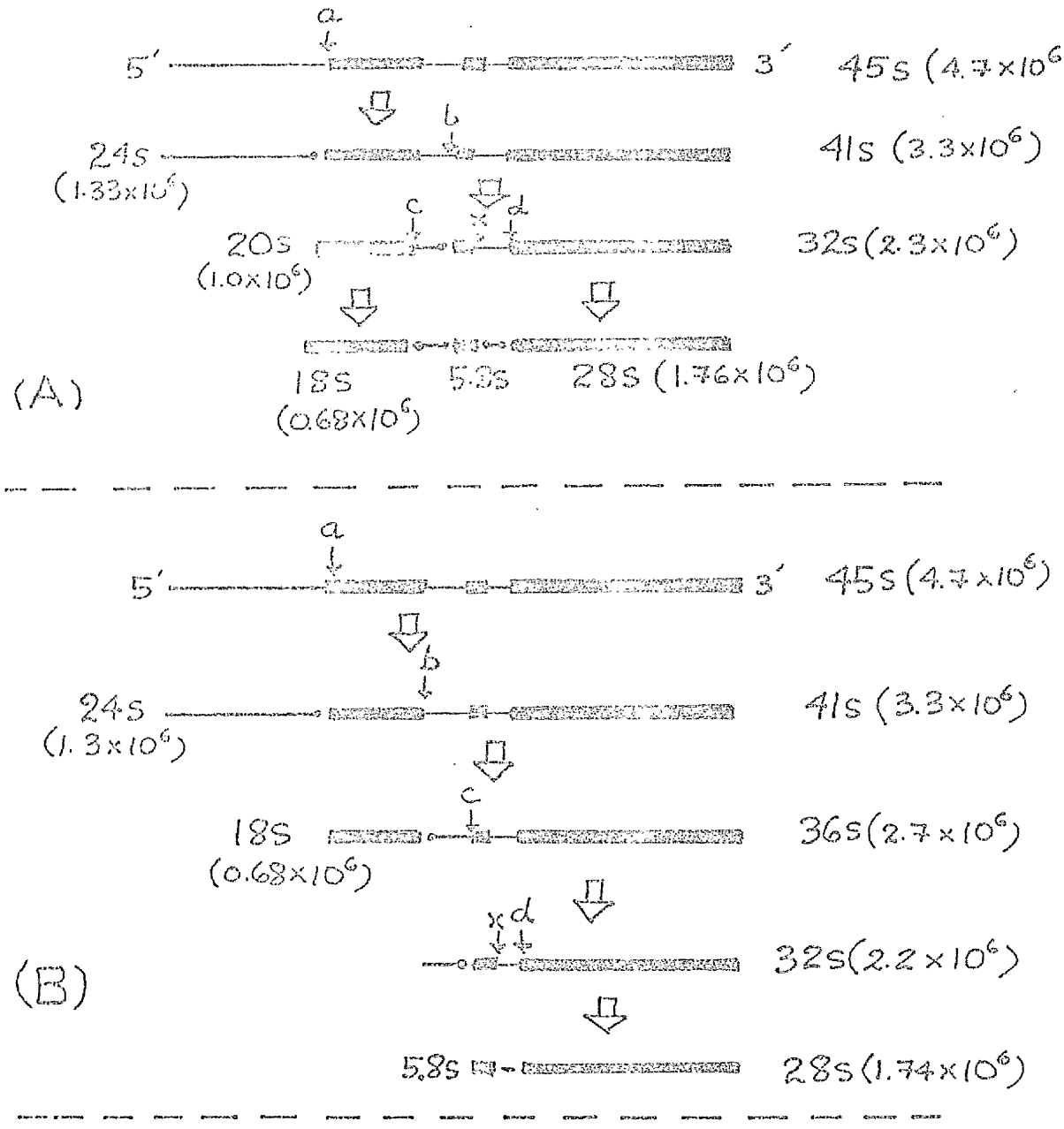


Fig 4 Scheme of Maturation of Primary Pre-rRNA in HeLa Cells (A) and Mouse L Cells (B)

Endoribonuclease activity is represented as four cleavages arbitrarily designated a,b, c and d to indicate order of attack. In addition a further cleavage at position X is required to release 5.8s rRNA.

a large spacer which is mainly non-transcribed (Pellegrini et al, 1977; Wellauer & Dawid, 1977; Wellauer et al, 1976). In addition ribosomal RNA transcription units in Drosophila contain another class of transcription unit in which the 28s sequence is interrupted by intervening sequences of between 0.5 to 6.0 kilobases in addition to the regular transcription units (Wellauer & Dawid, 1977; Glover & Hogness, 1977).

In mammalian cells the most rapidly labelled and largest pre-rRNA species is the 45s pre-rRNA (Scherrer & Darnell, 1962) which is a common precursor to 18s and 28s rRNA species (see Attardi & Amaldi, 1970; Maden, 1971). Further, cleavage of the 45s pre-rRNA in mammalian cells appears to occur at four major sites and depending upon the temporal order of cleavage at the two internal sites two pathways have been distinguished (Fig 4). The 5.8s rRNA is considered to be released during the conversion of 32s to 28s rRNA, but remains hydrogen bonded to the 28s species (Pene et al, 1968).

The enzymology of rRNA processing remains largely unknown. Although several groups have reported nucleolar-derived enzymatic activity that can fragment 45s rRNA (Prestayko et al, 1973; Boctor et al, 1974) and in some cases generate in vivo-sized products (Mirault & Scherrer, 1972; Winicov & Perry, 1974), it has been found that nucleolar preparations abound in several weak nucleases of poor specificity which can extensively fragment artificial polymers as well as rRNA (Kωan et al, 1974). In analogy with the double strand specific RNase III of E.coli and from the observations that HeLa 45s rRNA contains several regions of 2^{o} structure (Wellauer & Dawid, 1973) and that in vivo processing of rRNA is inhibited by agents that alter the properties of double-helical regions (Snyder et al, 1971), it has been thought that eukaryotic rRNA processing involves cleavage at specific duplex regions in precursor molecules. However, when highly purified E.coli RNase III was incubated with pure 45s rRNA from HeLa cell nuclei there was only limited cleavage evident (Gotoh et al, 1974; Nikolaev et al, 1975c).

Nucleoside modification occurs predominantly on the initial transcript and appears to be largely confined to the mature RNA sequences present in the transcript (see Perry, 1976). Methylation has been claimed to be necessary for correct cleavage of rRNA precursors (Vaughan et al, 1967), but the underlying mechanism remains obscure.

Pre-ribosomal RNA also appears to be associated with protein from the earliest stages of its formation (Miller & Hamkalo, 1972) while pre-ribosomal RNP particles have been isolated from a variety of cells and have been shown to contain 45s pre-rRNA and other rRNA processing intermediates (for review, see Hadjiolov & Nikolaev, 1976). In addition to a variety of proteins found in mature ribosomes, the protein complement of pre-rRNP particles contains a considerable number of stable nucleolar-restricted proteins which may be involved in the processing of pre-rRNP particles to mature ribosomes (Kumar & Subramanian, 1975). Certainly, processing is soon halted in the absence of a continuous protein supply (Craig & Perry, 1970; Willems et al, 1969).

2.2.4 mRNA

Until very recently understanding of the processing of mRNA in eukaryotes has been limited by the difficulty in establishing unequivocally the identity of RNA species which act as precursors to mRNA species. However evidence has accumulated which very strongly indicates that a class of molecules called heterogeneous nuclear RNA (hnRNA) serves at least in part as precursors to mRNA. Before discussing the biogenesis of mRNA the relationship of hnRNA to mRNA will be examined and also their association with protein.

3. HETEROGENEOUS NUCLEAR RNA: ITS RELATION TO mRNA

3.1 Structural properties

HnRNA molecules appear to possess a variety of structural features which are not necessarily common to all classes of hnRNA molecule. They include oligo U sequences, short oligo A tracts, double-stranded hairpin-like structures, 5'-capped structures (see below) and long poly A tracts at the 3' end. A number of these properties are shared by mRNA as discussed in individual sections.

3.1.1 Size of hnRNA and mRNA

The size of hnRNA molecules can be conveniently estimated by their sedimentation characteristics in the ultracentrifuge and by their mobilities in polyacrylamide gels. When, however, non-denaturing conditions are employed anomalously high estimates for the molecular weight of hnRNA species are obtained, reflecting the very strong tendency for the hnRNA molecules to aggregate under these conditions (Bramwell, 1972) due to the formation of intermolecular duplexes (Fedoroff et al, 1977). Under denaturing conditions e.g. in formamide (Spohr et al, 1976) or DMSO (Derman et al, 1976) a more modest sedimentation range of from 10s to over 70s is apparent which nevertheless considerably exceeds the range of 8s to 30s estimated for cytoplasmic mRNA (Penman et al, 1968; Perry et al, 1976).

3.1.2 Oligo U sequences

Oligo U sequences of between 30 to 40 nucleotides have been identified in hnRNA (Burdon & Shenkin, 1972; Molloy et al, 1972). They appear to be concentrated in the largest size classes being relatively sparse in small (<28s) hnRNA (Molloy et al, 1972). Although such sequences have been claimed to be essentially absent in mRNA (Molloy et al, 1972), Edmonds et al (1976) have furnished evidence for a cytoplasmic location for 20-25% of the total poly U sequences of HeLa cells, of which most were thought to be associated with mRNA.

In poly A-containing hnRNA at least, the oligo U sequences appear to be far removed from the 3' end.

3.1.3 Oligo A sequences

The presence of short oligo A sequences in hnRNA which arise through transcription of the DNA was first reported for HeLa hnRNA (Nakazato et al, 1973; Nakazato et al, 1974). They are present in all size classes of hnRNA (Nakazato et al, 1974), are about 25 nucleotides long (Edmonds et al, 1976) and appear to be absent from mRNA with the possible exception of mRNA from Dictyostelium discoideum (Jacobson et al, 1974).

3.1.4 Poly A tracts

Between 20 and 30% of HeLa hnRNA molecules contain a poly A segment of 200-250 nucleotides at their 3' end and sequences of this type are present in all size classes of hnRNA (Jelinek et al, 1973). The poly A segment is not transcribed from the DNA like the oligo A sequences but is added to hnRNA molecules post-transcriptionally (see 5.1.1). Approximately 70% to 90% of HeLa cytoplasmic mRNA molecules contain poly A tracts (Brawerman, 1974).

3.1.5 Capped structures and internal methylated sequences

Approximately 40% to 60% of hnRNA molecules and almost all mRNA species so far investigated contain unusual capped structures at the 5' terminal position (Perry et al, 1975). Structures of this type have the general formula $m^7G_{(5')}$pppXpYp with the unusual 5' to 5' triphosphate linkage effectively blocking the 5' terminus. Cap structures have been classified into 3 groups: cap O where bases X and Y are unmethylated; cap I where X is methylated in the 2'-O-ribose position; and cap II where both X and Y are methylated in the 2'-O-ribose position. Cap structures occur in all size classes of hnRNA but are confined to the type I variety whereas all three varieties are found in mRNA although type O caps seem to be more the province of lower eukaryotes (see Shatkin, 1976).

Internal methylated sequences of structure m^6Ap are found in both hnRNA and mRNA with a frequency of about 4-6 per molecule in very large hnRNA molecules while small poly A-containing hnRNA and mRNA species have between 1 and 2 on average per molecule (Salditt-Georgieff et al, 1976).

3.1.6 Repetitive sequences

Approximately 10-30% of hnRNA from mammalian cells is in the form of repetitive sequences (Melli et al, 1971; Perry et al, 1970; Pagoulatos & Darnell, 1970) while the corresponding mRNA fractions have a much lower content of these sequences (Darnell & Balint, 1970; Molloy et al, 1974; Goldberg et al, 1973). In addition, the repetitive sequence elements are approximately 200 nucleotides long on average and are interspersed with unique sequences (approximately 1100 nucleotides on average) in a large percentage of the RNA e.g. about 80% in rat ascites cells (Holmes & Bonner, 1974).

3.1.7 Double-stranded regions (dsRNA)

In HeLa cells the various size classes of hnRNA all contain about 3% of their mass as intra-molecular double-stranded RNA so that large hnRNA species contain several duplex RNA regions per molecule (Jelinek& Darnell, 1972). However cytoplasmic mRNA is comparatively deficient in such structures.

Thus hnRNA and mRNA classes share a considerable number of structural features such as capped structures at the 5' end, long poly A tracts at the 3' end, and internal m^6Ap and oligo U elements and these features also serve to distinguish the two RNA classes from other classes of RNA molecule. Also, the larger size of hnRNA molecules can accommodate a reductive processing scheme to generate the smaller mRNA species while both classes of RNA have a base composition generally resembling that of DNA (Soeiro et al, 1966; Girard et al, 1965; Latham & Darnell, 1965). Moreover, there is an overwhelming amount of evidence demonstrating sequence homology between hnRNA and mRNA (Ruiz-Carrillo et al, 1973;

Imaizumi et al, 1973; Georgiev et al, 1972; Spohr et al, 1974 inter alii). However although essentially all the poly A-containing cytoplasmic mRNA sequences are represented in the hnRNA population, a considerable proportion of the hnRNA sequences are not represented in cytoplasmic mRNA (Perry et al, 1976; Levy & McCarthy, 1976) while the sequence complexity (i.e. total length of diverse non-repetitive sequence) of hnRNA is usually 5 to 10 fold greater than that of polysomal mRNA (Getz et al, 1975; Hough et al, 1975).

3.2 Kinetic relationships

On the basis of purely static qualities, therefore, a considerable wealth of evidence has been amassed which suggests that hnRNA may serve, at least in part, as a precursor to mRNA. However, because of the rapid degradation of a large proportion of the hnRNA population (e.g. Penman et al, 1968) and because of difficulties with chase experiments it has been difficult to demonstrate unambiguously a kinetic relationship between hnRNA and mRNA (see Lewin, 1975). Also it has been recently demonstrated that hnRNA is heterogeneous with respect to its kinetic characteristics by exhibiting multiple decay rates in HeLa cells including a relatively prominent component with a half-life of 150 minutes. Therefore, various contingency approaches have been adopted including examination of the kinetics of labelling of defined segments (e.g. the 3' poly A tract and the 5' capped sequences) and of the kinetics of formation of specific mRNA species.

3.2.1 Kinetics of polyadenylation

The adenosine analogue, cordycepin, has been found to block the polyadenylation of hnRNA in the nucleus and also the appearance of mRNA in the cytoplasm (Darnell et al, 1971, Adesnik et al, 1972). Although this could be consistent with a precursor-product relationship between hnRNA and mRNA, the underlying assumption that cordycepin does not inhibit transcription has been strongly challenged by Beach & Ross (1978).

Until quite recently the kinetics of poly A synthesis and transport appeared to endorse a model whereby a considerable proportion of the nuclear poly A sequences turned over in the nucleus (Perry et al, 1974). However, revision of some assumptions, notably concerning the half-lives of eukaryotic mRNA, has prompted a new model which predicts the conservative transport of nuclear poly A sequences to the cytoplasm (Puckett et al, 1975; Puckett & Darnell, 1977). Such a model has been sustained by some experimental evidence e.g. in the case of the biogenesis of some Adenovirus-2-specified mRNAs (Nevins & Darnell, 1978).

3.2.2 Kinetics of methylation

Detailed kinetic analysis of the labelling of the methylated components of mouse L-cell hnRNA and mRNA has revealed a conservative flow of label from the 5' terminal cap structures of hnRNA to those of mRNA (Perry & Kelley, 1976). Thus the 5' terminal cap I structures appear to be derived from the cap I structures of hnRNA. However, the cap II structures, present in some mRNA species, probably arise by a secondary methylation that occurs after the mRNA has been transported to the cytoplasm (Perry & Kelley, 1976).

Initial kinetic analysis of the incorporation of methyl label into internal m^6Ap residues suggested that only a portion of such structures in nuclear RNA were transferred to the cytoplasm (Perry & Kelley, 1976). However it has recently been shown that m^6A is rapidly eliminated in the cytoplasm (Sommer et al, 1978). Accordingly, re-evaluation of the kinetic data favours the transport of at least the majority of the nuclear m^6Ap to the cytoplasm, a proposal which is strongly supported by nearest-neighbour sequence analysis and by estimation of m^6A per nucleotide ratios for hnRNA and mRNA (Schibler et al, 1977; Lavi et al, 1977).

3.2.3 Biogenesis of specific types of mRNA

The presence of specific mRNA sequences in hnRNA has been examined by two major approaches. One of these

employs molecular hybridization techniques using purified complementary DNA (cDNA - prepared from the purified specific mRNA using the enzyme reverse transcriptase - for review, see Verma, 1977) or a plasmid DNA, containing the relevant gene or restriction endonuclease fragments of such, to probe for sequences homologous to the original mRNA. The other approach is based on the identification of specific protein products whose synthesis is stimulated by translation of specific mRNA-containing RNA molecules in a cell-free translation system or in xenopus oocytes. By using these techniques investigators have claimed the presence in hnRNA of mRNA specific for globin (Williamson et al, 1973; Imaizumi et al, 1973), immunoglobulin (Rabbitts, 1978) histone (Melli et al, 1977), albumin (Strair et al, 1978), ovalbumin (Roop et al, 1978), and protamine (Iatrou & Dixon, 1978).

That large nuclear RNA species act as precursors to specific mRNA sequences has also been suggested by kinetic experiments. Generally these experiments use a pulse-label with a radioactive precursor to RNA to label cellular RNA for a few minutes, followed by a chase where further RNA labelling is prevented either by dilution of the radioactive label with a vast excess of unlabelled precursor or by actinomycin D-induced inhibition of RNA synthesis. By using hybridization probes to detect specific mRNA sequences the fate of the labelled nuclear RNA which contains specific mRNA sequences can be followed in the absence of further incorporation of label. Using these procedures several investigators have established the existence in erythroid cell nuclei of at least 2 RNA species, 15-16s and 10s which contain globin mRNA sequences (Ross, 1976; Curtis & Weissman, 1976; Bastos & Aviv, 1977; Kwan et al, 1977; Strair et al, 1977; Knöchel & Grundmann, 1978; Hayashi & Mikami, 1978). In addition a large 27s-28s nuclear RNA species which appears to contain globin mRNA sequences has been reported in erythroid cells (Bastos & Aviv, 1977; Strair et al, 1977; Niessing, 1978; Haynes et al, 1978)

while evidence has been presented for a processing scheme of the type: 27s → 15s → 10s (Bastos & Aviv, 1977). However Haynes et al (1978) have also shown that α-or β-globin cDNA-containing plasmids fail to hybridize to the 27s nuclear RNA species in Friend erythroleukaemic cells, thereby strongly suggesting the absence of globin mRNA sequences in this species.

Similar experiments have indicated the presence in some eukaryotic cell nuclei of discrete nuclear RNA species containing specific mRNA sequences including: 26s and 17s (mature size) precursors to albumin mRNA in rat liver and in a hepatoma cell line (Strair et al, 1978); 16-17s, 11s, 7.5s and 6s (mature size) precursors to protamine mRNA in trout testis (Iatrou & Dixon, 1978); 40s, 24s and 13s (mature size) precursors to immunoglobulin light chain mRNA in a mouse myeloma cell line (Gilmore-Herbert & Wall, 1978); and a variety of precursors to mRNAs coding for immunoglobulin γ_{2b} heavy chain, κ light chain and the constant region portion of a κ light chain in MPC-11 cells, including presumptive primary transcripts of respectively 11 kilobases (~40s), 5.3 Kb (~28s) and 3.3 Kb (~23s) (Schibler et al, 1978). Pulse-chase kinetics have established in many of the above cases the sequential appearance of label in the various precursors in order of decreasing size and Gilmore-Herbert & Wall (1978) have demonstrated that the 13s nuclear RNA species becomes labelled before the first appearance of newly synthesized κ light chain mRNA in the cytoplasm. Finally, similar pulse-chase experiments have shown that the hnRNA of adenovirus-2-infected cells contains adenovirus mRNA sequences which, during a chase, become progressively located on smaller molecules eventually reaching the size of mature viral mRNA (Bachenheimer & Darnell, 1975).

3.3 Transcription mapping

Another approach to examining the relationship between hnRNA and mRNA involves the identification of transcription units responsible for the synthesis of these two classes of RNA. For example, in an electron

microscopic study of Oncopeltus fasciatus embryos Foe et al (1976) were able to distinguish ribosomal transcripts from nuclear non-ribosomal transcripts on the basis of a number of parameters such as morphology of underlying chromatin (see 1.2.2), organization of transcription units (see 1.2.3) and the size of the transcriptional units. In particular the estimated sizes of non-ribosomal nuclear RNA transcription units were very large and included values up to 7 μm in length (~32 Kb) as was the case also for nuclear non-ribosomal transcription units in Drosophila melanogaster embryos (McKnight et al, 1978). Such very large values are consistent with the large sizes of some hnRNA species.

The investigation of specific hnRNA transcripts has of necessity been limited to a few special cases. For example in the highly specialized posterior silk gland cells of Bombyx mori over 80% of the protein synthesis is devoted to the production of silk fibroin, and a number of distinguishing characterisitcs have permitted electron microscopic visualization of the silk fibroin transcription unit (McKnight et al, 1976). The estimated size of these transcription units (~18,000 base pairs) however does not exceed the size of the very large fibroin mRNA by very much so that it is difficult to resolve a slightly larger RNA precursor to fibroin mRNA from the mature species (Lizardi, 1976).

In a different approach Darnell and his co-workers have been able to define the functional transcriptional units of adenovirus type 2 DNA early and late in infection by RNA-DNA hybridization studies (Evans et al, 1977; Weber et al, 1977; Goldberg et al, 1977a; Darnell et al, 1978). In particular it has been clearly shown that late in infection a very long transcriptional unit is responsible for coding a large primary transcript which is formed as the obligatory precursor to Ad-2-mRNA. In addition, in uninfected HeLa cells it appears that hnRNA molecules from 2 to >20 kilobases represent primary transcripts (Derman et al, 1976; Goldberg et al, 1977a) while UV-transcription mapping has defined target sizes

for mRNA which are considerably greater than their own length and therefore consistent with the proposal that hnRNA includes the primary transcripts for mRNA formation (Goldberg et al, 1977b; Goldberg et al, 1978).
Thus an overwhelming amount of evidence indicates that precursors to mRNA are to be found among the hnRNA populations. However, not all hnRNA sequences can be processed to mRNA and other sequences in hnRNA have been considered to be non-informational and rapidly degraded (Georgiev, 1969), to have a structural role (Herman et al, 1976; Herman et al, 1978) or to have a regulatory role (Likhtenstein & Shapot, 1976; Monahan & Hall, 1974; Kolodny, 1975; Davidson et al, 1977).

4. HETEROGENEOUS NUCLEAR RNA: ITS ASSOCIATION WITH PROTEIN

Evidence for an association of hnRNA with protein has been obtained from two major sources. On the one hand ultrastructural studies have been employed utilizing the techniques of light and electron microscopy in conjunction with specific chemical staining, specific enzymic digestion, autoradiography, immunofluorescence and in situ hybridization. On the other hand ribonucleoprotein complexes containing hnRNA (hnRNP particles) have been isolated from eukaryotic nuclei and a battery of analytical techniques has been engaged to study their structural properties.

4.1 Ultrastructural studies

4.1.1 Extraordinary chromosomal systems

Two extraordinary chromosomal systems, the lampbrush chromosomes of amphibian oocytes and the giant polytene chromosomes of dipteran salivary glands, have recommended themselves because of their large size which make them amenable to study by phase-contrast microscopy.

The lateral loops of lampshade chromosomes have been shown to consist of a DNA-protein axis surrounded by a matrix of RNP which increases in length progressively along each loop. When autoradiographic studies employing [^{3}H]-uridine as a RNA precursor were applied to this

system sequential labelling was evident along each loop (Gall & Callan, 1962). In addition, electron microscopic visualization of the dispersed chromosomes has clearly indicated the presence of long RNP fibrils transcribed from the DNA (Miller & Baaken, 1972). Moreover, the demonstrable presence of loops and RNA matrices at many loci throughout all the chromosomes served to identify the nascent RNA as hnRNA rather than rRNA (Callan & Lloyd, 1960). Finally, immunofluorescent methods have demonstrated that proteins from hnRNP particles prepared by biochemical techniques from the lampbrush chromosomes (see 4.2.) are associated specifically with the lateral loops (Scott & Sommerville, 1974).

The giant polytene chromosomes of dipteran salivary glands exhibit a series of bands when stained with dyes such as feulgen reagent, alternating with more lightly staining interband regions. Interband regions are defined by extended DNA fibres while the bands are comprised of folded DNA fibres which may be unwound to become available for transcription. The unwound regions are the sites of intensive RNA synthesis forming large RNA puffs, notably the relatively enormous Balbiani rings of Chironomus salivary gland chromosomes which are composed of a diffuse mass of electron opaque-granules (400-500 Å diameter), short threads (180-220 Å diameter) and associated fine chromatin fibrils. The granules appear to be formed from the short threads then released into the nucleoplasm en route to the nuclear envelope where many granules have been observed by electron microscopy to assume rod-like shapes (200 Å diameter) which appear to enter the nuclear pores (Stevens & Swift, 1966; Daneholt et al, 1976).

4.1.2 Other chromosomal systems

Less extraordinary chromosomal systems are not so amenable to ultrastructural investigation because the highly compacted chromatin structure precludes easy examination of associated RNP structures. The problem has been minimized by a number of approaches which include: careful dispersion of the chromatin complements

of gently-lysed nuclei as pioneered by Miller & Beatty (1969); use of chelating agents to bleach chromatin while maintaining the contrast of RNP structures in electron micrographs (Monneron & Bernhard, 1969); and extraction of the DNA from isolated nuclei using buffers containing high salt, frequently in conjunction with a DNase treatment.

The chromosome spreading technique has enjoyed considerable success in permitting direct visualization of the fine structure of individual genetic loci active in RNA synthesis. For example, electron micrographs of dispersed HeLa chromatin have disclosed attached RNP fibrils arranged along a loop axis (Miller & Bakken, 1972). Since >95% of all RNA being synthesized in the HeLa nucleus is hnRNA it has been concluded that very probably such fibrils contain nascent hnRNA molecules. Also, distinction between ribosomal and non-ribosomal transcription units has been possible in a variety of spread chromosomal preparations on the basis of chromatin morphology, proximity of adjacent transcription units, size of transcriptional units and other parameters (Foe et al, 1976; Foe, 1978; McKnight et al, 1978; Franke et al, 1978; Scheer, 1978). The estimated sizes of the non-nucleolar transcription units are consistent with the production of very large hnRNA molecules (see 3.3.).

The regressive staining procedure using chelating agents to bleach chromatin in electron micrographs has been successfully applied to the study of rat liver nuclei (Monneron & Bernhard, 1969; Puvion & Bernhard, 1975). Three major classes of extranucleolar RNP have been discerned in these nuclei: interchromatin granules (200-250 Å diameter) which turn over very slowly; perichromatin granules (400-450 Å diameter) which can be visualized in the channels leading to the nuclear pores where they seem to disappear; and perichromatin fibrils which are rapidly and intensively labelled with [^{3}H]-uridine which are thought to contain nascent hnRNA.

In addition, evidence has been presented in favour of a precursor-product relationship between perichromatin fibrils and granules via intermediate smaller perichromatin granules (Monneron & Bernhard, 1969). Most importantly however, there appeared to be structural connections between the various RNP components, chromatin, nucleoli and nuclear membrane.

Use of high salt buffers to remove the great majority of the DNA from isolated nuclei results in the maintenance of nuclear integrity while disclosing a fibro-granular network of RNP extending throughout the nucleus and apparently linking chromatin, nucleoli and nuclear membrane (Narayan et al, 1967). When the high salt treatment is supplemented by DNase digestion to reduce the residual DNA content of the extracted nuclei to <1% a similar type of RNP network is apparent (Herman et al, 1978; Faiferman & Pogo, 1975; Miller et al, 1978) while electron microscope autoradiography studies have confirmed that hnRNA is contained in such a nuclear RNP matrix (Herman et al, 1978). However, further exposure of the treated nuclei to RNase has indicated that the continued nuclear integrity is maintained by a nuclear protein matrix or skeleton with connections from an external proteinaceous lamina containing nuclear pore complexes through a fibrous protein network to residual nucelolar protein constituents (Berezney & Coffey, 1977).

HnRNP complexes have been shown to be bound to the nuclear membrane and only relatively drastic nuclear disruption protocols (sonication, disruption in the French press, etc.) are able to release the complexes as free hnRNP particles (Faiferman & Pogo, 1975), provided that endogenous proteolysis is inhibited (Miller et al, 1978). Moreover, if chromatin is removed as above, the RNP complexes remain bound to the nuclear protein matrix, yet if the nuclear skeleton is dissolved by detergents the RNP complexes remain attached to the chromatin cables (McKnight & Miller, 1976).

TABLE 2 Diagnostic Features of Isolated HnRNP Complexes

Diagnostic Feature	Characteristics of Feature	References
Heterogeneous sedimentation of HnRNP particles on sucrose density gradients	Commonly 30s->250s, although in tissues with high endogenous RNase activity the use of an RNase inhibitor is required to elicit normal profile. Controlled action of endogenous or exogenous RNase promotes formation of relatively homogeneous 30s-50s monoparticles	Georgiev & Samarina (1971); Pederson (1974)
Heterogeneous sedimentation of rapidly-labelled RNA extracted from the particles on SDS-sucrose density gradients	Commonly 5s-50s although in some tissues considerably higher values have been recorded. A correlation has been observed between the sedimentation coefficient of an hnRNP particle population and its component rapidly labelled RNA	Faiferman et al (1970); Niessing & Sekeris (1971b); Stévenin et al (1970)
Protein:RNA Ratio of approximately 4:1 or greater	Conveniently reflected by a buoyant density of about 1.40 $g.cm^{-3}$ in CsCl density gradients. Occasionally estimated by direct chemical analysis	Ducamp & Jeanteur (1973); Faiferman et al (1970)
Heterogeneous polypeptide profile on SDS-polyacrylamide gels	Generally a few major proteins from 30,000-45,000 daltons and several minor species >45,000	Beyer et al (1977); Kamm et al (1977); Pederson (1974)
Particulate structure upon electron microscopic examination	Polyparticle structures based upon a roughly spherical repeat unit, commonly about 200Å in diameter. A correlation exists between the sedimentation coefficient range of an hnRNP particle population and the number of repeating units	Samarina et al (1967a); Beyer et al (1977); Martin et al (1978); Samarina et al (1967b)

4.2 Biochemical studies

4.2.1 Isolation of hnRNP complexes

HnRNP complexes have been isolated from eukaryotic nuclei by two major methods: extraction of intact nuclei using isotonic pH 8.0 buffer as pioneered by Samarina et al (1966) or isolation from nuclei disrupted by mechanical means (e.g. sonication - Pederson, 1974; French press - Faiferman & Pogo, 1975; Nitrogen cavitation bomb - Faiferman & Pogo, 1975) or chemical means (e.g. by 0.5M NaCl - DNase treatment - Faiferman & Pogo, 1975; detergent - Stévenin & Jacob, 1972; hypotonic buffer - Moulé & Chauveau, 1968). Methods involving extraction of intact nuclei permit removal of pre-ribosomes by prior extraction in isotonic salt pH 7.0 buffer while methods entailing nuclear disruption are usually accompanied by brief sedimentation of the nuclear lysate to remove nucleoli, and prior exposure of cells to low (~0.04 μg/ml) concentrations of actinomycin D serves to inhibit pre-ribosome formation (Perry, 1963). Resolution of the hnRNP complexes is usually achieved by centrifugation through sucrose density gradients which may permit isolation of hnRNP complexes free from contaminating chromatin, nucleosolic proteins and nuclear membrane fragments (Pederson, 1974). Using these procedures experimenters have been able to isolate hnRNP particles from a wide variety of eukaryotic organisms including mammals (e.g. Albrecht & van Zyl, 1973), birds (Morel et al, 1971), insects (Armelin & Marques, 1972) amphibians (Sommerville, 1973), sea urchin (Wilt et al, 1973), slime mould (Firtel & Pederson, 1975) and higher plants (Ajtkhozin et al, 1975)and from a wide variety of cell types (Pederson, 1974; Martin et al, 1974; Matringe & Jacob, 1972 inter alii). Fulfillment of several diagnostic criteria identifies the material under study as hnRNP particles (see Table 2).

4.2.2 Structural aspects of hnRNP particles

The substructure of hnRNP complexes extracted from rat liver nuclei has been shown by electron microscopy

studies to be particulate in form with a correlation between the number of repeat units (monoparticles) in a specific polyparticle size class and the sedimentation coefficient exhibited by the polyparticle population, (Samarina et al, 1967a; Samarina et al, 1968). The monoparticles can be generated from a polyparticle preparation by the controlled action of endogenous or exogenous ribonuclease activity so that relatively large quantities of monoparticles can be prepared (Georgiev & Samarina, 1971). Closer electron microscopic examination of purified monoparticles extracted from rat liver nuclei indicates that they have a discoidal shape with dimensions of 180x180x60 Å (Samarina et al, 1967a; Lukanidin et al, 1972). However other investigators have produced evidence of size heterogeneity (200 Å - 300 Å diameter) for monoparticles from rat liver and other tissues (Monneron & Moulé, 1968; Martin et al, 1978; Beyer et al, 1977; Karn et al, 1977; Albrecht & van Zyl, 1973) while a monoparticle preparation from rat brain nuclei has been claimed to have different size classes ranging from 100 Å to 300 Å (Stévenin et al, 1976).

4.2.2.1 Properties of the RNA component

The rapidly labelled RNA isolated from HeLa hnRNP particles is heterogeneous in size (see Table 2) while there appears to be a correlation between the sedimentation coefficients of hnRNP particle populations and those of their corresponding extracted RNAs (Niessing & Sekeris, 1971b; Stévenin et al, 1970; Samarina et al, 1967a). Moreover the RNA associated with hnRNP particles appears to be heterogeneous in terms of radioactive labelling characteristics. In addition to the heterogeneous rapidly labelled RNA component several groups have produced evidence for a stable RNA component usually consisting of about 5 discrete species (estimated range of 4.5s to 8s) when analysed by polyacrylamide gel electrophoresis (Deimel et al, 1977; Northemann et al, 1977; Guimont-Ducamp et al, 1977; Howard, 1978).

Evidence that the rapidly labelled RNA component of hnRNP particles is equivalent to hnRNA has been obtained from several quarters, including: radioactive labelling kinetics and sensitivity to actinomycin D (Pederson, 1974; size (see Table 2); sensitivity to hormone treatment (Knowler, 1976); base composition (Georgiev & Samarina, 1971); distinctive structural elements e.g. 3' poly A tracts (Samarina et al, 1973; Quinlan et al, 1977), oligo A sequences (Kinniburgh & Martin, 1976a), oligo U sequences (Kish & Pederson, 1977) and dsRNA regions (Calvet & Pederson, 1977; Calvet & Pederson, 1978); and sequence complexity (Firtel & Pederson, 1975). Most importantly, hybridization studies have shown that sequences present in cytoplasmic poly $(A)^+$ mRNA are also represented in the RNA of nuclear hnRNP from mouse ascites cells (Kinniburgh & Martin, 1976b) and amphibian oocytes (Sommerville & Malcolm, 1976), while there appears to be a greater proportion of mRNA sequences in 30s monoparticles from rat liver nuclei than in 80s to 150s polyparticles (Alonso et al, 1978). Finally, the RNA from 30s hnRNP particles has been shown to contain nucleus-restricted sequences as does hnRNA (Martin & McCarthy, 1972). Although it has been claimed that the bulk or all of the hnRNA can be isolated as the free hnRNP particles (Georgiev & Samarina, 1971; Pederson, 1974) other investigators have found that a substantial proportion remains bound to the chromatin (Augenlicht & Lipkin, 1976; Kimmel et al, 1976).

4.2.2.2 Properties of the protein component

Although initial investigations suggested a very homogeneous protein complement in hnRNP particles consisting of a single polypeptide, informatin, (40000 daltons) (Krichevskaya & Georgiev, 1969) there now appears to be general consensus that there are a few major polypeptides of about 30,000 to 45,000 daltons and several minor species from 45,000 to 150,000 daltons or more (Pederson, 1974; Beyer et al, 1977; Karn et al, 1977). The smaller major polypeptide species, the "core" polypeptides, have

been suggested to function in the stabilization and packaging of hnRNA (Beyer et al, 1977) while the minor polypeptides have been variously considered to be contaminants (Patel & Holoubek, 1977) or to harbour possible processing enzymes which might nevertheless have only a transient association. Tissue specificity has been demonstrated for the polypeptide complements of hnRNP particles isolated from disrupted nuclei (Pederson, 1974). However other investigators have found that the polypeptide profiles of monoparticles extracted from various types of intact nuclei were almost identical (Martin et al, 1974), quite similar (Beyer et al, 1977) or indicative of a relatively close correspondence in the core polypeptides and considerable variation in the minor high molecular weight species (Karn et al, 1977).

The charge characteristics of hnRNP polypeptides are such that there are two major groups: a moderately acidic group accounting for 60% of the total in HeLa and including the majority of the high molecular weight polypeptides; and a slightly basic group including the majority of HeLa, and all of the rat liver, core polypeptides (Pederson, 1974; Beyer et al, 1977, Karn et al, 1977). In addition several hnRNP proteins are subject to post-synthetic modifications notably phosphorylation and methylation which give rise to phosphoserine, phosphothreonine and N^G,N^G-dimethylarginine in particular (Karn et al, 1977; Blanchard et al, 1978a).

Polypeptides associated specifically with the poly A segment of hnRNP.RNA usually include 2 to 3 major species, especially a polypeptide of about 75,000 daltons which is shared by the poly A sequence in mRNP.RNA, and a similar number of minor species (Kish & Pederson, 1975; Molnár & Samarina, 1975). The 75,000 dalton species may be bound to oligo U regions which have been suggested by Kish & Pederson (1977) to be base paired to a part of the poly A segment. However double-stranded RNA regions in hnRNP.RNA are almost devoid of protein (Calvet & Pederson, 1978).

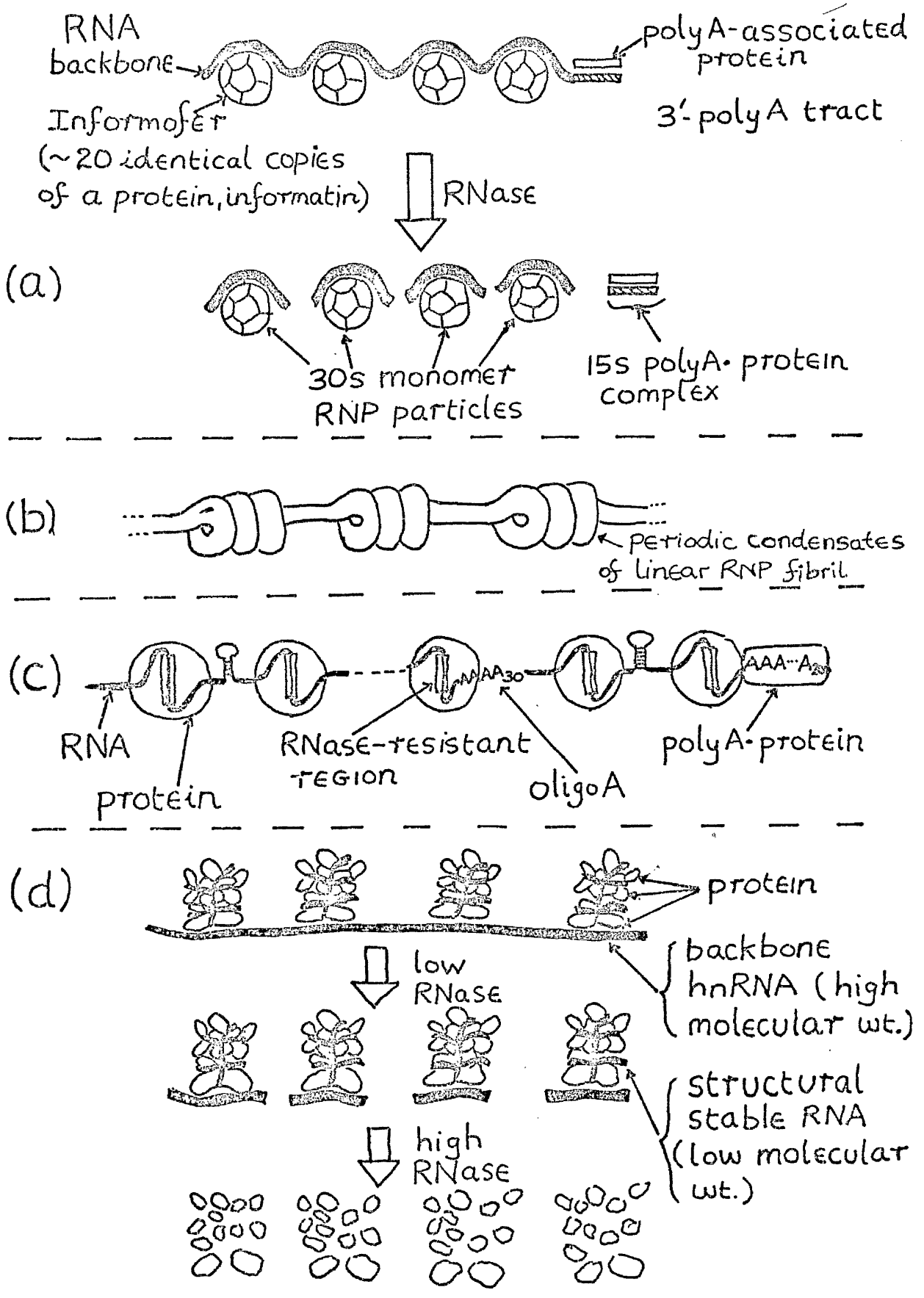


Fig 5 Models of HnRNP Structure

Models considered by (a) Samarina et al (1973); (b) Malcolm & Sommerville (1977); (c) Martin et al (1978); and (d) Sekeris & Niessing (1975)

4.3 Models of hnRNP structure

An initial model of hnRNP structure postulated by Samarina et al (1968) was an attempt to reconcile various observations including: a protein to RNA ratio of about 4:1 (see Table 2); a homogeneous protein complement containing one major polypeptide, informatin, of molecular weight 40,000; a correlation between hnRNP particle size, the size of the RNA that could be extracted from the particles and the number of repeating monomer units (see above); a reduction to relatively homogenous 30s monoparticles in response to mild RNase treatment; and the stability of the 30s monomers to relatively high RNase concentrations and of the protein complement to high salt concentrations (see Georgiev & Samarina, 1971). Consequently the large RNA component was envisaged to straddle several protein complexes, informofers, composed of about 20 identical copies of informatin, with the RNA being located largely on the surface of the protein complex while the predominant stabilizing force was conferred by protein-protein interactions (see Fig 5). Revision of the model has been prompted by the observation that the poly A component is not represented in 30s monoparticles but is released by mild RNase treatment of polyparticles as a separate poly A-protein complex sedimenting at about 15s (Samarina et al, 1973).

However this model has been challenged in many quarters, notably with respect to the demonstrable heterogeneity of the polypeptide complement (see 4.2.2.2 above). Also the release of proteins from the hnRNP particles in response to increasing salt concentrations or detergent treatment and the effect of RNase treatment of the particles has been suggested to reflect a folded RNP structure with the RNA largely located in the interior (Stévenin & Jacob, 1972; Stévenin et al, 1973; Stévenin & Jacob, 1974). Supportive evidence for a model of this type has been described by Malcolm & Sommerville (1977) who have concluded that the protein beads (informofers) of amphibian oocyte hnRNP particles have no existence

independent of the RNA and are formed by periodic condensates of linear RNP as a result of RNA secondary structure and/or protein-protein interaction (see Fig 5). However in this case no differential removal of proteins from RNP particles was evinced as in the case of hnRNP particles from HeLa (Pederson, 1974) and from rat brain (Stévenin & Jacob, 1974; Gallinaro-Matringe et al, 1975).

Recently a model of hnRNP structure has been proposed which is largely an extension of the schemes mentioned above but which also takes into account the low affinity of double-stranded RNA regions in hnRNP for protein and the presence of a reduced amount of protein associated with oligo A regions (Martin et al, 1978 - see Fig 5). However this model as with the others outlined above does not cater for the presence of small stable nuclear RNA species claimed by many to be associated with hnRNP particles (see 4.2.2.1 above). Nevertheless a model by Sekeris & Niessing (1975) has implicated stable RNA species in order to explain the results they obtained from digestion of hnRNP particles with high concentrations of RNase and from double labelling experiments. The authors have invoked a structural role for the stable RNA species (see Fig 5).

5. PROCESSING OF HnRNA

5.1 Mechanisms of hnRNA processing

5.1.1 Polyadenylation

Large polyadenylate tracts occur at the 3' end of about 20-30% of hnRNA and about 70%-90% of mRNA molecules in several cell types (see 3.1.4 above). The inability to detect "free" poly A in nuclei after very brief radioactive labelling e.g. 45 seconds (Jelinek et al, 1973) has suggested that the poly A sequence synthesized in the nucleus is incorporated at the 3' end of hnRNA in a series of sequential additions of AMP residues from substrate ATP, the reaction being catalysed by poly A synthetase (see 5.2.1 below). Poly A addition has been considered to be a post-transcriptional event since it has been shown that HeLa DNA does not contain oligo dT regions long enough to code for poly A (Birnboim et al, 1973). In addition, actinomycin D treatment of cells for 1-2 minutes arrested hnRNA synthesis while the synthesis of poly A on all sizes of hnRNA proceeded (Jelinek et al, 1973).

Poly A addition appears to be generally an event which occurs relatively early after transcription. Thus Derman & Darnell (1974) have obtained evidence that poly A is added to some hnRNA almost immediately after transcription although it also appeared to be added to some hnRNA, which may include cleavage products, some time after transcription. However, the discovery of rapid terminal poly A elongation reactions in the nucleus (Brawerman, 1976; Sawicki et al, 1977) has complicated the interpretation of these experiments. When specific mRNA precursors have been examined the evidence generally indicates that polyadenylation is a comparatively early processing event and precedes splicing in the biogenesis of certain immunoglobulin mRNA species (Schibler et al, 1978), β-globin mRNA (Kinniburgh et al, 1978), SV40-specified mRNA (Lai et al, 1978) and adenovirus-specified mRNA (Nevins & Darnell, 1978). However, in trout testis 16s-17s, 11s and 7.5s nuclear RNA species which contain protamine mRNA sequences do not appear to be polyadenylated

although a 6s nuclear RNA population, of the same size as mature protamine mRNA and which hybridized to protamine cDNA, contained polyadenylated sequences (Iatrou & Dixon, 1978).

Poly $(A)^+$ and poly $(A)^-$ hnRNA appear to be metabolically distinct populations with the poly $(A)^+$ hnRNA from Drosophila behaving as two kinetic components with half-lives of 20 minutes and 180 minutes whereas the poly $(A)^-$ hnRNA population decays with an initial half-life of 10-15 minutes (Levis & Penman, 1977). In addition, two functional classes of polyadenylated hnRNA have been distinguished in resting lymphocytes (Berger & Cooper, 1978). One of these included species which were labelled predominantly with exogenous radioactive precursors supplied during a brief labelling time and these appeared to be processed to polyadenylated mRNA. The other species primarily incorporated scavenged labelled precursors made available during a chase incubation and they appeared to be confined to the nucleus. Although previous studies on the kinetics of poly A synthesis and transport indicated that a considerable proportion of the nuclear poly A sequences turned over in the nucleus (see 3.2.1 below), it has been shown that 70-100% of the nuclear poly A sequences present in the polyadenylated nuclear RNA transcribed from the adenovirus 2 late transcription unit are transported to the cytoplasm (Nevins & Darnell, 1978).

5.1.2 Capping

Mechanisms involved in the formation of capped structures at the 5' termini of some hnRNA and mRNA species in eukaryotic systems have been most extensively investigated in the case of certain virus-specified mRNAs. In the case of vaccinia virus, reovirus and cytoplasmic polyhedrosis virus the capping reaction appears to involve the transfer of a guanosine monophosphate moiety from GTP to the 5'-diphosphate-terminated mRNA (ppX……) followed by methylation at the 7 position of the guanine, thereby generating a m^7GpppX…… terminus (see Shatkin, 1976). However, in the case of vesicular stomatitis virus a different mechanism involving capping of a 5'-monophosphate

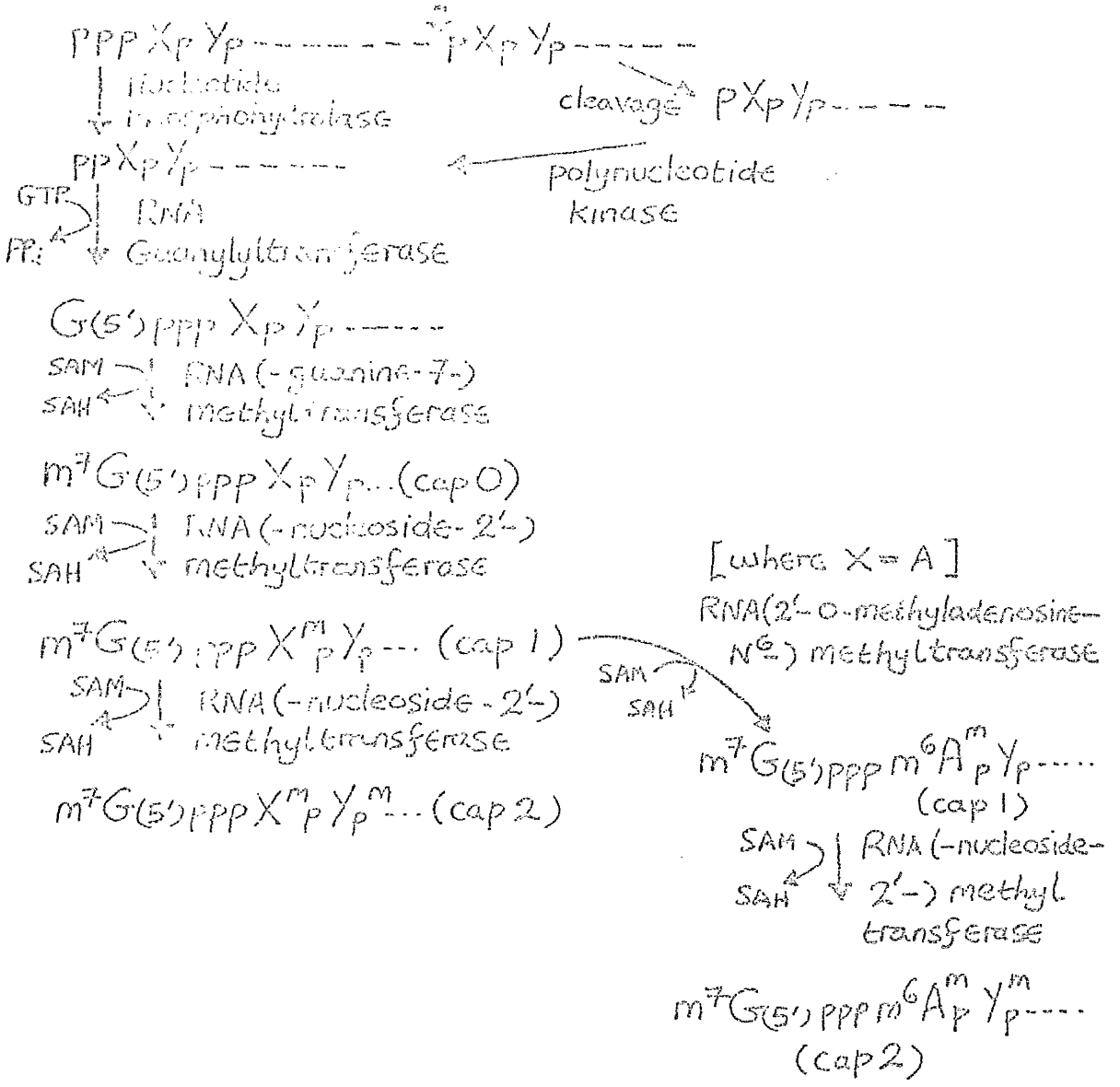


Fig 6 Possible Scheme for Biogenesis of 5' Terminal Cap Structures in HnRNA and mRNA in Eukaryotes

terminus (pX·····) appears to prevail (Abraham et al, 1975).

The penultimate nucleotide at the 5' terminus of capped viral mRNA appears to contain exclusively a purine base. Consequently, it is highly probable that capping of eukaryotic viral mRNA species occurs at the sequence corresponding to the point of initiation of transcription, a conclusion which is reinforced by the high degree of coincidence between the promoter site and capping site relevant in the biogenesis of some adenovirus-2-mRNA species (Ziff & Evans, 1978). In addition, inspection of the nucleotide sequences of a variety of different eukaryotic genes has revealed that there is a remarkable sequence homology based on a 12 base pair region which includes the cap site - as with genes specifying mouse β-globin mRNAs (Konkel et al, 1978), adenovirus mRNA (Ziff & Evans, 1978) and mouse immunoglobulin λ light chain mRNA (Tonegawa et al, 1978). Conceivably, therefore, such a sequence could constitute a eukaryotic RNA polymerase II promoter site in analogy to the well-documented sequence homology characterizing prokaryotic promoter sites (Pribnow, 1975; see Gilbert, 1976; Post et al, 1978).

In the case of eukaryotic cellular mRNA species the penultimate nucleotide at the 5' terminus is occasionally represented by a pyrimidine base in addition to the purine bases which more frequently occur at this position (e.g. Rottman et al, 1976). Accordingly, it is probable that in this case capping can occur at internal cleaved termini in addition to the sequence marking the initiation of transcription. Following an analysis of the 5' termini of mouse L cell hnRNA Schibler & Perry (1976) have envisaged a mechanism whereby 5' diphosphate termini serve as a common intermediate in the capping of sequences derived from the point of initiation of transcription and of sequences derived from internal cleavages (see Fig 6).

Capping reactions have been considered to be among the earliest hnRNA processing events and, in the case of certain eukaryotic viral mRNA species, may take place shortly after the very onset of transcription (Furuichi

et al, 1976; Furuichi, 1978). Moreover Levis & Penman (1978a) have shown that >75% of poly $(A)^+$ hnRNA and >50% of poly $(A)^-$ hnRNA from Drosophila bear capped structures which may suggest that capping occurs before or very soon after polyadenylation.

5.1.3 Cleavage and splicing

As detailed in 1.2.3 many of the genes for specific mRNAs in eukaryotes have their coding sequences interrupted by intervening sequences which are not represented in the mature mRNA. Of the various models advanced to explain the biogenesis of RNA from "split genes", a scheme featuring transcription of both coding and intervening sequences, excision of the transcribed intervening sequences from a large RNA precursor and ligation of the transcribed coding sequences is strongly supported by the current evidence e.g. in the case of mRNA specifying β-globin (Kinniburgh et al, 1978; Tilghman et al, 1978b), ovalbumin (Roop et al, 1978) and immunoglobulin (Rabbitts, 1978). In addition the splicing mechanism has been shown to occur following transcription of SV40 late genes (Horowitz et al, 1978b) and appears to take place subsequent to polyadenylation of some adenovirus-specified mRNA precursors (Nevins & Darnell, 1978).

5.2 Enzymology

5.2.1 Polyadenylation

Poly A synthetase activity (RNA terminal riboadenylate transferase E.C 2.7.7.19) has been reported in a wide variety of eukaryotic cells (for review, see Edmonds & Winters, 1976; Jacob & Rose, 1978), and large scale purification has been achieved in a few cases (Winters & Edmonds, 1973a; Tsiapalis et al, 1975; Rose & Jacob, 1976). Research into enzyme activities of this type was spurred on by the discovery of large poly A tracts at the 3' end of hnRNA and mRNA which appeared to be added post-transcriptionally (see 5.1.1). Consequently an assumption was evolved, and has since been widely entertained, that the addition of the poly A to hnRNA, and thereby mRNA, represented the cellular function of these enzymes.

Poly A synthetase activity from several cell types has been located in the nucleus although several workers have described cytoplasm-based activities (e.g. Mans & Stein, 1974). In rat liver nuclei no detectable poly A synthetase activity was apparent in the nucleoli (Jacob et al, 1976) while "free" and chromatin-bound forms could be distinguished and they appeared to exhibit different primer specificities (Jacob et al, 1976; Rose et al, 1976; Rose et al, 1977). In addition, two poly A synthetase activities, distinguishable on the basis of their ionic requirements, and a homoribopolymer synthetase have been reported in association with 30s rat liver nuclear RNP particles (Niessing & Sekeris, 1972; Niessing & Sekeris, 1973).

The poly A synthetase activities show an extreme specificity for ATP as a substrate while optimal activity is expressed in the presence of a divalent cation such as Mn^{2+} or Mg^{2+}. The enzyme-catalysed reaction is:

$$\text{RNA primer} + n\ \text{ATP} \xrightarrow{Mn^{2+}/Mg^{2+}} \text{RNA}.n\ \text{AMP} + n\text{PPi}$$

5.2.2 Capping

Enzyme activities which have been implicated in 5' terminal capping reactions have been extensively studied in viral systems and latterly in HeLa cells (for an outline of reactions catalysed by different enzyme activities, see processing scheme outlined in Fig 6). In the instance of viral mRNA cap formation three enzyme activities viz RNA guanylyltransferase, RNA(guanine-7-) methyltransferase and RNA(nucleoside-2'-)methyltransferase have been implied and have been isolated and purified from vaccinia virions (Ensinger et al, 1975; Martin et al, 1975; Martin & Moss, 1975; Barbarosa & Moss, 1978; Monroy et al, 1978). In addition, RNA guanylyltransferase activity has been detected by Wei & Moss (1977) in HeLa cell nuclei while RNA(guanine-7-)methyltransferase and RNA(2'-O-methyladenosine-N^6-) methyltransferase have been purified from HeLa cell cytoplasm (Ensinger & Moss, 1976; Keith et al, 1978). However, as yet, no evidence has been presented for the isolation from eukaryotic cells of a RNA(nucleoside-2') methyl-

transferase similar to the vaccinia enzyme.

The cellular locations of the processing enzyme remains to be established although a nuclear location for the guanylyltransferase reaction and a cytoplasmic location for the methylation of the third nucleoside at the 2-O' position are very probable (Wei & Moss, 1977; Perry & Kelley, 1976). Moreover guanylyltransferase and methyltransferase activities similar to the ones above have been claimed to exist in association with rat liver nuclear 30s hnRNP particles (Bajszár et al, 1978).

5.2.3 Cleavage and splicing

Until very recently the enzymic activities which process hnRNA transcripts by cleavage and splicing have been totally undefined. However Blanchard et al (1978b) have presented evidence which suggests that a cell-free extract of HeLa cells can mediate correct cleavage of a precursor to a specific Adenovirus-2-mRNA species and splicing of the appropriate fragments to generate a product of the same size as the mature mRNA. The simple addition of labelled virus-specific RNA to nuclear extracts did not produce any evidence of specific processing. However when nuclei were isolated from briefly labelled Adenovirus-2-infected HeLa cells and incubated in vitro it was possible to detect processing of a 5Kb.RNA species (~28s) to a 2Kb.RNA species (~20s), which appeared to involve specific cleavage and splicing, but only in the presence of a cytoplasmic extract (Blanchard et al, 1978b).

6. AIMS OF THE PROJECT

The strategy pursued in the present study concerned an investigation of the enzymic activities which process hnRNA in HeLa cells. Initially attention was devoted to a study of HeLa nuclear hnRNP particles in order to define their functional roles with regard to the processing of hnRNA. Latterly the compass of the project was extended to include an investigation of other subnuclear fractions of HeLa cells which could be involved in hnRNA processing. The enzymic activities investigated in this study include: exoribonuclease; endoribonuclease; double strand-specific ribonuclease; RNA guanylyltransferase; RNA ligase and poly A synthetase.

MATERIALS AND METHODS

1. MATERIALS

1.1 Chemicals:

Bio-Cult Laboratories Ltd., Paisley, SCOTLAND
- Calf Serum
- Amino acids
- Vitamins

Glaxo Pharmaceuticals, London, ENGLAND
- Penicillin
- Streptomycin

Koch-Light Laboratories Ltd., Colnbrooke, ENGLAND
- Triton X-114 (Scintillation Grade)
- Triton X-100
- Toluene (analar grade)
- Trichloroacetic acid
- Bromophenol Blue
- 2-Mercaptoethanol

Hopkin & Williams Ltd., Chadwell Heath, ENGLAND
- Caesium Chloride

Sigma Chemical Company, London, ENGLAND
- Phosphoenol pyruvate (sodium, crystalline)
- Dithiothreitol
- N-ethyl maleimide
- S-adenosyl-L-methionine
- Coomassie Brilliant Blue G250
- Adenosine 3'5' diphosphate, sodium salt
- Adenosine 2',3' monophosphate
- Adenosine 5' monophosphate
- Uridine 5' monophosphate, sodium salt
- Cytidine 5' monophosphate
- Guanosine 5' monophosphate

Boehringer-Mannheim, GERMANY
- Nicotinamide adenine dinucleotide

Fisons Scientific Apparatus, Loughborough, ENGLAND
- Hyamine Hydroxide (1M solution in methanol)

Calbiochem Ltd., Hereford, ENGLAND
- Actinomycin D

1.1 Chemicals (cont):

P-L Chemicals Inc., Wisconsin, U.S.A.

5'-Polyadenylic acid

5'-Polyinosinic acid

5'-Polyuridylic acid

Guanosine 5' triphosphate, sodium

Cytidine 5'-triphosphate, sodium

Uridine 5'-triphosphate, sodium

Adenosine 5'-triphosphate, sodium

H. Reeve-Angel & Co. Ltd., London, ENGLAND

Whatman DE 81 paper (46 cm x 57 cm)

Whatman 3 mm paper (46 cm x 57 cm)

Whatman 3 mm paper discs (2.5 cm, diameter)

Whatman GF/C glass fibre discs (2.5 cm, diameter)

Whatman DE 81 paper discs (2.5 cm, diameter)

Schleicher & Schüll,

BA 85 filters, 25 mm diameter, 0.45 μm pore size

Pharmacia, Uppsala, SWEDEN

Sephadex G50 (medium)

Bio-Rad Laboratories, Richmond, California, U.S.A.

AG 501-X8 Ion exchange mixed bed resin (20-50mesh, analytical grade)

1.2 Enzymes

Sigma Chemical Company, London, ENGLAND

Pyruvate Kinase (rabbit skeletal muscle)

Deoxyribonuclease I (DN-EP) electrophoretically purified

P_1 nuclease

Alkaline phosphate (E.coli)

Calbiochem. Ltd., Hereford, ENGLAND

Pancreatic Ribonuclease (chromatographically homogeneous)

T_1 ribonuclease

Pronase

1.3 Radiochemicals

Miles Laboratories Ltd., Stoke Poges, ENGLAND

[8-^{3}H]-Polyadenylate (6.35 μg/μCi)

[5-^{3}H]-Polyuridylate (8.2 μg/μCi)

[^{3}H]-Polycytidylate (8.2 μg/μCi)

1.3 Radiochemicals (cont.)

Radiochemical Centre, Amersham, ENGLAND

[5,6-^{3}H]-Uridine (46 Ci/mmol)

[5,6-^{3}H]-Uridine 5'-triphosphate (50 Ci/mmol)

[8-^{3}H]-Adenosine 5' triphosphate (23 Ci/mmol)

[8-^{14}C]-Adenosine 5' triphosphate (1 mCi/mmol)

[α-^{32}P]-Guanosine 5'-triphosphate (350 Ci/mmol)

[^{32}P] -orthophosphate (10 mCi/ml) carrier free

TABLE 3

Constituents of Eagle's Minimal Essential Medium (MEM) as used in the Department of Biochemistry, University of Glasgow

MEM amino acids	mg/L
L-arginine HCl	126.4
L-cysteine	24.0
L-glutamine	292.3
L-histidine HCl	41.9
L-isoleucine	52.5
L-leucine	52.5
L-lysine HCl	73.1
L-methionine	14.9
L-phenylalanine	33.0
L-threonine	47.6
L-tryptophan	10.2
L-tyrosine	36.2
L-valine	46.9

MEM vitamins	mg/L
D-calcium pantothenate	2.0
Choline chloride	2.0
Folic acid	2.0
i-inositol	4.0
Nicotinamide	2.0
Pyridoxal HCl	2.0
Riboflavin	0.2
Thiamine HCl	2.0

MEM inorganic salts and other components	mg/L
$CaCl_2.2H_2O$	264.9
KCl	400.0
$MgSO_4.7H_2O$	200.0
NaCl	6800.0
$NaH_2PO_4.2H_2O$	140.0
$NaHCO_3$	2240.0
Phenol Red (Na)	17.0
D-glucose	4500.0
Streptomycin Sulphate	100.0
Penicillin	10^5 units

2. STANDARD SOLUTIONS

2.1 Cell Culture Solutions

Versene (0.6M Na_2EDTA, 0.17M NaCl, 3.4mM KCl, 10mM Na_2HPO_4, 2.4M KH_2PO_4, 0.002% (w/v) Phenol Red)

Trypsin/Citrate (0.25% (w/v) Trypsin, 10.5mM NaCl, 1.0mM sodium citrate, 0.002% (w/v) Phenol Red pH 7.8)

Balanced Salts Solution (BSS: Earle, 1943) (0.116M NaCl, 5.4mM KCl, 1mM $MgSO_4$, 1mM NaH_2PO_4, 1.8mM $CaCl_2$, 0.002% (w/v) Phenol Red. pH of solution adjusted to 7.0 with 5.6% (w/v) $NaHCO_3$)

Minimal Essential Medium (MEM)
As used in the Department of Biochemistry, University of Glasgow, this contains the amino acids, vitamins, salts and other components outlined in Table 3.

2.2 Cell Fractionation and Analytical Solutions

LSLMT (Low-salt, low-Magnesium-Tris buffer i.e. 0.01M NaCl, 1.5mM $MgCl_2$, 0.01M Tris-HCl pH 7.0)

HSHMT (High-salt, high-magnesium-Tris buffer i.e. 0.5M NaCl, 0.05M $MgCl_2$, 0.01M Tris-HCl pH 7.0)

LETS (Lithium, EDTA, Tris-SDS buffer i.e. 0.1M LiCl, 0.01M EDTA, 0.1M Tris, 0.2% SDS, pH 7.0)

Other solutions are defined in individual figure legends.

3. METHODS

3.1 Cell culture methods

HeLa cells (Gey et al, 1952) and occasionally BHK21/C13 cells (Stoker, 1962) were used in this study. Cells were cultured as monolayers in Roux bottles or in rotating 80 oz Winchester bottles according to the technique of House and Wildy (1965). They were grown in an atmosphere of 5% CO_2 on modified Eagle's Minimal Essential Medium to which 10% (v/v) calf serum had been added (see Table 3). In addition the medium used to support the growth of BHK21/C13 cells was supplemented by the addition of tryptose phosphate broth. Generally cells were seeded at either 25×10^6 or 14×10^6 per 80 oz glass bottle and harvested after 2 or 3 days growth respectively at 37.5°. At this stage cells were in the exponential phase of growth.

3.1.1 Maintenance of cell lines

Cell lines were maintained by sub-culturing from confluent monolayers. The medium was replaced by about 20 ml of trypsin-versene solution (1:4, v/v) and the 80 oz glass bottle was rotated for one minute whereupon the solution was replaced by a further 20 ml trypsin-versene and the procedure was repeated. Most of the final trypsin-versene solution was poured off from the bottle leaving about 2-3 ml and the bottle was allowed to rotate at 37° for 5 minutes by which time the cells had become opaque. 20 ml of fresh warmed medium was added to the bottle with shaking. Detached cells were thoroughly resuspended before an aliquot was taken for estimation of the total cell number using a haemocytometer.

3.1.2 Contamination checks

Stock cultures were routinely examined for contamination by fungi and yeasts by monitoring growth of aliquots at 32° on Sabouraud's Medium. Bacterial contamination was checked by adding aliquots to blood agar plates and brain-heart infusion broth before incubation at 37°. Additionally, PPLO agar plates were seeded with passaged

cells by piercing the agar surface with a charged Pasteur pipette. The plates were grown in an atmosphere of 5% CO_2 in N_2 at 37° and examined microscopically for any indications of the characteristic "fried egg" appearance of PPLO colonies.

3.1.3 Cell harvesting

The medium was decanted from the 80 oz bottles and the cells were washed twice with ice-cold BSS (100 ml per bottle). 30 ml of ice-cold BSS were added to each 80 oz bottle and the cells were scraped from the glass using a rubber wiper mounted on a stainless steel frame. The resulting cell suspension was divided between 50-ml conical centrifuge tubes and the cells were collected in the form of a pellet by centrifugation at 450 g for 2 minutes.

3.2 Preparation of hnRNP particles (see Fig 7)

The procedures used to isolate HeLa hnRNP particles were essentially those described by Pederson (1974). To suppress, preferentially, rRNA synthesis, cell monolayers, in the exponential phase of growth, were treated for 30 minutes with actinomycin D at a concentration of 0.04 μg/ml. The treated cells were exposed for an additional 15 minutes to [^{3}H]-uridine (details in figure legends). Cells were harvested and washed with BSS as described in 3.1.3 above. Washed cells were resuspended in LSLMT buffer (i.e. low salt, low Mg^{2+}, tris buffer - 0.01M NaCl, 1.5mM $MgCl_2$, 0.01M Tris-HCl pH 7.0) at 0°-4° and all subsequent operations were performed within this temperature range. The cells were allowed to swell for 10 minutes before being homogenized in a stainless steel Dounce homogenizer (12 strokes; clearance 0.002 ins) to give greater than 99% cell lysis as judged by phase-contrast microscopy. Nuclei were obtained as a pellet following centrifugation of the cell lysate for 3 minutes at 1300 g and were washed three times by centrifugation and resuspension in LSLMT buffer.

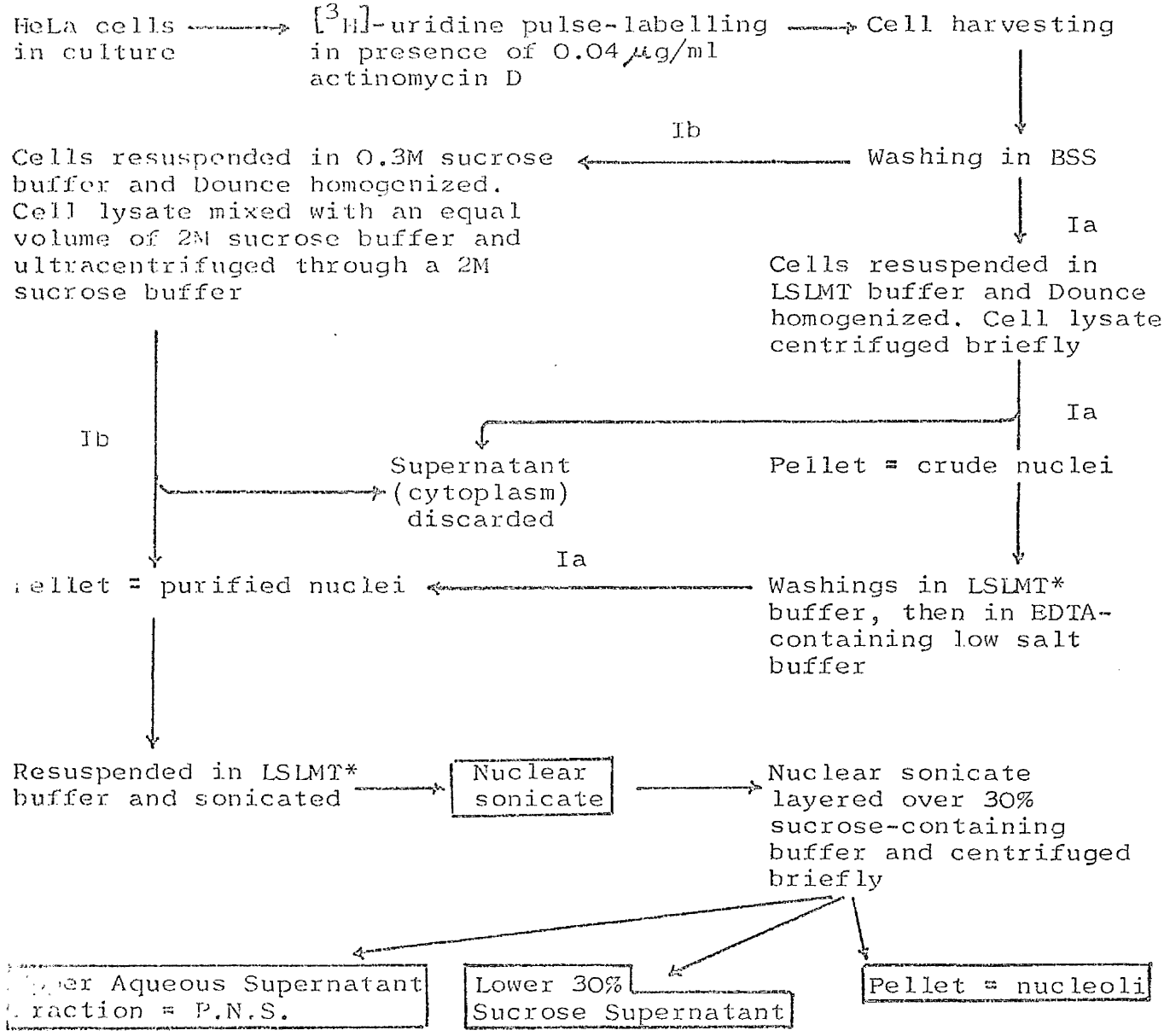
Two other alternative procedures for preparing nuclei were adopted from time to time as reported in

Figure 7

Preparation of HnRNP Particles and Associated Subnuclear Fractions from Sonicated HeLa Cell Nuclei

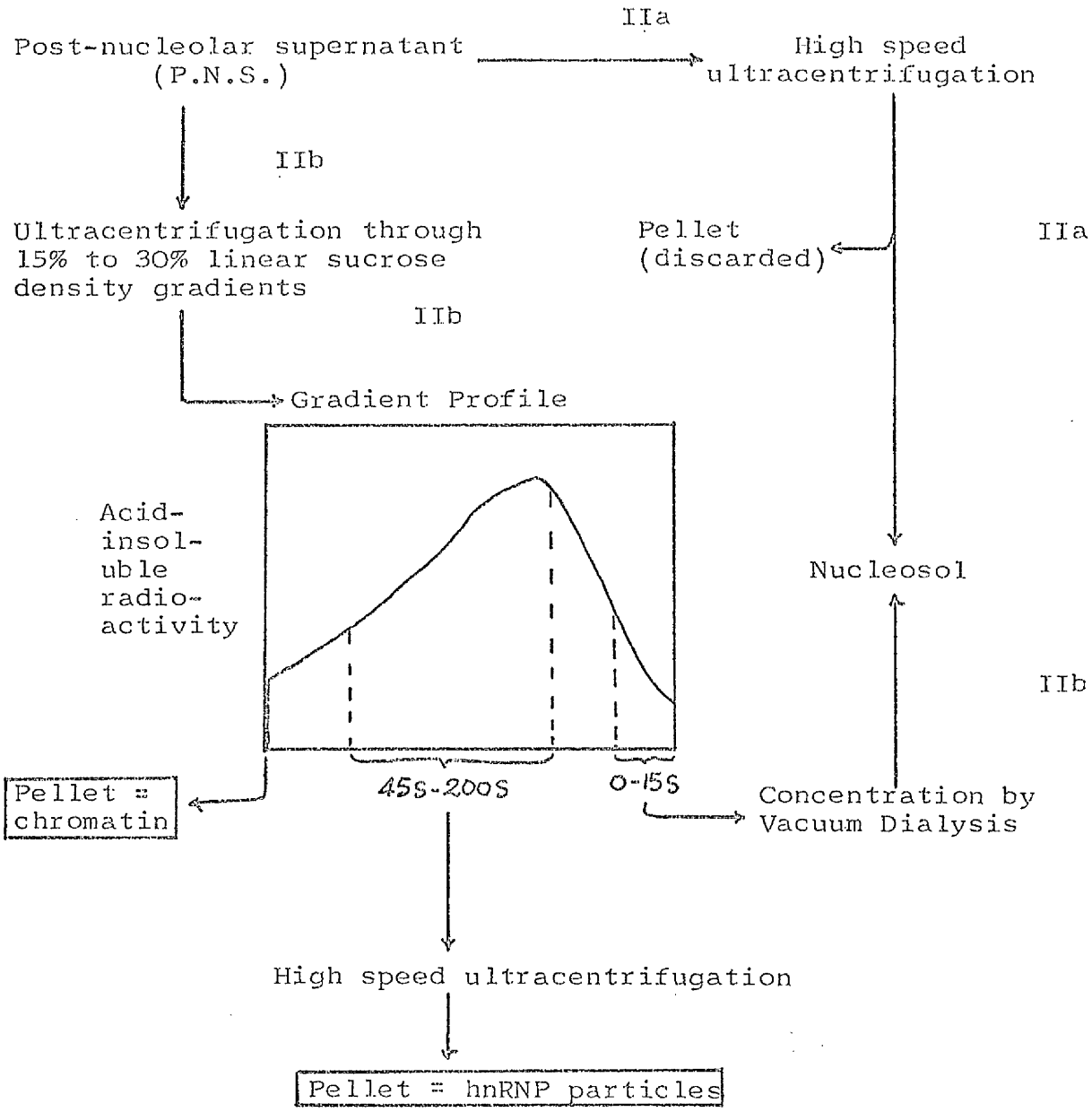
For convenience the procedures have been classified into two groups: (1) preparation of a post-nucleolar supernatant fraction or P.N.S. (Fig 7a); (2) resolution of the P.N.S. to give component subfractions of hnRNP particles, chromatin and nucleosol (Fig 7b). Two major alternative procedures were employed in the purification of HeLa cell nuclei i.e. Ia, Ib. Ia was employed when the diagnostic features characteristic of hnRNP particles were studied and when the various subnuclear fractions were examined for associated ribonuclease activity. Ib was employed when the HeLa subnuclear fractions were assayed for possible associated poly A synthetase, RNA ligase and RNA guanylyltransferase activities. In addition, preparation of a nucleosol fraction was initially achieved by route IIb and used for the study of associated ribonuclease activity but was more conveniently obtained by route IIa and monitored for the association of possible poly A synthetase, RNA ligase and RNA guanylyltransferase activities.

Fig 7a



* = 0.01M NaCl, 1.5mM $MgCl_2$, 0.01M Tris-HCl pH 7.0

Fig 7b


IIa
Post-nucleolar supernatant (P.N.S.)
High speed ultracentrifugation
IIb
Ultracentrifugation through 15% to 30% linear sucrose density gradients
Pellet (discarded)
IIa
IIb
Gradient Profile
Acid-insoluble radioactivity
Nucleosol
IIb
Pellet = chromatin
45S-200S
0-15S
Concentration by Vacuum Dialysis
High speed ultracentrifugation
Pellet = hnRNP particles

individual figure legends. The first of these simply required supplementing the protocol detailed above by two further nuclear washes in O.01M NaCl, 20mM EDTA, O.01M Tris-HCl pH 7.0 buffer before finally resuspending the washed nuclei in LSLMT buffer. The second method was that recommended by Sarma et al (1976) as modified by Detke et al (1978). Washed cells were resuspended in homogenizing medium (2mM magnesium acetate, O.5mM DTT i.e. dithiothreitol, O.3M sucrose, O.1% v/v triton X-100 and 10mM HEPES pH 7.6). The cells were disrupted by homogenization in a stainless steel Dounce homogenizer as above but using about 7-8 strokes to achieve 99% cell lysis as judged by phase-contrast microscopy. The cell homogenate was mixed with an equal volume of solution A (5mM magnesium acetate, O.5mM DTT, 2M sucrose, 10mM HEPES pH 7.6). The resulting mixture was layered in 10 ml quantities over 27 ml of solution A and centrifuged for 45 minutes at 20000 rpm in the Beckman SW 27 rotor. The supernatant wascarefully discarded, the tube was drained and the pellet was washed in LSLMT buffer before being resuspended in the same buffer.

Whichever method was used to prepare nuclei, the washed nuclei were resuspended in LSLMT buffer at a concentration of 4×10^7 nuclei/ml and disrupted by sonic oscillation in a MSE Sonicator (1 pulse of 10 seconds at 1.2A), resulting in 95% nuclear lysis as confirmed by phase contrast microscopy. Theensuing nuclear sonicate was layered in amounts up to 10 ml over 30% sucrose in LSLMT buffer and centrifuged briefly in the Beckman SW 27 rotor (4,500 g max, 15 minutes). The opalescent supernatant (post-nucleolar supernatant or P.N.S.) was removed by aspiration and layered in 2 ml aliquots over 15% to 30% linear sucrose gradients in LSLMT buffer and centrifuged in the SW 27 rotor for 17 hours (15000 rpm, 3^{o}). The gradients were fractionated using a peristaltic pump to withdraw the tube contents from the bottom of the tube into 2 ml fractions. Appropriate fractions representing hnRNP particles (sedimenting within the range 45s-200s - see Fig 7) were diluted with

an equal volume of LSLMT buffer and the diluate was submitted to high speed ultracentrifugation in the Beckman 60 Ti rotor (368000 g max, 5.5 hours) in order to collect the hnRNP particles in the form of a pellet. The pellet was mixed at 2° with the appropriate buffer and resuspended using a Pasteur pipette and then by carefully passing the moderately well resuspended liquid through a syringe needle (21 g x 5/8") three times.

The gradient fractions representing material sedimenting within the range 0-15s above were designated the nucleosol fraction and were concentrated by vacuum dialysis. An alternative, more convenient, method for preparing a nucleosol fraction was sometimes employed involving high-speed ultracentrifugation of the post-nucleolar supernatant in the Beckman 50Ti rotor (2 ml per polycarbonate tube, 50000 rpm, 2.5 hours, 3°). The supernatant fraction was designated the nucleosol fraction and embraced all material sedimenting from 0 to 15s.

The pellets recovered from the 15% to 30% linear sucrose gradients described above (i.e. SW 27 rotor, 15000 rpm, 17 hours) represented the chromatin fraction (Pederson, 1974 - see also Results Fig 14. The pellets were resuspended in the appropriate buffer by passage through a fine syringe needle (25 g x 5/8", 3 times).

3.3 Labelling and isolation of RNA

3.3.1 Ribosomal RNA

Ribosomal RNA was prepared from HeLa cells which had been seeded in rotating 80 oz glass bottles at a density of 3 x 10^7 cells per bottle, permitted to grow at 37.5° for 48 hours, then labelled with [5,6-^{3}H]-uridine (45 Ci/mmole; 150 μCi per bottle) for 16-17 hours. Cells were harvested as in 3.1.3 and washed cells were allowed to swell in LSLMT buffer for 10 minutes at 0°-4° before being disrupted by Dounce homogenization as in 3.2 above. The cell lysate was centrifuged for 3 minutes at 1300 g max and the supernatant fraction (cytoplasm) was carefully removed, treated with an equal volume of water-saturated phenol, shaken on a vortex mixer for

20 seconds, then centrifuged at room temperature (5000 g max, 10 minutes). The supernatant fraction was carefully removed by aspiration, replaced by an equal volume of LETS buffer and the procedure was repeated. The combined aqueous supernatant fractions were adjusted to 0.2M LiCl, then added to 2.5 volumes of cold absolute alcohol and stored at -20° overnight. The ensuing precipitate of RNA was collected by centrifuging at 12000 g max for 20 minutes at -10°. The pellet was resuspended in 1.0 ml LETS buffer, layered over 36.0 ml of a linear 10% to 25% sucrose density gradient in LETS buffer and centrifuged in the Beckman SW 27 rotor (22000 rpm, 17 hours, 20°). Appropriate fractions representing 28s rRNA or 18s rRNA were pooled (Fig 8). Where a particular rRNA species was to be offered as a substrate for assay of ribonuclease activity the RNA was submitted to a further 3 cycles of ethanol precipitation, centrifugation and resuspension as above in order to eliminate traces of phenol and SDS.

3.3.2 Heterogeneous nuclear RNA

HnRNA was prepared from nuclei of HeLa cells largely according to the method of Penman (1969). Exponentially growing cell cultures were treated with actinomycin D at low levels (0.04 μg/ml, 30 minutes) then briefly labelled with [5,6-^{3}H] uridine (45 Ci/mmol; 1 μCi/ml). Washed cells were resuspended in LSLMT buffer and allowed to swell for 10 minutes at 0° to 4° before being disrupted by Dounce homogenization as in 3.2 above. Nuclei were pelleted at 1300 g for 3 minutes, washed once in LSLMT buffer and resuspended in the same buffer. To the nuclear suspension was added 0.15 volumes of a combination of detergents (1 part of 10% sodium deoxycholate, 2 parts of a 10% solution of Tween 40). The resulting mixture was briefly subjected to vortex mixing before centrifugation at 1300 g for 3 minutes as before. For a pellet of purified nuclei containing approximately 2 x 10^8 nuclei, 4 ml of HSHMT buffer were added and the preparation was warmed to room temperature with mixing. Approximately 200 μg of electrophoretically purified pancreatic DNase I was added as a solution of 1 mg/ml in

Fig 8

Analysis of Cytoplasmic RNA from HeLa Cells on SDS-Sucrose Density Gradients

[^{3}H]-labelled cytoplasmic RNA prepared from HeLa cells as detailed in 3.3.1 was resuspended in 1.0 ml of LETS buffer and centrifuged through a linear 10% to 25% sucrose density gradient in LETS buffer (Beckman SW27 rotor, 22000 rpm, 17 hours, 20^{o}). Aliquots of gradient fractions were assayed for acid-insoluble radioactivity. 28s and 18s ribosomal RNA fractions were obtained by pooling fractions 6-9 and 13-15 respectively and submitting them to further purification as in 3.3.1.

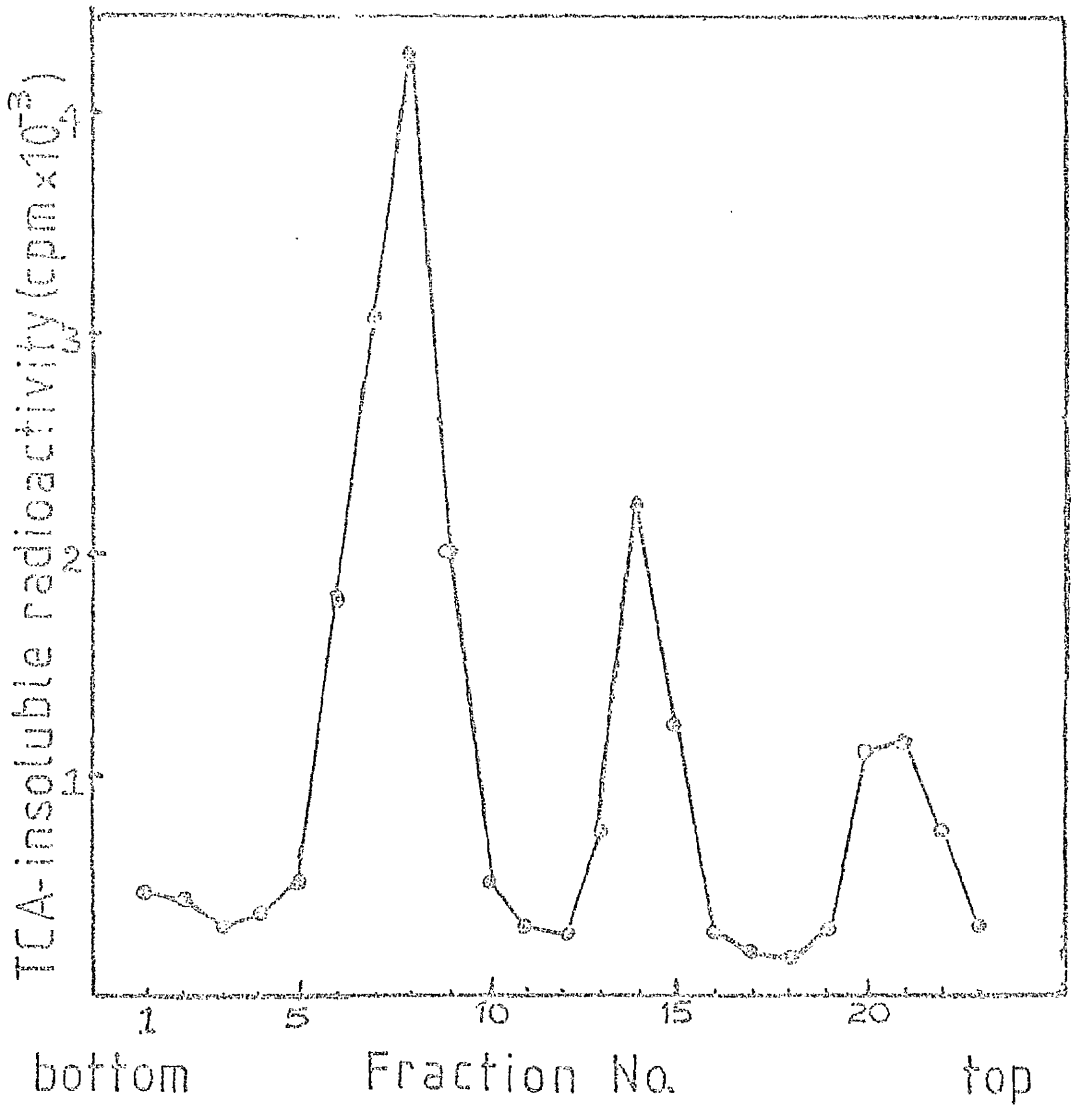

TCA-insoluble radioactivity (cpm x 10⁻³)
1
2
3
4
1
5
10
15
20
bottom
Fraction No.
top

Fig. 8

1mM $MgCl_2$, 5mM N-ethyl maleimide, 10mM Tris-HCl pH 7.6 and the mixture was stirred vigorously with a glass rod at room temperature until it was no longer viscous and there were no visible clumps (about 2 minutes). The presence of N-ethyl maleimide as a relatively potent and irreversible inhibitor of RNase was required following the demonstration by Lee, Penman and Malt (1973) that electrophoretically purified DNase was frequently associated with a contaminant endoribonuclease.

To the DNase-treated mixture were added SDS and a solution of sodium-EDTA pH 7.0 to final concentrations of 0.5% and 0.05M respectively. Water-saturated phenol (5 ml) was added and the combined liquids were briefly (20 seconds) subjected to vortex mixing, heated to 65° for 5 minutes and mixed again. A mixture of chloroform and isoamylalcohol (100:1, v/v) was added (5 ml), followed by another cycle of vortex mixing, heating to 65° and vortex mixing again. The final mixture was centrifuged at room temperature (5000 g max, 10 minutes) to separate the upper aqueous layer from the lower organic phrase. The lower phase was removed by aspiration taking care not to disturb the large flocculent precipitate which occupied a large portion of the upper layer. The procedure was repeated 3 times using the chloroform-isoamylalcohol mixture alone until only a very thin film of denatured protein remained at the interface. The aqueous phase from the last extraction was carefully removed, diluted 4-fold with sterile distilled water and then added to 2.5 volumes of cold absolute alcohol. The mixture was maintained at -20° overnight and the resulting precipitate of RNA was collected by centrifugation (12000 g max, 20 minutes, -10°).

When hnRNA was intended as a substrate for assay of ribonuclease the RNA so prepared was submitted to a further three cycles of ethanolic precipitation, centrifugation and resuspension as above. However it was found that a considerable quantity of DNA copurified with the hnRNA. Consequently some preparations of hnRNA

were subjected to a further treatment with DNase I folbwed by extraction with phenol-chloroform at 65° as above, then purification as before.

Analysis of hnRNA samples in non-denaturing and denaturing sucrose gradients is shown in Figs 9 and 10.

3.3.3 45s nucleolar RNA

45s rRNA was prepared from nucleoli of HeLa cells which had been labelled with [5,6-^{3}H]-uridine (45 Ci/mmole; 200μCi/burler) for 20 minutes prior to cell harvesting. Washed cells were disrupted by Dounce homogenization as in 3.2 above and nuclei were pelleted by centrifugation at 1300 g max for 3 minutes. Washed nuclei were resuspended in HSHMT buffer and treated with electrophoretically purified DNase I, added as a solution of 1 mg/ml in 1mM $MgCl_2$, 5mM N-ethylmaleimide, 10mM Tris-HCl pH 7.6. The mixture was stirred vigorously with a glass rod at room temperature until the mixture was no longer viscous (2 minutes). The digest was centrifuged at 4° (12000 g max, 5 minutes) and the supernatant fraction was discarded. The nucleolar pellet was resuspended in LETS buffer and extracted with phenol, then with phenol-chloroform at 65° as for hnRNA (3.3.2 above). The final aqueous supernatant fraction was combined with 2.5 volumes of absolute alcohol and maintained at -20° overnight. The ensuing precipitate was collected as a pellet by centrifugation (12000 g max, 20 minutes, -10°).

To separate the 45s rRNA from other nucleolar RNA species, the pellet of RNA was dissolved in 1 ml of LETS buffer and layered over 36 ml of a linear 15% to 30% sucrose gradient in LETS buffer. Centrifugation was conducted in the Beckman SW 27 rotor for 15 hours (19000 rpm, 20°). Appropriate gradient fractions representing 45s rRNA were pooled, ethanol-precipitated and purified by 3 cycles of ethanol precipitation, centrifugation and resuspension (Fig 11).

3.3.4 Double-stranded RNA

Double-stranded RNA was prepared as a sub-fraction

Fig 9

Analysis of HeLa HnRNA, on SDS-Sucrose Density Gradients

[^{3}H]-labelled hnRNA was isolated from HeLa cells as detailed in 3.3.2. The final ethanolic precipitate of hnRNA was collected by centrifugation as a pellet which was resuspended in 1.0 ml LETS buffer, then centrifuged through a 15% to 30% linear sucrose density gradient in LETS buffer (Beckman SW27 rotor, 15000 rpm, 17 hours, 20^o). Gradient fractions were assayed for acid-insoluble radioactivity and the positions of 18s and 28s rRNA markers were determined in a parallel gradient.

Fig 10

Analysis of HeLa HnRNA on Formamide-Sucrose Density Gradients

HeLa hnRNA, extracted as detailed in 3.3.2, was analysed on 8% to 20% linear sucrose density gradients in 98% formamide, 2mM EDTA, 10mM Tris-HCl as detailed in 3.7.1.2. Centrifugation was carried out in the Beckman SW56 rotor at 40000 rpm for 17 hours at 30^o. The positions of 28s, 18s and 4s RNA markers were determined by centrifuging a portion of [^{3}H]-labelled cytoplasmic RNA in a parallel gradient.

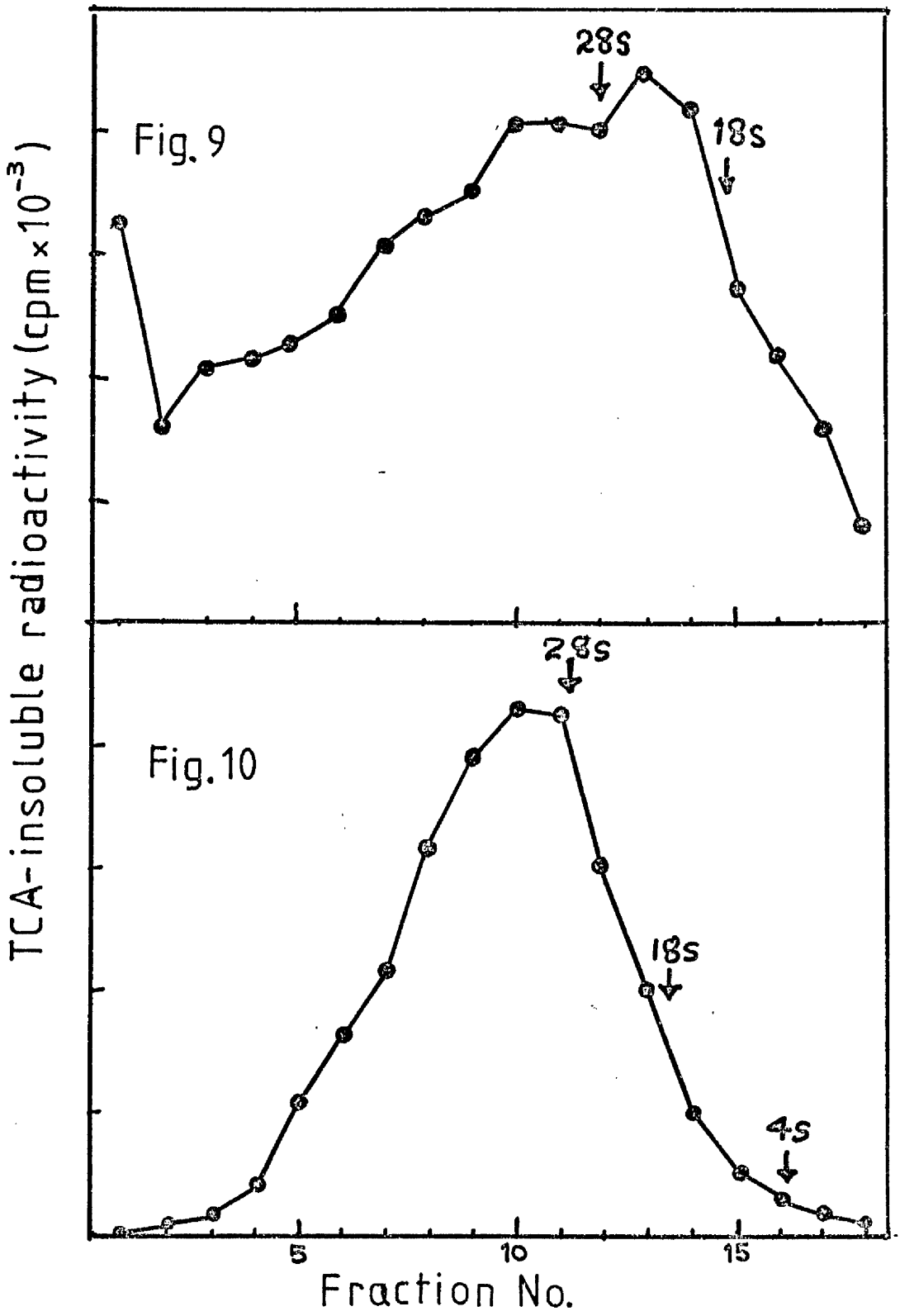


Fig. 9
28S
18S
Fig. 10
28S
18S
4S
TCA-insoluble radioactivity (cpm×10⁻³)
5
10
15
Fraction No.

Fig 11

Analysis of Nucleolar RNA from HeLa Cells on SDS-Sucrose Density Gradients

[^{3}H]-labelled nucleolar RNA prepared from HeLa cells as detailed in 3.3.3 was resuspended in 1.0 ml of LETS buffer and centrifuged through a linear 15% to 30% sucrose density gradient in LETS buffer (Beckman SW27 rotor, 19000rpm, 15 hours, 20^{o}). Aliquots of gradient fractions were assayed for acid-insoluble radioactivity. A 45s RNA fraction was obtained by pooling fractions 6-8 and submitting them to further purification as in 3.3.3.

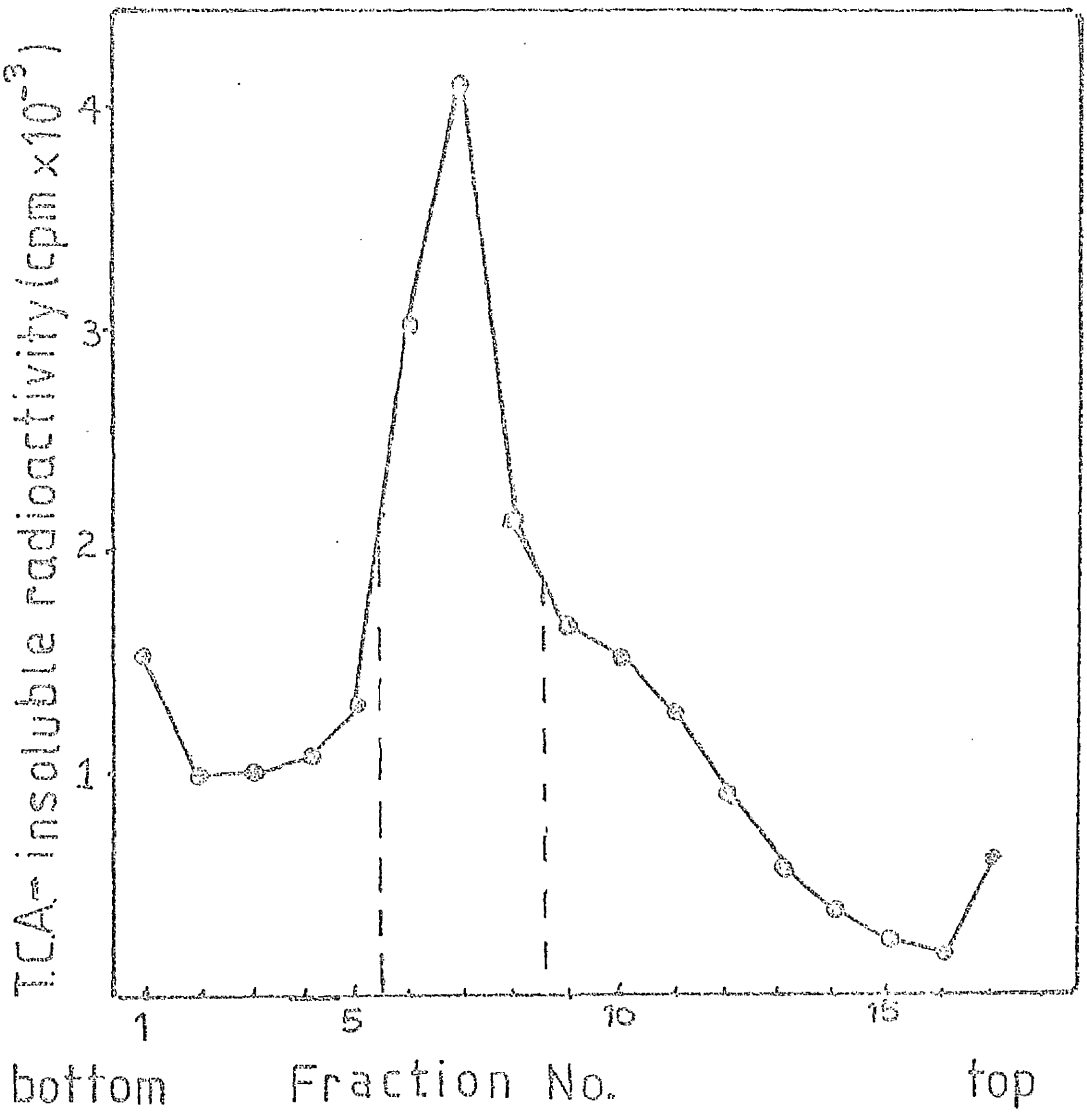

T.C.A.- insoluble radioactivity (cpm x 10^{-3})
4
3
2
1
1
5
10
15
bottom
Fraction No.
top

Fig. 11

of HeLa hnRNA by a method largely based on that of Ryskov et al (1976). HeLa hnRNA prepared as detailed in 3.3.2 above was treated with pancreatic DNase I at room temperature in 1mM $MgCl_2$, 10mM Tris-HCl pH 7.6 for 20 minutes. The reaction was stopped by addition of EDTA to a final concentration of 2mM. The mixture was adjusted to a final concentration of about 0.4M with respect to Na^+ by addition of 1/10 volume of 20XSSC (3M NaCl, 0.3M sodium citrate). Pancreatic ribonuclease A and T_1 ribonuclease were added to 50 μg/ml and 100 units/ml respectively and incubation was continued for 1 hour at 37°. The reaction was stopped by adding pronase to 200 μg/ml before continuing incubation for a further 30 minutes. The final mixture was adjusted to 0.5% in SDS before extraction with phenol-chloroform (1:1 v/v) at room temperature. The final aqueous supernatant fraction was precipitated from ethanol as above (e.g. 3.3.2) and, after centrifugation, (12000 g max, 20 minutes, ~10°) the pelleted precipitate of RNA was submitted to a further two cycles of ethanolic precipitation, centrifugation and resuspension as above.

3.4 Growth and purification of Vaccinia Virus

Baby hamster kidney cells BHK21/C13 (Stoker, 1962) were seeded at 25 x 10^6 per burler and growth was permitted at 37.5° in an atmosphere of 5% CO_2 using Modified Eagle's Minimal Essential Medium supplemented by the addition of tryptose phosphate broth and calf serum to 10% final concentration, i.e. ETC_{10} medium. After 3 days' growth the medium was removed and vaccinia virus (Evan's vaccine strain) in 15 ml of EC_{10} was added to each burler. Absorption of the virus was permitted for one hour at 37° using a multiplicity of infection of between 1:100 and 1:500. After this time a further 165 mls of medium containing 4% calf serum (EC_4) was added and growth was permitted for 60-70 hours until the maximum cytopathic effect had been achieved (MacCrae, 1975). The cells were harvested by using glass beads as an abrasive agent to assist in shaking cells off the glass. The cells were

collected by centrifuging at 1000 rpm, 4°, 15 minutes in 4 x 250 ml bottles and the cell pellet was resuspended in 60 ml 20mM Tris-HCl pH 8.5, 20mM EDTA. The cell suspension was submitted to sonication in a MSE sonicator for 1 minute using a wave amplitude of 6 microns. The sonicate was subjected to 3 cycles of freeze-thawing and large cell debris were removed by centrifugation in the Mistral 6L (250 g, 10 minutes, 4°). The supernatant fraction was decanted and centrifuged in the MSE 10 x 100 rotor at 20000 rpm (55000 g max) for 30 minutes at 4°. The resulting pellet was the source of further virus purification steps.

The pellet was resuspended in 45 ml of 50mM Tris-HCl pH 8.5 by sonication as above but for 30 seconds. To the sonicate was added 15 mls of concentrated NaCl and NaEDTA pH 7.0 solutions to give final concentrations of 0.5M and 50mM respectively. The resulting mixture was sonicated as above for a further 30 seconds. The sonicate was divided into 2 x 30 ml portions which were layered over 2 x 30 ml of 40% sucrose in 0.5M NaCl, 50mM EDTA, 50mM Tris-HCl pH 8.5 before centrifugation in the MSE 10 x 100 rotor at 20000 rpm at 4° for 130 minutes. The supernatant fraction was carefully discarded and the pellet was resuspended in 50mM Tris-HCl pH 8.5 as above, sonicated, adjusted to 0.5M NaCl, 50mMEDTA and sonicated again. The sonicate was divided into 4 x 3ml aliquots which were layered over 4 x 35 ml of a linear 20% to 45% sucrose gradient in 0.5M NaCl, 50mM EDTA, 50mM Tris-HCl pH 8.5. Centrifugation was conducted at 19000 rpm in the Beckman SW 27 rotor for 25 minutes at 4°. The prominent virus bands were observed at about the middle of the gradient and were collected, diluted with 50mM Tris-HCl pH 8.5 then centrifuged in the SW 27 rotor at 19000 rpm for 40 minutes at 4°. The virus pellet was resuspended in 2 ml 50mM Tris-HCl pH 8.5 and stored at -70°.

3.5 In vitro synthesis of Vaccinia Viral mRNA

Vaccinia virus (1.75 mg/ml in 50mM Tris-HCl pH 8.5)

was treated with Triton X-100 and mercaptoethanol at final concentrations of 0.1% and 32mM respectively, and incubated at room temperature for 15 minutes. Incubation was continued at 37° for one hour in a mixture containing the following constituents at final concentrations as indicated: 50mM Tris-HCl, pH 8.5; 7.5mM $MgCl_2$; 3mM ATP; 1mM CTP; 1mM GTP; 1mM UTP (or 0.1mM UTP + [^{3}H]-UTP); 7.2mM phosphoenolpyruvate and pyruvate kinase at 20 μg/ml. When [^{3}H]-UTP was included in the reaction mixture, aliquots of 10 μl were removed at various time intervals during incubation and spotted onto DE81 discs for estimation of DEAE-cellulose paper-bound radioactivity as in 3.7.4.2 below.

Following incubation at 37° for 1 hour the incubation mixture was adjusted to 50mM ammonium acetate and applied to a column (12 cm x 1.5 cm) of Sephadex G50 (medium grade) previously equilibrated in 50mM ammonium acetate. Material eluting in the excluded volume was adjusted to 250 mM ammonium acetate prior to addition of 2.5 volumes of absolute alcohol. The precipitate obtained after four hours at -20° was collected as a pellet (12000 g max, 20 minutes, -10°), dissolved in sterile distilled water, lyophilized, then redissolved in the appropriate buffer.

3.6 Enzyme assays

3.6.1 Assay of exoribonuclease activity

Radioactively labelled samples of RNA were incubated at 37° in 0.01M Tris-HCl pH 7.6 containing 0.01M $MgCl_2$ for 1-3 hours. After various times of incubation aliquots were removed and assayed for acid-insoluble radioactivity as in 3.7.4.1. The estimates obtained were expressed as a percentage of the original acid-insoluble radioactivity and plotted against time of incubation.

An alternative, and frequently supplementary, method required analysis of the fully digested sample by a chromatographic system based on DEAE-cellulose (Furlong, 1965). The method is detailed in 3.7.1.4.

3.6.2 Assay of endoribonuclease activity

Two types of experimental system were analysed for

endoribonuclease activity. In one of these the test sample (e.g. hnRNP particles) had been previously labelled radioactively in its RNA moiety and incubation was conducted at 37° for 3 hours in 0.01M Tris-HCl pH 7.6, 0.01M $MgCl_2$. In the other type of system the test sample was unlabelled and was incubated with radioactively-labelled RNA in 0.01M Tris-HCl pH 7.6, 0.01M $MgCl_2$ for 3 hours at 37°. Following incubation, the RNA component of the digest was extracted with phenol-chloroform as in 3.3.2 and the isolated RNA was analysed on sucrose gradients in 98% formamide (3.7.1.2) or on formamide-polyacrylamide gels (3.7.1.3). Time-dependent shifts to structures of lower sedimentation coefficient or increased mobility were monitored respectively in the absence of any detectable companion exoribonuclease activity (3.6.1).

3.6.3 Assay of double-stranded ribonuclease activity

Double-stranded specific ribonuclease activity was assayed using, as a substrate, either dsRNA prepared from HeLa hnRNA as detailed in 3.3.4, or a hybrid of [^{3}H]-poly C and poly I. In the latter case the hybrid was prepared by incubating [^{3}H]-poly C and a 30% molar excess of poly I at 37° for one hour in 0.1M NaCl, 0.01M Tris-HCl pH 7.6. Incubation of the double-stranded RNA with the test sample was conducted at 37° for 3 hours in 0.01M Tris-HCl pH 7.6, 0.01M $MgCl_2$. After various times of incubation aliquots were removed and assayed for acid-insoluble radioactivity which was expressed as a percentage of the original acid-insoluble radioactivity. Alternatively, after incubation, the RNA component of the digest was extracted with phenol-chloroform as in 3.3.2 and the isolated RNA was analysed by formamide-polyacrylamide gel electrophoresis (3.7.1.3).

3.6.4 Assay of poly A synthetase activity

The assay was based on that described by Rose et al (1977). Reaction mixtures (0.5 ml) contained 50mM Tris-HCl pH 8.5, 10mM DTT, 1mM $MnCl_2$, 500 μg poly A, 1.2 mg/ml phosphoenolpyruvate, 1 μg/ml pyruvate kinase, 0.04 ml [^{14}C]-ATP (1 mCi/mmol) or 0.04 ml [^{3}H]-ATP (23 Ci/mmol) + 0.2mM ATP, 30mM KCl and test sample. Incubation was

conducted at 37° and after various times of incubation aliquots of 0.05 ml were removed for estimation of TCA-insoluble radioactivity by the batch-washing method of filter discs in the presence of 0.1M sodium pyrophosphate (see 3.7.4.1).

3.6.5 Assay of RNA guanylyltransferase activity

The assay was based on the method of Wei & Moss (1977). Reaction mixtures (0.15 ml) contained 50mM Tris-HCl pH 7.9, 5mM $MgCl_2$, 0.5mM $MnCl_2$, 1mM ATP, 1mM DTT, 10% glycerol, 10 μg. of vaccinia viral mRNA prepared as in 3.5, 100 μm s-adenosyl methionine (50 μm added at t=0', and 50 μm at t=30'), 25 μCi of [α-^{32}P]-GTP (350 Ci/mmol) and test sample. Incubation was conducted at 30° for one hour. Following incubation, digests were combined with 1 ml of 50mM Tris-HCl pH 7.8, 0.5% SDS, 0.1mM GTP, 1mM EDTA containing 300 μg yeast tRNA and extracted with phenol/chloroform, then chloroform at room temperature. The final aqueous phase was combined with an equal volume of 10% TCA and placed on ice for 30 minutes. The ensuing precipitate was collected by centrifugation (12000 g, 12', 4°), redissolved in 10mM Tris-HCl pH 7.6 and precipitated using TCA as above twice more. The final precipitate was redissolved in 10mM sodium acetate pH 6.0, precipitated from TCA as above and redissolved in 0.075 ml 10mM sodium acetate pH 6.0, containing 50 μg nuclease P_1. Incubation was conducted at 37° for 2 hours after which 20 μg alkaline phosphatase in 0.075 ml 100mM Tris-HCl pH 8.6 was added and incubation was continued for a further 2 hours at 37°. Incubated reaction mixtures were spotted onto Whatman 3 MM chromatography paper and analysed by high voltage paper electrophoresis (3.7.1.5).

3.6.6 Assay of RNA ligase activity

Reaction mixtures contained in a final volume of 0.1 ml: 50mM Tris-HCl pH 7.6; 10mM $MgCl_2$; 1.5mM DTT; [^{32}P]-poly A, a gift of Miss J. Douglas and Dr. K. Vass, prepared by end labelling of poly A with [γ-^{32}P]-ATP using polynucleotide kinase; 0.1mM ATP or 0.15mM NAD and enzyme. Incubation was conducted at 37° for 30 minutes whereupon the volume was diluted to 0.5 ml with distilled water

and 15 μl of calf alkaline phosphatase was added and incubation was conducted for a further 30 minutes at 37°. The incubation was terminated by addition of 0.5 ml cold 10% TCA containing 0.1M sodium pyrophosphate. The mixtures were stored at 2° for at least 30 minutes and the ensuing precipitates were collected on nitrocellulose filters and washed with cold 5% TCA several times. Radioactivity bound to the filters was assayed as described in 3.7.4.1.

3.7 Analytical techniques

3.7.1 Techniques used to analyse RNA

3.7.1.1 Non-denaturing sucrose gradients

The appropriate RNA was dissolved in LETS buffer and layered in a quantity ($\leqslant$5% of the total gradient volume) over a linear sucrose gradient in LETS buffer, (for details of rotor speeds, time of centrifugation and sucrose concentrations see legends to individual figures). The gradient was fractionated by using a peristaltic pump to withdraw the tube contents from the bottom and an aliquot of each fraction was assayed for acid-insoluble radioactivity as in 3.7.4.1 below.

3.7.1.2 Formamide-sucrose gradients

The method adopted was that of Ross (1976). The appropriate RNA was dissolved in E-T buffer (2mM EDTA, 10mM Tris-HCl pH 7.2) and 0.075 ml of this solution were mixed at room temperature with 0.15 ml of 85% formamide in E-T buffer. The resulting mixture was maintained at 45° for 5 minutes before being layered on top of a linear sucrose gradient of 8% to 20% sucrose in 98% formamide in E-T buffer. The gradients were prepared in Beckman SW 56 polyallomer tubes by constructing 4 layers of 20% (0.7 ml), 16%(1 ml), 12% (1 ml) and 8% (0.75 ml) sucrose in 98% formamide E-T buffer and allowing the layers to diffuse at room temperature for 3 hours. Centrifugation was at 30° in the SW 56 rotor at the speeds and times indicated in individual figure legends. Fractions of approximately 0.19 ml (4 drops) were

collected dropwise from the bottom of each tube and the acid-insoluble radioactivity of each fraction was determined as in below.

3.7.1.3 Formamide-polyacrylamide gel electrophoresis

The method used was based on that of Duesberg & Vogt (1973). Formamide (Fluka Ltd) was deionized by adding 5 g of BioRad AG501-X8 mixed bed ion exchange resin per 100 mls formamide and stirring for one hour at room temperature before filtering. The filtrate was buffered to pH 7.0 by the addition of Na_2HPO_4 and NaH_2PO_4 to a final concentration of 0.01M in each case. Polyacrylamide gels were cast with a final acrylamide concentration of 5% by adding to 29.5 mls formamide pH 7.0, 1.5 g acrylamide and 0.261 g bis-acrylamide followed by 0.375 ml of a 10% solution of ammonium persulphate and 60 μl of TEMED. The resulting solution was poured into glass tubes (0.7 cm x 12 cm) which had been sealed at the bottom with dialysis tubing and the acrylamide solution was overlaid with about 50 μl of distilled water. Polymerization of the acrylamide was achieved within 20 minutes at room temperature.

The appropriate RNA sample was dissolved in 25 μl of distilled water to which was added 50 μl of a 50% solution of glycerol in formamide buffered to pH 7.0 and also containing bromophenol blue at a final concentration of 0.005%. Individual tubes were included in an electrophoresis tank with a reservoir buffer of 0.04M sodium phosphate pH 7.0. Electrophoresis was conducted at room temperature for about 4-5 hours at 120V. After electrophoresis extruded gels were sliced into 1mm gel slices which were processed as detailed in 3.7.4.4 below.

3.7.1.4 DEAE-cellulose paper chromatography

The appropriate RNA sample was spotted in quantities up to 50 μl onto Whatman DE81 DEAE-cellulose chromatography paper. Spots were dried in a stream of cold air and the dried chromatogram was placed in a Shandon chromatography tank containing 0.75M NH_4HCO_3 pH 8.6. Chromatography

was conducted in the ascending mode until the solvent front had migrated about 20 cm along the paper. The developed chromatogram was allowed to dry in air overnight and cut into 5 cm x 20 cm pieces representing the course of migration of individual samples. These pieces were further cut transversely into 20 1-cm x 5-cm strips each of which were cut into four pieces and placed in a scintillation vial and processed as detailed in 3.7.4.3 in order to assay radioactivity.

3.7.1.5 High-voltage paper electrophoresis

Appropriate samples were spotted in quantities up to 50 μl onto Whatman 3MM paper. Electrophoresis was conducted at 4600V for 25 minutes in a solvent of 0.5% pyridine, 5% acetic acid, 1mM EDTA pH 3.5. Dried electrophoretograms were processed for estimation of radioactivity as detailed in 3.7.4.3.

3.7.2 Techniques used to analyse RNP particles

3.7.2.1 Analysis on CsCl density gradients

The method employed was based on that followed by Ducamp & Jeanteur (1973). Fixation of particles was achieved by adding glutaraldehyde (previously neutralized to pH 7.0 by the addition of 1M $NaHCO_3$) to a concentration of 6%. The sample was placed on ice for 8 hours to ensure complete fixation of the particles. Individual samples were included in a 4 ml preformed CsCl gradient containing 0.5% Brij 58. Centrifugation was performed in nitrocellulose tubes in the Beckman SW 50.1 rotor for 60 hours at 40000 rpm at 2°. Tubes were fractionated by dripping from the bottom and samples were taken at intervals to estimate the refractive index by means of an Abbé refractometer. The densities of the various tested samples were then computed from standard tables. Aliquots were also assayed for acid-insoluble radioactivity as in 3.7.4.1 below.

3.7.3 Techniques used to analyse protein

3.7.3.1 SDS-polyacrylamide gel electrophoresis

The method of Maizel (1971) was followed. The pelleted hnRNP particles were resuspended in 0.2 ml of

2% SDS, 2% 2-mercaptoethanol, 0.01M sodium phosphate pH 7.0 and heated to 100° for 4 minutes. Samples were weighted with sucrose crystals and a few droplets of 0.1% Bromophenol Blue were added as a tracker dye. The samples were loaded in a volume of up to 150 µl. The gel system comprised 6 x 75 mm cylindrical gels of 7.5% acrylamide in 0.1% SDS, 0.5M urea, 5mM EDTA, 0.1M sodium phosphate pH 7.0 overlaid with a 20 mm spacer gel of 2.5% acrylamide prepared in the same polymerization solution but with 0.01M sodium phosphate buffer pH 6.0. Electrophoresis was conducted at room temperature until the dye had reached the bottom 5 mm of the 7.5% gel (7.5 hours) with the current set to give 8 mA/gel. The extruded gels were fixed in 50% methanol, 10% acetic acid overnight and stained with 0.25% Coomassie Brilliant Blue in 50% methanol, 10% acetic acid for 2 hours. Destaining of the gels was accomplished using 10% acetic acid.

3.7.3.2 Protein estimation

Protein was estimated using the method described by Bradford (1976). The protein reagent was prepared by dissolving 100 mg of Coomassie Brilliant Blue G250 in 50 ml. 95% ethanol, adding 100 ml 85% (w/v) phosphoric acid and diluting with water to one litre. For estimation of protein in the range 10-100 µg, protein solution in 0.1 ml buffer was mixed with 5 ml of protein reagent and the absorbance at 595 nm was measured. For assay of less than 10 µg protein protein solution in 0.1 ml buffer was mixed with 1 ml of protein reagent before measuring the absorbance at 595 nm. Reference blanks were prepared by mixing 0.1 ml of buffer with the appropriate volume of protein reagent. Protein standards contained bovine serum albumin in a final volume of 0.1 ml and at final concentrations between 0.001% to 0.1% as based on the extinction coefficient at 260 nm for a 1% solution of bovine serum albumin which was taken to be 6.6.

3.7.4 Estimation of radioactivity

3.7.4.1 Acid-insoluble radioactivity

Acid-insoluble radioactivity was determined by several methods. In some instances samples were brought to a final concentration of 5% in TCA and maintained on ice for at least 30 minutes. The ensuing precipitate was collected by means of suction on cellulose nitrate filter discs or on Whatman GF/C glass fibre discs (in the latter case samples had been supplemented by the addition of 100 μg bovine serum albumin as carrier prior to addition of TCA). The discs were washed twice with cold 5% TCA, placed individually in scintillation vials and oven-dried (60^{o}, 2 hours). To each vial was added 5 ml of a 0.5% solution of PPO in toluene and radioactivity was determined by liquid scintillation spectrometry.

In other cases samples of up to 0.1 ml in volume were spotted directly onto Whatman 3 MM filter paper discs. The filters were allowed to dry briefly whereupon they were subjected to a batch-washing procedure. Filters were immersed in cold 5% TCA (10 ml per filter) and maintained on ice, with occasional swirling, for 15 minutes after which the liquid was replaced by fresh cold 5% TCA. Three further washes in cold 5% TCA were followed by washing in absolute alcohol (5 minutes, room temperature) then in diethyl ether (5 minutes, room temperature). Ether-washed filters were air dried and placed in scintillation vials to which was added 0.5 ml of a 1M solution of hyamine hydroxide in methanol. The vials were capped and heated to 60^{o} for 25 minutes. 5 ml of a 0.5% solution of PPO in toluene was placed in each vial and radioactivity was determined by liquid scintillation spectrometry.

In situations where radioactivity labelled nucleoside triphosphates were present in the test-sample the method of batch-washing of filters was applied in the presence of 0.1M sodium pyrophosphate. Filters were washed 6 times in cold 5% TCA-0.1M sodium pyrophosphate, then twice in cold 5% TCA, once in absolute alcohol, once in ether and the air-dried filters were heated to 60^{o} for

25 minutes in the presence of hyamine hydroxide as above. Toluene-PPO was added and radioactivity was determined by liquid scintillation spectrometry as above.

3.7.4.2 DEAE-cellulose bound radioactivity

Test samples were spotted onto Whatman DE 81 discs in 0.05 ml quantities and the filters were allowed to dry briefly in air. The filters were washed 6x in 5% sodium pyrophosphate (5 minutes), 2x in distilled water (3 minutes), 2x in absolute alcohol (3 minutes), 1x in diethyl ether (3 minutes), all operations being carried out at room temperature. The filters were dried in air and submitted to the hyamine hydroxide-toluene-PPO treatment described in 3.7.4.1.

3.7.4.3 Assay of radioactivity present on chromatograms and electrophoretograms

Chromatogram or electrophoretogram strips (5 cm x 20 cm or 5 cm x 40 cm) representing the course of migration of individual samples were cut transversely (in relation to the direction of migration of the sample) into 20 or 40 strips of 1 cm x 5 cm. Each of these strips was sliced into four pieces which were placed in a scintillation vial and submitted to the hyamine hydroxide-toluene PPO treatment described in 3.7.4.1.

3.7.4.4 Assay of radioactivity present in polyacrylamide gel slices

Gel slices were placed in scintillation vials (2 x 1 mm slices per vial) to which was added 0.2 ml 0.3M NaOH. Vials were capped and heated to 60^{o} for 15 hours after which the vial contents were neutralized with 0.2 ml 0.3M HCl and 4 ml of a solution containing 0.5% PPO, 0.05% p-Bis-(o-methylstyryl)benzene in Triton X114-Toluene (350:650 v/v) was added to each vial. The vial contents were thoroughly mixed and allowed to clarify prior to estimation of radioactivity by liquid scintillation spectrometry.

3.8 Sterility precautions

To eliminate contaminating ribonuclease activity

all glassware was baked in an oven at 200^{o} for a minimum of three hours. Solutions were autoclaved at 15 lb in^{-2} for 25 minutes or, if they contained sucrose, at 5 lb in^{-2} for 45 to 50 minutes and in some instances diethyl pyrocarbonate was added to solutions to a final concentration of 0.1% prior to autoclaving. Wherever possible, relevant items of experimental apparatus were autoclaved as above or, failing that, were treated with boiling 1% SDS prior to exhaustive rinsing with sterile distilled water.

RESULTS

Initial attention was devoted to hnRNP particles as a possible source of enzyme activities which might process hnRNA. The hnRNP particles were prepared from HeLa cell nuclei by a method essentially that described by Pederson (1974). Purified HeLa cell nuclei were disrupted by sonication and the nuclear lysate was layered over buffer containing 30% sucrose and centrifuged briefly in order to pellet the nucleoli. The resulting aqueous supernatant fraction (i.e. post-nucleolar supernatant or P.N.S.) was collected from above the sucrose layer and contained nuclear membrane fragments, chromatin, RNP particles and soluble nuclear proteins and these could be resolved by centrifugation through linear sucrose density gradients (see Materials & Methods, Fig 7 - routes Ia, IIb).

1. Isolation and characterization of HeLa hnRNP particles

1.1 Sedimentation properties of particles

When the post-nucleolar supernatant fraction (P.N.S.) from HeLa cells which had been pulse-labelled with [^{3}H]-uridine in the presence of low concentrations of actinomycin D (to suppress labelling of rRNA) was sedimented through linear sucrose density gradients, a heterogeneous profile of acid-insoluble radioactivity was disclosed which reflected structures ranging in sedimentation coefficients from Os to >250s (Fig 12). The profile obtained bears a very close resemblance to that described for HeLa cells by Pederson (1974), but differs in at least two respects. In the present study the profile shows a peak at about 45s compared with about 50s to 60s for that described by Pederson (1974). Also, in the latter work, there is little evidence of radioactively-labelled structures sedimenting at s values below 10-15s whereas they are more generously represented in this study. Possibly the relatively time-consuming methods required to harvest monolayer cultures of cells invites increased degradation of hnRNP particles during their isolation, compared with that evident during the isolation

Fig 12

Sucrose Density Gradient Centrifugation of HeLa hnRNP Particles

$3x10^8$ HeLa cells in the exponential phase of growth in culture were exposed to actinomycin D (0.04μg/ml) for 30 minutes followed by [5,6-^{3}H]-uridine (51 Ci/mmol; 1μCi/ml) for a further 15 minutes. Cells were harvested, washed twice with balanced salts solution (BSS) and Dounce homogenized in LSLMT buffer (0.01M NaCl, 1.5mM $MgCl_2$, 0.01M Tris-HCl pH 7.0). Nuclei were washed three times in the same buffer and processed as detailed in Materials and Methods (see Fig 7). The post-nucleolar supernatant (P.N.S.) was centrifuged in a 15% to 30% linear sucrose density gradient in LSLMT buffer (Beckman SW27 rotor, 15000 rpm, 17 hours, 3^o). Gradient fractions were assayed for acid-precipitable radioactivity.

The position of 76s single ribosomes was determined by centrifuging, on a parallel gradient, a portion of the cytoplasm which had been made 0.5% in sodium deoxycholate. In addition 50s and 30s markers were provided by centrifuging a portion of cytoplasm, which was made 0.05M in EDTA, on 15% to 30% linear sucrose density gradients in LSET buffer (0.01M NaCl, 0.01M EDTA, 0.01M Tris-HCl pH 7.0).

The individual fractions included in areas A (45s-200s) and B (0-15s) were pooled to provide respectively hnRNP particles and nucleosol fractions while the pelleted material obtained from the gradient yielded a chromatin fraction (see text).

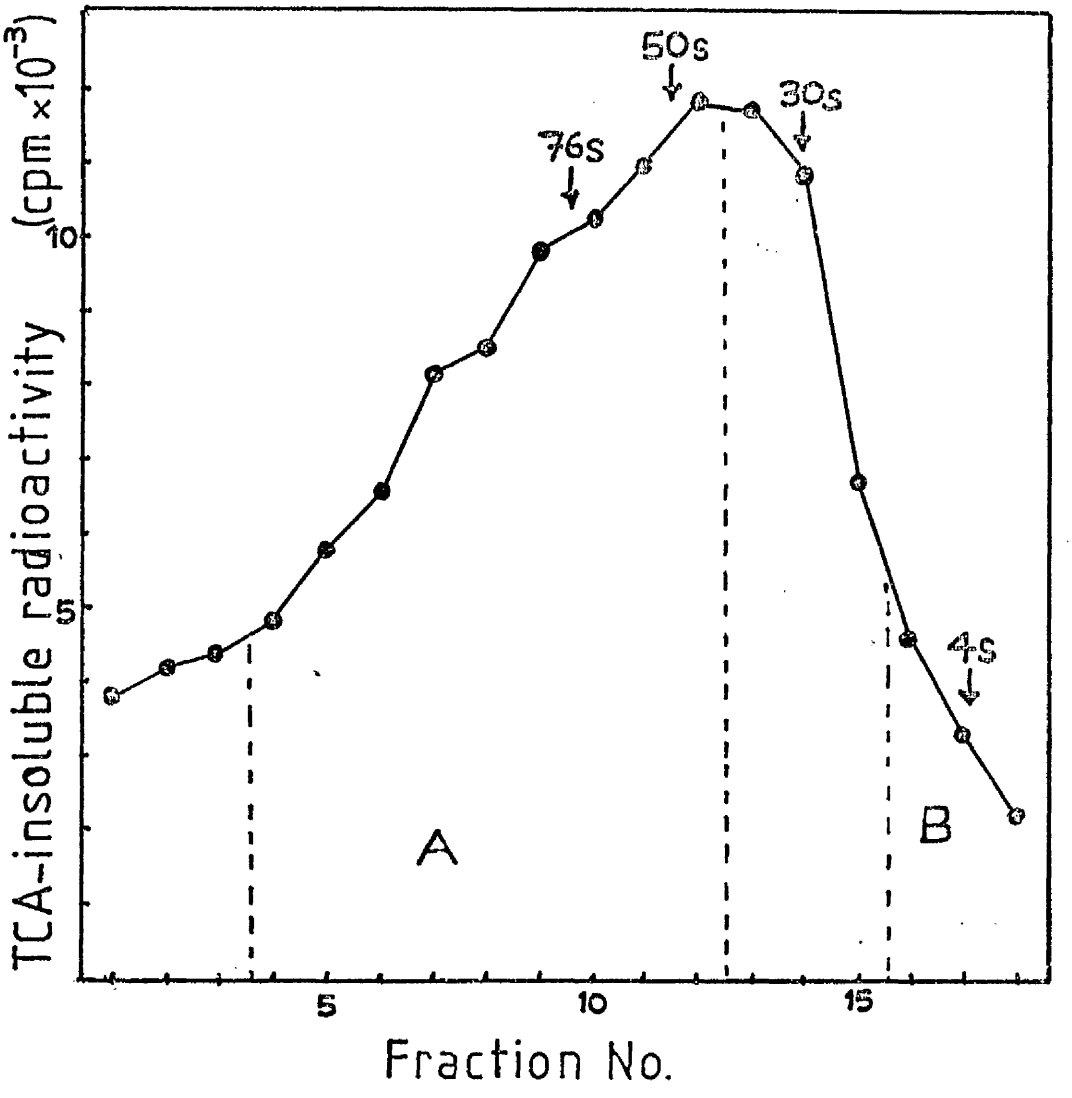

TCA-insoluble radioactivity (cpm ×10⁻³)
10
5
50s
30s
76s
4s
A
B
5
10
15
Fraction No.

Fig. 12

of particles from equivalent suspension cultures. Alternatively the anomalies may reflect genuine differences in the metabolic properties of monolayer cultures (this study) and equivalent suspension cultures of HeLa cells (Pederson, 1974).

Generally, when hnRNP particles are prepared either by nuclear sonication (as in the present study) or alternatively by the pH 8.0 isotonic salt nuclear extraction (e.g. Stévenin et al, 1970), from cells pulse-labelled with a radioactive precursor to RNA, a heterogeneous sedimentation profile of acid-insoluble radioactivity characterizes the hnRNP complexes. However, in certain tissues, e.g. rat liver, a more homogeneous profile displaying a peak at about 30s to 40s is normally obtained unless RNase inhibitor is present, whereupon the more heterogeneous profile including very high sedimentation values is evident (Faiferman et al, 1970). This has been considered to reflect the relatively vigorous endogenous RNase activity in tissues such as rat liver. By comparison, neoplastic tissues are often deficient in such activity (Daoust & Damirande, 1975) and the endogenous ribonuclease activity in HeLa cells is certainly low when compared to that from rat liver. However, extraction of isolated HeLa cell nuclei in isotonic salt pH 8.0 buffer at elevated temperatures (20^{o}, 37^{o}) has resulted in a relatively homogeneous hnRNP particle population of sedimentation coefficient ~40s, presumably due to the increased activity of endogenous RNase at the elevated temperatures (Pederson, 1974; Beyer et al, 1977).

1.2 Analysis on CsCl density gradients

HnRNP particles were obtained by pooling gradient fractions representing structures sedimenting between 45s and about 200s (pool A in Fig 12). An aliquot was taken and dialysed against LSLMT buffer, fixed with glutaraldehyde, and centrifuged to equilibrium through a preformed CsCl density gradient. Following centrifugation, a sharp peak of acid-insoluble radioactivity was evident

at a buoyant density of 1.39 $g.cm^{-3}$ (Fig 13) confirming the value obtained by Ducamp & Jeanteur (1973) for HeLa hnRNP particles while Pederson (1974) has claimed a slightly higher value of 1.43 $g.cm^{-3}$ for the same particles.

A buoyant density of about 1.4 $g.cm^{-3}$ in CsCl density gradients characterizes hnRNP particles from a wide variety of tissues (e.g. Niessing & Sekeris, 1971b; Augenlicht & Lipkin, 1976; Martin & McCarthy, 1972) and also hnRNP particles of different sedimentation coefficient from one tissue (Niessing & Sekeris, 1971b; Samarina et al, 1968). In accordance with an empirical formula proposed by Spirin (1969) this reflects a protein: RNA ratio of about 4:1. A more accurate estimation of the protein:RNA ratio in hnRNP particles can probably be achieved by direct chemical quantitation measurements of protein and of RNA resulting in values of about 4:1 for rat liver hnRNP particles (Faiferman et al, 1970; Samarina et al, 1967; Patel & Holoubek, 1977). However, other authors have reported considerably higher protein:RNA ratios (up to 8:1) following chemical analysis of rat liver hnRNP particles (Northemann et al, 1977; Moulé & Chauveau, 1968). In addition, nuclear RNP particles from amphibian oocytes which display many properties reminiscent of hnRNP particles appear to have very high protein:RNA ratios - probably in excess of 20:1 (Sommerville, 1973).

The value of 1.4 $g.cm^{-3}$ for the buoyant density of aldehyde-fixed hnRNP particles in CsCl density gradients serves to distinguish them from ribosomes and nuclear pre-ribosomal particles which are found to have buoyant densities within the range of 1.53-1.57 $g.cm^{-3}$, reflecting a protein:RNA ratio of approximately 1:1 (Kumar & Pederson, 1975; Kumar & Warner, 1972; Bachellerie et al, 1975). However cytoplasmic informosomes (i.e. free RNP particles containing non-ribosomal RNA) and mRNP particles (which are released upon dissociation of polysomes) can also demonstrate prominent peaks at about 1.4 $g.cm^{-3}$ following CsCl density gradient analysis of the fixed particles (Henshaw, 1968; Kumar & Pederson, 1975).

Fig 13

CsCl Density Gradient Analysis of HeLa HnRNP Particles

Pooled sucrose gradient fractions representing HeLa hnRNP particles as in Fig 12 were dialysed against LSLMT buffer at 4^{o} then fixed in 6% glutaraldehyde (pH 7.0) for 8 hours at 0^{o}-4^{o}. The fixed particles were layered over a preformed CsCl density gradient containing 0.5% Brij 58 and centrifuged in the Beckman SW50.1 rotor at 2^{o} for 60 hours at 40000 rpm. Gradient fractions were assayed for acid-insoluble radioactivity while aliquots were taken at intervals for estimation of density by means of refractometry.

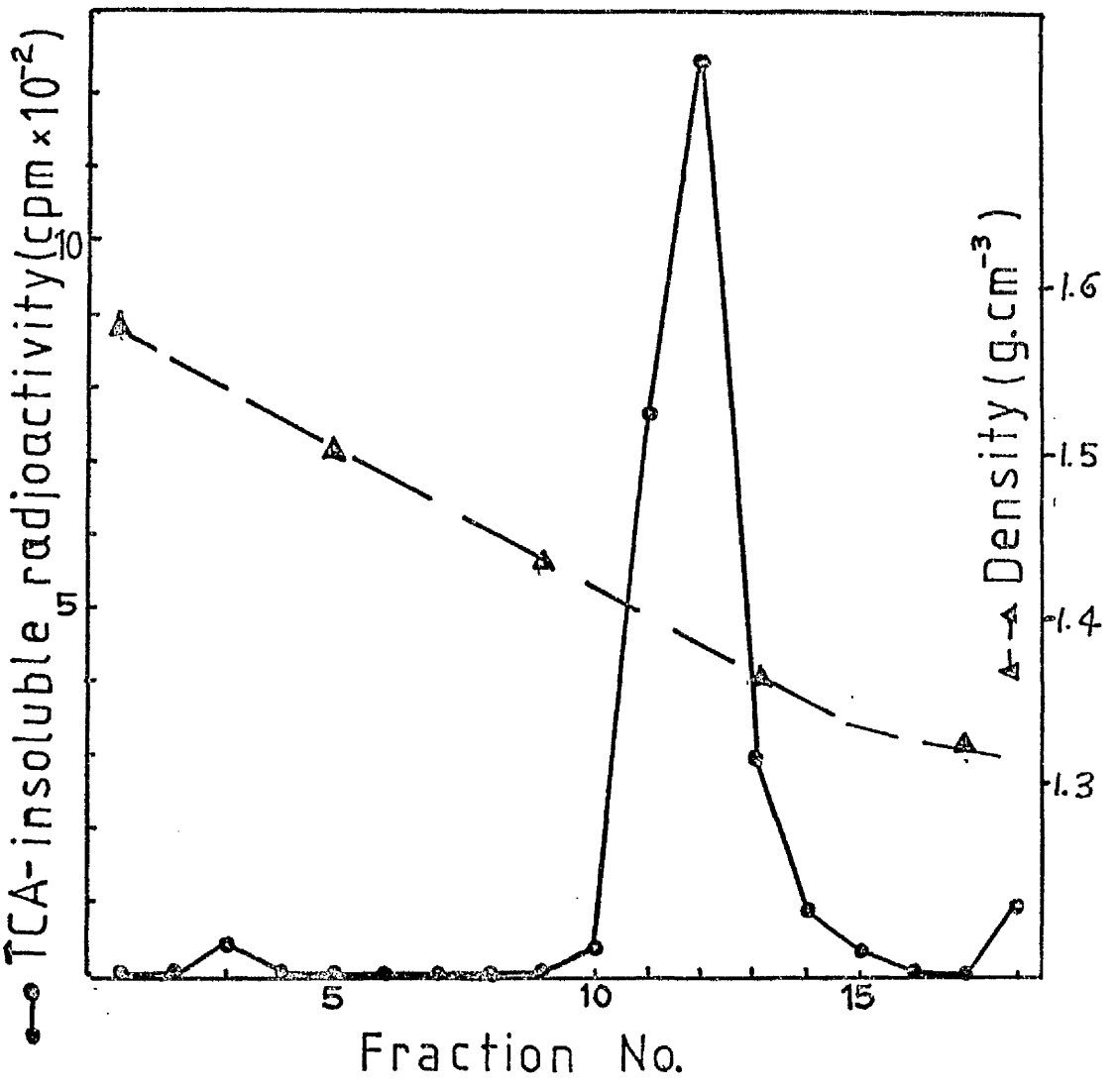

TCA-insoluble radioactivity (cpm x 10⁻²)
10
5
Density (g.cm⁻³)
1.6
1.5
1.4
1.3
5
10
15
Fraction No.

Fig.13

1.3 Analysis on SDS-polyacrylamide gels

The polypeptide complement of RNP complexes can be resolved by means of briefly boiling the sample in the presence of relatively high concentrations of SDS and a reducing agent such as β-mercaptoethanol, and thereafter subjecting the treated sample to electrophoresis through a polyacrylamide medium in the presence of SDS-containing buffer. When HeLa hnRNP particles were subjected to this protocol extensive heterogeneity was apparent in the electrophoretic mobilities of species which could be stained by the protein-specific dye, Coomassie Brilliant Blue (Fig 14). A minimum of 20 different polypeptide species can be discerned by this one-dimensional resolving procedure and the polypeptide profile obtained verifies that described for HeLa hnRNP particles by Pederson (1974).

The electrophoretic mobility of a polypeptide species through a polyacrylamide medium in the presence of SDS has been shown to be in direct correlation with the logarithm of its molecular weight (Weber & Osborn, 1969). The observed mobilities of several marker polypeptide species of known molecular weight provided the basis for the scale of estimated molecular weights flanking the gels in Fig 14. It can be seen that the polypeptide profile of HeLa hnRNP particles embraces species ranging from about 40,000 daltons to greater than 150,000 daltons with a particularly conspicuous component of about 40,000 daltons. Most importantly, it vindicates the ability to discriminate on sucrose density gradients between the hnRNP particles and the chromatin fraction: low molecular weight histone species which are abundantly evident in the chromatin sample are nearly completely absent in the polypeptide profile of hnRNP particles (Fig 14). Also, HeLa ribosomal structural proteins which generally are found in the 15,000 to 55,000 daltons molecular weight range (Kumar & Warner, 1972) are not evident. Further, the polypeptide profile is distinct from that exhibited by HeLa mRNP which consists of only a limited number of different polypeptide species (Kumar & Pederson, 1975).

Fig 14

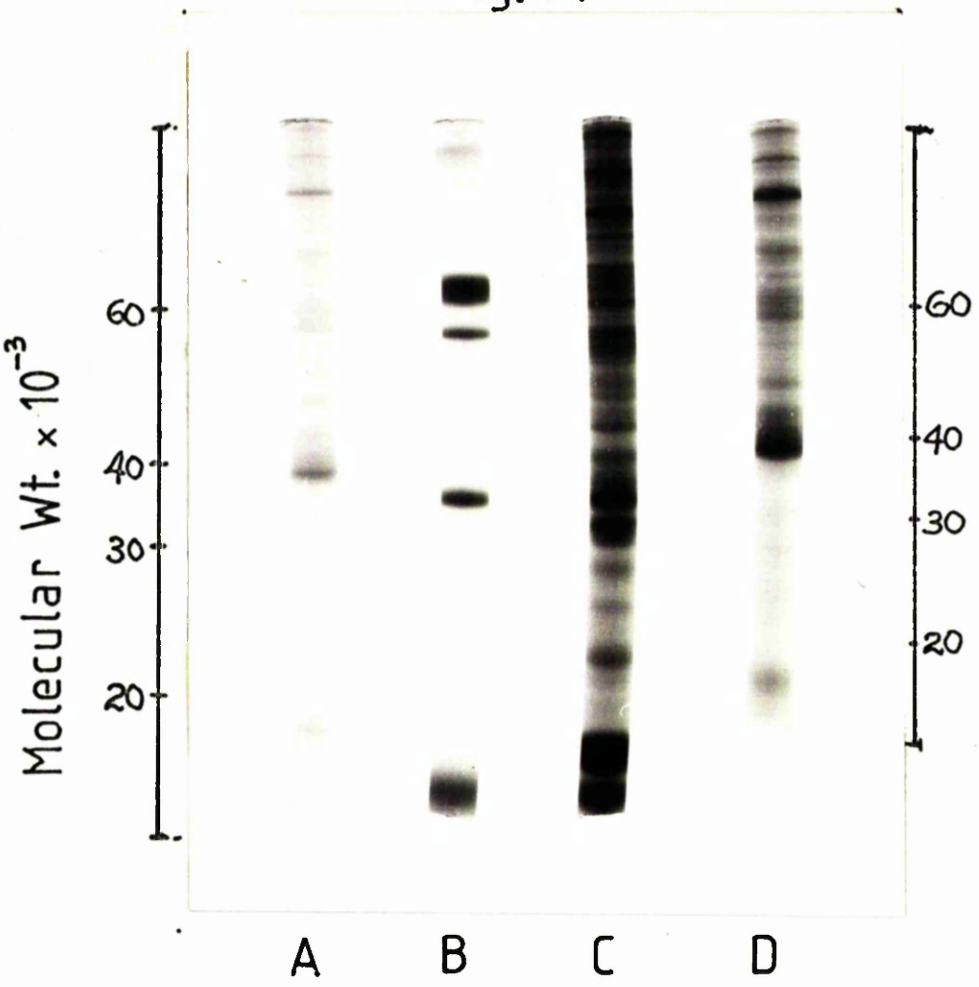
SDS-polyacrylamide Gel Electrophoresis of HeLa HnRNP Particle Proteins

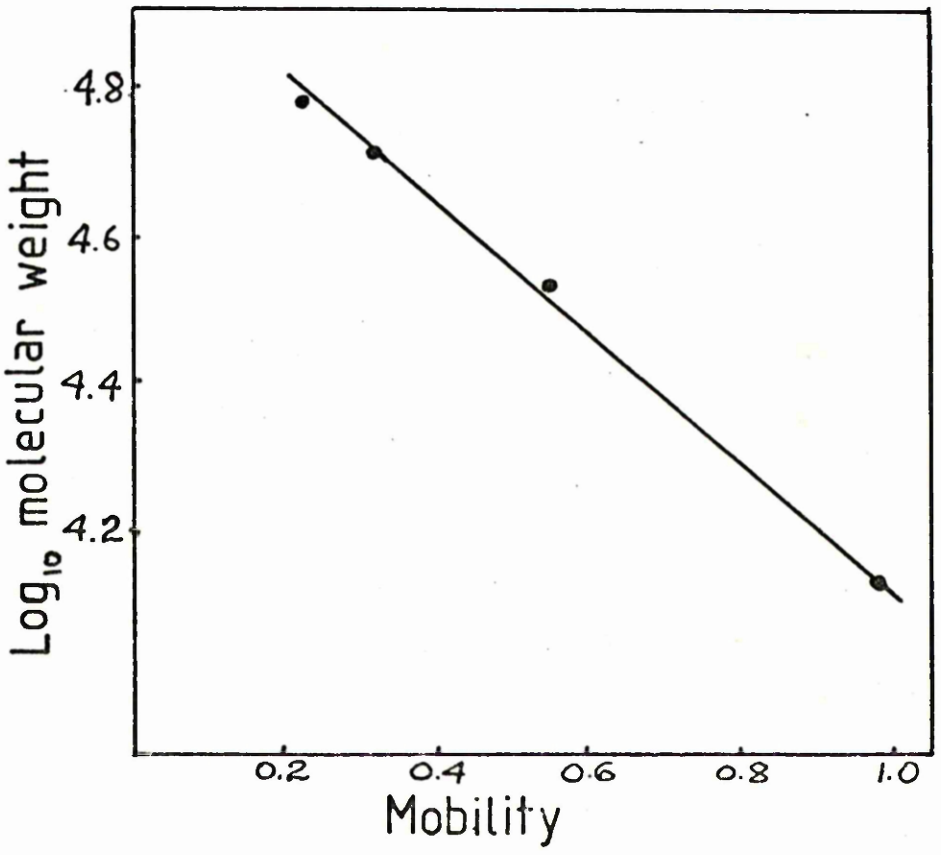
Pooled sucrose gradient fractions representing HeLa hnRNP particles (area A in Fig 12) were diluted with an equal volume of LSLMT buffer and centrifuged in the Beckman 60Ti rotor (368000 g max, 5.5 hours, 3°). The resulting pellet was resuspended in 0.3 ml of 1% SDS, 1% 2-mercaptoethanol, 0.01M sodium phosphate pH 7.0. In addition, the pellet obtained from the sucrose density gradient in Fig 12 was separately resuspended in the same buffer. Samples in volumes up to 150 µl were heated at 100° for 4 minutes then mixed with a few crystals of sucrose and some Bromophenol Blue tracking dye prior to sample application to the gel electrophoresis system (cylindrical gels consisting of a spacer gel of 2.5% acrylamide, and a running gel of 7.5% acrylamide in 0.01M phosphate buffer). Electrophoresis was conducted at room temperature for about 7.5 hours. Extruded gels were subjected to fixing, staining and destaining procedures described in Materials and Methods.

Gels represent from left to right:- A) 5 µg hnRNP particle protein; B) mixture of marker samples including bovine serum albumin (68000 daltons), glutamate dehydrogenase (53000 daltons), lactate dehydrogenase (36000 daltons) and cytochrome C (13500 daltons); C) pellet from sucrose density gradient in Fig 12; D) 35 µg hnRNP particle protein.

From the observed mobilities of the marker proteins a graph of log molecular weight versus mobility provided the basis for the scales of estimated molecular weight values flanking the gels.

Fig. 14


Molecular Wt. x 10^{-3}
60
40
30
20
60
40
30
20
A
B
C
D


Log_{10} molecular weight
4.8
4.6
4.4
4.2
0.2
0.4
0.6
0.8
1.0
Mobility

When hnRNP particles extracted from isolated HeLa cell nuclei in isotonic buffer pH 8.0 at 37°, are analysed by a similar SDS-polyacrylamide gel electrophoretic system, a polypeptide profile is disclosed which is comparable to that manifested by hnRNP particles obtained from sonicated HeLa cell nuclei (Pederson, 1974). However, using a different SDS-polyacrylamide gel electrophoretic system, Beyer et al (1977) have found a different polypeptide profile for hnRNP particles prepared by extracting isolated HeLa cell nuclei at 20° and then at 37°. In this case there appeared to be three pairs of closely spaced (i.e. in terms of electrophoretic migration) bands which dominated the 30,000 to 45,000 daltons zone with much less evidence of the higher molecular weight (i.e. > 45,000 daltons) species. It is possible therefore that the superior resolving power of the electrophoretic system employed by Beyer et al (1977) permitted greater resolution of the relatively broad 40,000 dalton species found by experimenters using alternative electrophoretic systems. In addition, the proportion of the higher molecular weight species appears to be strikingly dependent on the temperature of extraction of the isolated HeLa nuclei (Pederson, 1974). Accordingly, the much more thorough 37° extraction method employed by Pederson (1974) would be expected to be accompanied by a more generous representation of higher molecular weight species than is evident in the RNP particles prepared by Beyer et al (1977). However Beyer et al (1977) have also resolved HeLa hnRNP particles on sucrose density gradients and then analysed individual gradient fractions in terms of their polypeptide profiles on SDS-polyacrylamide gels. Their results have indicated that many of the high molecular weight polypeptide species associated with the 40s HeLa hnRNP particles were not specifically concentrated in that region of the gradient corresponding to hnRNP particles (see Discussion).

The polypeptide profile from hnRNP complexes from a wide variety of eukaryotic tissues has previously been

the subject of some acrimonious debate. The total number of polypeptide species associated with hnRNP particles from a variety of eukaryotic tissues has been estimated to be one (Krichevskaya & Georgiev, 1969; Lukanidin et al, 1971), less than 8 (Niessing & Sekeris, 1970; McParland et al, 1972; Martin et al, 1974; Morel et al, 1971; Matringe & Jacob, 1972; Sommerville, 1973), between 10 and 30 (Augenlicht & Lipkin, 1976; Pederson, 1974; Ducamp & Jeanteur, 1973; Niessing & Sekeris, 1971a; Faiferman et al, 1971; Albrecht & van Zyl, 1973; Beyer et al, 1977; Karn et al, 1977; Patel & Holoubek, 1977), or between 30 and 50 (Pagoulatos & Yaniv, 1977). However, it is now recognised that the variety of electrophoretic procedures employed by these investigators has contributed to the confusion regarding the vastly different polypeptide profiles for hnRNP particles prepared from a particular tissue type by similar experimental protocols. In particular, polyacrylamide gel electrophoresis based on acetic acid pH 4.5 - urea has been shown to be deficient in that it does not resolve proteins of low isoelectric point notably the phosphorylated polypeptides (Gallinaro-Matringe & Jacob, 1974). Consequently the technique of SDS-polyacrylamide gel electrophoresis which resolves component polypeptide species in terms of size rather than charge has largely superseded the acid-urea system as the method of choice. When this technique is used the polypeptide profiles from a considerable variety of eukaryotic tissues are markedly heterogeneous and approximate a pattern of a few major proteins in the 30,000 to 45,000 daltons molecular weight range and many minor high molecular weight species (Beyer et al, 1977; Karn et al, 1977; Patel & Holoubek, 1977).

Recently two-dimensional polyacrylamide gel electrophoretic systems have been applied to the study of hnRNP particle polypeptides. In particular they have facilitated the identification of various species present in rat liver 40s hnRNP particles (Patel & Holoubek, 1977; Karn et al, 1977). However the resolving power of such

systems does not appear to be very much greater than that of one-dimensional SDS-polyacrylamide gel electrophoresis when used to study hnRNP particle proteins (Pagoulatos & Yaniv, 1977; Suria & Liew, 1979).

1.4 Analysis of the rapidly labelled RNA component

Extraction of the RNA component of hnRNP particles present in appropriate sucrose density gradient fractions was achieved using the SDS-phenol-chloroform technique recommended by Penman (1969). When the extracted RNA was submitted to ultracentrifugation through linear SDS-sucrose density gradients a heterogeneous profile of acid-insoluble radioactivity was disclosed spanning sedimentation coefficients from 4s to greater than 40s with a median value of about 18s (Fig 15). As with the lower average sedimentation coefficient of hnRNP particles prepared from HeLa monolayer cell cultures in the current investigation in relation to those prepared from HeLa S_3 cells, the rapidly labelled RNA extracted from the hnRNP particles of the monolayer cultured cells was of lower average sedimentation coefficient than that from the particles obtained from HeLa S_3 cells (Pederson, 1974). Again this may reflect increased degradation during isolation of hnRNP particles from monolayer cultures of HeLa cells or genuine metabolic differences between HeLa cells in monolayer and suspension cultures.

In summary, the hnRNP particles isolated from HeLa cell nuclei in the present study were identified by the following criteria: subcellular location; rapid incorporation of a labelled RNA precursor in the presence of low concentrations of actinomycin D which preferentially suppress rRNA synthesis (Perry, 1963); heterogeneous sedimentation in sucrose density gradients; a protein:RNA ratio of about 4:1 as testified by their buoyant density in CsCl density gradients; heterogeneous sedimentation of the RNA component in SDS-sucrose density gradients; and a characteristic heterogeneous polypeptide profile when examined by SDS-polyacrylamide gel electrophoresis.

Fig 15

Analysis on SDS-Sucrose Density Gradients of RNA Extracted from HnRNP Particles and Chromatin of HeLa Cells

Pooled sucrose density gradient fractions representing hnRNP particles and the sucrose density gradient pellet representing the chromatin fraction, both as in Fig 12, were adjusted or resuspended respectively to give solutions containing 0.5M NaCl, 50mM $MgCl_2$, 10mM Tris-HCl pH 7.0. To each solution was added 100 μg pancreatic DNase I in 5mM N-ethyl maleimide, 10mM Tris-HCl pH 7.0 and incubation was conducted at room temperature with vigorous stirring for about 2 minutes. Thereafter SDS was added to 0.5% and sodium EDTA pH 7.0 to a final concentration of 60mM. Phenolic extractions were performed at room temperature, then at 65^{o} using a phenol-chloroform mixture as detailed in Materials and Methods 3.3.2. RNA was precipitated from the final aqueous supernatants by means of cold ethanol and the pelleted precipitates were resuspended in 1.0 ml LETS buffer and centrifuged through a linear 10% to 25% sucrose density gradient in LETS buffer (Beckman SW27 rotor, 24000 rpm, 17 hours, 20^{o}). Gradient fractions were assayed for TCA-precipitable radioactivity and the positions of 18s rRNA and cytoplasmic 4s RNA markers were determined in a parallel gradient.

●——● chromatin RNA; ▲-----▲ hnRNP particle RNA

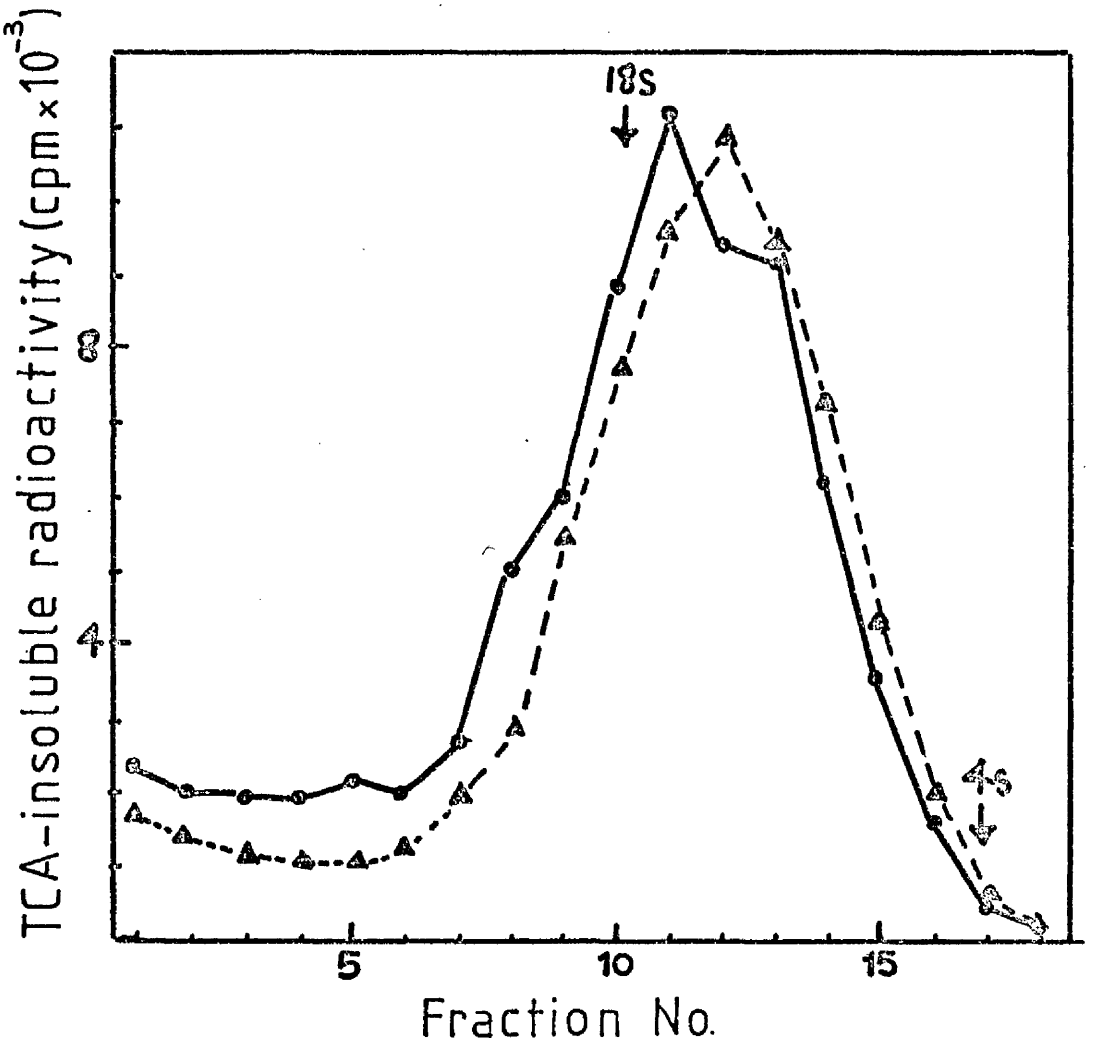

TCA-insoluble radioactivity (cpm×10⁻³)
8
4
18S
4S
5
10
15
Fraction No.

Fig. 15

Because the primary concern of this study was an investigation of the functional attributes of HeLa hnRNP particles rather than their structural definition these criteria were considered adequate evidence for establishing the identity of the isolated hnRNP particles. Consequently other properties of isolated HeLa hnRNP particles which have been examined by Pederson (1974) including their "reality" i.e. their equivalence to structures found in vivo in HeLa cell nuclei (see Discussion) have been assumed to be shared by the hnRNP complexes isolated in this study.

1.5 Investigation of the method for isolating hnRNP particles

It seems from the foregoing data that the fractionation technique pioneered by Pederson and co-workers (Bhorjee & Pederson, 1973; Pederson, 1974) for the isolation of hnRNP particles free from chromatin is relatively successful, as judged by the absence of contaminant histone species in the polypeptide profile of the particles. In addition, by exposing cells to low concentrations of actinomycin D for some time prior to harvesting and by removing nucleoli from the nuclear lysate prior to resolution of the particles in sucrose density gradients there is little scope for contamination by ribosomes and pre-ribosomal particles.

In order to examine further the basis of the fractionation scheme employed to isolate HeLa hnRNP particles, the fate of structures in the nuclei of HeLa cells previously pulse-labelled with [^{3}H]-uridine in the presence of low concentrations of actinomycin D was followed. By removing aliquots at various stages in the fractionation procedure for assay of acid-insoluble radioactivity it was possible to relate the distribution of radioactivity between the various sub-nuclear fractions using the nuclear lysate as a reference (Table 4). Even when HeLa nuclei were sonicated at relatively low densities (40 x 10^6 per ml) it was found that a very considerable proportion of the total acid-insoluble radioactivity of the sonicated nuclei could be traced to structures of very rapid sedimentation coefficient.

When the nuclear lysate was layered over 30% sucrose-containing buffer and centrifuged at low speed for a short time, only about 50% of the total acid-insoluble radioactivity could be recovered from the aqueous supernatant fraction (P.N.S.), the remainder being distributed between the 30% sucrose supernatant fraction (38%) and the nucleolar pellet (10%) (Table 4). In addition, when the P.N.S. was subsequently fractionated on 15% to 30% linear sucrose density gradients approximately 70% of the acid-insoluble radioactivity (i.e. 36% of the total for the nuclear lysate) was recovered in the supernatant fraction whereas as much as 30% was found in the chromatin pellet.

The amount of radioactive label incorporated by rRNA during a brief exposure of cells to [^{3}H]-uridine in the presence of 0.04 μg/ml actinomycin D is almost negligible. Accordingly, the acid-insoluble radioactivity associated with the nucleolar pellet and the post-nucleolar 30% sucrose supernatant fraction may be attributed to the presence of aggregated chromatin structures following nuclear sonication (Bhorjee & Pederson, 1973). Consequently the total chromatin contribution (i.e. aggregated forms and the more dispersed components sedimenting at the bottom of the 15% to 30% linear sucrose density gradients) accounts for about 65% of the rapidly labelled RNA of the nuclei of HeLa cells which had been briefly exposed to [^{3}H]-uridine in the presence of low concentrations of actinomycin D. This observation contradicts Pederson (1974) who has claimed that material sedimenting at 40s to 250s comprises about 95% of the total hnRNP when using the fractionation scheme he and his co-workers have devised for isolating hnRNP particles free from chromatin contamination (Bhorjee & Pederson, 1973; Pederson, 1974). However, the observation reported here endorses the work of Augenlicht & Lipkin (1976) who have employed the Pederson fractionation scheme to prepare chromatin and hnRNP particle fractions from human HT-29 cells. They have found that the bulk of the non-nucleolar nuclear rapidly

TABLE 4 Subnuclear Distribution of Acid-insoluble Radioactivity during the Isolation of HnRNP Particles from Sonicated HeLa Cell Nuclei

Subnuclear Fraction	Percentage of Acid-insoluble Radioactivity of Nuclear Sonicate
Nuclear Sonicate	100
Nucleolar Pellet	10 (±5)
30% Sucrose Supernatant	38 (±4)
Aqueous Supernatant (≈ P.N.S.)	51 (±8)
Chromatin Pellet	16 (±3)
HnRNP Particles	36 (±4)

HnRNP particles were isolated from HeLa cells as outlined in Materials and Methods Fig 7 (routes Ia, IIb). At stages during the fractionation aliquots were removed from various subnuclear fractions (i.e. boxed titles in Fig 7) and assayed for acid-insoluble radioactivity. Results were expressed as a percentage of the total value for the nuclear sonicate and represent the mean of three independent estimations.

labelled RNA of HT-29 cells is found in association with the chromatin component while the remainder is associated with the hnRNP particles. In addition, alternative subnuclear fractionation schemes have generated chromatin fractions which harbour a large part of the rapidly labelled non-nucleolar nuclear RNA (Tata & Baker, 1974a,b; Monahan & Hall, 1975; Kimmel et al, 1976).

The rapidly labelled RNA extracted from chromatin and hnRNP particles prepared from HeLa cells in this study disclosed quite similar sedimentation profiles on SDS-containing sucrose density gradients, although the chromatin-associated RNA tended to be more generously represented in the higher sedimentation coefficients (Fig 15). Similarly in HT-29 cells the rapidly labelled RNA extracted from the chromatin fraction boasts components of higher average s value than the equivalent hnRNP-extracted RNA (Augenlicht & Lipkin, 1976). This observation together with the kinetics of appearance of radioactive label in the two fractions following brief exposure of the cells to [^{3}H]-uridine in the presence of low concentrations of actinomycin D, has been interpreted by the authors to suggest that some of the chromatin-associated RNA is a precursor to the hnRNP-RNA.

2. Identification of possible RNA processing enzymes in HeLa cell nuclei

Initially the isolated HeLa hnRNP particles were assayed for possible associated hnRNA processing enzymes. Latterly, the search for such enzyme activities was extended to other non-nucleolar, subnuclear fractions which were by-products of the hnRNP isolation procedure, notably chromatin and nucleosol (Materials & Methods, Fig 7).

2.1 Ribonuclease

Endoribonuclease and exoribonuclease assays were performed in conjunction, either in terms of self-incubation of a pre-labelled subnuclear fraction or by incubating subnuclear fractions prepared from unlabelled cells with various radioactively-labelled RNA species.

2.1.1 Exoribonuclease

During the course of investigation of the adopted subnuclear fractionation scheme it was found that the RNA in the P.N.S. fraction from HeLa cells, which had been briefly exposed to [^{3}H]-uridine in the presence of low concentrations of actinomycin D prior to cell harvesting, was unstable following incubation at 37^o with a rapid decline of initial acid-insoluble radioactivity (Fig 16A; Fig 17). To investigate the source of this activity the P.N.S. fraction was resolved into constituent hnRNP particle, chromatin and nucleosolic sub-fractions.

When hnRNP particles were isolated from the nuclei of HeLa cells which had been exposed briefly to [^{3}H]-uridine in the presence of low concentrations of actinomycin D immediately before cell harvesting, and incubated in low-salt buffer at 37^o there was no evidence for exonucleolytic digestion of the labelled RNA moiety of the particles. The total acid-insoluble radioactivity of the labelled particles during the course of incubation remained at values close to 100% of that of the particles prior to incubation (Fig 16B; Table 5). Moreover, following incubation, the particles did not appear to contain any molecular species co-migrating with a UMP

Fig 16

Exoribonuclease Activity in Non-nucleolar HeLa Cell Subnuclear Fractions

A post-nucleolar supernatant fraction (P.N.S.) was obtained by the procedure detailed in Materials and Methods 3.2 from HeLa cells which had been exposed to [^{3}H]-uridine for 15 minutes in the presence of 0.04 μg/ml actinomycin D, immediately before harvesting. One portion of this fraction (obtained from 5×10^7 HeLa cell nuclei) was incubated in LSLMT buffer at 37° for 3 hours. The remainder was resolved on sucrose density gradients to provide chromatin, hnRNP particles and nucleosol fractions as outlined in Materials and Methods 3.2. The resulting fractions, representing material from 5×10^7 HeLa cell nuclei, were individually incubated in LSLMT buffer at 2° or 37° for 3 hours. At intervals during the course of incubation aliquots were removed from each incubation mixture and assayed for acid-insoluble [^{3}H]-radioactivity. A - P.N.S.; B - hnRNP particles; C - nucleosol; D - chromatin.

●——● incubation at 2°

▲- - - -▲ incubation at 37°

Fig. 16

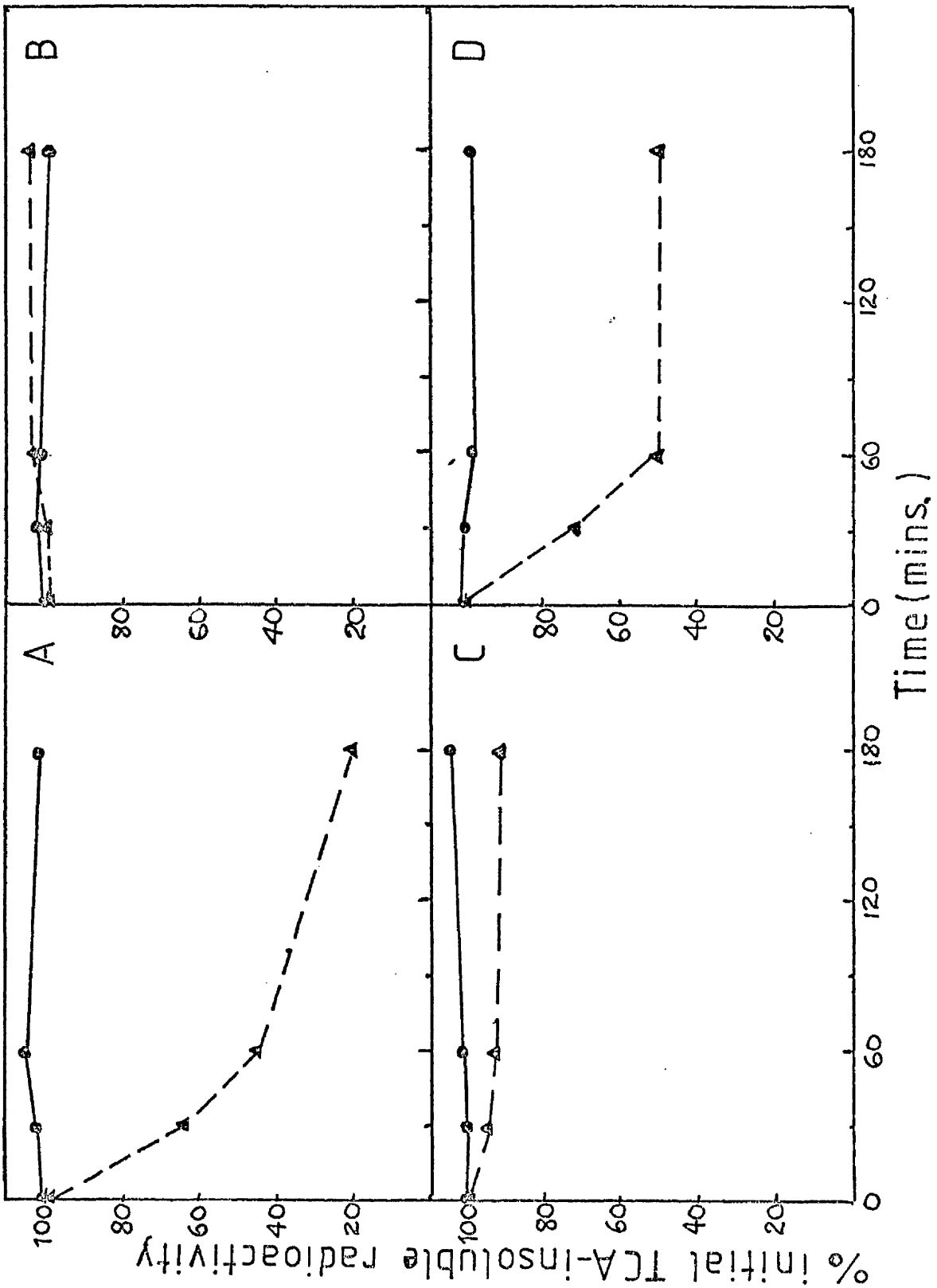

A
B
C
D
% initial TCA-insoluble radioactivity
Time (mins.)
0
60
120
180
20
40
60
80
100

Fig 17

Exoribonuclease Activity in the Post-nucleolar Supernatant Fraction from HeLa Cells

A post-nucleolar supernatant fraction (P.N.S.) was obtained by the procedure detailed in Materials and Methods 3.2 from HeLa cells which had been exposed to [^{3}H]-uridine for 15 minutes in the presence of 0.04 μg/ml actinomycin D, immediately before harvesting. The P.N.S. fraction, obtained from 5×10^7 HeLa cell nuclei, was incubated in LSLMT buffer at 37^o and at intervals 50 μl aliquots were removed and immediately frozen. Thawed digests were applied to Whatman DE 81 DEAE-cellulose paper chromatograms and chromatography was conducted in the ascending mode in a solvent of 0.75M NH_4HCO_3 pH 8.6 to permit the detection of any mononucleotide products. Developed chromatograms were fractionated and assayed for radioactivity as in Materials and Methods 3.7.4.3. The mobility of a marker species uridine 5'monophosphate was assessed by examining the developed chromatogram under UV-irradiation. The positions of the origin and solvent front coincided with fraction numbers 4 and 21 respectively. Aliquots were removed and assayed by DEAE-cellulose paper chromatography after incubation for: A) - 0 mins; B) - 60 mins; c) - 180 mins.

Fig.17

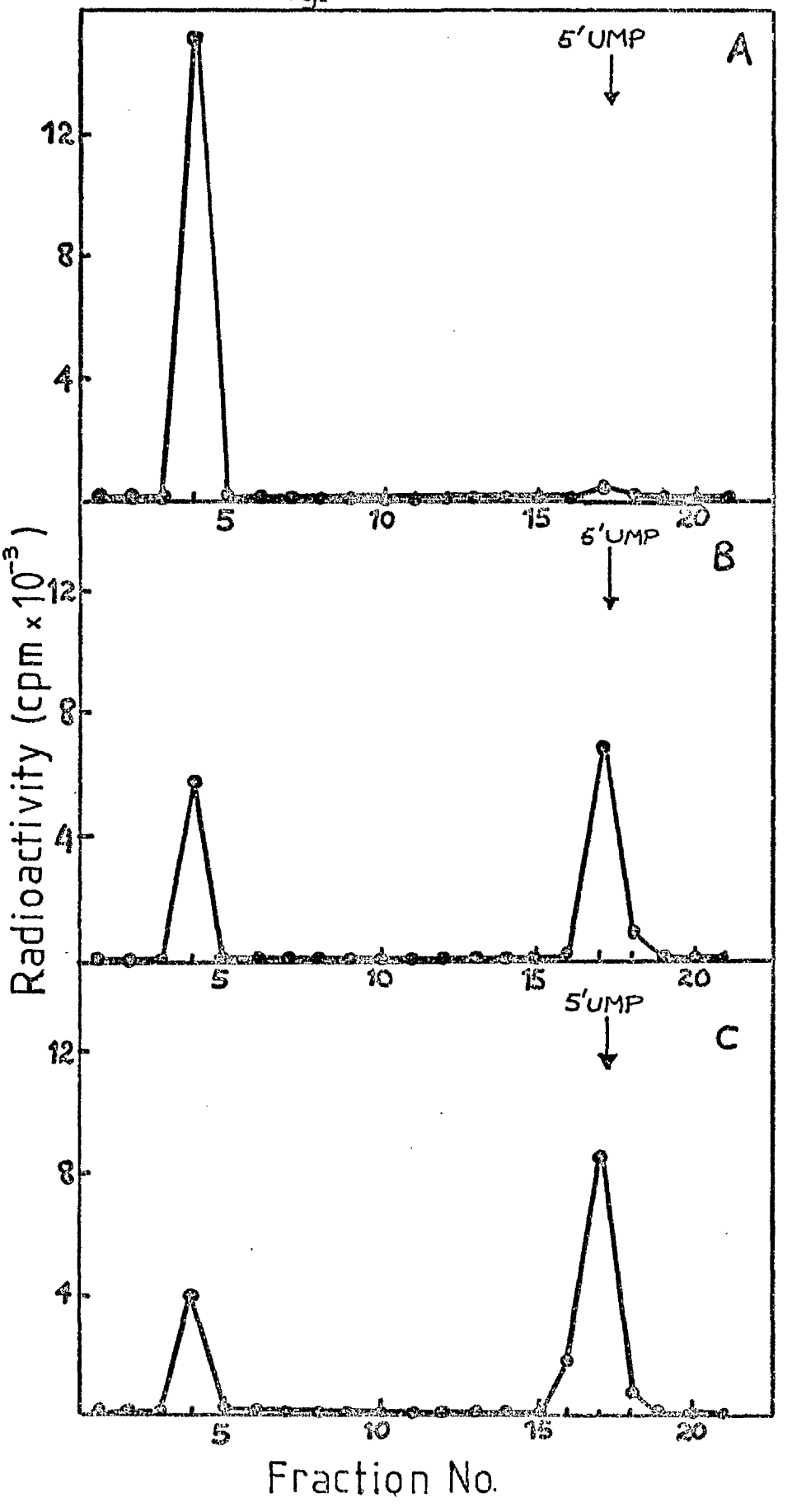

5'UMP
A
12
8
4
5
10
15
20
5'UMP
B
12
8
4
5
10
15
20
5'UMP
C
12
8
4
5
10
15
20
Radioactivity (cpm x 10⁻³)
Fraction No.

TABLE 5 Assay of Exoribonuclease Activity Associated with HeLa HnRNP Particles

Nature of Incubation of HnRNP Particles at 37° for 3 hours	Percentage of Initial Total Acid-insoluble Radioactivity remaining after incubation	Percentage of Radioactivity remaining at origin of a DEAE-cellulose paper chromatography following development in 0.75M NH_4HCO_3 pH 8.6
[^{3}H]-labelled hnRNP particles in LSLMT buffer	104 (±3)	98
Unlabelled hnRNP particles in 0.01M Tris-HCl pH 7.8, 10mM $MgCl_2$ buffer with:-		
0.5μg [^{3}H]-poly C (8.2μg/μCi)	103 (±6)	96
0.5μg [^{3}H]poly A (6.4μg/μCi)	105 (±4)	98
0.5μg [^{3}H] poly U (8.2μg/μCi)	106 (±4)	99
2μg [^{3}H]-hnRNA (10^4 cpm/μg)	103 (±5)	100
10μg [^{3}H]-28s rRNA (2×10^3 cpm/μg)	105 (±3)	99

Possible exoribonuclease activity associated with HeLa hnRNP particles was assayed either by incubation of [^{3}H]-labelled hnRNP particles (prepared as described in Materials and Methods Fig 7 Routes Ia, IIb) in LSLMT buffer or by incubation of unlabelled hnRNP particles (20μg protein) with a variety of [^{3}H]-labelled RNA species in 0.01M Tris-HCl pH 7.8, 10mM $MgCl_2$. Exoribonuclease activity was monitored as a time-dependent decline in initial acid-insoluble radioactivity or by use of a DEAE-cellulose paper chromatography system (Materials and Methods 3.7.1.4) to detect the presence in the digests of mononucleotide species.

marker species on DEAE-cellulose paper chromatograms (Table 5). Confirmation of an absence of exonuclease activity associated with the particles was obtained following incubation of unlabelled hnRNP particles with a variety of radioactively-labelled RNA species including the artificial ribohomopolymers [^{3}H]-poly U, [^{3}H]-poly C and [^{3}H]-poly A and also HeLa [^{3}H]-28s rRNA (prepared as in Materials & Methods 3.3.1) and [^{3}H]-hnRNA (Materials & Methods 3.3.2) (Table 5).

However, equivalent analyses using a HeLa chromatin fraction produced evidence of a relatively vigorous exoribonuclease activity. Both self-incubation of pre-labelled chromatin and incubation of unlabelled chromatin with a variety of radioactively labelled RNA species resulted in degradation of the radioactively-labelled species which was expressed as a time-dependent decline in total acid-insoluble radioactivity or in the appearance of molecular species which co-migrated with marker mononucleotides in a DEAE-cellulose paper chromatography system (Figs 16D, 18, 19A, 19C). Incubation was normally conducted in the presence of a 10mM NaCl buffer containing 10mM Mg^{2+} ions. However when incubation was carried out in a buffer in which the 10mM Mg^{2+} was replaced by 10mM EDTA exonucleolytic degradation of the RNA substrate was abolished (Figs 19B, 19D).

The nucleosol compartment also harboured a similar exoribonuclease activity although in this case the activity was less evident than with that expressed in the chromatin fraction (Fig 16C; Table 6).

2.1.2 Endoribonuclease

One consequence of the EDTA-induced abolition of HeLa nuclear exoribonuclease activity was that it permitted a means of assaying endoribonuclease activity of the chromatin and nucleosolic fractions in the absence of a potentially masking exoribonuclease activity. However since there was no evidence for exoribonuclease activity associated with HeLa hnRNP particles, in agreement with similar findings by McParland et al (1972) and Northemann

Fig 18

Exoribonuclease Activity in a Chromatin Fraction from HeLa Cells

A chromatin fraction was obtained by the procedures detailed in Materials & Methods 3.2 from HeLa cells which, immediately before harvesting, had been exposed to [^{3}H]-uridine for 15 minutes in the presence of 0.04 μg/ml actinomycin D. The chromatin fraction obtained from 5 x 10^7 HeLa cell nuclei was incubated in LSLMT buffer at 37^o and, at intervals, 50 μl aliquots were removed and analysed by DEAE-cellulose paper chromatography using a solvent of 0.75M NH_4HCO_3 pH 8.6 (Materials & Methods 3.7.1.4 and 3.7.4.3). The positions of the origin and solvent front coincided with fraction numbers 4 and 21 respectively while the position of a marker uridine 5' monophosphate species was assessed by examining the developed chromatogram under UV-irradiation. Analysed samples were obtained following incubation for: A) - 0 mins; B) - 60 mins; C) - 180 mins.

Fig. 18

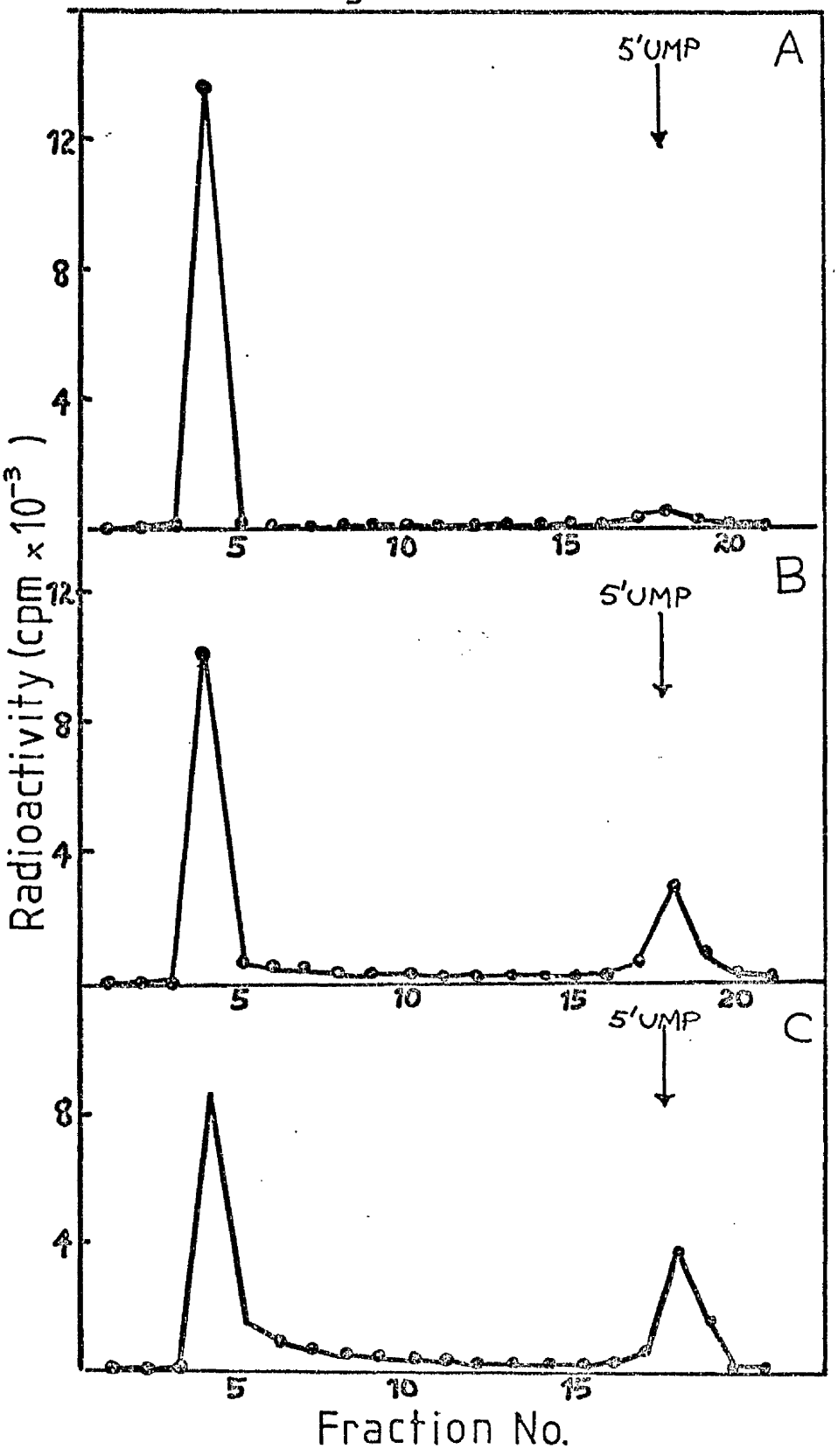

5'UMP
A
12
8
4
5
10
15
20
B
5'UMP
12
8
4
5
10
15
20
5'UMP
C
8
4
5
10
15
Radioactivity (cpm x 10⁻³)
Fraction No.

Fig 19

Sensitivity to EDTA of an Exoribonuclease Activity Associated with a Chromatin Fraction from HeLa Cells

A chromatin fraction was isolated from unlabelled HeLa cells by the procedures detailed in Materials and Methods 3.2. The chromatin pellet obtained from about 1.6×10^8 HeLa cell nuclei was resuspended in 0.01M Tris-HCl pH 7.8 and the solution was divided into four portions. To two portions was added $MgCl_2$ to a final concentration of 10mM while to the remaining portions was added EDTA to give a final concentration of 10mM. An RNA substrate of about 0.5μg of either [^{3}H]-poly C (8.2μg/μCi) or [^{3}H]-poly A (6.35μg/μCi) was provided and incubation was conducted at 37^o for 3 hours. Following incubation aliquots of individual reaction mixtures were applied to DEAE-cellulose paper chromatograms and subjected to ascending chromatography as detailed in Materials and Methods 3.7.1.4. Developed chromatograms were fractionated and assayed for [^{3}H]-containing readioactivity. The positions of marker mononucleotides cytidine 5' monophosphate and adenosine 5' monophosphate were obtained by examining the developed chromatogram under UV-irradiation. The positions of the origin and the solvent front coincided with fractions 4 and 20 respectively. Chromatograms represent incubations employing [^{3}H]-poly C (A,B) or [^{3}H]-poly A (C,D) in the presence of 10mM $MgCl_2$ (A,C) or 10mM EDTA (B,D).

Fig.19

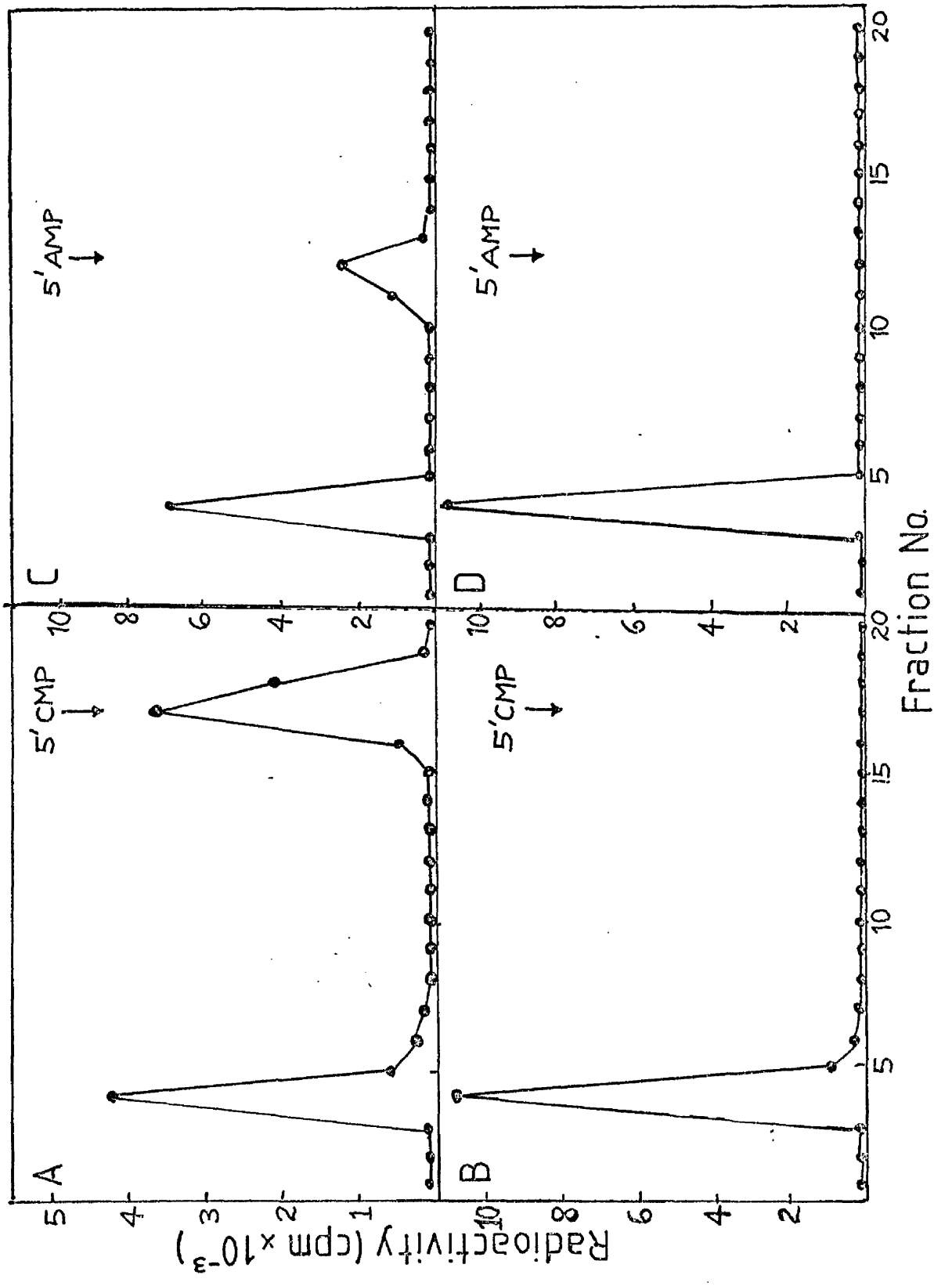


TABLE 6 Assay of Exoribonuclease Activity Associated with a HeLa Nucleosol Fraction

Nature of incubation of nucelosol fraction at 37° for 3 hours	Percentage of initial total acid-insoluble radioactivity remaining after incubation	Percentage of radioactivity of digests which migrates at the same position as marker mononucleotide species on DEAE-cellulose paper chromatograms
[^{3}H]-labelled nucleosol in LSLMT buffer	87 (±4)	n.d.
Unlabelled nucleosol in O.01M Tris-HCl pH 7.8, 10mM $MgCl_2$ with:-		
0.5μg [^{3}H]-poly C (8.2μg/μCi)	91 (±5)	11
0.5μg [^{3}H]-poly A (6.4μg/μCi)	96 (±2)	5
2μg [^{3}H]-hnRNA (10^4 cpm/μg)	92 (±4)	7

Exoribonuclease activity associated with a HeLa nucleosol fraction obtained from $5x10^7$ HeLa cell nuclei was assayed in a procedure exactly analogous to that described in Table 5.

et al (1978) for rat liver 30s hnRNP particles, no restrictions were imposed on an assay for endoribonuclease activity associated with HeLa hnRNP particles.

To assay for HeLa hnRNP particle-associated endoribonuclease activity hnRNP particles were prepared from cells which had been briefly exposed immediately before harvesting to [^{3}H]-uridine in the presence of 0.04 μg/ml actinomycin D and the resulting particles were incubated at 37^o in a buffer containing 10mM NaCl and 10mM $MgCl_2$. When the RNA was extracted following incubation and analysed on denaturing gradients the acid-insoluble radioactivity profile revealed a spectrum of sedimentation coefficients which were considerably lower than those for unincubated particles (Fig 20). Since the incubation was not accompanied by any exoribonuclease activity (see 2.1.1 above) the degradation of the RNA component is thus presumed to be mediated by an endonucleolytic mechanism.

The sedimentation profile on denaturing gradients of the rapidly-labelled RNA extracted from hnRNP particles prior to incubation reveals species of lower average sedimentation coefficient than that expressed by mRNA on comparable gradients (Perry, 1976). Also the chromatin-associated rapidly-labelled RNA encompasses a range of sedimentation coefficients (Fig 15) which are lower on average than that evinced by rapidly labelled RNA extracted directly from nuclei (Materials & Methods; Figs 9, 10). Therefore, in agreement with the findings of Augenlicht & Lipkin (1976) who used a similar nuclear sonication method for the preparation of HT-29 hnRNP particles there is clearly RNA breakdown during preparation. Further, since the total acid-insoluble radioactivity remained constant during the isolation of the HeLa hnRNP particles the RNA degradation would appear to be exerted by an endonucleolytic mechanism - again in agreement with Augenlicht & Lipkin (1976).

RNA degradation during the protracted procedures for isolating hnRNP particles may therefore be inevitable, possibly because of the close association of the RNA with an endoribonuclease activity responsible for its processing.

Fig 20

Digestion of Endogenous RNA following Self-Incubation of HeLa HnRNP Particles

[^{3}H]-labelled HeLa hnRNP particles (100 μg. protein) were incubated in LSLMT buffer at 37^o for 0 hours or 3 hours. Following incubation RNA was extracted from the digests and analysed on 98% formamide - containing 8% to 20% linear sucrose density gradients (Beckman SW56 rotor, 50000 rpm, 30^o, 16 hours). Aliquots of gradient fractions were analysed for acid-insoluble radioactivity and the positions of [^{3}H]-labelled cytoplasmic RNA species were determined on a parallel gradient.

●——● 0 hours incubation

▲------▲ 3 hours incubation

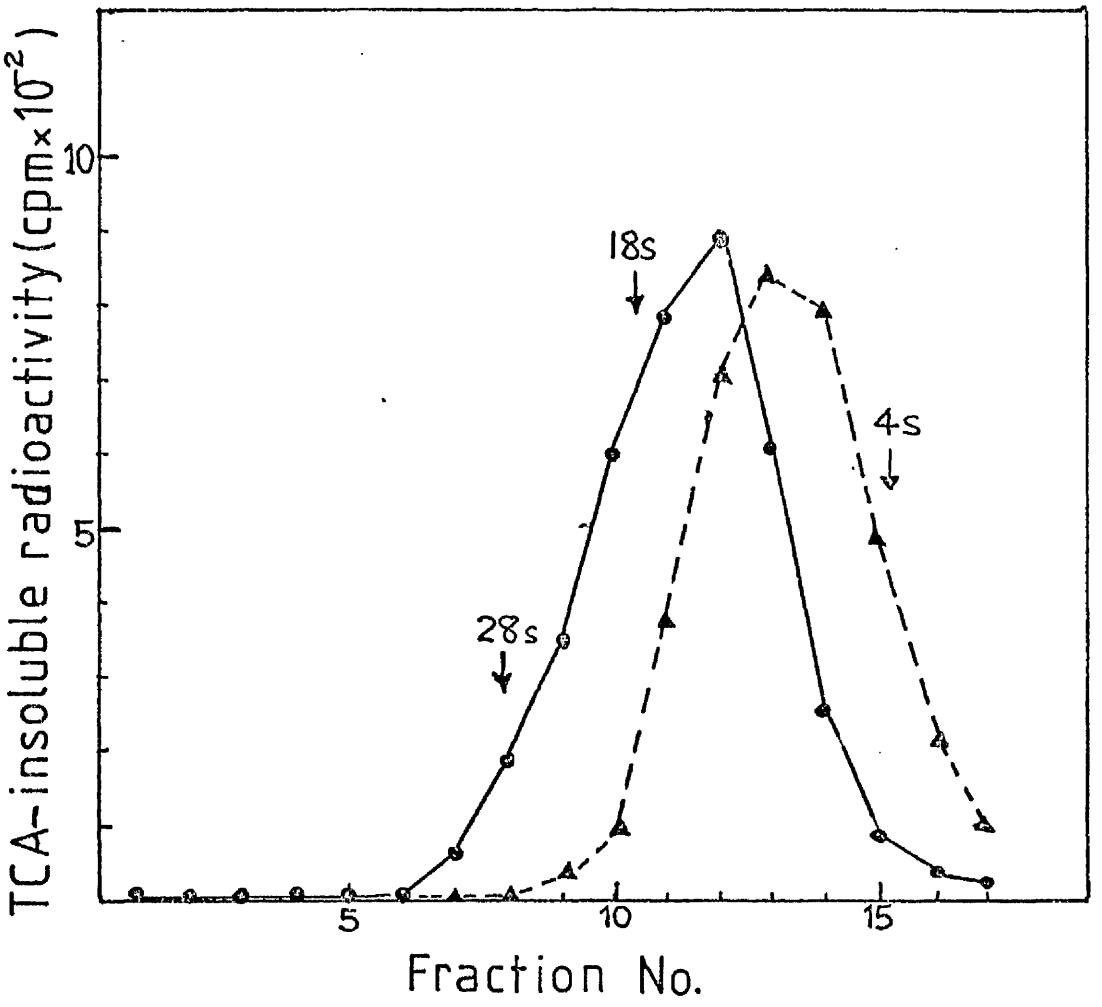

TCA-insoluble radioactivity (cpm×10⁻²)
10
5
18s
4s
28s
5
10
15
Fraction No.

Fig. 20

However, since the endoribonuclease activity associated with the HeLa hnRNP particles generated RNA products considerably smaller than mRNA some extra element of specificity is probably lacking, if indeed the associated endoribonuclease activity is specifically involved in hnRNA processing. Nevertheless in this case one would expect a considerable proportion of the products to be smaller than mRNA species because of the established occurrence of intervening sequences in a variety of mRNA precursors, which are smaller than the mature mRNA size.

Incubation of unlabelled HeLa hnRNP particles with [^{3}H]-labelled hnRNA (prepared from HeLa cells as in Materials & Methods 3.3.2) also results in degradation of the RNA moiety when analysed on denaturing gradients (Fig 21). Because there is no evidence of accompanying exoribonuclease activity (see 2.1.1) the hydrolysis of the RNA is mediated by an endoribonucleolytic mechanism. Increasing amounts of hnRNP particles provoked degradation of the RNA to structures of considerably lower average sedimentation coefficient than that which characterizes HeLa mRNA. Again, therefore, if the hnRNP particle-associated endoribonuclease is featured in the processing of hnRNA *in vivo* the experimental conditions employed probably do not cater for the specificity such an enzyme would be expected to exert *in vivo*. Further, the endoribonuclease activity is capable of degrading a variety of RNA substrates including [^{3}H]-HeLa 45s nucleolar RNA (Fig 22) [^{3}H]-HeLa cytoplasmic RNA (Fig 27) and to some extent [^{3}H]-poly C (Fig 23). Consequently under the experimental conditions employed the endoribonuclease activity does not appear to exert great substrate specificity.

The endoribonuclease activity appears to be stimulated by 10mM Mg^{2+} but there is no absolute requirement for the presence of Mg^{2+} ions judging by the considerable degradation obtained when EDTA replaces Mg^{2+} in the incubation buffer (Fig 24). Quantitation of the enzyme activity however proved to be difficult because of a variety

Fig 21

Analysis on Formamide-containing Sucrose Density Gradients of HeLa hnRNA following Incubation with HeLa HnRNP Particles

HnRNP particles were prepared from EDTA-washed HeLa cell nuclei as detailed in Materials and Methods 3.2 and resuspended in 0.01M Tris-HCl pH 7.8, 10mM $MgCl_2$. The particles (25 μg.protein or 40 μg.protein) were incubated with 5 μg [^{3}H] -HeLa hnRNA (10^4 cpm/μg) in 0.25 ml 0.01M Tris-HCl pH 7.8, 10mM $MgCl_2$ at 37^o for 0 hours or 3 hours. A parallel incubation of [^{3}H]-hnRNA was conducted at 37^o for 3 hours in the absence of hnRNP particles. Following incubation the RNA components were extracted and analysed on 98% formamide-containing 8% to 20% linear sucrose density gradients. Centrifugation was in the Beckman SW56 rotor at 50000 rpm for 16 hours at 30^o. Gradient fractions were assayed for acid-insoluble radioactivity. The positions of [^{3}H]-labelled cytoplasmic RNA markers were determined in a parallel gradient. Incubation was carried out at 37^o for 0 minutes in the presence of 25 μg protein hnRNP particles(o----o) or for 3 hours in the presence of various amounts of hnRNP particles, i.e. 0 μg protein (●——●); 25 μg protein (▲·····▲); 40 μg protein (x----x).

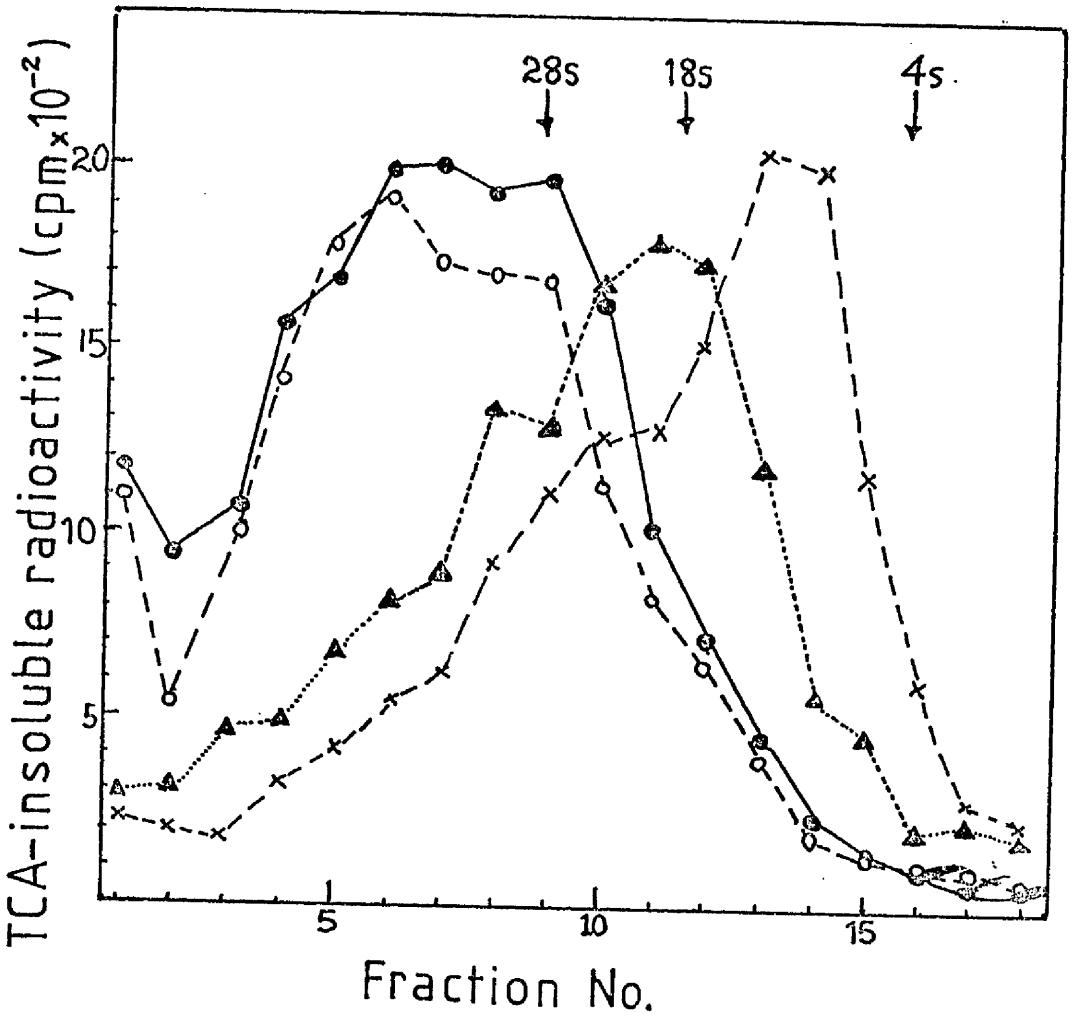

28s
18s
4s
TCA-insoluble radioactivity (cpm×10⁻²)
20
15
10
5
5
10
15
Fraction No.

Fig. 21

Fig 22

Analysis on Formamide-containing Sucrose Density Gradients of HeLa 45s rRNA following Incubation with HeLa HnRNP Particles

HnRNP particles (50μg protein) were prepared from EDTA-washed HeLa cell nuclei as detailed in Materials and Methods 3.2 and incubated with 5μg [^{3}H]-45s rRNA prepared from HeLa cells (Materials and Methods 3.3.3) in 0.25 ml 0.01M Tris-HCl pH 7.8, 10mM $MgCl_2$ at 37^o for 0 hours or 3 hours. In a parallel incubation 5μg [^{3}H]-45s rRNA were incubated for 3 hours at 37^o in the same buffer but in the absence of hnRNP particles. Following incubation, RNA was extracted and analysed on 98% formamide-containing 8% to 20% linear sucrose density gradients (Beckman SW56 rotor, 43000 rpm, 17 hours, 30^o). Gradient fractions were assayed for acid-insoluble radioactivity while the positions of [^{3}H]-labelled cytoplasmic RNA markers were determined in a parallel gradient. Incubation of the 45s rRNA was carried out at 37^o for 3 hours in the absence of hnRNP particles (x- - - -x) or in the presence of hnRNP particles (50μg protein) for 0 hours (●———●) or 3 hours (▲.....▲).

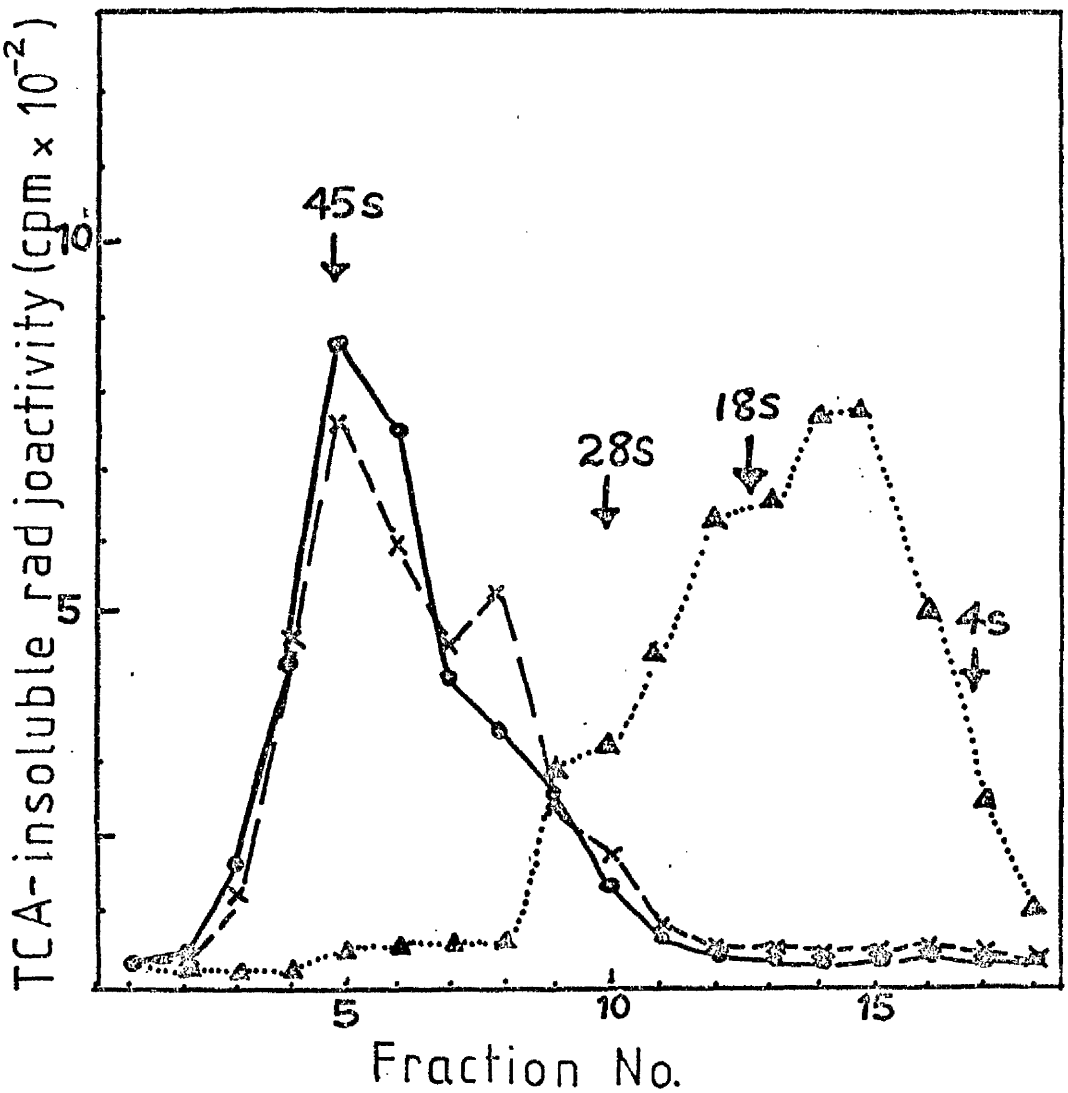

TCA-insoluble radioactivity (cpm x 10^{-2})
10
5
45s
28s
18s
4s
5
10
15
Fraction No.

Fig. 22

Fig 23

Analysis by Formamide-Polyacrylamide Gel Electrophoresis of [^{3}H]-poly C following Incubation with HeLa HnRNP Particles

HnRNP particles (30 μg protein) were prepared from EDTA-washed HeLa cell nuclei as detailed in Materials and Methods 3.2 and incubated with 0.5 μg [^{3}H]-poly C (8.2 μg/μCi) in 0.25 ml 0.01M Tris-HCl pH 7.8, 10mM $MgCl_2$ at 37^o for 0 hours or 3 hours. In a parallel incubation [^{3}H]-poly C was incubated for 3 hours at 37^o in the same buffer but in the absence of hnRNP particles. Following incubation, extracted RNA was analysed on 5% polyacrylamide gels containing formamide.

A - Incubation for 0 hours + hnRNP particles
B - Incubation for 3 hours - hnRNP particles
C - Incubation for 3 hours + hnRNP particles
BPB - Position of bromophenol blue tracker dye

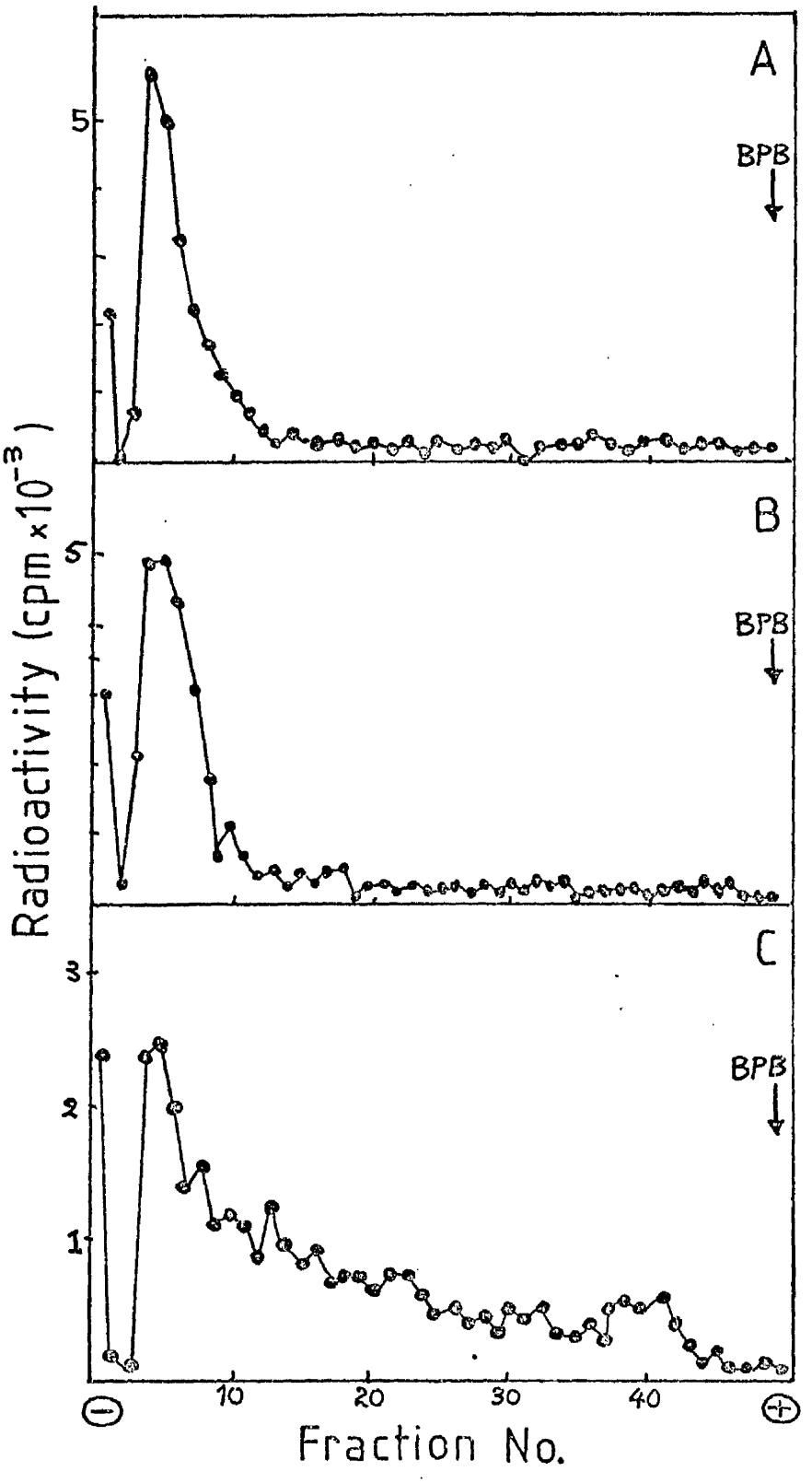

A
B
C
BPB
BPB
BPB
5
5
3
2
1
Radioactivity (cpm×10⁻³)
10
20
30
40
⊖
⊕
Fraction No.

Fig. 23

Fig 24

Effect of EDTA on Endoribonuclease Activity Associated with HeLa HnRNP Particles

HeLa hnRNP particles (40 μg protein) prepared from EDTA-washed HeLa cell nuclei (Materials and Methods 3.2) were incubated with [^{3}H]-hnRNA (prepared from HeLa cells - Materials and Methods 3.3.2) in 0.01M Tris-HCl pH 7.8 containing either 0.01M EDTA or 0.01M $MgCl_2$ for 0 or 3 hours at 37°. Following incubation, RNA was extracted and analysed on linear 8% to 20% sucrose density gradients in 98% formamide-containing buffer (Beckman SW56 rotor, 50000 rpm, 16 hours, 30°). Gradient fractions were assayed for TCA-precipitable radioactivity. Incubations were for 0 hours (●——●) or 3 hours in 0.01M EDTA (x- - - -x) or 0.01M $MgCl_2$ (▲·····▲).

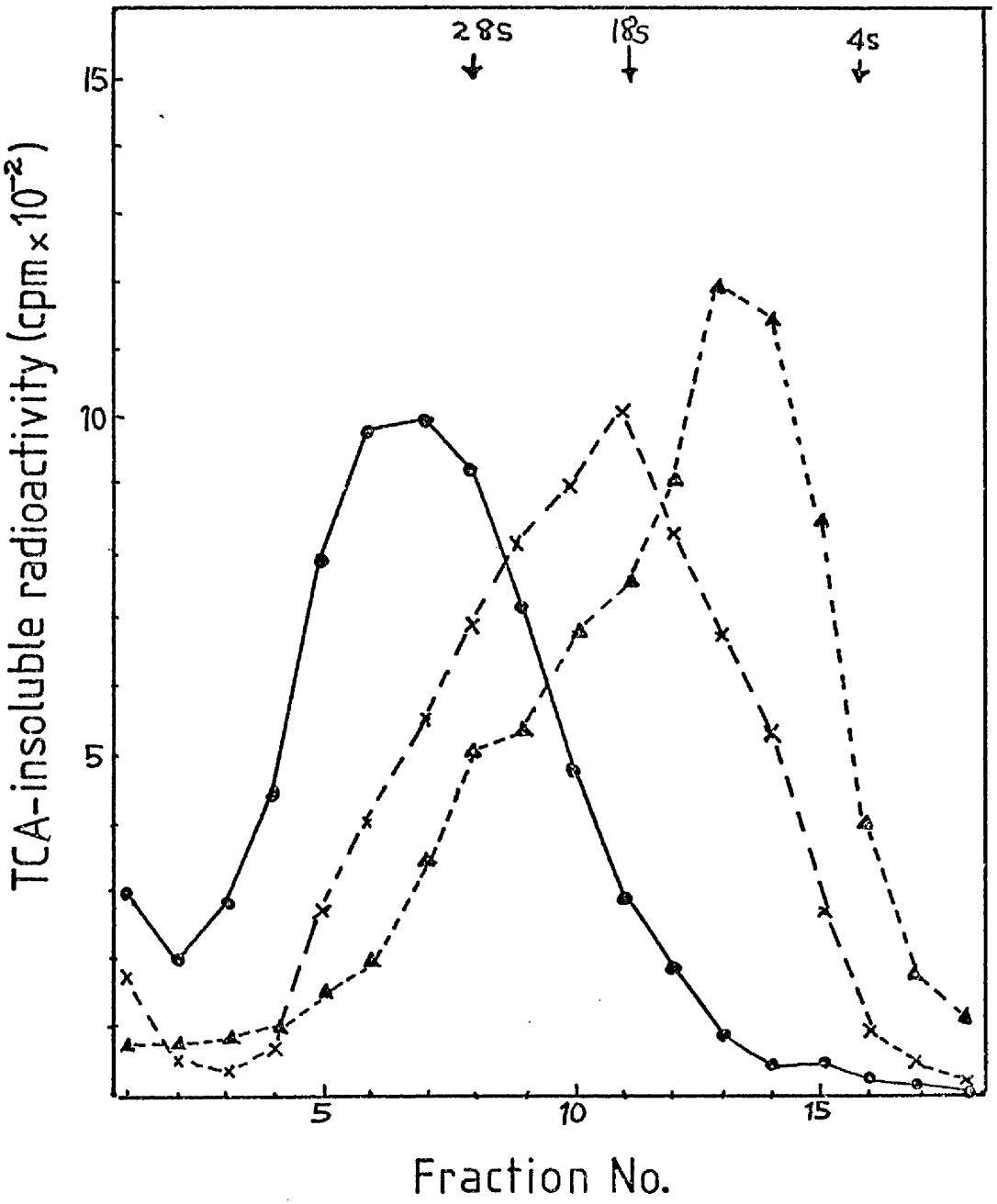

28s
18s
4s
15
10
5
TCA-insoluble radioactivity (cpm × 10⁻²)
5
10
15
Fraction No.

Fig. 24

of factors. The rate of degradation mediated by the enzyme is relatively modest when compared to the endoribonuclease activity reported in association with rat liver 30s hnRNP particles. Further, the production of acid-insoluble degradation products is not apparent after relatively long incubation periods. To complicate matters the enzyme activity was extremely labile to storage even at low temperatures.

To investigate endoribonuclease activity associated with chromatin and nucleosolic fractions the incubation buffer contained 5mM EDTA and no Mg^{2+} ions in order to suppress associated exoribonuclease activity (see 2.1.1 above). In both cases endoribonuclease activity was disclosed which appeared to exert little substrate specificity (Figs 25, 26, 28, 29). Again, however, the endoribonuclease activity displayed modest degradative capabilities and there was very little production of acid-insoluble components from the RNA substrates tested.

2.1.3 Double-strand specific ribonuclease

Following the analogy of the double-strand specific E.coli RNase III which has been shown to be involved in the initial processing cleavages of viral mRNA and bacterial rRNA precursors (Nikolaev et al, 1973; Dunn & Studier, 1973b) it has been widely considered that similar types of activity may be implicated in the processing of hnRNA in eukaryotic systems. In support of such a claim a number of investigators have documented evidence in favour of the existence of double-stranded segments in pulse-labelled nuclear RNA (Jelinek & Darnell, 1972; Ryskov et al, 1973; Monckton & Naora, 1974) while in the present study approximately 2.5% of HeLa hnRNA was estimated to consist of double-stranded regions (Materials & Methods 3.3.4). Therefore it was decided to investigate possible double-strand specific RNase activity in sub-nuclear fractions of HeLa cells with particular reference to HeLa hnRNP particles.

To assay for double-strand specific RNase activity a hybrid of two complementary artificial ribohomopolymers ($[^3H]$-poly C.poly I) was used as a substrate and, occasionally,

Fig 25

Incubation of [^{3}H]-HeLa Cytoplasmic RNA with a Chromatin Fraction from HeLa Cells

20μg of [^{3}H]-HeLa cytoplasmic RNA (2×10^3 cpm/μg - prepared as outlined in Materials and Methods 3.3.1) were incubated at 37° in 0.01M Tris-HCl pH 7.8, 10mM $MgCl_2$ with a chromatin fraction (30μg protein) prepared from HeLa cells as detailed in Materials and Methods 3.2. Extracted RNA was analysed on 98% formamide-containing 8% to 20% linear sucrose density gradients (Beckman SW60 rotor, 55000 rpm, 17 hours, 30°). Incubations: ●——● 0 hours+chromatin; ○——○ 3 hours-chromatin; ▲- - -▲ 3 hours+chromatin.

Fig 26

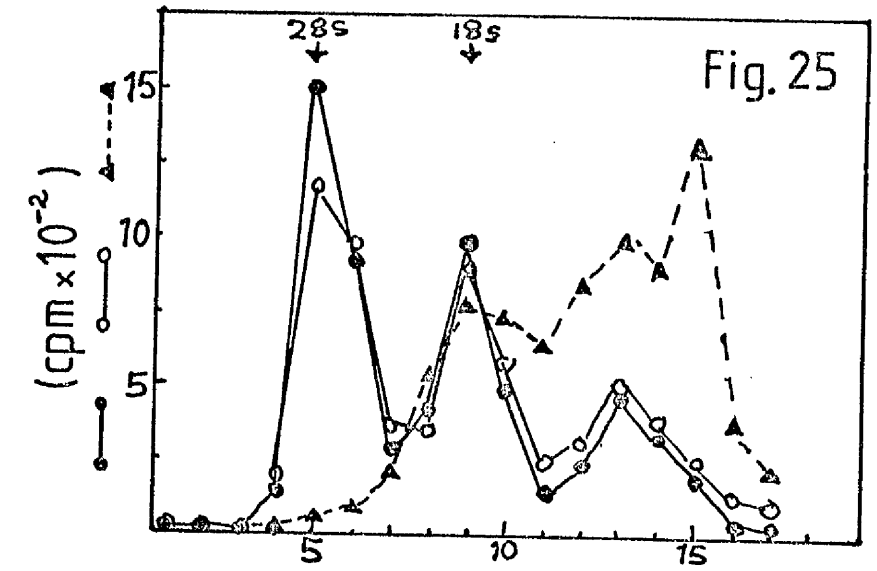
Incubation of [^{3}H]-HeLa Cytoplasmic RNA with a Nucleosol Fraction from HeLa Cells

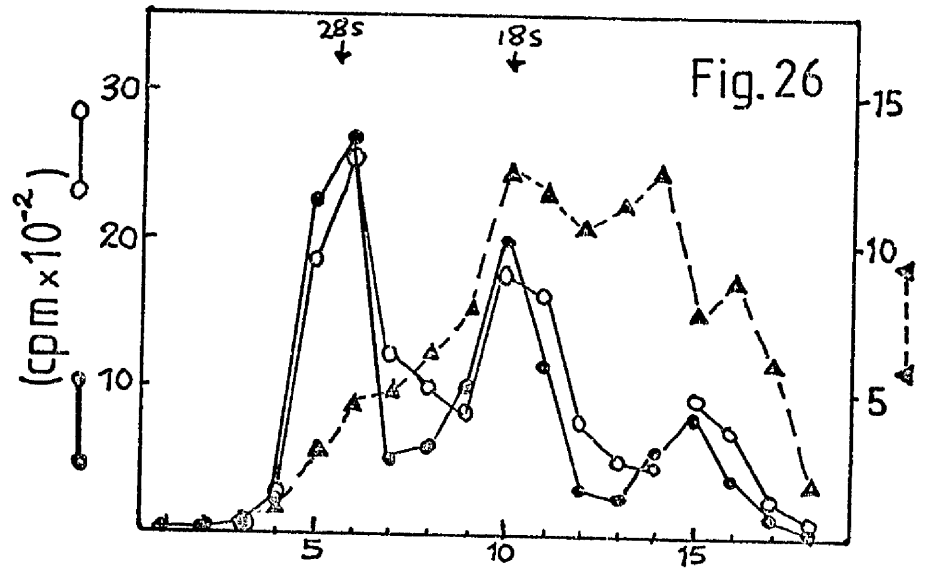
20μg of [^{3}H]-HeLa cytoplasmic RNA (2×10^3 cpm/μg) were incubated at 37° in 0.01M Tris-HCl pH 7.8, 10mM $MgCl_2$ with a nucleosol fraction prepared from HeLa cells (Materials and Methods - Fig 7 Routes Ia, IIa). Extracted RNA was analysed on 98% formamide-containing sucrose density gradients (Beckman SW56 rotor, 55000 rpm, 17 hours, 30°). Incubations: ●——● 0 hours+nucleosol; ○——○ 3 hours-nucleosol; ▲----▲ 3 hours+nucleosol.

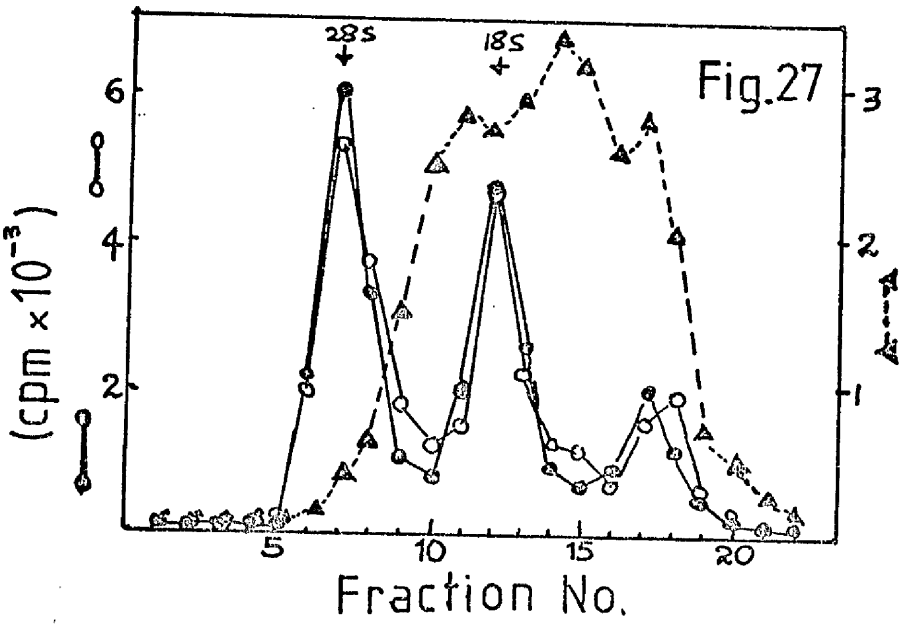
Fig 27

Incubation of [^{3}H]-HeLa Cytoplasmic RNA with HeLa HnRNP Particles

20μg of [^{3}H]-HeLa cytoplasmic RNA (2×10^3 cpm/μg) were incubated at 37° in 0.01M Tris-HCl pH 7.8, 10mM $MgCl_2$ with HeLa hnRNP particles (30μg protein). Extracted RNA was analysed on formamide-containing sucrose density gradients (Beckman SW56 rotor, 55000 rpm, 17 hours, 30°). Incubations: ●——● 0 hours-hnRNP particles; ○——○ 3 hours-hnRNP particles; ▲----▲ 3 hours+hnRNP particles.







TCA-insoluble radioactivity

Fig 28

Endoribonuclease Activity Associated with a Nucleosol Fraction from HeLa Cells

A nucleosol fraction from HeLa cells (Materials and Methods - Fig 7 Routes Ia, IIa) was incubated with ~5μg [^{3}H]-HeLa hnRNA (~10^4 cpm/μg) in 0.01M Tris-HCl pH 7.9, 2mM EDTA for 0 or 3 hours at 37°. Following incubation, RNA was extracted and analysed on linear 8% to 20% sucrose density gradients in 98% formamide-containing buffer (Beckman SW56 rotor, 40000 rpm, 30°, 17hours). Gradient fractions were assayed for acid-insoluble radioactivity and the positions of [^{3}H]-labelled cytoplasmic RNA marker species were determined from a parallel gradient. Incubation was carried out at 37° for 0 hours (o———o) or 3 hours in the absence (x- - - -x) or presence (●———●) of HeLa nucleosol.

Fig 29

Endoribonuclease Activity Associated with a Chromatin Fraction from HeLa Cells

A chromatin fraction from HeLa cells (Materials and Methods - Fig 7 Route Ia) was incubated with ~5μg [^{3}H]-HeLa hnRNA (~10^4 cpm/μg) in 0.01M Tris-HCl pH 7.9, 2mM EDTA for 0 or 3 hours at 37°. Following incubation RNA was extracted and analysed on formamide-containing sucrose density gradients as above (Beckman SW56 rotor, 50000 rpm, 30°, 16 hours). Incubation was carried out at 37° for 0 hours (o———o) or 3 hours in the absence (x- - - -x) or presence (●———●) of HeLa chromatin.

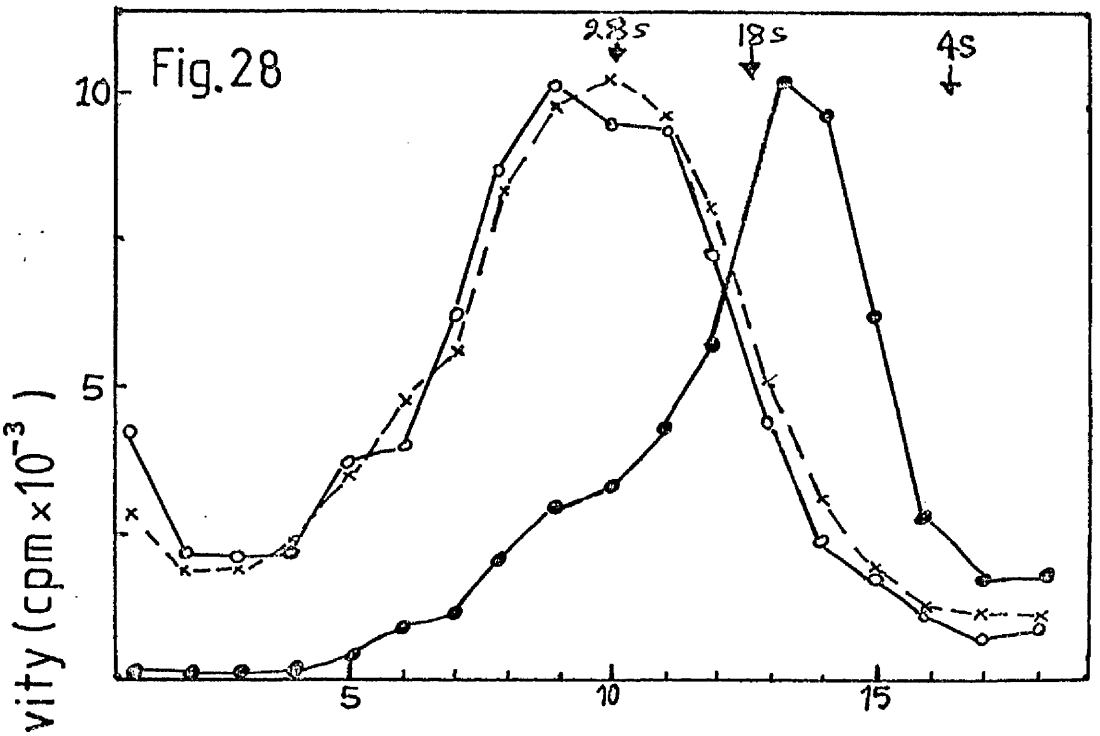


Fig. 28
28S
18S
4S
10
5
5
10
15

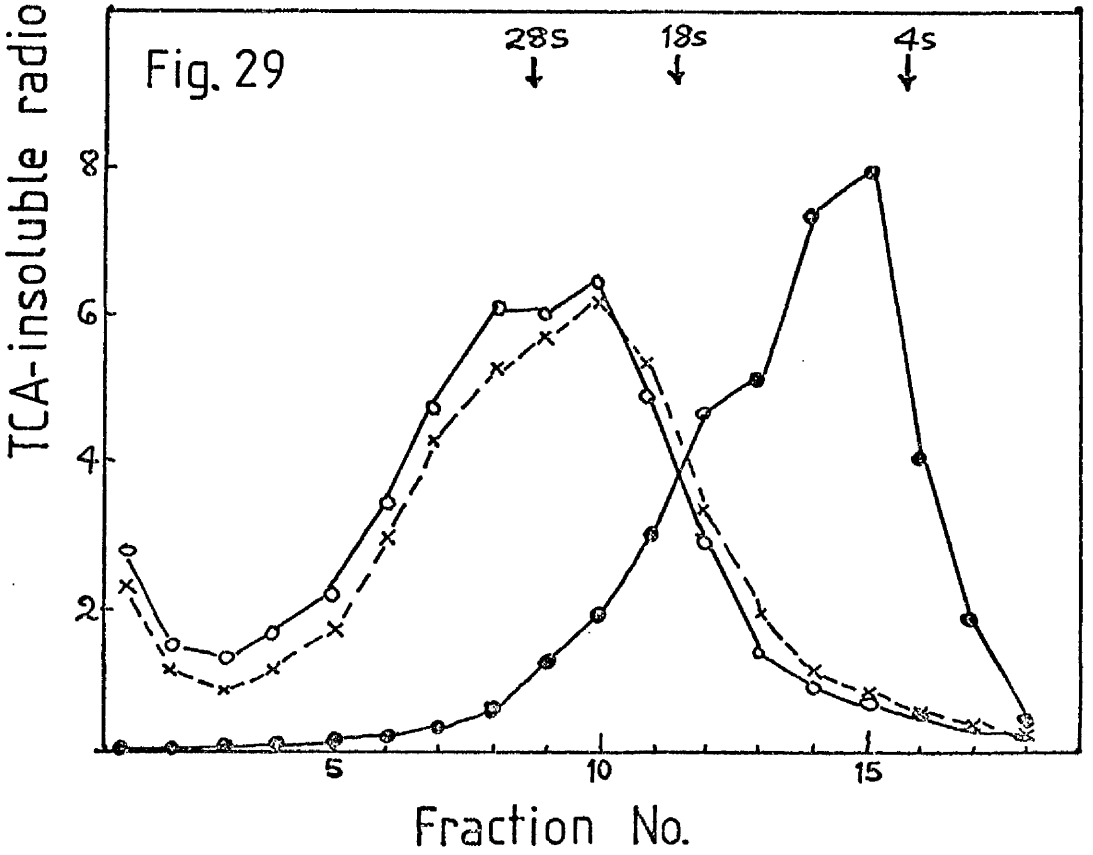


Fig. 29
28S
18S
4S
8
6
4
2
5
10
15
TCA-insoluble radioactivity (cpm×10⁻³)
Fraction No.

also [^{3}H]-double-stranded RNA prepared by pancreatic RNase A + T_1 digestion of [^{3}H]-HeLa hnRNA. When digestion of the double-stranded RNA substrate was monitored in terms of the production of acid-soluble components there was no detectable double-strand specific RNase associated with HeLa hnRNP particles (Table 7). However a nucleosol fraction from HeLa cells and, to a much lesser extent, the chromatin fraction of HeLa cells were able to effect some degradation of both RNA substrates (Table 7). That the [^{3}H]-poly C:poly I hybrid was indeed present in a double-stranded form was indicated by the inability of pancreatic RNase A to produce radioactively labelled acid-soluble fragments under identical experimental conditions although poly C was readily degraded under the same conditions.

To determine if the HeLa hnRNP particle-associated endoribonuclease could also be capable of fragmenting a double-stranded RNA substrate without production of acid-soluble products, HeLa hnRNP particles were incubated with [^{3}H]-poly C:poly I and the RNA was extracted following incubation and analysed on denaturing polyacrylamide gels (Fig 30). However, in this case no degradation of the radioactively labelled component was evident.

2.2 RNA Guanylyltransferase

The purified virions of some animal viruses have been profitably employed to support investigations into the nature of the capping reaction catalysed by RNA guanylyltransferase. The purified virions (or the virus cores obtained by detergent or proteolytic digestion of the virions) of a number of animal viruses can support *in vitro* synthesis of viral mRNA, which, in the presence of GTP and SAM demonstrates capped and methylated structures at the 5' end which are similar to those found *in vivo* as with the double-stranded DNA virus, vaccinia (Wei & Moss, 1975; Urushibara et al, 1975), the double-stranded RNA viruses, reovirus (Furuichi et al, 1975), wound tumour virus (Rhodes et al, 1977) and

TABLE 7 Double-strand RNA-specific RNase in HeLa Cell Nuclei

Substrate	HeLa Subnuclear Fraction	Percentage of initial acid-insoluble radioactivity remaining after incubation
1 μg dsRNA prepared by pancreatic A+T_1 RNase digestion of [^{3}H]-HeLa hnRNA (~10^4 cpm/μg)	None	102 ($\pm$3)
	HnRNP particles	100 ($\pm$3)
	Chromatin	93 ($\pm$3)
	Nucleosol	79 ($\pm$4)
1.5 μg [^{3}H]poly C•poly I (~19 μg/μCi)	None	103 ($\pm$4)
	(+Pancreatic RNase A)	98 ($\pm$4)
	HnRNP Particles	101 ($\pm$4)
	Chromatin	90 ($\pm$3)
	Nucleosol	72 ($\pm$2)

A post-nucleolar supernatant fraction (P.N.S.) was obtained from HeLa cell nuclei and resolved to give constituent subfractions of hnRNP particles, chromatin and nucleosol (Materials and Methods Fig 7 Routes Ia, IIb). Individual subnuclear fractions (obtained from 6×10^7 HeLa cell nuclei) were incubated in 0.01M Tris-HCl pH 7.8, 10mM $MgCl_2$ with 10000 cpm double-stranded RNA (~10^4 cpm/μg) prepared from [^{3}H]-hnRNA (Materials and Methods 3.3.4) or with an artificial hybrid of [^{3}H]-poly C•poly I prepared as detailed in Materials and Methods 3.6.3. After incubation for 3 hours at 37^o aliquots were assayed for acid-insoluble radioactivity and results (mean of two determinations) were expressed as a percentage of the initial acid-insoluble radioactivity prior to incubation.

Fig 30

Assay of Double-strand RNA-specific RNase Activity Associated with HeLa HnRNP Particles

HeLa hnRNP particles (20 μg protein) were incubated at 37^{o} in 0.15 ml 0.01M Tris-HCl pH 7.8, 10mM $MgCl_2$ with ~1.2 μg [^{3}H]-poly C.poly I (~19 μg/μCi) prepared as detailed in Materials and Methods 3.6.3. Incubation was carried out at 37^{o} for 0 or 3 hours while in a parallel incubation the RNA substrate was incubated for 3 hours in the absence of hnRNP particles. Following incubation, the RNA was extracted and analysed on 5% polyacrylamide gels containing formamide.

A - Incubation for 0 hours + hnRNP particles
B - Incubation for 3 hours - hnRNP particles
C - Incubation for 3 hours + hnRNP particles
BPB - Position of bromophenol blue tracker dye

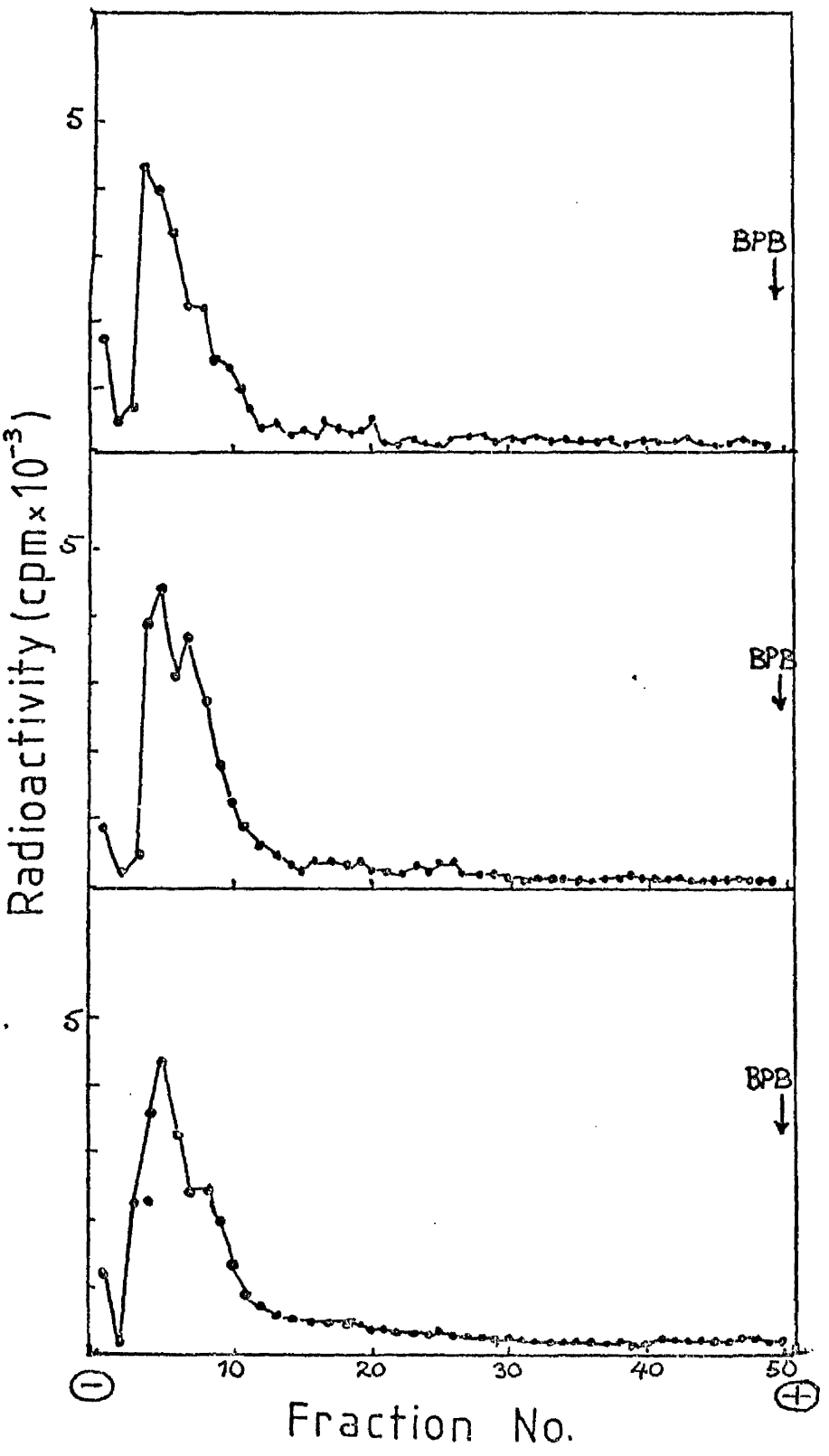


Fig. 30

cytoplasmic polyhedrosis virus (Furuichi & Muira, 1975) and the single-stranded RNA virus vesicular stomatitis virus (Abraham et al, 1975). Of these the enzymes that synthesize vaccinia mRNA caps have been solubilized and purified (Martin, Paoletti & Moss, 1975; Martin & Moss (1975) while recently the vaccinia mRNA guanylyl-transferase has been purified free from associated mRNA (guanine-7-)methyl transferase activity (Monroy et al, 1978). In eukaryotic cells a mRNA guanylyl-transferase activity has only recently been detected in HeLa cell nuclei (Wei & Moss, 1977) while a preliminary report has claimed the existence of a similar type of activity in calf thymus (Laycock, 1977). It was of interest therefore to examine the subnuclear distribution of the HeLa mRNA guanylyltransferase activity and, in particular, to investigate a possible association with HeLa hnRNP particles.

To assay for RNA guanylyltransferase activity vaccinia viral mRNA synthesized in vitro was used as a substrate. Purified vaccinia virions were disrupted by detergent treatment in the presence of a sulphydryl reagent and the liberated virus cores were incubated at 37° in the presence of the ribonucleoside triphosphates ATP, CTP, GTP and UTP and also Mg^{2+} ions. When [^{3}H]-UTP was included in the reaction mixtures there was a time-dependent incorporation of radioactive label into structures which could bind strongly to DEAE-cellulose paper filters (Fig 31). The incorporation was stimulated strongly by the presence of an energy regenerating system based on phosphoenolpyruvate and pyruvate kinase, while the presence of a RNase-inhibitor, macaloid, also produced evidence of higher amounts of incorporation (Fig 31). In addition, extraction of the RNA following incubation, and analysis on SDS-containing sucrose density gradients revealed a relatively broad spectrum of [^{3}H]-acid-insoluble radioactivity which embraces species from 4s to >28s with a peak at about 11s (Fig 32).

The size of the vaccinia viral mRNA synthesized in vitro compares favourably with that estimated by

Fig 31

In vitro Synthesis of Vaccinia Viral mRNA

Vaccinia virus cores were prepared by treating purified vaccinia virus with Triton X-100 and mercaptoethanol as detailed in Materials and Methods 3.5. In vitro synthesis of vaccinia viral mRNA was conducted in reaction mixtures containing: 50mM Tris-HCl pH 8.5; 7.5mM $MgCl_2$; 3mM ATP; 1mM CTP; 1mM GTP; 0.1mM UTP; 2 μCi [^{3}H]-UTP (50 Ci/mmole); 20 μg vaccinia virus core particles. Optional additions included 75 μg/ml macaloid and an energy regenerating system consisting of 7.2mM phosphoenolpyruvate and 20 μg/ml pyruvate kinase. Reaction mixtures were incubated for 1 hour at 37°. At various time intervals during incubation aliquots were removed and spotted onto Whatman DE 81 discs for estimation of DEAE-cellulose paper-bound radioactivity.

o——o Minus energy regenerating system

x——x Minus macaloid

▲——▲ Complete system

Fig 32

Analysis on SDS-containing Sucrose Density Gradients of Vaccinia Viral mRNA synthesized in vitro

Vaccinia viral mRNA was synthesized in vitro as described in Fig 31 above. The RNA product was extracted with phenol:chloroform (1:1) and precipitated from ethanol. The precipitate was collected in the form of a pellet by centrifugation whereupon the pellet was resuspended in 1.0 ml of LETS buffer and layered over 16.0 ml of a 10% to 25% linear sucrose density gradient. Centrifugation was conducted in the Beckman SW27.1 rotor at 25000 rpm for 16 hours at 20°. Gradient fractions were assayed for TCA-insoluble radioactivity. The positions of cytoplasmic RNA markers were determined in a parallel gradient.

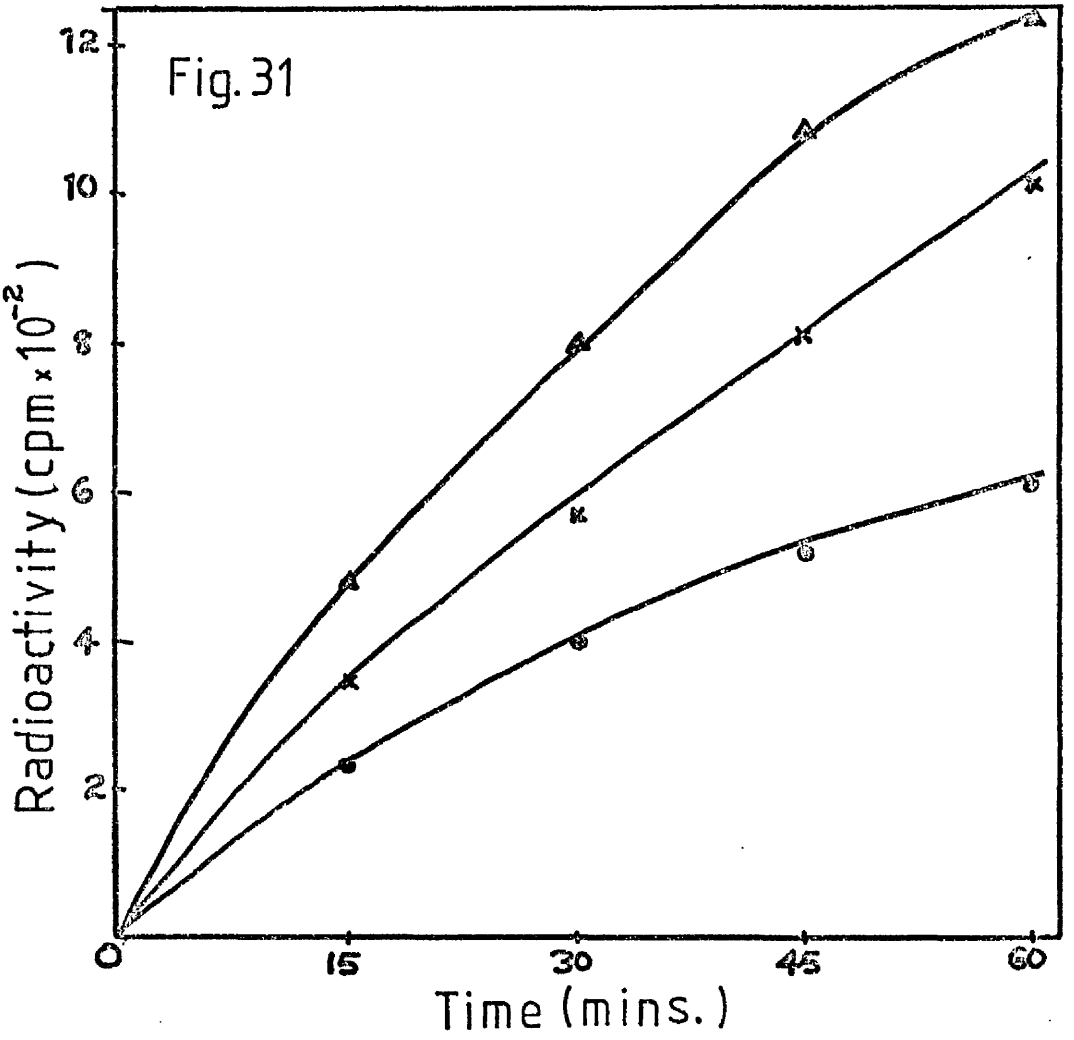


Fig. 31
Radioactivity (cpm × 10⁻²)
12
10
8
6
4
2
0
15
30
45
60
Time (mins.)

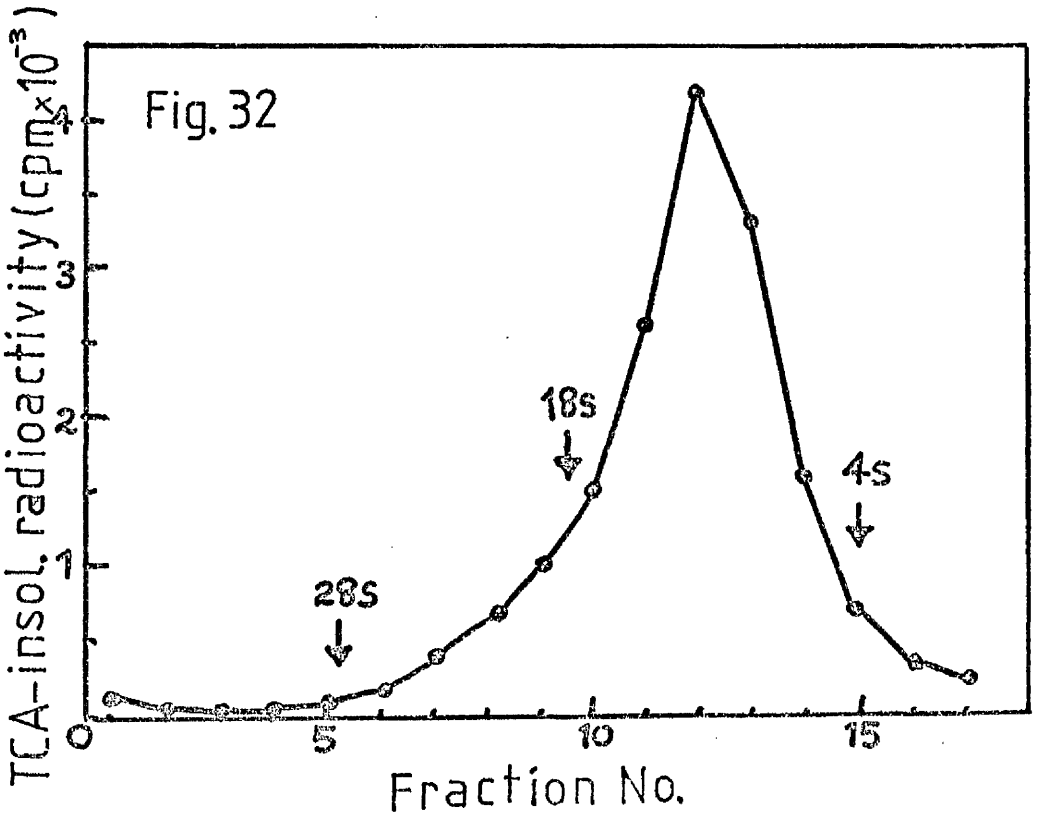


Fig. 32
TCA-insol. radioactivity (cpm × 10⁻³)
4
3
2
1
0
28S
18S
4S
5
10
15
Fraction No.

Kates & Beeson (1970). In addition, when the in vitro synthesis is conducted in the absence of s-adenosyl methionine the vaccinia viral mRNA products boast 5' termini of ppA and ppG as well as G(5')pppG and G(5')pppA (Moss et al, 1976) and these may serve as substrates for capping and methylating enzymes. Therefore, to assay for RNA guanylyltransferase activity test samples were incubated with purified vaccinia viral mRNA, synthesized in vitro in the absence of SAM, and with [α-^{32}P]-GTP in a suitable buffer. Double digestion of the reaction product with nuclease P_1 (cleaves all 3'-5' phosphodiester linkages in RNA or DNA) followed by alkaline phosphatase treatment permits distinction of [^{32}P]-labelled capped species on a suitable electrophoretic system (Fig 33). Of the various subnuclear fractions tested, only the nucleosol fraction from HeLa cells generated a reaction product which migrated with the same mobility as $m^7GpppG^{(m)}$ and $m^7GpppA^{(m)}$ although some species of slightly greater electrophoretic mobility, possibly GpppG and GpppA, were also just evident. The low incorporation of the [^{32}P]-radioactivity precludes confident interpretation of analogously migrating species obtained using HeLa chromatin as a possible source of guanylyltransferase activity.

The results are therefore consistent with a nucleosolic location for HeLa nuclear RNA guanylyltransferase activity while also indicating a similar location for the RNA(guanine-7) methyl transferase activity which has been detected in both nuclear and cytoplasmic fractions of HeLa cells (Ensinger & Moss, 1976). Very recently a report by Bajszár et al (1978) has claimed the existence of enzyme activities in association with rat liver 30s hnRNP particles which can promote the synthesis of a capped structure $m^7G(5')pppG^mpC$ using as substrates ppGpC, GTP and SAM. However it is uncertain to what extent the association of these activities with hnRNP particles reflects the in vivo situation (see Discussion).

2.3 RNA ligase

The recent discovery that several viral and cellular

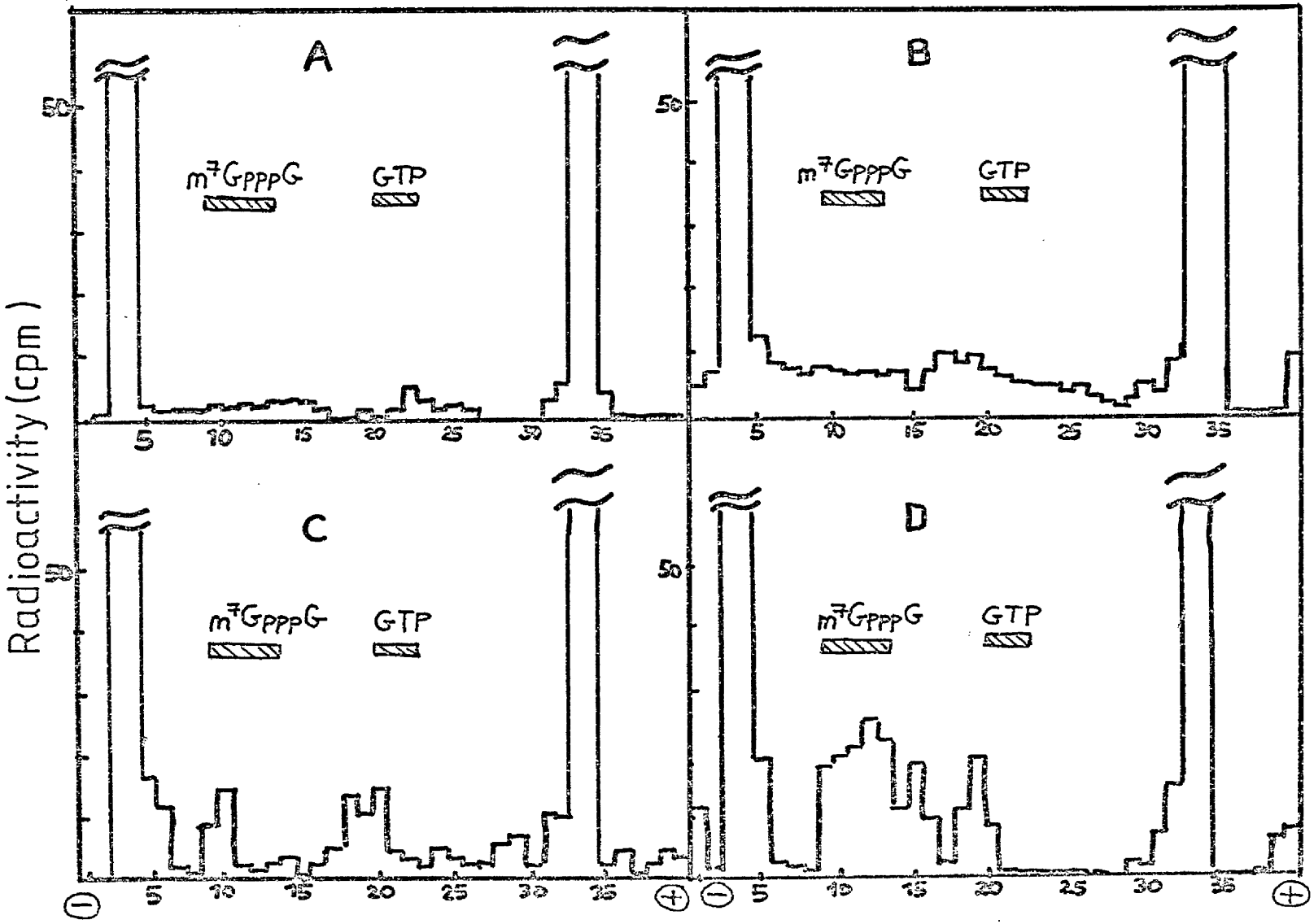
Fig 33

RNA Guanylyltransferase Activity in HeLa Cell Nuclei

HeLa cell nuclei were used to provide subnuclear fractions of nucleosol (Materials and Methods Fig 7 Routes Ib, IIa), chromatin (Materials and Methods Fig 7 Routes Ib, IIb) and hnRNP particles (Materials and Methods Fig 7 Routes Ib, IIb).

RNA guanylyltransferase activity was assayed in reaction mixtures of 0.15 ml containing: 50mM Tris-HCl pH 7.9, 5mM $MgCl_2$; 0.5mM $MnCl_2$; 1mM ATP; 1mM DTT; 10% glycerol; 10 μg of vaccinia viral mRNA synthesized *in vitro* (Figs 31, 32 above); 100 μM S-adenosyl-methionine; 25 μCi of [α-^{32}P]-GTP (350 Ci/mmol) and subnuclear fraction from 6×10^7 HeLa cells. Incubation at 30° for 1 hour was followed by RNA extraction and purification prior to digestion of the RNA by nuclease P_1, then alkaline phosphatase (Materials and Methods 3.6.5). Finally, the digests were analysed by high-voltage paper electrophoresis (Materials and Methods 3.7.1.5). The developed electrophoretogram was fractionated and assayed for [^{32}P]-radioactivity. A background rate of 22 cpm was subtracted from individual values prior to expressing the results as a histogram. The origin was located between fractions 3 and 4. Individual graphs represent assay of enzyme activity in: A) absence of subnucleolar fraction; B) hnRNP particles; C) chromatin; D) nucleosol. Peaks not accomodated by the radio-activity scale represent material bound at the origin, i.e. fraction nos. 3-5 (total cpm respectively of: A - 195; B - 260; C - 186; D - 312) and inorganic ^{32}P i.e. fraction nos. 33-35 (total cpm respectively of: A - 2065; B - 1872; C - 1065; D - 3460).

Fig. 33



mRNA species are composed of segments coded by disparate regions of the genome has prompted re-evaluation of theoretical mechanisms pertinent to the processing of hnRNA. One possibility that could be entertained features excision of transcribed intervening non-coding sequences present in the primary transcript followed by ligation of the coding sequences. Although it might be expected that the ligation reactions in this case would be catalysed by enzymes displaying high degrees of substrate specificity the specific RNA probes necessary for detecting such activity were not available in this study. However it was of interest to determine whether relatively non-specific RNA ligase activity could be detected in HeLa cell nuclei in analogy to the bacteriophage T_4-induced RNA ligase activity (Silber et al, 1972; Last & Anderson, 1976).

Although the existence of RNA ligase activity in eukaryotic cells has been questioned by Bedows et al (1975) other investigators have reported some evidence which might suggest the presence of RNA ligase activity in some eukaryotic systems (Linné et al, 1974; Yin, 1977; Koliais & Dimmock, 1978). In addition, Kolodny (1977) has presented some evidence which possibly indicates the en bloc incorporation of exogenous oligonucleotides into HeLa cell RNA which may therefore require a RNA ligase activity. On a different tack Edmonds et al (1976) and Kinniburgh & Martin (1976b) have suggested that internal oligo A regions in hnRNA may serve as a recognition signal for determining the site of addition of the 3' poly A segment. Although the available evidence suggests that the poly A addition is completed by sequential addition of AMP residues the possibility has not been eliminated that poly adenylate units may be added en bloc possibly to the 3'-adenylate terminal residue of a cleaved internal oligo A site.

To investigate these possibilities, subnuclear fractions from HeLa cells were assayed for their ability to convert a [^{32}P]-labelled monophosphate ester group located at the 5' terminus of a poly A substrate (prepared by end-labelling of the poly A with [γ-^{32}P]ATP and

polynucleotide kinase following alkaline phosphatase treatment) to an internal 3'-5'-phosphodiester bridge, either by intramolecular cyclization or intermolecular ligation. Resistance of the [^{32}P]-label to hydrolysis by alkaline phosphatase treatment could be taken to reflect an internal phosphodiester bridge location for the [^{32}P]-label. When various HeLa subnuclear fractions were assayed in this manner there appeared to be a positive response in each case when compared to the appropriate controls while the reaction did not appear to depend on the presence of ATP or NAD (Tables 8, 9).

To investigate the nature of the increased resistance of the [^{32}P]-label to alkaline phosphatase treatment appropriate incubated samples were subjected to several cycles of ethanolic precipitation in the cold, centrifugation and resuspension and then hydrolysed in alkali (0.3N NaOH, 22 hours at 37^{o}). Following these treatments internal [^{32}P]-label would be expected to be liberated as [^{32}P]-adenosine 2'(or 3') monophosphate while external [^{32}P]-label surviving alkaline phosphatase treatment would be liberated as $\overset{*}{p}$Ap (where $\overset{*}{p}$=^{32}P). Discrimination between these possibilities could be met by the appropriate paper chromatography system (Fig 34). However when analysed by this protocol it was found that the additional alkaline-phosphatase resistant [^{32}P]-label migrated in a position identical to that of an inorganic orthophosphate marker species. Possibly incubation of the subnuclear fractions generated some factor antagonistic to the action of alkaline phosphatase.

2.4 Poly A synthetase

Poly A synthetase activity has been partially purified and characterised from a cytoplasmic fraction of HeLa cells (Mans & Stein, 1974). In addition Jelinek (1974) has demonstrated that isolated intact HeLa cell nuclei can support poly A synthesis in vitro. The compass of the present study was therefore expanded to include extension of these studies. In particular, some attention was devoted to examination of the subnuclear distribution and reaction properties of the HeLa nuclear poly A synthetase activity.

TABLE 8

Assay of ATP-dependent RNA Ligase Activity in HeLa Cell nuclei

Reaction Conditions			% Acid-insoluble Radioactivity of Product		
Ligase Incubation	Presence of 0.1mM ATP	Alkaline Phosphatase treatment	P.N.S.	Chromatin	HnRNP Particles
-	-	-	100	100	100
+	-	-	112(±11)	108(±6)	98(±7)
+	+	-	60(± 7)	63(±4)	95(±5)
-	-	+	7(± 1)	7(±2)	10(±2)
+	-	+	35(± 4)	25(±7)	35(±5)
+	+	+	26(± 4)	19(±1)	33(±5)

Post-nucleolar supernatant (P.N.S.), chromatin and hnRNP particle fractions were prepared from EDTA-washed HeLa cell nuclei as detailed in Materials & Methods 3.2 and assayed for possible RNA ligase activity as described in Materials & Methods 3.6.6. Reaction mixtures of 0.1 ml contained: 50mM Tris-HCl pH 7.6; 10mM $MgCl_2$; 1.5mM DTT; 10^5 cpm $[^{32}P]$ -5'-end labelled poly A; enzyme source (obtained from 10^8 HeLa cells and occasionally 0.1mM ATP. Incubation at 37^o for 30 minutes was followed by alkaline phosphatase treatment so that RNA ligase activity could be quantitated as the percentage of $[^{32}P]$-radioactivity rendered resistant to alkaline phosphatase treatment.

TABLE 9

Assay of NAD-dependent RNA Ligase Activity in HeLa Cell Nuclei

Reaction Conditions			% Acid-insoluble Radioactivity of Product		
Ligase Incubation	Presence of 0.15mM NAD	Alkaline Phosphatase treatment	P.N.S.	Chromatin	HnRNP Particles
-	-	-	100	100	100
+	-	-	108(±5)	115(±10)	98(±7)
+	+	-	70(±4)	75(± 7)	92
-	-	+	6(±2)	7(± 1)	12(±3)
+	-	+	28(±4)	25(± 7)	20(±5)
+	+	+	26(±5)	20(± 7)	18(±7)

Post-nucleolar supernatant (P.N.S.), chromatin and hnRNP particle fractions were prepared from EDTA-washed HeLa cell nuclei as detailed in Materials & Methods Fig 7, and assayed for possible RNA ligase activity as described in Materials & Methods 3.6.6. Reaction mixtures of 0.1 ml contained: 50mM Tris-HCl pH 7.6; 10mM $MgCl_2$; 1.5mM DTT; 10^5 cpm [^{32}P]-5' end-labelled poly A; enzyme source (obtained from 10^8 HeLa cells) and occasionally 0.15mM NAD. Incubation at 37^o for 30 minutes was followed by alkaline phosphatase treatment (see Materials & Methods 3.6.6) so that RNA ligase activity could be quantitated as the percentage of [^{32}P].radioactivity rendered resistant to alkaline phosphatase treatment.

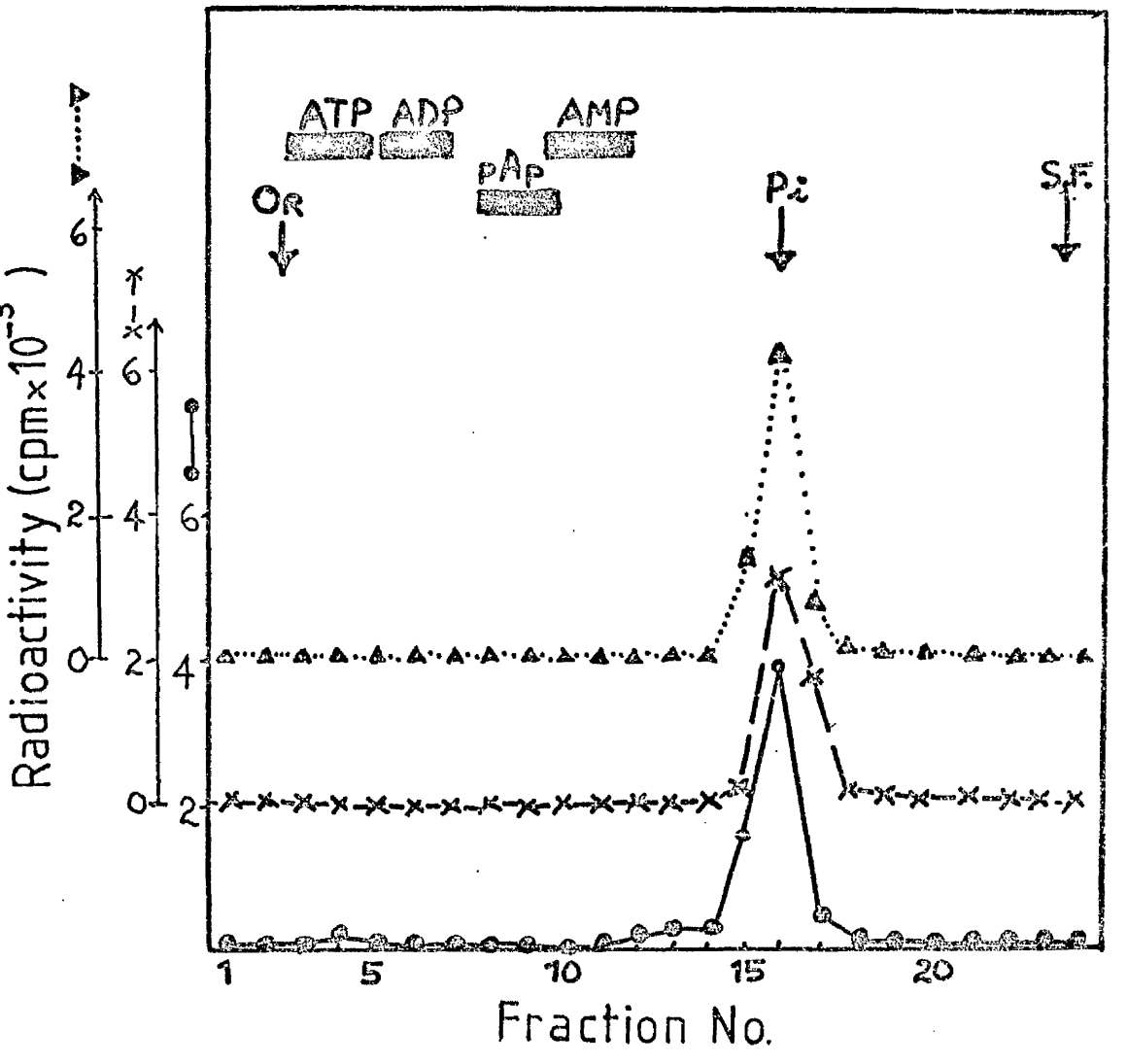
Fig 34

Chromatographic Analysis of Products of RNA Ligase Assays of HeLa Cell Subnuclear Fractions

RNA ligase activity was assayed in HeLa chromatin, hnRNP particles and P.N.S. fractions in the absence of ATP and followed by alkaline phosphatase treatment exactly as outlined in Table 8 (see row 5). Reaction products were adjusted to 0.2M NaCl precipitated from ethanol and resuspended in 1.0 ml of 0.2M NaCl, 10mM Tris-HCl pH 7.0 followed by a further two cycles of ethanolic precipitation and resuspension. The final ethanol precipitates were resuspended in 0.1 ml H_2O, adjusted to 0.3M NaOH and incubated at 37^{o} for 20 hours. Alkaline hydrolysates were analysed by paper chromatography (Whatman 3 MM paper, ascending mode, solvent of acetone/formic acid/H_2O in proportions of 60:14:26 by volume). In addition, alkaline-treated marker species of AMP, ADP, ATP, adenosine 3',5'-diphosphate (pAp) and $^{32}P_i$ were chromatographed in the same system. Following chromatography, the developed chromatograms were viewed under UV-irradiation to locate unlabelled marker species. The chromatogram was fractionated and assayed for [^{32}P]-radioactivity as described in Materials and Methods 3.7.4.3. Chromatographic analysis was carried out of RNA ligase assays involving the following subnuclear fractions: P.N.S. A------A; chromatin X-----X; hnRNP particles ●———●

O_R = origin S.F. = chromatographic solvent front

Fig. 34


ATP
ADP
AMP
pAp
OR
Pi
S.F.
Radioactivity (cpm×10⁻³)
Fraction No.
1
5
10
15
20

Various subnuclear fractions from HeLa cells i.e. chromatin, hnRNP particles and nucleosol were assayed for the presence of poly A synthetase activity supported by endogenous primer molecules or in the additional presence of exogenously provided poly A primer (Fig 35). No activity could be detected in association with hnRNP particles which promoted the time-dependent incorporation of $[^{14}C]$-radioactivity from $[^{14}C]$-ATP into acid-insoluble material even when a primer of poly A was available in high concentrations (Fig 35A). The chromatin fraction exhibited a modest activity utilizing endogenous primer molecules while the presence of exogenously added poly A primer boosted the reaction rate to much higher levels (Fig 35B). However by far the most vigorous rate of incorporation was experienced with the nucleosol fraction and this reaction appeared to be totally dependent on exogenously added poly A primer (Fig 35C).

The characteristics of the nucleosol-mediated poly A-dependent reactions were investigated further. When the concentration of the added poly A primer was increased the rate of incorporation of $[^{14}C]$ATP into acid-insoluble radioactivity was correspondingly enhanced (Fig 36). In addition, provision of an energy regenerating system of phosphoenolpyruvate and pyruvate kinase in the company of appropriate cationic requirements served to boost the reaction rate especially after 60 minutes when otherwise it began to flag considerably (Fig 37). Therefore an energy regenerating system and a relatively high concentration of poly A primer were adopted routinely.

Substrate and primer specificities of the nucleosol-mediated poly A synthetase reaction have also been investigated (Table 10). It was found that UTP could not replace ATP as a substrate for the enzymic activity. In addition various other ribohomopolymers e.g. poly U, poly C or polydeoxyribonucleotides e.g. poly dA, salmon sperm DNA were totally ineffective in replacing poly A as a primer for the poly A synthetase reaction. Of the various alternative primer substrates tested only yeast tRNA which has a 3'-CCA terminal proved to be relatively effective (15% of optimal values using poly A as a primer).

Fig 35

Poly A Synthetase Activity in HeLa Cell Nuclei

HeLa cell nuclei were prepared as described in Fig 33 and the purified nuclei were fractionated to yield nucleosol, chromatin and hnRNP particles. Sub-nuclear fractions obtained from 3×10^7 HeLa cells were individually included in incubation mixtures containing: 50mM Tris-HCl pH 8.5; 10mM DTT; 1mM $MnCl_2$; 1mM [^{14}C]-ATP (0.6 mCi/mmol); 1.2 mg/ml phosphoenolpyruvate; 1 μg/ml pyruvate kinase; 30mM KCl and occasionally a primer of 600 μg/ml poly A. Incubation was conducted at 37° for 2 hours and poly A synthetase activity was quantitated in terms of the time-dependent incorporation of [^{14}C]-radioactivity into acid-insoluble material. Incubations were conducted in the absence (o——o) or presence (●——●) of primer poly A and in the presence of hnRNP particles (A), chromatin (B) or nucleosol (C) fractions.

Fig. 35

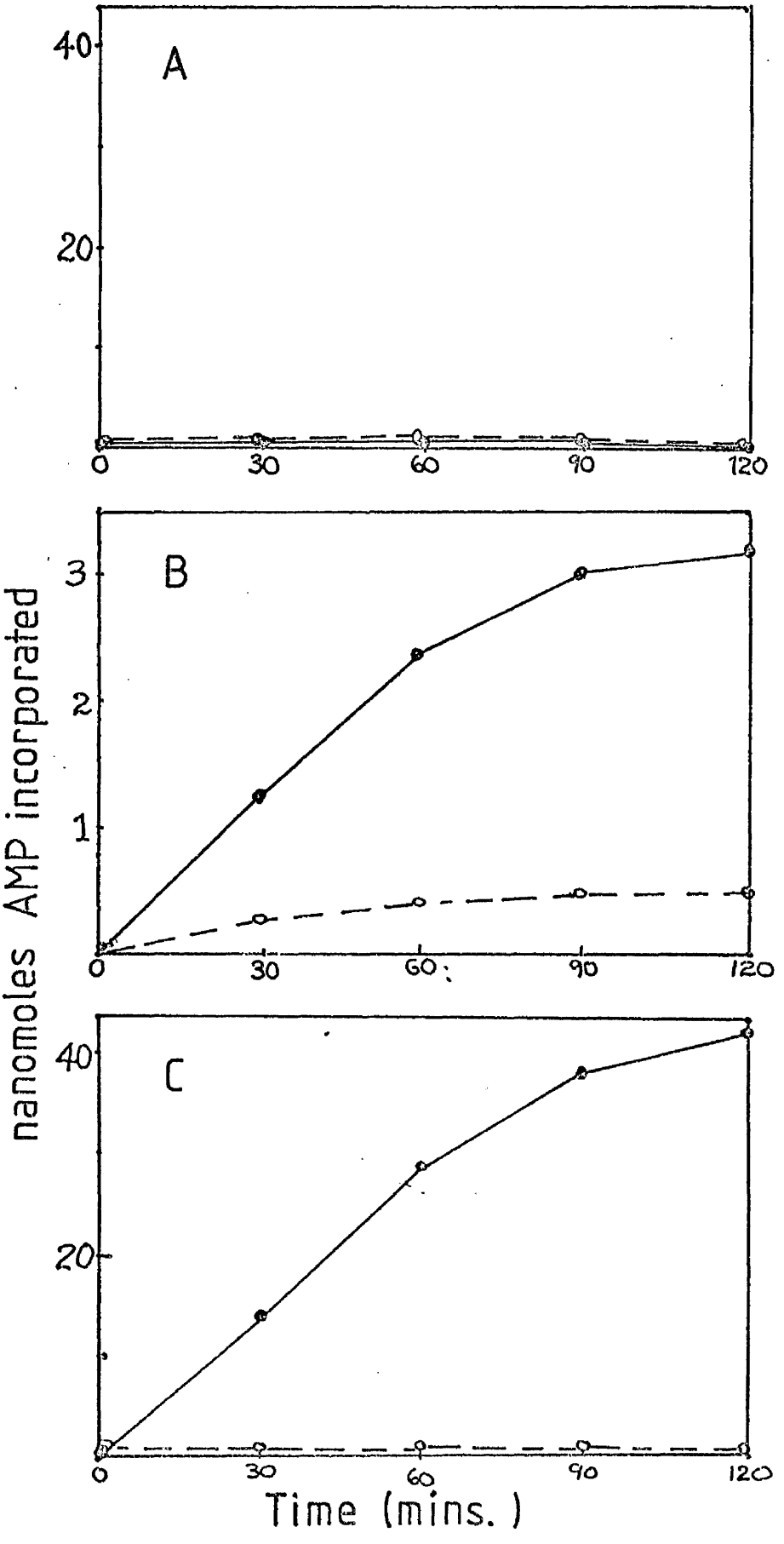

A
B
C
40
20
3
2
1
0
30
60
90
120
nanomoles AMP incorporated
Time (mins.)

Fig 36

Dependence of Poly A Synthetase Activity from HeLa Cell Nuclei on Concentration of Poly A Primer

Poly A synthetase activity was assayed as in Fig 35 using a HeLa nucleosol fraction (Materials and Methods Fig 7 Routes Ib, IIa) except that the poly A primer was present in the incubation mixture at a concentration of 150 μg/ml (o——o), 300 μg/ml (▲- - -▲) or 600 μg/ml (●——●).

Fig 37

Dependence of Poly A Synthetase Activity from HeLa Cell Nuclei on an Energy Regenerating System

Poly A synthetase activity was assayed as in Fig 35 using a HeLa nucleosol fraction (Materials and Methods Fig 7 Routes Ib, IIa) except that an energy regenerating system of 1.2 mg/ml phosphoenolpyruvate, 1 g/ml pyruvate kinase and 30mM KCl was either absent (▲- - - -▲) or present (●——●) in the incubation mixture.

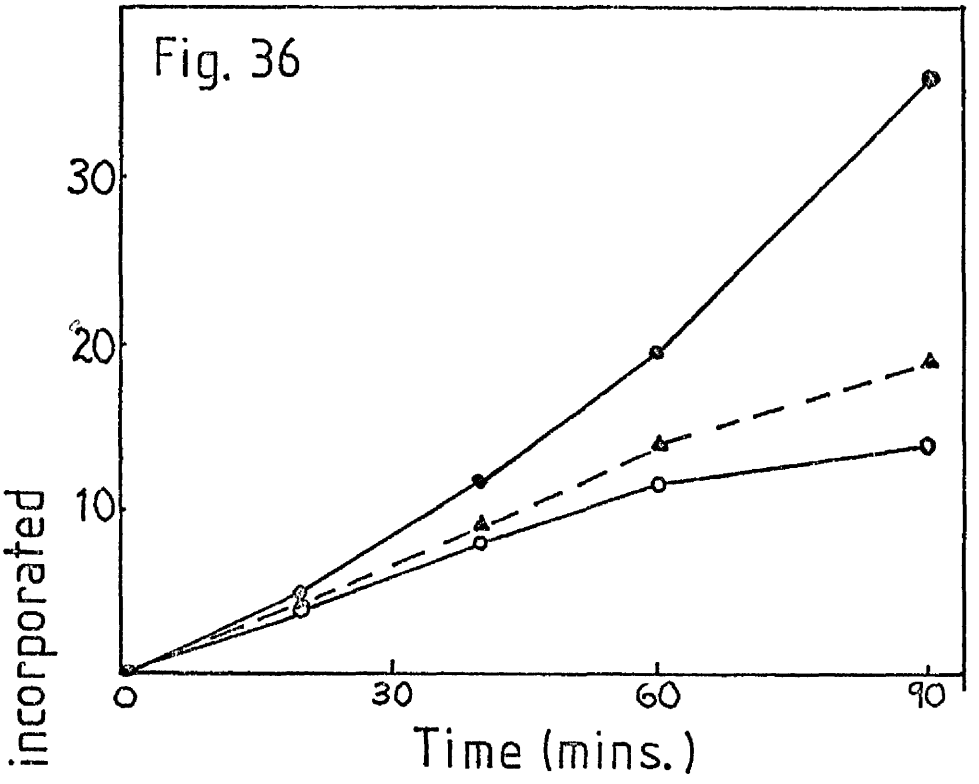


Fig. 36
30
20
10
0
30
60
90
Time (mins.)
nanomoles AMP incorporated

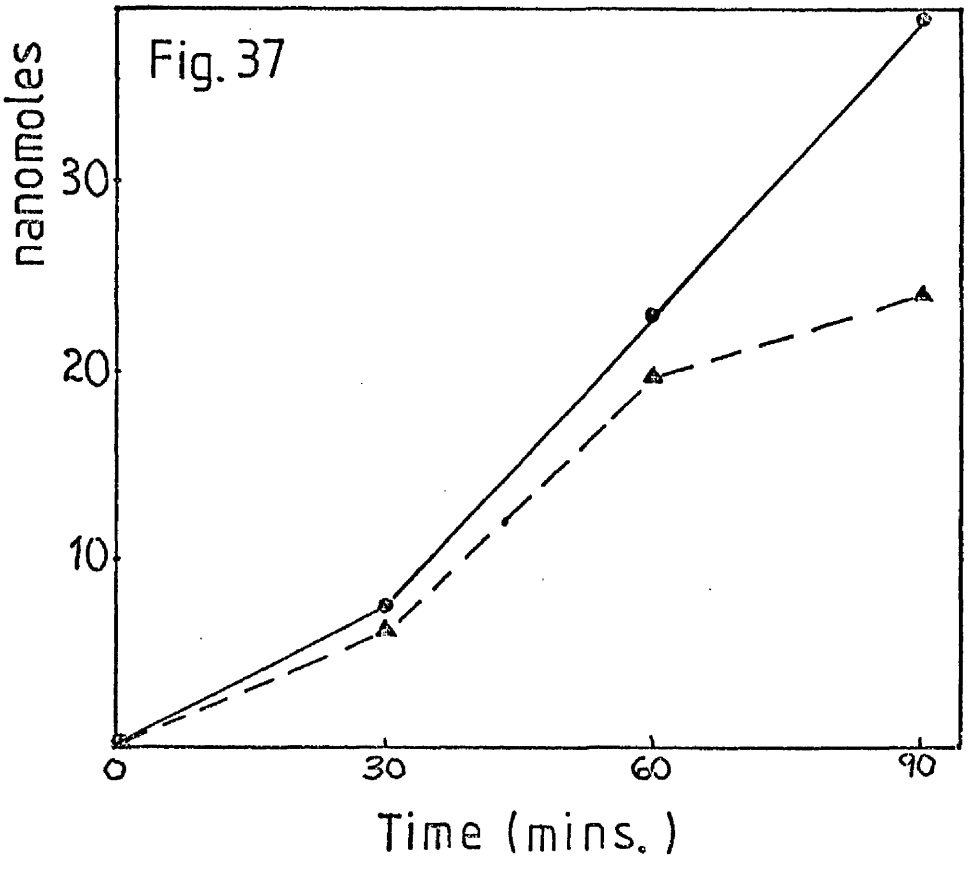


Fig. 37
30
20
10
0
30
60
90
Time (mins.)

TABLE 10 Substrate and Primer Specificities of HeLa Nuclear Poly A Synthetase

Substrate	Primer	Percentage of Optimal Enzyme Activity
ATP	Poly A	100
UTP	Poly A	1
ATP	Poly U	1.5
ATP	Poly C	5.0
ATP	Poly dA	2.5
ATP	Yeast tRNA	15.5
ATP	Salmon sperm DNA	1.5

Poly A synthetase activity was assayed in a reaction mixture containing: 50mM Tris-HCl pH 8.5; 10mM DTT; 1mM $MnCl_2$; 1 mg/ml primer; 1.2 mg/ml phosphoenolpyruvate; 1 μg/ml pyruvate kinase; [^{3}H] ATP (23 Ci/mmol) or [^{3}H]-UTP (50 Ci/mmol) and nucleosol (50 μg protein). Following incubation triplicate aliquots were applied to Whatman 3 MM paper discs and assayed for acid-precipitable radioactivity (Materials and Methods, 3.7.4.1). Results were expressed as percentages of an optimal value using ATP as a substrate and poly A as a primer for the poly A synthetase reaction.

Finally, to identify the enzyme activity as a poly A synthetase, it was necessary to characterize the reaction products. The incorporation of [^{14}C]-radioactivity into cold TCA-insoluble material was normally monitored. However, when an aliquot of the incubated mixture was heated to 90^{o} for 20 minutes in TCA virtually all the radioactivity was converted to a soluble form thereby suggesting that incorporation of radioactivity into protein was negligible (Table 11). Such a conclusion was endorsed by the failure of pronase digestion of the reaction products to convert significantly large quantities of radioactively labelled material into an acid-soluble form.

Digestion of the reaction products with pancreatic DNase I succeeded in reducing the amount of acid-insoluble radioactivity to 78% of the control value. However digestion with RNase T_2 which cleaves preferentially after adenine residues succeeded in liberating the great majority of the radioactivity into an acid-soluble form. Therefore, it would appear that the [^{14}C]-radioactivity from the [^{14}C]-ATP substrate is being incorporated into a molecular species of similar properties to those of poly A. Possibly the difficulty in satisfactorily reducing the pH of the buffer to the very low pH which is optimal for the T_2 ribonuclease reaction (pH 4.5) accounted for the failure to effect complete digestion as suggested by the similar amount of degradation of an internal [^{3}H]-poly A control (results not shown).

TABLE 11

Evidence for HeLa Nucleosol-mediated Incorporation of Radioactivity from [^{14}C]-ATP into a Poly A Product

Treatment	Percentage of Control Radioactivity
Cold TCA (control)	100
Hot TCA	1
Pronase Digestion	92($\pm$5)
DNase Digestion	78($\pm$4)
T_2 RNase Digestion	22($\pm$4)

Poly A synthetase activity was quantitated as the incorporation of [^{14}C]-radioactivity from [^{14}C]-ATP into acid insoluble material in a reaction mixture containing 50mM Tris-HCl pH 8.5; 10mM DTT; 1mM $MnCl_2$; 1 mg/ml poly A; 1.2 mg/ml phosphoenolpyruvate; 1 μg/ml pyruvate kinase; [^{14}C]-ATP (1 mCi/mmol) and HeLa nucleosol (50 μg protein). Following incubation at 37° for 90 minutes the reaction mixture was divided into five equal portions. From one of these, duplicate aliquots were applied to paper discs and assayed for cold TCA-insoluble radioactivity (control). From a second portion duplicate aliquots were applied to paper discs, maintained in TCA for 20 minutes at 90°, washed in cold TCA and assayed for radioactivity remaining bound to the filter. The remaining three portions were incubated for one hour at 37° with either pronase (100 μg/ml), pancreatic DNase I (100 μg/ml) or T_2 RNase (50 units/ml) and then aliquots were applied to paper discs and assayed for cold TCA-precipitate radioactivity. Results were expressed as a percentage of the control value.

DISCUSSION

1. THE SIGNIFICANCE OF ISOLATED HnRNP PARTICLES

Initially attention was devoted to the study of HeLa hnRNP particles as a possible source of enzymes which process hnRNA. Such an approach was encouraged by several observations. In particular the polypeptide complement of hnRNP particles from a wide variety of sources reveals extensive heterogeneity when examined by SDS-polyacrylamide gel electrophoresis. In addition to a major group of a few polypeptides between 30,000 and 45,000 daltons the typical polypeptide complement includes very many minor high molecular weight species which appear to exhibit a considerable degree of tissue specificity (e.g. Pederson, 1974; Karn et al, 1977). Moreover, since hnRNA appears to be bound to protein *in vivo* from the moment it is formed on the chromatin template (Miller & Hamkalo, 1972; Augenlicht & Lipkin, 1976) then hnRNP complexes would be expected to serve as substrates for even the earliest hnRNA processing events which could effectively be mediated by some of the proteins bound to hnRNA. Finally, the work of Niessing & Sekeris (1970, 1972, 1973) had suggested that enzyme activities which could conceivably be involved in hnRNA processing appeared to be bound to rat liver hnRNP particles.

1.1 The "reality" of isolated hnRNP particles

Before embarking on a discussion of the functional significance of isolated hnRNP particles it is necessary to consider their relevance to the *in vivo* situation. Indeed, an important, but often neglected, premise on which the strategy of research into hnRNP particles is founded, is that the proteins found attached to hnRNA in isolated hnRNP particles are specifically associated with hnRNA *in vivo*. The reality of hnRNP complexes *in vivo* has been substantiated by a vast array of ultrastructural evidence (see Introduction 4.1) and appears to be beyond doubt. What remains contentious, however, is the possibility that during attempts to isolate the genuine hnRNP complexes there is considerable scope for non-specific binding by cellular protein to the complexes and/or rearrangements in the original association between hnRNA and proteins.

A notable cause for concern has stemmed from the finding that eukaryotic cells contain appreciable quantities of "binding factor" proteins which are capable of forming non-specific complexes with exogenous RNA (Baltimore & Huang, 1970). Although binding factor components appear to be largely confined to the cytoplasmic compartment, they appear to include some species exhibiting electrophoretic mobilities similar to those of proteins associated with mRNA (Liautard et al, 1976). However the non-specific binding is apparent only at low ionic strength and it has been shown that hnRNP particles prepared from HeLa cells under conditions of low (0.01M NaCl) and moderate (0.15M NaCl) ionic strength exhibit identical profiles in SDS-polyacrylamide gels (Pederson, 1974).

The reality of isolated hnRNP particles could also be impeached by the finding that many of the polypeptide species associated with hnRNP complexes from rat liver and rat brain are similar in electrophoretic mobility to species expressed in other nuclear compartments such as chromatin and nucleosol (Stévenin et al, 1978). Similarly, inspection of the polypeptide profiles of hnRNP particles and of chromatin prepared from HeLa cells in the present study reveals considerable overlap of co-migrating species. Although it is quite feasible that the similarity in electrophoretic mobilities of components in a one-dimensional resolving system is purely fortuitous, a certain degree of identity of species persists following examination of the polypeptide components of hnRNP particles and other subnuclear fractions from monkey kidney cells in a 2-dimensional electrophoretic resolving system (Pagoulatos & Yaniv, 1977). However it is possible that certain proteins, possibly involved in regulatory functions, may shuttle back and forth between different subnuclear compartments in vivo and would therefore be expected to be associated with more than one subnuclear fraction following isolation procedures. Secondly, contaminating molecular species which could co-purify with isolated hnRNP particles may contribute polypeptide species which would appear to be associated with hnRNP particles (see 1.2.1 below).

Certain characteristics of isolated hnRNP particles argue against non-specific binding of protein during isolation procedures. For example, when hnRNP particles are isolated from a large variety of eukaryotic tissues by employing widely different experimental protocols (see Introduction 4.), there appears to be a fundamental constancy in the buoyant density of the aldehyde-fixed particles in CsCl density gradients. In all cases the expressed banding density falls within a remarkably narrow range with a maximum at about 1.40 $g.cm^{-3}$ reflecting a protein:RNA ratio of approximately 4:1. In addition, the polypeptide profiles of hnRNP complexes extracted from intact nuclei or isolated from mechanically disrupted nuclei of the same tissue type appear to be similar when judged by the criterion of electrophoretic mobility in one-dimensional SDS-polyacrylamide gels (Albrecht & van Zyl, 1973; Pederson, 1974; Karn et al, 1977). Moreover, the association of protein with RNA in isolated hnRNP particles is not on a purely random basis with respect to the sequence of the RNA e.g. the 3' poly A tract appears to be associated with a specific subset of proteins including a notable 76,000 dalton species (e.g. Kish & Pederson, 1975) while double-stranded RNA structures are almost totally devoid of protein (Calvet & Pederson, 1978).

Several attempts to resolve any doubts attendant upon the reality of isolated hnRNP particles have been reported. They take the form of <u>in vitro</u> reconstruction experiments whereby deproteinized hnRNA is added to nuclei or subnuclear fractions in an attempt to generate hnRNP complexes with the same characteristics as those obtained by the methods normally used. When [^{3}H]-labelled RNA extracted from HeLa hnRNP particles is added to unlabelled HeLa cell nuclei before or after sonication and the non-nucleolar nuclear contents are analysed on sucrose density gradients there appeared to be no change in the sedimentation characteristics of the labelled RNA (Pederson, 1974). However this finding does not exclude the possibility that proteins can bind non-specifically to hnRNP particles

during their isolation, a possibility which is enhanced by the observation that protein-protein interactions are among the most important stabilizing forces in hnRNP complexes (see Georgiev & Samarina, 1971; Martin et al, 1978).

Comparable experiments featuring nuclear sap protein and hnRNA isolated from rat liver (Ishikawa et al, 1974) or rat brain (Zawislak et al, 1974) have been successful in generating RNP complexes *in vitro*. Although the artificially constructed rat liver complexes never exceeded a sedimentation coefficient of 20s the rat brain complexes included particles of about 40s, the size of the complex appeared to be related to the size of the RNA and the buoyant density of the fixed particles in CsCl density gradient was close to 1.40 $g.cm^{-3}$ at saturation. It is possible therefore that complexes with some of the characteristic of the "native" particles can be generated during such *in vitro* reconstitution experiments. However, there may be little specificity in the binding of protein to hnRNA in the *in vitro* reconstitution experiments, compared to the ordered process that presumably characterises the formation of hnRNP complexes *in vivo*. Such a proposal is endorsed by the relative fragility (e.g. to increasing NaCl concentrations) of the artificially generated complexes (Zawislak et al, 1974).

Thus, while there is some circumstantial evidence that isolated hnRNP complexes reflect an association of RNA and protein which is not purely adventitious and may reflect the normal hnRNA-protein interaction *in vivo* the question of the reality of the isolated hnRNP particles remains unsolved. The question is a difficult one to answer, however. Simple *in vitro* reconstitution experiments of the type described above suffer from a series of deficiencies imposed by the elaborate structural connections between hnRNA-containing hnRNP complexes and various elements of the nuclear architecture (e.g. Faiferman & Pogo, 1975; Herman et al, 1978 - see also Introduction). Some of the proteins associated with RNA in isolated hnRNP particles appear to become bound to the RNA *in vivo*

while the RNA is still firmly bound to its chromatin template (Augenlicht & Lipkin, 1976). Consequently the microenvironment of the RNA in vivo is complex and assuredly plays a substantial role in dictating the specificity of the protein attachment, a role which ill befits the necessarily unsophisticated structural organisation of isolated nuclear sap proteins.

Another difficulty is that all in vitro reconstitution experiments use deproteinized hnRNA as a probe for detecting non-specific binding of nuclear proteins. However, such an experimental design is inadequate in that it does not cater for the detection of non-specific binding of nuclear proteins to genuine hnRNP complexes. The hnRNP organisation in vivo could be characterised by the association of a limited number of proteins at a few specific sites on the hnRNA. However, during attempts to isolate natural hnRNP complexes some nuclear proteins could perhaps become bound adventitiously to proteins already bound to the hnRNA, as well as to other possible sites, thereby generating RNA complexes of a high protein:RNA ratio. Certainly the isolated hnRNP complexes are characterised by a high protein:RNA ratio of at least 4:1 whereas other natural nucleoprotein complexes have lower proportions of protein (~50% in ribosomes, ~65% in chromatin). Conversely, the presumed processing products of hnRNP particles, cytoplasmic informosomes and polysomal mRNP particles, include complexes of similar protein:RNA ratios as those present in the parent hnRNP particle populations. Finally, in vitro reconstitution experiments have not accomodated the recently acknowledged existence of small stable RNA in hnRNP particles which might have a structural role (Sekeris & Niessing, 1975).

One direct way of approaching the question of the reality of isolated hnRNP particles is to combine biochemical techniques with ultrastructural studies. Convergence of these two experimental techniques has however generally only been possible in certain exceptional systems which are amenable to investigation. One of these is the lampbrush chromosomes of amphibian oocytes. Immunofluorescent techniques have shown that at least some of the proteins

present in nuclear RNP complexes isolated from amphibian oocytes are located on lampbrush chromosomes (Scott & Sommerville, 1974).

1.2 Heterogeneity of isolated hnRNP particles

Although knowledge of the heterogeneity of isolated hnRNP particles is crucial to our understanding of their properties such a subject has not been thoroughly investigated. The heterogeneity of isolated hnRNP particles can manifest itself in two major ways:

(1) the isolated particles may be contaminated by non-hnRNP structures which co-purify with hnRNP particles.

(2) the hnRNP population itself may be heterogenous, embracing a variety of different complexes.

Therefore, before the functional characteristics of isolated hnRNP particles can be assessed it is necessary to assess the limitations imposed by the heterogeneity and impurity of isolated hnRNP particles.

1.2.1 Contamination of isolated hnRNP particles by non-hnRNP structures

Possible contaminants of isolated hnRNP particles include chromatin fragments, soluble proteins, nuclear membrane fragments, ribosomes and pre-ribosomal particles. In the present study isolated nuclei have been purified by EDTA-washes or triton X-100 treatment followed by sedimentation through a 2M sucrose-containing buffer in order to minimize cytoplasmic contamination, especially with regard to ribosomes attached to the outer nuclear membranes. Such measures have been supplemented by exposure of cells for 30 minutes, immediately prior to harvesting, to concentrations of actinomycin D known to suppress selectively rRNA synthesis and by removal of nucleoli prior to discrimination of hnRNP particles on sucrose density gradients. However the possibility that soluble proteins or chromatin fragments can contaminate isolated hnRNP particles is a more formidable problem.

To test for the possibility of free proteins which could aggregate and co-sediment with HeLa hnRNP particles Pederson (1974) digested HeLa hnRNP particles, isolated from sucrose density gradients, with ribonuclease, then

collected any possible RNase-resistant complexes as a pellet by high-speed ultracentrifugation under conditions just sufficient to sediment structures of the same sedimentation coefficient as the particles. Electrophoretic analysis of the resulting pellet revealed the complete absence of the polypeptide species normally associated with the hnRNP particles. However, in a similar type of experiment Pagoulatos & Yaniv (1977) have obtained evidence suggesting that actin and tubulin are present as high molecular weight polymers that co-sediment with hnRNP particles from monkey kidney cells. Moreover, ribonuclease digestion of rat brain hnRNP particles which had been previously radioactively labelled in their amino acid moieties revealed a very considerable portion of labelled RNase-resistant material especially in the case of the smaller (30s-40s) sized particles (Stévenin & Jacob, 1974).

Consequently, contamination by non-RNA-bound protein could perhaps be a problem especially for the hnRNP particles of lower sedimentation coefficient. Such observations may be consistent with doubts expressed about the origin of many of the high molecular weight polypeptide species apparently associated with hnRNP particles. For example, Beyer et al, (1977) have shown that many of the high molecular weight polypeptide species which characterise 40s HeLa hnRNP particles are not specifically enriched in that zone of sedimentation in a sucrosedensity gradient which contains the 40s particles. Furthermore, purification of rat liver or rat ascites cell 30s hnRNP particles by gel filtration and further density gradient centrifugation selectively removes the minor high molecular weight polypeptide species from the RNP particles at the expense of their major low molecular weight counterparts (Patel & Holoubek, 1977; Martin et al, 1974).

Another major contender as a possible contaminant of hnRNP particles, especially those prepared from sonicated nuclei, may be chromatin fragments. When, however, the polypeptides of hnRNP particles prepared from HeLa cells in the present study were examined there was almost negligible evidence of histone contamination in agreement with the results of others (e.g. Pederson, 1974). The proportion

of DNA to RNA in hnRNP particles from rat liver and rat brain has been estimated to be 0.05 and between 0.05 and 0.1 respectively while the proportions of histones to total particle proteins were 0.01 and 0.03 respectively (Faiferman et al, 1970; Northemann et al, 1978; Gallinaro-Matringe et al, 1975). In addition, Augenlicht & Lipkin (1976) have calculated that the hnRNP particles obtained from the nuclei of human HT-29 cells contain 6% of the total nuclear DNA while the contamination of hnRNP particle protein by chromatin protein was estimated to be about 10%. Although the extensive heterogeneity of chromosomal and hnRNP particle proteins provides ample scope for coincidental overlap by species of similar electrophoretic mobilities in one dimension (present study) there seems to be less identity of species from monkey kidney cell subnuclear fractions when examined by a 2-dimensional electrophoretic procedure (Pagoulatos & Yaniv, 1977).

1.2.2 Intrinsic heterogeneity of hnRNP particles

In addition to possible contamination of hnRNP particles by non-hnRNP structures the hnRNP particles themselves may possibly consist of a variety of different particle types. Certainly there is extensive structural heterogeneity of isolated hnRNP particles when care is exercised in minimizing degradation events during isolation, with particles ranging in sedimentation coefficients from 30s to well over 250s. Such heterogeneity has been deemed to reflect a variety of different integral repeats of an underlying monoparticle structure (see Introduction). However, the expected integral peaks are very poorly resolved on sucrose density gradients (e.g. see Results - Fig 12; Augenlicht & Lipkin, 1976 etc.) while, in addition, the monoparticles themselves are not excessively homogenous in sedimentation characteristics (Gattoni et al, 1978).

Electron microscopic examination of isolated hnRNP particle monomers has also indicated a limited degree of heterogeneity of particle types. Although some investigators have considered the rat liver particles to be of uniform dimensions (Samarina et al, 1967a) other workers have

considered that equivalent monomeric hnRNP particle preparations from rat liver and other tissues are heterogeneous in structure, generally 200Å-300Å in diameter (Monneron & Moulé, 1968; Martin et al, 1974; Albrecht & van Zyl, 1973; Karn et al, 1977; Beyer et al, 1977) and size classes ranging from 100Å to 300Å have been claimed for rat brain particles (Stévenin et al, 1976). In addition, Martin et al (1978) have attempted classification of the particles in terms of their varied morphologies in negatively stained preparations viewed under the electron microscope. However the foundations for such a classification cannot be very stable when one considers the potential contribution of artefacts deriving from sample preparation for electron microscopy and from the distinct possibility that a unique 3-dimensional particle structure may project a variety of different 2-dimensional profiles. It is quite possible also that the limited degree of structural heterogeneity of the particles may also reflect varying degrees of proteolytic hydrolysis of a single type of particle structure as possibly suggested by the frequent presence of disintegrating particle structures in the preparations studied. However, although endogenous protease activity could scarcely be detected in rat brain nuclei (Stévenin et al, 1977a) rat brain hnRNP monoparticle preparations revealed considerable structural heterogeneity as mentioned above.

Evidence for the structural complexity of hnRNP monoparticle populations has also been obtained by biochemical approaches. By means of controlled pancreatic RNase digestion of rat brain hnRNP particles Stévenin et al (1977b) have suggested that in addition to the large heterogenous-sized particles there are least two classes of monoparticles i.e. M_α which appears to be relatively insensitive to RNase treatment and primarily contains low molecular weight proteins, and M_β which is more sensitive to RNase and is comparatively enriched in high molecular weight protein species. The functional significance of such heterogeneity remains to be assessed.

1.3 Suitability of isolated hnRNP particles as a possible source for investigation of functional aspects of hnRNA processing

The evidence presented above, although largely circumstantial, favours the "reality" of isolated hnRNP particles. Moreover, the hnRNP particle population, although almost inevitably contaminated by small amounts of chromatin fragments, may plausibly be investigated as a potential source of hnRNA processing enzymes with two provisos:

(1) the hnRNP particle population should be selected so as to minimize contamination by nuclear soluble proteins

(2) association of a particular enzyme activity cannot immediately be accepted as evidence that the activity is an integral component of the hnRNP particle population.

In the present study the hnRNP particle population did not include structures of sedimentation coefficient less than 45s to minimize contamination by nucleosol proteins. However the subnuclear fractionation scheme adopted herein can only be regarded as a relatively crude fractionation of subnuclear components. It seems quite probable, for example, that the chromatin fraction is contaminated to some extent by large hnRNP particles. The original yardstick for measuring successful separation of these two subnuclear fractions, following nuclear sonication, was the reduction in chromatin of the presence of a 40,000 dalton polypeptide species characteristic of hnRNP particles compared with that evinced in more conventional chromatin preparations (Bhorjee & Pederson, 1973). Nevertheless the same polypeptide species remains in quite substantial amounts in the chromatin prepared from sonicated nuclei, although the possibility of coincidental electrophoretic mobility of two different polypeptide species in a one-dimensional resolving system has not been eliminated.

Another possibility of cross-contamination between subnuclear fractions concerns the possibility that nucleosol proteins bind non-specifically to chromatin during isolation procedures, while degradation of chromatin and hnRNP particles during isolation procedures may be expected to liberate proteins to the nucleosol fraction (Stévenin et al, 1978). Therefore, association of a potential RNA

processing enzyme activity with one particular subnuclear fraction, obtained by the procedures adopted in the present study, cannot be unequivocally demonstrated. However, if the particular activity is considerably enriched in a particular fraction compared to the other available subnuclear locations, some confidence can be entrusted in its reported location.

2. SUBNUCLEAR FRACTIONATION AND THE ENZYMOLOGY OF HnRNA PROCESSING

2.1 Ribonuclease

The nuclear RNA species which act as precursors to cytoplasmic mRNA species are frequently found to be of considerably higher molecular weight than the mature mRNAs (see Introduction). Accordingly, a reductive scheme of processing could operate in the biogenesis of mRNA with endoribonuclease-mediated cleavage at specific sites. The non-conserved sequences could then possibly be degraded by non-specific exoribonuclease and/or endoribonuclease activities.

2.1.1 Exoribonuclease

Exoribonuclease activity in the HeLa cell nuclei investigated in the present study was found to be confined to a chromatin fraction and, to a lesser extent, to a nucleosol fraction. No activity of this type could be detected in association with HeLa hnRNP particles in agreement with similar findings in the case of rat liver 30s-40s hnRNP particles as reported by McParland et al (1972) and Northemann et al (1978). However Molnár et al (1978) have presented evidence which could suggest that a 5' exoribonuclease may be associated with rat liver 30s hnRNP particles although the specificity of such an association remains to be determined.

Exoribonuclease activity has been characterised from the nuclei of a few eukaryotic tissues (Lazarus & Sporn, 1967; Perry & Kelley, 1972; Kwan et al, 1974; Skridonenko, 1975; Skridonenko et al, 1975). In all cases there appeared to be a strict requirement for Mg^{2+} ions, a property which also characterised the HeLa chromatin-associated enzyme in the present study. In addition, there appeared to be very little substrate specificity for the HeLa chromatin-associated activity, a feature which is shared by the other reported nuclear exoribonuclease activities. Very probably the enzyme activity studied herein corresponds at least in part to the processive exoribonuclease designated NuB which has been detected by Kwan et al (1974) in HeLa cell nucleoplasm.

The subnuclear distributions of the eukaryotic exoribonuclease activities have not been defined, but such activity has been reported in nucleoli (Perry & Kelley, 1972; Kwan et al, 1974), nuclear membranes (Skridonenko et al, 1975) and non-nucleolar nucleoplasm (Kwan et al, 1974). Ribonuclease activity in association with chromatin preparations has been reported by a number of investigators (e.g. Dahmus & Bonner, 1965; Dati & Maurer, 1971; Nikonova et al, 1977) although attempts to distinguish between exo-nucleolytic and endonucleolytic mechanisms attendant on these activities have rarely been carried out. However the observed heterogeneity of RNase activity associated with rat thymus chromatin could well accomodate exo-ribonuclease activity (Umansky et al, 1974; Nikonova et al, 1977).

The significance of eukaryotic nuclear exoribonuclease activity remains elusive. In all cases, where studied, such activities have been shown to produce nucleoside 5' monophosphates and to act processively in a 3'-5' direction (e.g. Lazarus & Sporn, 1967; Kwan et al, 1974). Consequently it is possible to envisage a situation whereby cleavage enzymes involved in RNA processing could generate 3'OH termini. These could in turn serve as substrates for degradation by the nuclear exoribonuclease activities in analogy with E.coli RNase II which is a Mg^{2+}-dependent exoribonuclease producing 5' mononucleotide species by a processive mechanism beginning at a 3'OH terminus and which has been considered to be implicated in tRNA processing (see Introduction 2.).

The significance of a variety of subnuclear locations for eukaryotic exoribonuclease activity is also uncertain. Possibly this reflects the situation <u>in vivo</u> while it is conceivable that it is an indication of, as yet unappreciated, functional heterogeneity within the nuclear exoribonuclease population. Alternatively it may result from adventitious subnuclear reassortment of exoribonuclease activity during isolation procedures. For example, nucleosol exoribonuclease activity may become fortuitously bound to chromatin com-ponents following sonication of HeLa cell nuclei in analogy

to the artefactual association of E.coli RNase I with isolated ribosomes following cell disruption (Neu & Heppel, 1964). In this regard it would be interesting to examine which polypeptides in the various subnuclear fractions from HeLa cells were responsible for the observed ribonuclease activity following their resolution on SDS-polyacrylamide gels using the technique of Huet et al (1978) or Rosenthal & Lacks (1977).

2.1.2 Endoribonuclease

Although reactions catalysed by exoribonuclease activities would be expected to be no more than tributary to the mainstream of hnRNA processing events, endoribonuclease activity would be expected to feature prominently in analogy to the endoribonuclease activities known to be implicated in RNA processing in prokaryotes (see Introduction 2.). In the present study endoribonuclease activity has been detected in hnRNP particles, chromatin and nucleosol fractions from HeLa cells under conditions selected to suppress companion exoribonuclease activity, if any.

Endoribonuclease activity which appears to promote limited fragmentation of rat liver hnRNA has been reported in association with rat liver 30s hnRNP particles (Niessing & Sekeris, 1970). Although the substrate specificity of this activity was not investigated the equivalent HeLa enzyme in the present study displayed a poor degree of substrate specificity. Moreover, incubation with high quantities of the hnRNP particles or prolonged digestion in the presence of the particles, elicited degradation products from HeLa hnRNA which were considerably lower in sedimentation coefficient, on average, than the HeLa mRNA population. Although some of the smaller degradation products during mRNA biogenesis *in vivo* may be accounted for by excised intervening sequences, the lack of substrate specificity for the HeLa hnRNP particle-associated enzyme ill befits the properties of a cleavage enzyme involved in hnRNA processing.

A variety of endoribonuclease activities have been reported in eukaryotic cell nuclei, none of which convincingly displays the degree of substrate specificity expected of a

cleavage enzyme involved in RNA maturation (Table 12). However it is quite feasible that the experimental design in many, if not all, these cases has been inadequate in that it placed emphasis on artificially deproteinized RNA substrates. Certainly, in the case of nuclear precursor species to mRNA and rRNA a ribonucleoprotein organisation appears to prevail in vivo (see Introduction). In addition, when endoribonuclease activities purified from HeLa cell nucleoli (Mirault & Scherrer, 1972; Kwan et al, 1974) or L cell nucleoli (Winicov & Perry, 1974) are incubated with 80s pre-ribosomal particles there appears to be limited fragmentation of the RNA moiety to generate RNA products similar to those observed during in vivo processing. However the same enzyme activities can extensively fragment artificially deproteinized RNA substrates. Further, some of the cleavage enzymes thought to be involved in RNA processing in prokaryotes, e.g. the activities involved in the maturation of E.coli 16s rRNA (Hayes & Vasseur, 1976; Dahlberg et al, 1978) are dependent for their high degree of substrate specificity on a RNP organisation for their substrate.

It is conceivable therefore that the proteins associated with hnRNA may serve, in part, to confer a considerable degree of substrate specificity on otherwise undistinguished endoribonuclease activity. Possibly in this way, enzymic access is limited only to a few exposed specific cleavage sites while association with a RNP organisation may serve to alter the conformation of the enzyme and thereby its catalytic properties. However, it is likely that additional factors are involved as suggested by the ability of isolated HeLa hnRNP particles to promote, upon self-digestion, degradation of the RNA moiety to molecular species of considerably lower average sedimentation coefficient than that of the mRNA population. In addition, experiments involving incubation of pre-ribosomal particles with purified nucleolar endoribonuclease (Mirault & Scherrer, 1972; Kwan et al, 1974; Winicov & Perry, 1974) can be criticised in that identification of degradation products as processing intermediates observed in vivo was assessed

TABLE 12

Endoribonuclease Activities in Eukaryotic Cell Nuclei
(see also Table 13)

Source	Substrate Specificity	Mg^{2+}-sensitivity
Pig liver nuclei	poly A, poly I, DNA	+
Mouse liver nuclei	poly A, poly U, poly C, denatured DNA	+
Rat liver nuclei	poly A, poly C, poly U, rRNA	+
HeLa nucleoli	poly C, poly U, rRNA	-
L-cell nucleoli	poly C, hnRNA, mRNA, rRNA	-
Novikoff hepatoma nucleoli	poly C, poly U	-
Rat liver nucleoli	poly U, poly A, rRNA	
Beef brain nuclei (alkaline optimum)	poly C, poly U, rRNA	
Beef brain nuclei (acidic optimum)	poly A, poly U, poly C	-
Mouse kidney nuclei	poly A, poly C, rRNA	
Rat thymus chromatin	poly U, nuclear RNA, cytoplasmic RNA, DNA	-
Rat liver hnRNP particles	hnRNA	
Rat mammary gland and tumour nuclei	poly U, poly C	- (by Ca^{2+})
Mouse myeloma nuclei	4.5s RNA	

+ stimulation of activity - Inhibition of activity

Product	Reference
Oligonucleotide 5' phosphate	Heppel (1966)
Oligonucleotide 5' phosphate	Cordis et al (1975)
Oligonucleotide 5' phosphate	Gorchakova et al (1976)
Oligonucleotide 5' phosphate	Kwan (1976)
Oligonucleotide 3' phosphate	Winicov & Perry (1974)
	Prestayko (1973)
4s products	Boctor et al (1974)
	Niedergang et al (1974)
Oligonucleotide 3' phosphate	Ittel et al (1975)
	Warnick & Lazarus (1977)
Oligonucleotide 3' phosphate	Nikonova et al (1977)
mRNA-sized fragments	Niessing & Sekeris (1970)
Oligonucleotide 3' phosphate; 2',3'cyclic mononucleotides	Liu et al (1977)
4s RNA from 4.5s RNA	Marzluff et al (1974)

merely by a broad similarity in electrophoretic mobilities while no attempts were made to establish the products of limit digests.

An alternative view is that endoribonuclease associated with hnRNP particles may play a subsidiary part in hnRNA processing by serving to degrade excised intervening sequences possibly with the assistance of non-specific exoribonuclease activity. In its insensitivity to inhibition by Mg^{2+} ions the HeLa hnRNP particle-associated enzyme differs from an endoribonuclease which was purified from HeLa cell nucleoli but which was also found in substantial amounts in HeLa cell nucleoplasm. Instead, its insensitivity to Mg^{2+}-induced inhibition and its lack of substrate specificity align it more readily with a group of non-specific endoribonuclease activities of similar properties (Heppel, 1966; Cordis et al, 1975; Gorchakova et al, 1976; Warnick & Lazarus, 1977).

Endoribonuclease activities capable of degrading a variety of RNA substrates were also detected in nucleosol and chromatin fractions from HeLa cells in the present study. The significance of the heterogeneity of RNase activity within the nuclei of a single cell type (e.g. Kwan et al, 1974) or within a particular subnuclear fraction e.g. nucleoli (Kwan et al, 1974; Prestayko et al 1973), nucleoplasm (Kwan et al, 1974; Kwan, 1976) and chromatin (Umansky et al, 1974; Nikonova et al, 1977) remains obscure. It is quite possible that certain endoribonuclease activities which have been detected in different subnuclear fractions are identical (e.g. Kwan, 1976) and this could reflect the <u>in vivo</u> situation or may be a consequence of adventitious rearrangements during isolation procedures. It is therefore possible that the endoribonuclease activities found in association with hnRNP particles from HeLa (present study) and rat liver (Niessing & Sekeris, 1970) represent nucleosol-based activities which become fortuitously associated with hnRNP particles during their isolation. In the absence of an easily applicable quantitative assay for either of these two enzymic activities (due to the lack of acid-soluble products) it remains difficult to refute this suggestion.

2.1.3 Double-strand specific RNase

Following the implication of the double-strand RNA-specific E.coli RNase III in the maturation of some bacteriophage-specified mRNA and also rRNA in prokaryotic systems (Nikolaev et al, 1973; Dunn & Studier, 1973b), the presence of double-stranded RNA sequences in hnRNA (e.g. Jelinek & Darnell, 1972) and the ability of highly purified E.coli RNase III to degrade 45s rRNA and hnRNA in a fashion which appears to mimic *in vivo* processing, (Gotoh et al, 1974; Nikolaev et al, 1975c; Robertson & Dickson, 1975), it has been widely assumed that dsRNA-specific RNase activities feature in hnRNA processing. Such activities have been characterised in a few types of eukaryotic cell (Table 13).

The initial promise offered by some encouraging reports of an activity from HeLa cell nuclei which displayed some properties similar to E.coli RNase III (Birge & Schlessinger, 1974; Nikolaev et al, 1975c) has largely been unfulfilled by subsequent research. The two types of double strand-specific RNase in HeLa cell nuclei which have been studied by Saha & Schlessinger (1977, 1978) do not appear to be convincing processing candidates, one being an exoribonuclease while the other exhibits activity towards a variety of single-stranded RNAs, single and double-stranded DNA, and DNA-RNA hybrids in addition to dsRNA. In addition, other eukaryotic dsRNA-specific RNase activities (see Table 13) appear to be accompanied by significant and in some cases overwhelming activity towards single-strand RNA substrates. Even the extensively purified activity from calf thymus which has been shown to cleave rat liver 45s rRNA and duck reticulocyte hnRNA to products of similar sizes to those observed during *in vivo* processing, is still accompanied by quite significant hydrolytic activity towards single-stranded RNA (Ohtsuki et al, 1977).

In the present study attention was confined to an evaluation of the subnucleoplasmic distribution of dsRNase in HeLa cell nuclei. Although no activity of this type could be detected in association with HeLa hnRNP particles

TABLE 13

Double-strand Specific RNase in Eukaryotes

Source	Substrate Specificity	Mg^{2+}-Requirement
HeLa nuclei	None	+
HeLa nuclei	Reovirus dsRNA, rRNA	+
Chick embryo	poly rA•poly dT, poly rC, poly rG•poly rC	+
Chick embryo	poly rC, poly rC•poly rG	+
Calf thymus (nuclei + cytoplasm)	Reovirus dsRNA, poly C• poly I, poly C	+
Krebs ascites cytoplasm	poly rC•poly rG, Qβ-dsRNA - also poly rA•poly dT	
Mouse embryo HMPE cell cytoplasm	poly rA•poly rU, poly rA• poly dT, poly A, poly U	
KB cell cytoplasmic membranes, crude nucleoli	Reovirus dsRNA	
Rat liver 30s hnRNP particles	dsRNA prepared from hnRNA	

Note: Acid-thermostable RNase activities from some mammalian sources display appreciable activity against dsRNA (see Bardon et al, 1976).

Mode of hydrolysis	Reference
Endonucleolytic	Saha & Schlessinger (1977, 1978)
Exonucleolytic	Saha & Schlessinger (1977, 1978)
Endonucleolytic	Hall & Crouch (1977)
Endonucleolytic	Hall & Crouch (1977)
Endonculeolytic	Ohtsuki et al (1977); Busen & Hausen (1975)
	Rech et al (1976) Robertson & Mathews (1973)
	Shanmugam (1978)
	Bothwell & Altman (1975)
Endonucleolytic	Molnár & Samarina (1976); Molnár et al (1978)

considerable activity was found in a nucleosol fraction and to a lesser extent in the chromatin fraction from HeLa cells. However, evidence has been presented in favour of the existence of a dsRNase activity bound to 30s hnRNP particles from rat liver (Molnár & Samarina, 1976; Molnár et al, 1978). As yet, it remains to be determined to what extent such an activity is adventitiously associated with the hnRNP particles or represents contaminating structures in the hnRNP particle population.

Double-stranded RNA sequences have been indicated in the RNA component of hnRNP particles (Molnár et al, 1975; Kinniburgh et al, 1976; Calvet & Pederson, 1977) while a number of criteria suggest that such sequences are almost entirely devoid of protein (Calvet & Pederson, 1978). Consequently some of these structures might be expected to take up relatively exposed positions in hnRNP particles and therefore be eminently accessible to the attention of cleavage enzymes.

2.2 RNA ligase

The existence in HeLa cell nuclei of an RNA ligase activity similar to the well-established bacteriophage T_4-specified activity (Silber et al, 1972; Last & Anderson, 1976) has not been demonstrated in the present study. Initial reports on eukaryotic RNA ligase activity suggested the presence of this activity in the cytoplasmic compartment of a variety of cultured mammalian cell lines (Linné et al, 1974; Cranston et al, 1974). However Bedows et al (1975) have questioned the validity of T_4-type RNA ligase activity in eukaryotic cells and the current data have supported such a viewpoint although it remains possible that T_4-type RNA ligase activity occurs in certain virus-infected animal cells (Yin, 1977; Koliais & Dimmock, 1978).

Very recently a new significance for postulated RNA ligase activity in eukaryotic systems has stemmed from the discovery of "split" genes in eukaryotes and the accumulating evidence in favour of a model whereby intervening sequences are transcribed along with coding regions

to give a large RNA precursor species from which non-informational sequences are excised prior to ligation of the individual coding segments. In this case the RNA ligase activity might be expected to be very specific. Present evidence for such activity is rather limited. However, extracts of yeast cells have been shown to be capable of removing intervening sequences in precursor RNA species to a few varieties of tRNA prior to ligation of the sequences present in the mature tRNA species (Knapp et al, 1978; O'Farrell et al, 1978). In addition, incubation of nuclei prepared from adenovirus-infected cells with cell extracts affords conversion of a pre-labelled RNA precursor species containing transcribed intervening sequences to a product of the same size and sequence composition as the authentic mRNA species (Blanchard et al, 1978b).

2.3 RNA guanylyltransferase

Present information on enzymes which participate in the formation of capped and methylated structures found at the 5' termini of some hnRNA and also mRNA species is rudimentary. The most thoroughly characterised of such activities have been derived from vaccinia virions (Martin & Moss, 1975; Barbarosa & Moss, 1978; Monroy et al, 1978) while, recently, analogous activities have been characterised in mammalian cells including RNA guanylyltransferase (Wei & Moss, 1977), RNA(guanine-7-)methyltransferase (Ensinger & Moss, 1976) and RNA(2'-O-methyladenosine-N^6-)methyltransferase (Keith et al, 1978).

In the present study attention was confined to examination of the subnucleoplasmic distribution of HeLa RNA guanylyltransferase activity. Within the limits of detection available, such activity appeared to be restricted to the nucleosol compartment. In contrast the presence of RNA guanylyltransferase, RNA(guanine-7-)methyltransferase and RNA 2'-O-methyltransferase activities in association with the 30s hnRNP particles from rat liver has recently been inferred by Bajszár et al (1978). These investigators have described evidence which suggests that the rat liver hnRNP particles can mediate the conversion of the synthetic substrate ppGpC to G(5')pppGpC

in the presence of GTP and to $m^7G(5')pppG^mpC$ in the additional presence of SAM. However, the authors have conceded that the great majority of such RNA guanylyltransferase and methyltransferase activities reside in a 4s-6s nuclear fraction. While simple contamination between nucleosol and hnRNP particles is unlikely to be significant in that an intermediate 14s RNP fraction did not appear to disclose such activities, it remains quite feasible that the appropriate enzymic activities could become non-specifically bound to elements present in the 30s RNP particles during their isolation (see 3.1 below).

2.4 Poly A synthetase

In the current investigation the subnucleoplasmic distribution and reaction properties of HeLa nuclear poly A synthetase activity have been examined. In agreement with analogous findings for rat liver nuclear poly A synthetase activity (Rose et al, 1977) it was found that the bulk of the HeLa nuclear poly A synthetase activity was located in a nucleosol fraction and was dependent on the presence of exogenously added poly A as a primer for the enzymic activity while a small percentage of the nuclear activity could be traced to a chromatin fraction. In addition, the HeLa chromatin fraction was capable of supporting a poly A synthetase activity in the absence of exogenously added primer and such activity was stimulated about 5-fold by the addition of exogenously-provided poly A primer in agreement with the results of Rose et al (1977) for the rat liver chromatin-based activity. Accordingly, the suggestion by Rose et al (1977) that there are two functional states for rat liver nuclear poly A synthetase activity may also be applicable to the HeLa nuclear activity with the chromatin-based activity being responsible for addition of adenylate residues to hnRNA while the nucleosol-based activity may serve to extend the poly A segment of hnRNA molecules initially polyadenylated by the chromatin enzyme.

In the current study no poly A synthetase activity could be detected in association with HeLa hnRNP particles. In the case of rat liver Jacob et al (1976) have reported that the great majority of the nuclear poly A synthetase activity sediments in glycerol gradients at about 4s. Conversely, Sekeris and co-workers have consistently claimed that poly A synthetase activity is associated with rat liver hnRNP particles (Niessing & Sekeris, 1972, 1973, 1974; Niessing, 1975; Louis et al, 1978). The possibility remains therefore that a portion of a predominantly nucleosol-located poly A synthetase activity could become non-specifically bound to hnRNP particles during their isolation. In the case of HeLa cell nuclei where only a modest nuclesol poly A synthetase activity was expressed, non-specific binding of a small portion of the nucleosol activity to isolated hnRNP particles may have escaped the limits of delection.

Currently, poly A synthetase activity has been characterised from a wide variety of eukaryotic tissues including nuclei or nuclear extracts from rat liver (Niessing & Sekeris, 1973; Rose et al, 1977), calf thymus (Winters & Edmonds, 1973a,b), guinea pig (Kato & Kurokawa, 1970), sea urchin (Hyatt, 1967) and quail oviduct (Müller et al, 1975). Generally the poly A synthetase activities are characterised by a marked specificity for ATP as a substrate, requirement for Mg^{2+} or Mn^{2+} ions and a relative lack of primer specificity, all of which are represented in the HeLa nuclear activity investigated in this study.

The poly A synthetase prepared from a cytoplasmic fraction of HeLa cells (Mans & Stein, 1974) is similar in properties to the HeLa nuclear enzyme of the current investigation but appears to have a broader primer specificity. Because of the method of isolating and purifying HeLa cell nuclei in the current investigation (homogenization in isotonic sucrose buffer containing 0.1% Triton X-100 and sedimentation through buffer containing 2M sucrose) it is unlikely that the poly A synthetase activity studied derives from cytoplasmic contaminants. However the hypotonic swelling procedure employed by Mans & Stein (1974) to disrupt

HeLa cells could perhaps have been accompanied by leakage of some of the nuclear activity into the cytoplasmic compartment in analogy with the ready extractability of the rat liver nuclear activity (Rose et al, 1976).

During the course of the present investigation Nevins & Joklik (1977) have described poly A synthetase activity in uninfected and vaccinia virus-infected HeLa cells. In uninfected HeLa cells they were able to distinguish a minor primarily nuclear activity which was stimulated by Mn^{2+} and also by Mg^{2+} and which was able to use HeLa cell RNA but not oligo A as a primer, and a major predominantly cytoplasmic activity which was absolutely dependent on the presence of Mn^{2+} and was able to use both HeLa cell RNA and oligo A as a primer. Although the authors did not test the ability of exogenously added poly A to serve as a primer for the poly A synthetase activity it appears highly probable that their primarily nuclear-restricted activity is equivalent to the poly A synthetase activity characterised from HeLa nucleosol in the present study.

The significance of multiple forms of poly A synthetase activity in eukaryotic nuclei remains obscure. As in the case of HeLa poly A synthetase activities (Nevins & Joklik, 1977), so there are analogous activities in calf thymus i.e. a predominantly nuclear activity which is stimulated by both Mn^{2+} and Mg^{2+} and cannot readily use oligo A as a primer (although poly A was an effective primer) and a predominantly cytoplasmic activity which is strictly dependent on Mn^{2+} ions and can utilise a variety of RNA primers including oligo A (Winters & Edmonds, 1973a,b; Tsiapalis et al, 1975). Because of the compatatively restricted occurrence of poly A sequences on certain classes of RNA, it seems inescapable that at least some of the eukaryotic poly A synthetase activities, presumably the nuclear-located activities, serve to synthesize the poly A sequences found at the 3' termini of hnRNA and mRNA. However, even the most highly purified poly A synthetase activities demonstrate a notable lack of primer specificity and appear to be incapable of synthesising

poly A tracts of the lengths (~200 adenylate units) normally found attached to nuclear RNA *in vivo*.

Another complication is provided by the apparent occurrence of poly A elongation mechanisms in both nuclear and cytoplasmic compartments which are distinct from *de novo* poly A synthesis (Sawicki et al, 1977). While the nuclear poly A elongations occur on large sized poly A tracts the cytoplasmic poly A elongations can occur on long poly A sequences and also on short poly A sequences produced by cytoplasmic poly A degradation events. Such an observation is consistent with the reported abilities of some nuclear poly A synthetase activities to utilise poly A but not oligo A as a primer while corresponding cytoplasmic activities can utilise both types of primer.

The significance of multiple poly A synthetase activities in rat liver nuclei which can be distinguished by their differential response to Mg^{2+} and Mn^{2+} ions (Niessing & Sekeris, 1973) is also uncertain. The Mg^{2+}-stimulated and Mn^{2+}-stimulated activities have been reported to be separable by chromatographic procedures (Niessing & Sekeris, 1974). The Mn^{2+}-dependent activity appears to be optimally active with a polyadenylated RNA primer (Niessing, 1975) while the Mg^{2+}-dependent enzyme appears to utilise polyadenylated and non-adenylated RNA primers equally. Thus it has been considered that the Mn^{2+}-dependent activity serves to transfer adenylate residues to RNA previously adenylated by the Mg^{2+}-stimulated activity, a conclusion which may be supported by the apparently different subnuclear locations for the two types of activity evident from electron microscopic autoradiography studies (Louis et al, 1978). In the present study the Mn^{2+}-stimulated poly A synthetase activity from HeLa cell nuclei was optimally active with a poly A primer and may therefore be involved in nuclear poly A elongation mechanisms *in vivo*.

3. IDENTIFICATION OF EUKARYOTIC RNA PROCESSING ENZYMES

In the current investigation the experimental strategy adopted was largely addressed to investigating the functional significance of hnRNP complexes with regard to the processing of hnRNA in HeLa cells. Because of the previous claims for endoribonuclease and poly A synthetase activities in association with rat liver 30s hnRNP particles (Niessing & Sekeris, 1970, 1973, 1974) and because it appeared highly probable that a RNP organisation prevailed as the structural substrate for hnRNA processing events, a likely starting point for the identification of HeLa hnRNA processing enzymes appeared to be provided by studying HeLa hnRNP particles. However, of the various enzyme activities examined during the current investigation, only endoribonuclease activity could be detected in association with HeLa hnRNP particles while the great majority of the nuclear enzymic activities probed in this study appeared to be present in a nucleosol fraction. Consequently it is possible also that the analogous enzyme activities reported in association with rat liver 30s hnRNP particles may derive from contamination of the particle preparation by soluble components. If this is the case then two immediate questions arise: (1) what is the functional significance of hnRNP particles? (2) how should future research into the identification of hnRNA processing enzymes be directed?

3.1 The functional significance of hnRNP particles

The fulcrum upon which rests the validity of the experimental approach involved in identifying an association of hnRNA processing enzymes with hnRNP particles, is defined by favourable answers to two important questions:
(1) Are the reported enzymic activities intimately associated with the hnRNP particles?
(2) Are the reported enzymic activities functional in the sense that they are involved in the maturation of mRNA? *in vivo*?

3.1.1 The nature of the association of enzymic activities with hnRNP particles

The association of enzymic activities with isolated hnRNP particles can reflect a variety of possible interactions.

The enzymes may: (a) be present as tightly bound integral components of the hnRNP particles; (b) be bound loosely to the particles in an association which reflects a transient binding of the enzymes to hnRNP complexes *in vivo*; (c) become bound non-specifically to the particles during isolation procedures or (d) be integral components of structures which co-purify with hnRNP particles.

The vast majority of possible hnRNA processing enzyme activities reported in association with hnRNP particles (Table 14) have been demonstrated using 30s monomeric hnRNP particles from rat liver. Because of their low sedimentation coefficient they are particularly vulnerable to contamination by nucleosol components in the manner proposed by Stévenin & Jacob (1974). In particular, the majority of nuclear RNA guanylyltransferase, RNA methyltransferase and poly A synthetase activity appears to be located in the nucleosol fraction (see 2.3, 2.4 above). Although simple contamination by nucleosol components is unlikely in the case of the enzymic activities responsible for capping and methylation events (Bajszár et al, 1978), it remains quite feasible that there is some non-specific binding of the nucleosol activities to the 30s hnRNP particles during their isolation. In the present study it is possible that in the case of RNA guanylyl-transferase and poly A synthetase activities the non-specific binding of a small portion of nucleosol activities to the HeLa hnRNP particles escapes the limits of detection of such activities. Alternatively, the deliberate selection of the HeLa hnRNP particle population to exclude structures of sedimentation coefficients $< 45s$ may have pre-empted possible nucleosol contamination.

Investigation of the nature of the association of the reported enzyme activities with hnRNP particles has rarely been attempted. Although it must be noted that resedimentation of the rat liver 30s hnRNP particles in sucrose density gradients was accompanied by a loss of 65% of their original RNA methyltransferase activity (Bajszár et al, 1978), almost all of the corresponding poly A

TABLE 14 Enzymic Activities Associated with HnRNP Particles

Enzyme Activity	Source, Method of Preparation, and Size of HnRNP Particles	Properties of Enzymic Activity	Reference
Endoribonuclease	Rat liver, pH 8.0 extraction, 30s	Cleaves hnRNA from rat liver but other substrates not tested	Niessing & Sekeris (1970)
	Rat liver, pH 8.0 extraction, 30s	Cleaves dsRNA present in hnRNP particles or prepared from hnRNA	Molnár & Samarina (1976); Molnár et al (1978)
	HeLa, nuclear sonication, 45s-200s	Non-specific; cleaves hnRNA but also rRNA and poly C	This work
Exoribonuclease	Rat liver, pH 8.0 extraction, 30s	Produces nucleoside 5' monophosphates	Molnár et al (1978)
Poly A Synthetase	Rat liver, pH 8.0 extraction, 30s and >30s	At least 2 different activities of different ionic requirements	Niessing & Sekeris (1972, 1973, 1974) Louis et al (1978)
RNA Guanylyltransferase; RNA(Guanine-7-)methyl transferase; RNA-2'-O-methyl transferase	Rat liver, pH 8.0 extraction, 30s	Individual activities not characterized but assumed from ability of particles to mediate conversion of ppGpC to $m^7GpppGp^mC$	Bajszár et al (1978)
Protein Kinase	Rat liver, pH 8.0 extraction, 30s		Schweiger & Schmidt (1974); Karn et al (1977)
	HeLa, nuclear sonication		Blanchard et al (1975, 1977)
Phosphoprotein phosphatase	HeLa, nuclear sonication		Periasamy et al (1977)
Protease	Rat liver, nuclear sonication, 35-150s		Stévenin et al (1977a)

synthetase activity appeared to be retained (Niessing & Sekeris, 1972).

The evidence that the enzyme activities are specifically bound to hnRNP particles is therefore far from convincing. Perhaps the most persuasive case for a specific association of an enzyme activity with hnRNP particles has been furnished by Blanchard et al (1977). After taking some care to exclude possible contamination by other subcellular fractions these authors have demonstrated that HeLa hnRNP particles and their companion protein kinase activity are inseparable with regard to their profiles on sucrose density gradients, metrizamide equilibrium density gradients and their binding characteristics on oligo dT-cellulose columns.

3.1.2 Functional significance of enzyme activities reported in association with hnRNP particles

The functional significance of hnRNP particle-associated enzymic activities remains unclear largely because of the general difficulty in identifying RNA processing enzymes in eukaryotic cells. Although the substrate specificity of the endoribonuclease reported in association with rat liver 30s hnRNP particles has not been tested, the poor degree of substrate specificity of the equivalent HeLa enzyme in this study does not mark it as a suitable processing candidate. However it is uncertain to what extent this reflects an inadequacy of the experimental design (see 2.1.2 above). In addition, the functional significance of a double-strand RNA-specific endoribonuclease and a 5'-exonuclease reported in association with 30s hnRNP particles from rat liver (Molnár & Samarina, 1976; Molnár et al, 1978) remains to be assessed.

Because of the comparative rarity of capped 5' termini and 3' polyadenylate tracts in eukaryotic RNA species, the RNA guanylyltransferase, RNA methyltransferase and poly A synthetase activities reported in association with rat liver 30s hnRNP particles hold the most promise in terms of a functional role in mRNA maturation. As yet, however, the individual RNA guanylyltransferase and RNA methyltransferase activities have not been investigated but rather their combined action is inferred from the ability of the particles

to promote the conversion of the artificial substrate ppGpC to m^7GpppG^mpC in the presence of GTP and SAM (Bajszár et al, 1978). In addition, the significance of the heterogeneity of poly A synthetase activities detected in association with rat liver 30s hnRNP particles remains uncertain (Niessing & Sekeris, 1974). Certainly, however, poly A synthetase activity characterised from subnuclear fractions from eukaryotic cells does not manifest properties expected of an hnRNA processing activity e.g. there appears to be a relative lack of primer specificity as exhibited by the rat liver hnRNP particle-associated enzyme activities. In addition both Mn^{2+}- and Mg^{2+}-dependent activities reported in association with rat liver hnRNP particles are accompanied by very significant activity using ADP as a substrate (Niessing & Sekeris, 1974).

3.1.3 Other functional aspects of hnRNP particles

In addition to harbouring possible hnRNA processing enzymes hnRNP particles may include other possible functional activities. As mentioned above, some of the proteins may function to contribute to the specificity of cleavage by the appropriate endoribonuclease function. Certainly the double-strand RNA duplexes which may act as signals for cleavage are almost entirely devoid of protein and therefore accessible to possible cleavage enzymes (Calvet & Pederson, 1978).

Another possibility is that some of the major low molecular weight proteins may function in the packaging of hnRNA transcripts (Beyer et al, 1977). Presently, the evidence for such a role is largely circumstantial. The polypeptides in question consist of a small number of predominantly basic species which are subject to a variety of post-synthetic modifications (which in turn involve electronic charge differences), which are of similar amino acid composition and which do not appear to be tissue-specific. Thus these proteins which account for the majority of the protein associated with hnRNP particles may serve to condense the unwieldy large primary transcripts in structural analogy to the condensation of DNA by the histones.

Conceivably some of the proteins bound to hnRNA may act in a regulatory capacity with respect to the transport of mRNA precursors. Regulation of transport could possibly be expressed at several levels including detachment of RNA species from the chromosomal site of synthesis, selection of processing intermediates and transport through the pores of the nuclear membrane. Currently there is a dearth of information regarding these possibilities although a 75,000 dalton polypeptide species which appears to be bound to the 3' polyadenylate segment of HeLa hnRNA and mRNA has been implicated in the regulation of transport of mRNA from the nucleus to the cytoplasmic compartment (Schwarz and Darnell, 1976).

3.2 Current progress in the identification of eukaryotic RNA processing enzymes

From the previous discussion it would appear that the initial promise offered by the study of hnRNP particles in identifying associated hnRNA processing enzymes remains, as yet, largely unfulfilled. A major obstacle derives from the overall difficulty in identifying hnRNA processing enzymes or even eukaryotic RNA processing enzymes in general.

3.2.1 Requirements for the detection of eukaryotic RNA processing enzymes

3.2.1.1 Utilisation of a suitable RNA substrate

In order to assay for a particular RNA processing enzyme it is highly desirable to utilise as substrates only molecular species identical to those which serve as substrates for *in vivo* RNA processing enzymes. In eukaryotic systems such an ideal situation has not been possible for a variety of reasons. A major obstacle concerns the difficulty in isolating specific precursor RNA species. Even more discouraging is our considerable ignorance of the identity of primary transcripts which are ultimately processed to generate mature eukaryotic cellular RNA species. Thus, although it is now widely recognised that the precursors to eukaryotic mRNA are to be found within the hnRNA population, in no specific instance has it been possible to identify unambiguously a primary transcript

which acts as a precursor to a specific mRNA. Admittedly, however, considerable information has been obtained concerning a 16s nuclear RNA precursor to β-globin which may represent a primary transcript (Curtis & Weissman, 1976; Haynes et al, 1978; Kinniburgh et al, 1978; Tilghman et al, 1978b). In this regard a major difficulty arises from our ignorance of the origin and termination points of transcription although an encouraging future line of research is provided by the demonstration of the close coincidence of promoter and cap sites relevant to the biogenesis of some adenovirus-specified mRNAs (Ziff & Evans, 1978).

Similarly, although the rapidly labelled 45s rRNA in mammalian cell nucleoli has been assumed to be among the initial products of rDNA transcription there is no proof that it is the primary transcript of the ribosomal genes. However, very recently it has been shown that a 40s-pre-rRNA from Xenopus (Reeder et al, 1977), a 34s pre-rRNA from Drosophila (Levis & Penman, 1978b), a 37s pre-rRNA from yeast (Nikolaev et al, 1979; Klootwjik et al, 1979) and a 35s pre-rRNA from Tetrahymena (Niles, 1978) all bear triphosphorylated purine residues at their 5' termini as expected of primary transcripts. In the case of eukaryotic tRNA processing De Robertis & Olson (1979) have, by following the transcription of cloned yeast $tRNA^{Tyr}$ genes injected into Xenopus oocytes, been able to identify a 108 base pair tRNA precursor which represents the largest and most rapidly labelled tRNA precursor obtained in this system. Such a precursor appears to bear a triphosphorylated adenosine nucleotide at its 5' terminus and because of the known DNA sequence of the cloned gene it has been possible to map this presumptive primary transcript. Finally, in the case of 5s rRNA it appears to be generally the case that the 5' terminus of the mature RNA species retains the triphosphorylated nucleotide marking the initiation of transcription.

In conclusion, therefore, some progress has been made recently in identifying presumptive primary transcripts to certain tRNA and rRNA species. In order to assay for

eukaryotic RNA processing enzymes it would be ideal to be able to isolate unprocessed RNA precursors which could then be used as substrates to monitor possible RNA processing enzyme activities. In prokaryotes isolation of temperature-sensitive mutants deficient in particular RNA processing enzyme activities has greatly facilitated the identification of precursor RNA species as well as permitting elucidation of mechanisms of RNA processing (Dunn & Studier, 1973b; Schedl & Primakoff, 1973; Ghora & Apirion, 1978). However, generally, eukaryotic systems are less amenable to such genetic studies and even in the instance of the well-studied Drosophila and yeast systems there has been a dearth of recognised mutants affecting RNA maturation. Nevertheless Hopper et al (1978) have succeeded in isolating a yeast temperature sensitive mutant deficient in tRNA biosynthesis which has been of immediate benefit in characterisation of a tRNA processing enzyme activity (see 3.2.2 below).

Another means of isolating unprocessed RNA precursors is to utilise *in vitro* transcription processes in order to synthesize primary RNA products identical to those manufactured *in vivo*. Again such a procedure has been effectively employed in prokaryotic systems using the highly purified E.coli RNA polymerase to transcribe in accurate fashion a prokaryotic DNA template (e.g. Dunn & Studier, 1973a). However, in eukaryotic systems a comparable level of success has not been enjoyed. Only in the case of RNA polymerase III has it been possible to reproduce *in vitro* with a high degree of fidelity the transcriptional processes occurring *in vivo* (e.g. Parker & Roeder, 1977; Yamamoto et al, 1977). At least in the case of 5s RNA and tRNA species an important development has been the demonstration that cloned genes for such RNA species can be accurately transcribed following injection into Xenopus oocytes,(Brown & Gurdon, 1978; Kressman et al, 1978) or by means of extracts from Xenopus germinal vesicles (Birkenmeier et al, 1978; Schmidt et al, 1978). Consequently at least in these instances the future looks bright for the isolation of precursor RNA species both as

substrates for potential RNA processing enzymes and as an aid in characterising eukaryotic mutants defective in RNA biosynthesis.

3.2.1.2 Establishment of identity between products of *in vivo* and *in vitro* processing

In order to define a eukaryotic RNA processing enzyme it is also necessary to characterise the reaction products of the *in vitro* assay and demonstrate their equivalence to those obtained during *in vivo* processing events. Moreover, if such a condition is fulfilled when utilising the ideal RNA substrate then the next step would be to purify the activity free from contaminating activities which could utilise substrates not expected to be prey to processing enzymes *in vivo*. Assessment of these criteria will be discussed in the next section.

3.2.2 Candidate RNA processing enzymes in eukaryotes

Generally, isolation of specific eukaryotic RNA precursors for assay of RNA processing enzymes has not been possible. However in the case of ribosomal RNA isolation of a 45s precursor species which exhibits only a very limited degree of molecular heterogeneity has been possible from mammalian cell nucleoli. Use of such a comparatively well-defined RNA substrate to assay for potential cleavage enzymes from eukaryotic cell nucleoli has resulted in non-specific degradation of the RNA substrate to small fragments (e.g. Winicov & Perry, 1974) thereby suggesting that an element of specificity is lacking in the *in vitro* processing systems. However a purified endoribonuclease from calf thymus which demonstrates great specificity towards double-stranded RNA regions apparently mediates the production of 33s, 29s and 19s fragments from 45s rRNA although in this case identity between *in vivo* and *in vitro* processing products was only established by a very rough correspondence of electrophoretic mobilities on polyacrylamide gels (Ohtsuki et al, 1977).

Because of the complexity of the mRNA and tRNA populations, isolation of specific RNA precursors has proved to be a formidable obstacle and certain contingency

plans have been adopted:

(1) Use of prokaryotic RNA precursor substrates. This can only be justified where divergence in structure between prokaryotic RNA precursors and their analogous eukaryotic counterparts is comparatively slight. For example, Koski et al (1976) have used an E.coli $tRNA^{Tyr}$ precursor to purify an endoribonuclease from human KB cells which resembles E.coli RNase P in both substrate specificity and identity of specific cleavage products. The ability of this activity to reduce unfractionated KB cell tRNA precursor species to mature tRNA-sized molecules is also consistent with its postulated role as a RNA processing enzyme.

(2) Use of heterogenous RNA precursor substrates. Such an approach has frequently been dictated by the previous inability to isolate specific tRNA and mRNA precursors from eukaryotes but suffers from an enormous disadvantage in that it precludes identification of specific reaction products. Thus an activity has been described from mouse myeloma cell nuclei which has been considered to be involved in tRNA biogenesis because it exerts considerable specificity towards a 4.5s RNA population synthesized *in vitro* thereby degrading it to a 4s (mature tRNA-sized) form (Marzluff et al, 1974). In addition none of the wide variety of endoribonuclease activities described in eukaryotic cells, including the one studied in the present investigation demonstrate the substrate specificity expected of an enzyme involved in eukaryotic mRNA biogenesis.

(3) Use of RNP substrates. In E.coli the experimental evidence favours a situation where although the specificity of early rRNA processing events is determined only by RNase III and the structure of pre-rRNA, the specificity of later cleavage events appears to be dependent on an RNP organisation for the substrate (Hayes & Vasseur, 1976; Dahlberg et al, 1978). In eukaryotes the evidence for correct processing of deproteinized hnRNA or pre-rRNA *in vitro* is very weak. However several experimenters have described endoribonuclease activities purified from mammalian cell nucleoli which appear to mediate limited fragmentation of the rRNA in pre-ribosomes to give inter-

mediates apparently resembling those obtained during *in vivo* processing. Again, however, these experiments can be criticised in that correspondence between *in vivo* and *in vitro* processing intermediates was established in terms of a rough similarity of electrophoretic mobilities in polyacrylamide gels. In the present study self-incubation of isolated hnRNP particles provoked degradation of the endogenous RNA to species of low sedimentation coefficient which may suggest that an extra element of specificity is still required in such an *in vitro* processing system.

(4) Examination of relatively unique RNA processing enzymes

A variety of structural features are commonly found on only a few varieties of eukaryotic RNA e.g. capped structures at the 5' terminus and 3' poly A adenylate tracts are largely confined to the mRNA population while tRNA species commonly bear nucleotides which are very seldom found in other classes of RNA. Therefore certain RNA processing enzyme activities involved in the formation of these structures have been probed in the absence of specific RNA precursor substrates. In particular, poly A synthetase activity has been purified from a wide variety of eukaryotic tissues but there appears to be a considerable lack of primer specificity while even the most highly purified preparations are incapable of synthesising the large (~200 adenylate units) poly A tracts found *in vivo* at the 3' termini of hnRNA species, although isolated HeLa cell nuclei have been shown to be capable of such synthesis (Jelinek, 1974).

Enzymes involved in the synthesis of methylated capped structures have only been recently described in eukaryotic cells and in these cases the substrates used were provided by suitably modified vaccinia viral mRNA synthesised *in vitro* or by artificial oligonucleotides or polynucleotides (Wei & Moss, 1977; Ensinger & Moss, 1976; Keith et al, 1978). Groner et al (1978) have described some evidence which suggests that capping of endogenous RNA species by HeLa nuclear homogenates is largely confined to RNA polymerase II transcripts. However the authors have also observed the formation in the same *in vitro* system of diphosphate capped structures which may represent an artefactual consequence of *in vitro* incubation.

Regarding the various RNA modifying enzymes a signal success has been achieved very recently with the purification to homogeneity of considerable amounts of tRNA guanine insertase from rabbit reticulocytes (Howes & Farkas, 1978). However, for a variety of reasons, a corresponding degree of success has generally not been achieved regarding purification of the various RNA methylases.

Finally, novel enzyme activities which engage in splicing reactions have only recently been identified. An activity from crude yeast extracts, which features in the maturation of yeast $tRNA^{Tyr}$ & $tRNA^{Phe}$ species by excision of a short intervening RNA sequence and ligation of the informational sequences has been identified by O'Farrell et al (1978) and Knapp et al (1978). Also a comparable activity involved in the maturation of an adenovirus mRNA species has been detected in an *in vitro* system based on isolated nuclei and even then addition of a cytoplasmic component appeared to be a pre-requisite for the accurate reproduction *in vitro* of such *in vivo* processing events (Blanchard et al, 1978b).

In conclusion, the most reliable evidence for the definition of eukaryotic RNA processing enzymes has been achieved in the case of enzymes which process tRNA whose precursors, unlike those for rRNA and mRNA, appear to be devoid of a RNP organisation. Especially in the case of mRNA maturation a major problem concerns isolation and utilisation of suitable precursor substrates for assay of the processing enzyme activity. Because of the difficulty in isolating specific precursors to mRNA it has been usual to use the ill-defined hnRNA population from a particular tissue type as an alternative, if far from ideal, substrate. In such a system it would be possible to follow the processing of several particular mRNA species using molecular hybridization with the appropriate cDNA probe. However as possibly suggested from the present work and by others (see above) it may well be that an RNP organisation for the substrate precursor RNA may be essential for permitting the required specificity of *in vitro* processing. Consequently such a system would not be amenable to molecular hybridization techniques to follow

the processing of a specific class of mRNA.

Accordingly, there are still some considerable difficulties attendant upon investigation of hnRNA processing enzyme activities. One must hope that the day is not far off when *in vitro* transcription systems are capable of accurate transcription of genes specifiying mRNA in order to permit isolation of specific mRNA precursors. In this context one would hope to utilise cloning technology in order to amplify suitably constructed plasmids in E.coli thereby generating large amounts of specific genes coding for a particular type of mRNA which could be transcribed in a suitable *in vitro* system. Alternatively it might be possible to obtain suitable RNA precursors from transcription of genes within suitably constructed plasmids in E.coli.

A further refinement would be to investigate possible *in vitro* reconstitution systems utilising the specific mRNA precursor and dissociated proteins representative of the hnRNP particle population in order to permit an artificial reconstruction of the RNP organisation relevant to a specific mRNA precursor species *in vivo*. However, it may well be that additional factors are involved. For example, evidence has accumulated suggesting the presence of some low molecular weight, stable RNA species associated with hnRNP particles (Deimel et al, 1977; Northemann et al, 1977; Guimont-Ducamp et al, 1977; Howard, 1978), while it has also been shown that similar RNA species are found in base-pairing association with nuclear and cytoplasmic poly $(A)^+$RNA species (Jelinek & Leinwand, 1978). Thus it may be the case that such species may play a role in mRNA maturation events. In addition, some soluble protein factors which may have an ephemeral association with the RNP substrate could play a role in the maturation of mRNA and rRNA. Certainly, more accurate simulation of *in vivo* processing events culminating in the biogenesis of mRNA appears to be possible using isolated nuclei rather than subnuclear fractions (see above) while there are some indications that in certain cases cytoplasmic components may also be necessary to convey accurate eukaryotic RNA processing in *in vitro* systems (Blanchard et al, 1978b) Knapp et al, 1978).

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