SEQUENCE STUDIES ON THE HIGH MOLECULAR WEIGHT RNA OF HeLa CELLS

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ABBREVIATIONS

Sequences of nucleotides are written N-N-N-Np, with hyphens for internal phosphate and p for terminal phosphates.

mRNA	messenger RNA
HnRNA	heterogeneous nuclear RNA
tRNA	transfer RNA
RNase	ribonuclease
DNase	deoxyribonuclease
DEAE	diethylaminoethylcellulose
EDTA	ethylenediaminetetra-acetate
poly (A)	polyadenylic acid
poly (U)	polyuridylic acid
SDS	sodium dodecyl sulphate

C-O-N-T-E-N-T-S

LIST OF FIGURES

LIST OF TABLES

SUMMARY

When growing cultures of mammalian cells are exposed to radioactive RNA precursors, two general types of very high molecular weight nuclear RNA can be recognised; ribosomal precursor RNA and a series of molecules, with molecular weight varying between 1×10^6 and 20×10^6, which have been collectively termed heterogeneous nuclear RNA (HnRNA). It is commonly believed that cytoplasmic messenger RNA is formed from nuclear HnRNA by post-transcriptional modification. The aim of this work was to examine the relationship between HnRNA and mRNA by studying short sequences present in them.

1) It was found that HeLa cells had to be treated with low levels of actinomycin D before radiochemically pure HnRNA or mRNA could be isolated.

2) Fingerprints of HnRNA clearly showed the sequence complexity and apparent lack of methylated products in HnRNA compared with ribosomal RNA.

3) Specific labelling of methylated nucleotides with L- [^{14}C-methyl] methionine however showed the possibility of HnRNA methylation.

4) Double stranded HnRNA fragments were richer in guanine and cytosine than total HnRNA.

5) HnRNA, labelled for 3 hours with $^{32}PO_4$ in the presence of low levels of actinomycin D, revealed a low frequency of oligonucleotides terminated by the dinucleotide C-Gp. The relative molar oligonucleotide frequencies obtained from this RNA were similar to those expected from a general transcript of the nuclear DNA.

6) Fingerprints of mRNA, labelled under similar conditions, showed a less marked C-Gp sequence deficiency.

7) When HnRNA was labelled for 16 hours in the presence of actinomycin D it had a relative molar oligonucleotide frequency different from that labelled for 3 hours but similar to mRNA.

8) Only a small difference in relative molar oligonucleotide frequencies was observed between large and small (mRNA sized) HnRNA molecules fractionated on sucrose density gradients.

9) Comparison of large HnRNA labelled for 3 hours and 16 hours in the presence of actinomycin D, by sucrose-formamide density gradient analysis, showed that although the former was large in size, the latter consisted mainly of aggregated molecules.

10) Homopolymeric sequence containing HnRNA showed no difference from total HnRNA with respect to relative molar oligonucleotide frequency.

11) The small HnRNA, rich in polyadenylic acid, and isolated from chromatin by extraction with 0.4M ammonium sulphate, 0.03M Tris-HCl pH 7.4, was shown to be similar to mRNA in terms of relative molar oligonucleotide frequency data.

The results of these sequence studies of high molecular weight RNA are discussed in terms of the relationship of HnRNA to mRNA.

INTRODUCTION

1. TRANSCRIPTION

The genetic information which codes for the development and functioning of organisms is encoded in the sequence of deoxyribonucleotides that constitute its DNA. Transcription is the process whereby this genetic information is transferred to polyribonucleic acid (RNA) molecules and it occurs at specific regions of the DNA molecule known as genes. The process is catalysed by an RNA polymerase enzyme which links together ribonucleotide molecules in an order complementary to the base sequence of a DNA template. As we are interested in studying RNA it is of use to know something of the RNA polymerase enzyme and how it synthesises RNA.

1.1. RNA Polymerase

RNA polymerase (E.C.2.7.7.6.) catalyses the polymerisation of the four ribonucleotides (ATP, GTP, CTP and UTP) to form RNA using DNA as a template.

DNA dependent RNA polymerase activity was first demonstrated in 1959 in rat liver nuclei (Weiss and Gladstone, 1959; Weiss, 1960). However until ten years later most of the studies on animal enzymes were carried out on unpurified chromatin ("aggregate enzyme") rather than on purified soluble enzyme. About the same time Huang et al. (1960) isolated a similar enzyme from plant tissue, and Hurwitz et al. (1960) from bacteria. In contrast with the eukaryotic RNA polymerase, the bacterial enzyme has been found to be comparatively easy to purify.

Much work has been done to characterise the enzyme of E. coli (Chamberlin and Berg, 1962). Two enzymatically active forms of the

enzyme are known. These are (a) core enzyme consisting of two α subunits, and a β and β' subunit and (b) holoenzyme consists of the core enzyme plus a σ subunit (Burgess, 1969). Holoenzyme has a molecular weight of about 500×10^3 daltons. The difference in the function of the two forms lies in their ability to bind to DNA and initiate RNA chains.

The simplest RNA polymerase known is the eukaryotic mitochondrial RNA polymerase (Chamberlin et al., 1970). It has a molecular weight of about 64×10^3 daltons. T_7 phage RNA polymerase, another small polymerase, has a molecular weight of 100×10^3 daltons. These enzymes are less than one quarter of the size of the E. coli enzyme. However unlike the RNA polymerase of E. coli these enzymes only have one polypeptide chain and this may reflect the simplicity of the genes they transcribe.

1.2 Mechanism of Transcription

Transcription can be divided into three stages, initiation, elongation and termination.

The molecular mechanism involved in the initiation of RNA synthesis in vitro was first suggested by Zillig and his workers (Fuch et al., 1967) and subsequently confirmed and elaborated on by several workers (Bautz et al., 1972; Hinkle and Chamberlin, 1972). After random interactions with the template DNA, the RNA polymerase holoenzyme "recognises" a specific structure or sequence within a promoter region. This recognition, probably due to the sigma factor, enables the polymerase to bind to the DNA in this region at least an order of magnitude more strongly than the non-specific interactions.

The binding of the enzyme causes a change in the promoter region of the DNA which is both co-operative and reversible. Furthermore, once this has occurred polymerisation of ribonucleotides may occur rapidly (Travers, Baillie and Pederson, 1973; Mangel and Chamberlin, 1974). It is generally argued that the vital stage of the initiation of RNA synthesis is the opening of the promoter. This is said to occur when the energy made available by the initial tight binding of the RNA polymerase enzyme to the promoter, is greater than that required to melt a short region of the DNA double helix at or close to the initial binding site.

Regulatory proteins may bind close to the promoter site either raising or lowering the energy required to cause the vital opening of the DNA and in this way effect control. Several regulatory factors have been discovered. Probably the best understood is the catabolite gene activator protein or cyclic AMP binding protein (CAP factor) as it is sometimes known as. This factor is required by E. coli for the induction of catabolic enzymes (Zubay et al., 1970). In order to obtain maximal synthesis of the enzymes of the "lac operon", not only must the repressor be removed from the operator site, but also the transcription must be activated by a cyclic AMP-protein (CAP factor) complex.

The polymerisation of ribonucleotides appears to begin by the coupling of ATP or GTP with a second ribonucleoside triphosphate to eliminate inorganic pyrophosphate and generate a dinucleoside tetraphosphate of the structure ppp.Pu.p.X (Maitra et al., 1967). RNA chain elongation proceeds by the addition of successive nucleoside monophosphate residues, from nucleoside triphosphates, to the initial dinucleotide tetraphosphate at its 3'-OH terminus (Bremer et al., 1965). Thus the polymerisation

reaction proceeds from the 3' end to the 5' end of the DNA strand and growth of the RNA chain proceeds from the 5' terminal triphosphate to the 3' hydroxyl terminus.

The specificity of the selection process which governs the incorporation of a nucleotide during chain elongation is determined by the corresponding base in the DNA template, the structure of the base in the nucleoside triphosphate substrate, and also by the structure of the RNA polymerase itself. The normal selection process most probably involves Watson-Crick base pairing between the bases of the template and substrate molecules. Nevertheless, the importance of RNA polymerase itself in the selection process has been shown by damaging the enzyme. When damaged with X-rays the extent of incorrect incorporation, estimated to be less than 1 in 3,000 (Chamberlin et al., 1963; Bujard and Heidelberger, 1966) is enhanced (Strniste et al., 1973).

Much evidence on the sequences complementarity between the product of transcription and its DNA template have been gained by the use of the hybridisation technique. In this technique, double stranded DNA is denatured and then slowly cooled in the presence of RNA. This annealing process leads to the formation of artificial DNA-RNA hybrid molecules only if the RNA sequences are complementary to those of the DNA. By making use of the fact that RNA and DNA differ in density the products of the hybridisation reaction can be separated on buoyant density gradients. This technique was used by Hall and Spiegelman (1961) to show that RNA synthesis in vivo after infection of E. coli with phage T_2 is complementary to the phage DNA. Geiduschek et al. (1961), using a similar technique, demonstrated that RNA formed in vitro by micrococcus

RNA polymerase has a high complementarity for the primer DNA.

Other techniques have also been used to elucidate this point. The base ratio of RNA synthesised by E. coli RNA polymerase, using DNA primers from various sources, has been shown to be complementary to the base ratio of the primer DNA (Furth et al., 1961). Furthermore, Hurwitz et al. (1962) demonstrated that the nearest neighbour nucleotide frequencies of both primer and product were similar.

The final stage of the transcription reaction is chain termination and release, in which the nacent chain and the RNA polymerase are released from the template. This is probably the least well understood area of the polymerase reaction. Nevertheless several kinds of termination have been noted in the in vitro systems. These include sequence induced termination, factor dependent termination and [illegible] induced termination.

Termination of RNA synthesis in vivo has been shown to be different from that in vitro in several well characterised cases. In vitro transcription of T_3 and T_4 DNA continue past their in vivo termination points (Brody and Geidushek, 1970; Brody et al., 1970; et al., 1972; Milanesi et al., 1970). Also the two largest transcripts of λDNA appear to be read through their normal termination sites. This probably encouraged the search for accessory termination factors which would enhance correct RNA chain termination during transcription. Such a factor has been isolated from E. coli and designated the ρ factor (Roberts, 1969).

1.3. Eukaryotic RNA Polymerase

Unlike the prokaryotic RNA polymerase, the eukaryotic enzyme is tightly bound to a complex of DNA, histone, acidic protein and RNA. Consequently in most early studies either whole nuclei or chromatin were used.

Isolated nuclei at low ionic strength were shown to synthesise a GC rich ribosomal RNA and at high ionic strength, more DNA-like RNA was made (Widnell and Tata, 1964; 1966). In low ionic strength conditions RNA was mainly synthesised in the nucleolus, while in high ionic strength extranucleolar synthesis of RNA was predominant (Maul and Hamilton, 1967; Pogo *et al.*, 1967). These results suggested that there were at least two RNA polymerase activities present in eukaryotic cells. Further indication of the presence of multiple forms of the enzyme came from the observation that α amanitin (a toxin from the toadstool *Amanita phaloides*) specifically inhibited RNA synthesis catalysed under high ionic strength conditions in mouse liver nuclei (Stirpe and Fume, 1967).

Roder and Rutter (1970) were probably the first workers to isolate several different polymerase activities. They used DEAE Sephadex to separate three activities from sea urchin embryos and rat liver. One activity appeared to be responsible for transcribing rRNA, one for mRNA and possibly a third for tRNA (Pogo, 1969; Roder and Rutter, 1970). Since then many other enzyme sources have been used including HeLa cells (Sugden and Sambrook, 1970).

The different classes of RNA polymerase all have similar molecular weights but different subunit structures. Values of

$550 \times 10^3 \pm 10\%$, $600 \times 10^3 \pm 10\%$ and $570 \times 10^3 \pm 10\%$ have been found for three calf thymus enzymes (Chambon et al., 1972; Kedinger et al., 1974). The subunit pattern of these three enzymes has been examined and it seems that the basic structure of the mammalian enzyme is similar to that of the prokaryotic enzyme in that they consist of two large subunits accompanied by several small subunits (Chambon et al., 1972; 1973; Gissinger and Chambon, 1972; Kedinger and Chambon, 1972).

2. HIGH MOLECULAR WEIGHT RNA IN EUKARYOTES

The previous section outlined current ideas on the enzymic mechanism whereby RNA is synthesised and the means whereby the process can be controlled. In this next section the various types of high molecular weight RNA known to exist in the eukaryote cell will be discussed.

Eukaryotic cells contain many RNA species ranging in size from about 2.5×10^4 to more than 20×10^6 daltons. When growing cultures of mammalian cells are exposed to radioactive precursors, two general types of high molecular weight nuclear RNA can be recognised. One is a precursor of ribosomal RNA and the other is a series of molecules of high molecular weight (0.1×10^6 - 20×10^6 daltons) which are collectively called heterogeneous nuclear RNA (HnRNA).

Some of the HnRNA is widely believed to be the precursor of mRNA which is found in the cytoplasm. It is possible that HnRNA undergoes a maturation process similar to that undergone by precursor rRNA to yield mature rRNA.

2.1. Ribosomal RNA

Ribosomes are ribonucleoprotein particles found in the cytoplasm of eukaryotic cells where they play a central role in the synthesis of protein molecules. They consist of approximately 50% RNA and 50% protein and are formed from two subunits of unequal size. The RNA component of the large subunit (50S) has a sedimentation value of 28S and that of the small subunit (30S) is 18S.

Both these rRNA molecules are formed by the maturation of a much larger (45S) precursor molecule which is thought to be the primary product of transcription (Weinberg et al., 1967). The genes for rRNA are believed to be clustered in the nucleolus (McConkey and Hopkins, 1964).

Post transcriptional modification of 45S rRNA consists of methylation followed by a series of cleavages to produce the mature rRNA (Weinberg and Penman, 1970; Maden, 1971). The rRNA is then exported to the cytoplasm in the form of ribonucleoprotein particles (Warner, 1966). Recently the processing of HeLa cell rRNA has been studied by electron microscopy. Wellaur and Dawid (1973) have prepared highly reproducible secondary structure maps from which features such as hairpin loops can be seen. From examination of 45S RNA electronmicrographs the positions of the 28S and 18S rRNA sequences can be detected. Hybridisation experiments suggest that probably 50% of the 45S precursor molecule is represented by non-ribosomal RNA sequences (Jeanteur et al., 1968; Jeanteur and Attardi, 1969).

The base composition of highly purified rRNA and its precursors have been determined (Amaldi and Attardi, 1968; Jeanteur et al., 1968; Willems et al., 1968). The compositions suggested that 45S precursor RNA was significantly higher in G+C content than would be expected from 28S plus 18S rRNA. This suggests that the RNA degraded from the 45S rRNA molecule to produce mature rRNA, known as transcribed spacer RNA is very high in G+C content.

It now seems probable that the 28S rRNA has a small 5.8S RNA molecule associated with it (Pene et al., 1968; Rubin, 1973).

Maden and Robertson (1974) have shown that the 5.8S RNA is transcribed as part of the 45S precursor rRNA molecule and is probably covalently attached to the 32S rRNA intermediate. In fact, the 5.8S RNA sequence is thought to lie between the 28S and the 18S region of 45S RNA molecule.

Associated with the large ribosomal subunit is a 5S RNA species. The chromosomal site of the 5S RNA genes is apparently unlinked to the nucleoli (Brown and Webber, 1968; Aloni et al., 1971). There appears to be five times as many 5S genes as there are 45S genes in HeLa cells (Halten and Attardi, 1971; Jeanteur and Attardi, 1969). Furthermore the 5S rRNA appears to be synthesised in four times the amount required for ribosomal subunits and the excess 5S rRNA is broken down in the nucleus (Leibowitz et al., 1973). No explanation of this phenomenon has been offered so far.

2.2 Heterogeneous Nuclear RNA

Heterogeneous nuclear RNA (HnRNA) molecules are characterised by a heterogeneity in size (from 0.1×10^6 to 20×10^6 daltons) with no detectable discrete RNA species (Darnell, 1968). This contrasts with the nucleolar precursor of rRNA and its mature products, which form a class of homogeneous discrete species.

It has been estimated that approximately 1 - 3% of the cellular RNA is HnRNA (Darnell, 1968). Moreover, cell fraction studies have shown that the HnRNA is largely if not completely unassociated with the nucleolus (Penman, 1968). However at the most, only 10% of this nucleoplasmic HnRNA is destined to be exported to the cytoplasm (Darnell, 1968).

Several structural features have been shown to occur in HnRNA. These include 1) a polyadenylic acid (poly (A)) region, 2) oligoadenylic acid (oligo (A)) regions, 3) an oligouridylic acid (oligo (U)) sequence, 4) a 5' terminal triphosphate, 5) double stranded regions.

2.2.1. Polyadenylic Acid

A polyadenylic acid (poly (A)) region, approximately 10S in size was shown to be attached to rat polysomes by Hadjivassiliou and Brawerman (1966). The association of Poly (A) with high molecular weight RNA was shown 3 years later by Edmonds and Caramela (1969) using oligo (dT) cellulose chromatography.

The experiments of several workers have shown that it is the relatively short segment of the HnRNA molecule adjacent to, and containing the poly (A) sequence, which is eventually transported to the cytoplasm to act as mRNA (Darnell et al., 1971A; Darnell et al., 1972; Adesnik et al., 1972; Perry et al., 1974; Latorre and Perry, 1973; Edmonds et al., 1971; Jelinek et al., 1973).

The poly (A) region of HeLa cells has been shown to have approximately one adenosine residue for every 200 adenylic acid residues released by alkaline hydrolysis (Mendecki et al., 1972). The existence of the adenosine residue means that the poly (A) region is at the 3' end of the molecule. Jelinek has shown that while 30 - 40% of the large HnRNA molecules contain poly (A) regions only 10 - 20% of the small HnRNA molecules contain poly (A) tracts (Jelinek et al., 1973).

A heterogeneity of nucleotide sequences adjacent to the poly (A) sequence of mouse L cell mRNA has been shown by Nichols and Eiden (1974). This would seem to indicate that the nuclear poly (A) synthesising enzyme does not recognise a specific oligonucleotide sequence, or that the recognition site is further removed from the 3' terminus of the transcription unit.

2.2.2. Oligoadenylic Acid

Nakazato et al. (1973) has shown that besides the poly (A) sequence added post-transcriptionally there are shorter oligo (A) regions probably of 20 - 30 nucleotides, which are not at the 3' terminal end of the HnRNA molecule. Work by Jacobson et al. (1974) has shown that an oligo (A) region of 25 nucleotides exists in slime moulds. Furthermore there appear to be oligo (dT) tracts in the DNA of these cells, suggesting that oligo (A) regions are (unlike poly (A) regions) transcribed from regions of the DNA.

2.2.3. Oligouridylic Acid

HnRNA has been shown to contain an oligo (U) tract (Burdon and Shenkin, 1972). Molloy et al. (1972) have shown that this oligo (U) region is about 30 nucleotides long in HeLa cells with one G at the 3' end and 2 - 3 Cs and 2 - 3 As located along its length.

Hybridisation experiments showed that the oligo (U) region was transcribed from repetitive regions of the DNA (Molloy et al., 1972). The oligo (U) region was also shown to be located predominantly in the larger (70S - 90S) HnRNA, and essentially absent from mRNA. Later work by Molloy et al. (1974) showed that the oligo (U) regions occur near the

5' end of HnRNA. Furthermore 2 - 3 oligo (U) regions seemed to be present in poly (A) terminated HnRNA molecules of 20,000 nucleotides in length.

2.2.4. 5' Terminal Triphosphate

If one hydrolyses an RNA molecule, the 5' terminal nucleotide should be released as a tetraphosphate (pppXp). Georgiev's group in Moscow have found these polyphosphates only in HnRNA molecules larger than 30S and thus suggest that the 5' end of HnRNA molecules is lost on processing to smaller molecules (Ryskov et al., 1971; Georgiev et al., 1972).

2.2.5. Double Stranded Regions

As well as the RNase resistant poly (A) tail of HnRNA, there is also a region of mixed base composition which is resistant because of its double stranded nature.

While 90% of the RNase resistance of small HnRNA molecules are derived from poly (A) regions, less than 33% of the RNase resistance of large (>35S) HnRNA molecules of Ehrlich ascites carcinoma cells is derived from poly (A) regions (Ryskov et al., 1972). The remainder is due to double stranded RNA. Two types of double stranded sequences have been detected in Ehrlich ascites cells; longer (A+U) rich sequences and shorter, very (G+C) rich sequences. Both types of sequence had symmetrical base composition. A value of 74% for the A+U content of the longer sequences and a value of 50 - 55% for the G+C content has been given (Ryskov et al., 1972).

Although only 1% of Ehrlich ascites HnRNA was shown to be double stranded (Ryskov et al., 1972), 3% of HeLa cell HnRNA has been shown to be double stranded (Jelinek and Darnell, 1972). The size of HeLa cell double stranded sequences was found to be approximately 3 - 5S from polyacrylamide gels. Furthermore work on the reannealing properties of these double stranded sequences shows snap-back kinetics, indicating that they probably exist as hairpin configurations (Ryskov et al., 1972; Jelinek and Darnell, 1972). Recently, DNA-RNA hybridisation studies suggest that these double stranded regions appear to be transcribed from repetitive sequences of the DNA (Jelinek et al., 1974).

Jelinek and Darnell (1972), using HeLa cells, report having found one sixth as much double stranded RNA in mRNA as in HnRNA. However, in the sea urchin, double stranded RNA has not been detected in association with polysomes Kronenberg and Humphreys, 1972).

2.2.6. HnRNA-DNA Hybridisation Studies

Hybridisation of radioactive HnRNA or mRNA in the presence of non-radioactive competing RNA from either source show that there probably exists considerable overlap between the two species (Birnboim et al., 1967; Soeiro and Darnell, 1969). It was also shown that both types of RNA can, when in vast excess, combine with as much as 5 - 10% of the total DNA. However in these early experiments hybrids were formed mainly with fast reannealing, repetitive sequence regions of the genome.

To further investigate the RNA molecules transcribed from these reiterated sites, and to develop a procedure for ridding any RNA

sample of such sequences, Pagoulatos and Darnell (1970) evolved the technique of "exhaustive hybridisation". In this technique RNA from reiterated sequences hybridises faster than RNA from non-reiterated sequences thus reducing, in the unattached RNA, the proportion of sequences from reiterated regions. By studying rates of hybridisation, Pagoulatos and Darnell (1970) have concluded that HnRNA consists of a spectrum of reiterated sequences ranging in reiteration from 0.5 to 100 times that of rRNA (rRNA has about 400 sites per haploid genome in HeLa cells). However the majority of HnRNA molecules appeared to be unique or at least considerably less reiterated than rRNA.

Using the technique of "exhaustive hybridisation" to separate RNA into unique and repetitive transcripts, Darnell and Balint (1970) repeated the earlier experiments on the overlapping of sites for mRNA and HnRNA. They found that the repetitive transcripts of HnRNA hybridised to DNA three times faster than those of mRNA. Thus they concluded that highly reiterated sequences occur only in HnRNA.

The role of these reiterated sequences of HnRNA, which are not present in mRNA may be regulatory. Such a role for some RNA has been proposed by Britten and Davidson (1969) though there is no evidence to support this suggestion.

2.3. Messenger RNA

The earliest detection of a minor RNA component with properties related to a specific DNA is generally credited to Volkin and Astrachan (1957), who studied RNA synthesised immediately after infection of *E. coli* with phage T_2DNA.

A small proportion of the RNA in the cytoplasma of HeLa cells was first suggested as mRNA because it associated with polysomes, had sedimentation properties corresponding to the right order of size to code for proteins, and had a base composition resembling that of DNA (Girard et al., 1965; Latham and Darnell, 1965). A technique for specifically labelling mRNA in the cytoplasm was developed by Perry and Kelley (1968) who showed that short pulses (5 - 10 min) of radioactively labelled RNA precursor labelled mRNA but not rRNA.

Great advances in the field of mRNA study have been made since the discovery that a poly (A) sequence was present in most eukaryotic mRNA molecules. It has allowed purification of the active messenger RNA fraction from other RNA species found in polysomes.

2.3.1. Polyadenylic Acid

Studies of the steady state population of mRNA and its polyadenylic acid (poly (A)) segment in mammalian cells by Jeffery and Brawerman (1974) have shown that in mouse sarcoma cells poly (A) sequences are heterogeneous in size, with an average of 130 residues. Nevertheless 2 hr pulse labelled mRNA was shown to contain a homogeneous poly (A) sequence of about 170 residues in length.

These differences are said to be characteristic of the ageing of Poly (A) sequences attached to mRNA. Sheiness and Darnell (1973) have shown that the ageing of HeLa cell poly (A) sequences, attached to mRNA, is not controlled by protein synthesis - the termination of protein synthesis did not stop poly (A) ageing. Furthermore the shortening process did not seem to be related to the number of protein

molecules translated. However, Diez and Brawerman (1974) have shown the occurrence of a cytoplasmic poly (A) polymerase activity capable of the limited addition of 7 - 8 adenosine monophosphate residues to poly (A) sequences already on mRNA. This mechanism could serve as a control for poly (A) length.

The presence of poly (A) sequences in the mRNA of viruses such as polio (Yogo and Wimmer, 1972) and vaccinia (Kates, 1972) which replicate exclusively in the cytoplasm suggest that transport of RNA from the nucleus to the cytoplasm may not be the function of poly (A) sequences. Furthermore poly (A) sequences in mRNA do not contribute to secondary structure required for protein synthesis as complexes of poly (A) mRNA plus poly (U) show no difference in protein synthesising power (Munoz and Darnell, 1974). Poly (A) binding proteins have been reported in both cytoplasm and nucleoplasm. These proteins which may eventually clarify the function of poly (A) are mentioned in section 5, "Ribonucleoprotein Particles".

There is evidence that histone mRNA does not contain poly (A) sequences (Adesnik and Darnell, 1972; Adesnik et al., 1972b). Evidence that both transcriptional and translational control of histone mRNA is governed by the cell cycle has been found by Borum et al. (1975). If we assume that the mode of synthesis of poly (A) containing mRNA and histone mRNA are different, this could explain why histone mRNA enters the cytoplasm more quickly than poly (A) containing mRNA.

The isolation of 30% of total HeLa cell mRNA molecules apparently lacking in poly (A) tails by Milcarek (1974) suggests that histone mRNA is not the only class of mRNA lacking in poly (A) regions.

By preparing cDNA to poly (A) containing mRNA she showed that the non-poly (A) containing mRNA does not arise from poly (A) containing mRNA by de-adenylation. Furthermore while 3' deoxyadenosine almost completely inhibits the synthesis of poly (A) containing mRNA (95% inhibition), it only partially inhibits the synthesis of non-poly (A) containing mRNA (60% inhibition). Nevertheless the kinetics of labelling of both types of mRNA are similar.

2.3.2. Stability of mRNA

Compared with rRNA, mRNA molecules are very short lived. Work on mouse 3T3 and 3T6 cells has shown a half-life of 9 hr for mRNA (Abelson et al., 1974). However this is very long lived compared to prokaryote mRNA (it is interesting to note that the half-life of mRNAs from the Gal operon of E. coli are epimerase - 1.0 min, transferase - 0.6 min, kinase - 1.5 min (Achord and Kennell, 1974)).

Spradling et al. (1975) have shown that there are two very different kinetic components of mRNA present in an insect cell line. One has a half-life of 20 hr and the other has a half-life of 1.2 hr; a 15 fold difference. This compares with HeLa cell mRNA which has a half-life of 24 hr and 7 hr according to Singer and Penman (1973). In the presence of actinomycin D, often used to inhibit rRNA synthesis, the half-life was reduced to 12 hr and 4 hr respectively. In the steady state cell, labelled for a long time with radioactive RNA precursors, mRNA equals approximately 5% of the rRNA and the long and short half-life mRNAs comprise approximately 33% and 67% of the total mRNA (Singer and Penman, 1973). Recently, Pucket et al. (1975) have shown the existence of a third shorter half-life mRNA species with a half-life of 1 - 2 hr and accounting for 35 - 50% of mRNA from pulse labelled cells.

2.3.3. Specific mRNA

Certain cells are highly differentiated and produce mainly one type of protein. These cells are most useful for studying individual mRNA species and may provide useful models for total mRNA production.

Probably the most commonly used system is the reticulocyte which specialises in producing globin. Rabbit globin mRNA has been fractionated on sucrose/formamide gradients into α globin mRNA and β globin mRNA. The α globin protein is composed of 141 amino acids and thus requires 423 nucleotides to code for it. However 630 nucleotides are found in α globin mRNA (Hamlyn and Gould, 1975). β globin has 146 amino acids and therefore strictly requires 438 nucleotides. Nevertheless β globin mRNA has 710 nucleotides, only 50 of which can be accounted for by a poly (A) region (Hamlyn and Gould, 1975). However it should be noted that a poly (A) region of 150 nucleotides (10S in size) has been isolated from duck haemoglobin mRNA (Pemberton and Baglioni, 1972).

Isolation of mRNA from membrane bound polysomes (10% of total polysomes) and free ribosomes from rabbit reticulocytes has shown that the major species of both free and bound ribosomes was globin mRNA (Brennessel and Goldstein, 1975). Work on various tissues suggests that the synthesis of proteins which are secreted from cells takes place almost exclusively on membrane-bound polysomes (Rolleston, 1974). The absence of mRNA for casein in free polysomes of lactating ewe mammary glands has been shown recently (Houdelbine and Gaye, 1975). However mRNA for casein was found on the membrane-bound polysomes of these cells.

The list of mRNAs which have been identified and purified from various systems is long. It includes the mRNA coding for silk fibroin from Bombyse mori (Suzuki and Brown, 1972), collagen mRNA (Boedtker et al., 1974), calf lens mRNA (Lavers et al., 1974; Piperno et al., 1974) which includes a 50 nucleotide poly (A) sequence, δ crystallin mRNA from embryonic chick lens fibers (Zelenka and Piatigorski, 1974), and actin mRNA from cultures of differentiating embryonic chick skeletal muscle (Patterson et al., 1974).

Immunoglobulin mRNA coding for the immunoglobulin light chain has actually been fingerprinted (Milstein et al., 1974). Furthermore, Proudfoot and Brownlee (1974A)have shown that the 3' end of globin mRNA shows some homology with immunoglobulin light chain mRNA. Purification of immunoglobulin light chain mRNA by Mach et al. (1973) has shown it to be 14S in size with 1100 nucleotides. Only 65% of this mRNA appears to be translated to produce a precursor of light chain immunoglobulin protein which is 20 amino acids longer than the immunoglobulin light chain protein (Mach et al., 1973).

Ovalbumin mRNA has been prepared 95% pure by a sequential combination of techniques of sizing and selective purification of poly (A) containing mRNA (Rosen et al., 1975). It was shown to have 1600 nucleotides (520×10^3 molecular weight) by formamide/sucrose density gradient analysis, including a sequence of Poly (A) 70 nucleotides long (Rosen et al., 1975). Ovalbumin protein has only 387 amino acids, therefore of the 1600 nucleotides, 550 are extra sequences.

Thus it seems that mRNAs for specific proteins require approximately 33% more nucleotides than the number required to code for the translated amino acid chain.

2.3.4. DNA Sequences Coding for mRNA

In order to understand the way in which the information contained in cellular DNA is used, it is of interest to know which parts of the DNA code for protein and which parts have control functions. Much use has been made of the hybridisation reaction to study this problem. However, there have been several problems to solve. An early one was the preparation of highly pure mRNA and this was partially overcome with the advent of poly (A) containing RNA purification techniques.

A second problem has been repetitive sequence elements present in the DNA which tend to hybridise with related sequences. This gives mismatched hybrid molecules and produces falsely high results (Britten and Kohne, 1968). Kohne (1968) overcame this problem by removing the repetitive fraction of DNA by binding the mismatched repetitive DNA molecules to hydroxylapatite. The unique fraction of DNA which does not readily form hybrids is not retained on hydroxylapatite. Using this technique, it has been shown that approximately 10% of non-repetitive mouse DNA is complementary to total RNA found in brain (Hahn and Laird, 1971; Brown and Church, 1971; Grouse et al., 1972). Experiments with normal and virus transformed mouse cells have shown an increase in unique sequence expression in transformed cells from 8.3 to 15% (Grady and Campbell, 1973). However only 0.9% of non-repetitive Xenopus DNA is represented in transcripts of mature oocytes (Davidson and Hough, 1971).

The interspersion of unique and intermediately repetitive sequence elements of DNA has been shown to occur in mammals, insects,

molluscs and certain lower vertebrates (Davidson et al., 1974). In the genome of the sea urchin Stronglyocentrotus purpuratus (Graham et al., 1974) and Xenopus laevis (Davidson et al., 1973) short repetitive DNA sequence elements averaging 300 nucleotides in length are extensively interspersed among non-repetitive sequences of one to several thousand nucleotides. As much as 70% - 80% of the DNA may be organised in this manner, according to Davidson et al. (1975). Recently unique and repetitive sequences in multiple genes for feather keratin have been examined (Kemp, 1975). Complementary DNA (cDNA) was prepared using the mRNA coding for feather keratin proteins as a template, and using the reverse transcription enzyme of avian myoblastosis virus. Studies on the kinetics of hybridisation and reannealing of cDNA indicate that there are 25 - 35 different keratin mRNA species in the embryonic chick feather, and a total of 100 - 240 keratin genes in the chick genome. Each keratin gene was found to contain both unique and repetitive sequences. Kemp (1975) proposes that the repetitive sequences are the keratin coding sequences and that the unique sequences correspond to untranslated regions.

The advent of cDNA, synthetised on an mRNA template by the reverse transcriptase enzyme, helped to overcome the problem of gaining information on repetition frequencies of mRNA sequences in DNA. Hybridisation studies between HeLa mRNA and cDNA (prepared on a HeLa cell poly (A) containing mRNA template) have been used by Bishop et al. (1974) to show 3 abundance classes of mRNA. These are as follows:

number of sequences	number of copies
36, 250	8
330	440
18	8000

From Bishop's data we can see that there are many copies of a few genes and a few copies of many genes.

The distribution of repetitive and unique sequence transcripts within mRNA molecules has been studied by Dina et al. (1973) who suggest that each molecule of mRNA has a small repetitive sequence tag covalently joined to the main part of the molecule which is transcribed from unique DNA. These studies have been done in developing Xenopus embryos and contradict the results of Klein et al. (1974) who have studied the distribution of repetitive and non-repetitive sequence transcripts in HeLa cell mRNA. According to the experiments of Klein et al. (1974) HeLa cell mRNA does not contain repetitive sequence elements linked to non-repetitive sequences. However a small fraction of the HeLa mRNA does seem to be completely transcribed from repetitive DNA sequences (6%).

3. EVIDENCE FOR A PRECURSOR-PRODUCT RELATIONSHIP BETWEEN HnRNA AND mRNA

It is not yet certain how mRNA is derived from its presumed precursor, HnRNA. Several different approaches have been used to test whether large heterogeneous RNA is a precursor of cytoplasmic RNA or not.

In 1966, Soeiro et al. showed that HnRNA had a base composition that resembled DNA. That is, it had a G+C content of 43 - 47% unlike ribosomal precursor RNA which has a much higher G+C content.

Results consistent with the possibility that virus specific mRNA molecules in transformed cells may be derived through selective cleavage of cell specific sequences from HnRNA which contain both viral and cellular sequences, have been gained by Wall and Darnell (1971) using mouse 3T3 cells transformed with SV40.

The finding of apparently identical nucleotide sequences at the 3'-OH termini of both the mRNA and some HnRNA molecules in mammalian cells has given strong support to the idea that mRNA is derived from the 3'-OH terminal portions of at least some of the HnRNA molecules (Georgiev, 1972). The polyadenylation appears to be a post-transcriptional event important for the effective processing of mRNA and/or its transport to the cytoplasmic ribosomes (Darnell et al., 1972; Adesnik et al., 1972). Kinetic studies show that polyadenylation is a relatively late post-transcriptional event (Perry et al., 1974).

Latorre and Perry (1973) have estimated the relative amounts of non-adenylated and polyadenylated precursor of mRNA in mouse L cells

by use of the actinomycin D and cordycepin. The results of their experiments suggest that the pool of non-adenylated pre-mRNA is approximately twice that of polyadenylated pre-mRNA. Furthermore the amount of Poly (A) lost from the nuclei during a chase experiment is significantly greater than that gained in the polyribosomes (by about 20 fold), suggesting the possibility of an intracellular turnover of poly (A). Part of this discrepancy can be accounted for by the large difference in size between HnRNA and mRNA molecules (approximately 5 - 10 fold). However Pucket et al. (1975) have recently shown the existence of a class of very short half-life mRNA which they use to explain the "lost" poly (A) noticed in the experiments of Latorre and Perry (1973).

Competition hybridisation with DNA has shown that there is a degree of similarity between HnRNA and mRNA molecules (Birnboim et al., 1967; Arion and Georgiev, 1967; Shearer and McCarthy, 1967; Soeiro and Darnell, 1970). The conditions used in most of these experiments permitted only the reiterated fraction of DNA or RNA to hybridise. Thus the results represented only a fraction of the total sequences present, and there is the possibility of mismatching of sequences in the hybrids.

Using conditions which would allow the hybridisation of some of the less repetitive sequences of DNA, Scherrer et al. (1970) concluded that there is a precursor product relationship between large HnRNA molecules and cytoplasmic mRNA. Scherrer's results are likely to be more meaningful because he is looking at some of the more unique sequences of DNA which code for the largest portion of the mRNA (Bishop et al., 1974; Klein et al., 1974). Scherrer et al. (1970) found that HnRNA hybridises

to 5 - 10% of the DNA whereas polyribosomal mRNA and cytoplasmic messenger-like RNA hybridises with only about 0.5 to 1%. He concluded that cytoplasmic mRNA may be homologous to ten times less DNA than the nascent nuclear HnRNA.

Melli and Pemberton (1972) used an unusual technique to study the 10S mRNA from anaemic duck erythrocytes. They used Micrococcus lysodeikticus RNA polymerase to produce "anti mRNA" on a mRNA template. This "anti message", radioactively labelled to high specific activity, was used to test fractions of nuclear RNA for sequence complementarity. Their results suggest that HnRNA does contain mRNA sequences and thus is a precursor to mRNA.

Imaizumi et al. (1973) used the reverse transcriptase enzyme of avian myoblastosis virus to synthesise DNA chains using oligo (dT) primers and duck globin mRNA as template for the reaction. This complementary DNA (cDNA) provides a sequence of over 500 bases complementary to the 3' terminal region of mRNA adjacent to the poly (A) region. In these experiments nuclear RNA molecules of several size classes (> 28S, 20 - 28S, < 20S) appeared to contain the globin mRNA sequence.

Aggregation of HnRNA molecules has been known for some time (Bramwell and Harris, 1967). When Imaizumi et al. (1973) compared the results of sucrose and dimethylsulphoxide/sucrose gradient analysis of nuclear RNA they concluded that there was considerable aggregation. Experiments by Macnaughton et al. (1974) using the same system showed that duck erythroblast HnRNA on sucrose gradients mostly sediments at a rate faster than 50S. After treatment with formamide, a broad peak was found

between 10S and 40S with little RNA sedimenting faster than 50S. By using formamide/sucrose density gradients Macnaughton et al. (1974) were able to isolate a single peak containing RNA that hybridises to globin cDNA with a S value of 14. This sedimentation value corresponds to a molecular weight about 3 times larger than that of globin mRNA and is much smaller than any other estimate for the size of the precursor. It remains to be seen whether this is the primary transcript from the DNA or a processing intermediate. A small precursor for hen ovalbumin mRNA has also been reported (McKnight and Schimke, 1974).

Although small precursors for globin and ovalbumin mRNA have been identified, it is notable that dimethyl sulphoxide/sucrose density gradients of rat ascites cell HnRNA (Holmes and Bonner, 1973), and HeLa cell HnRNA (Derman and Darnell, 1974) show very large molecules of HnRNA do exist and are not the result of aggregation.

Recently, a subfraction of HeLa cell HnRNA molecules, small in size, which shows some properties that would be expected of a mRNA precursor molecule has been isolated (Price et al., 1974). This interesting fraction of HnRNA was prepared by lysing nuclei at low ionic strength and centrifuging gently. This procedure pellets the chromatin and all of the HnRNA. On treating the pellet with 0.4 - 0.7 M ammonium sulphate, approximately 10% of the HnRNA is released from the pellet in the form of a ribonucleoprotein particle. The HnRNA extracted from these ribonucleoprotein particles sediments around 28S. Approximately three times larger than HeLa cell mRNA. This fraction of HnRNA was shown to contain about 40% of the nuclear poly (A) sequences and, after one hour chase with 3-deoxyadenosine (cordycepin) the poly (A) in this fraction

disappeared completely. However more than half of the poly (A) in the rest of the HnRNA still remained in the nucleus. Thus it seems likely that only a small fraction of HeLa cell HnRNA molecules code poly (A) containing mRNA.

4. CONTROL OF TRANSCRIPTION

We have already mentioned the transcription process whereby RNA is formed from a DNA template; looked at the different types of high molecular weight RNA produced by this process; and have reviewed evidence for the post-transcriptional processing of some HnRNA into mRNA. In this section we shall mention the possible roles played by the chromosomal structure in influencing which parts of the information, contained in the DNA, is transcribed. Furthermore mention will be made of several models of post-transcriptional modification and control.

4.1. Models of Chromosome Structure with Relation to Gene Activity

As well as the highly organised helical structure of DNA, it seems that the packaging of DNA into the nuclei of mammalian cells involves complex folding of the molecule. The basic unit of this final level of organisation is the chromosome.

Chromosomes probably contain one DNA molecule (Hubbard, 1973) in association with two classes of protein. These are histone and non-histone protein. Histone proteins are basic proteins and are found bound to the DNA of most eukaryotic chromosomes. The histone proteins can be subdivided into five major classes according to their amino acid composition (Rasmussen et al., 1962; Johns and Butler, 1962). One important feature of histone protein structure is the packing of nearly all the basic amino acid residues into one half of the molecule. Most likely it is this half of the molecule which interacts with the nucleic acid.

Those remaining proteins associated with chromosomal material

are known as non-histone proteins. Unlike histone proteins they form a variable proportion of the mass of the chromosomes. Purified from calf thymus chromosomes they represent 3% of the histone mass (Goodwin and Johns, 1972). Nevertheless they appear to be much more heterogeneous in composition than the histone proteins (Elgin et al., 1971; Teng et al., 1971), and many of them probably have enzymic functions (Elgin et al., 1971).

Microscopy studies of certain chromosomes, like the giant polytene chromosomes of Diptera have revealed a banding pattern. Several workers have proposed models of chromosomal structure based on this observation. Crick (1971) has suggested that the chromosomal DNA is divided into two classes; a globular fraction which contains unpaired DNA regions for control, and a much smaller fraction consisting of fibrous DNA which codes for protein. Within the globular regions, Crick suggests that there are hairpin loops of DNA, which are split into single strands at their ends due to an untwisting effect which is generated by the stem of the hairpin. Regulatory proteins could bind to these regions and provide initiation points for RNA transcription. Transcription of structural genes would then proceed into the interband regions (of fibrous DNA) of the chromosome.

Paul (1972) has proposed a different model of transcriptional control based on the same microscopy studies. In Paul's model, the binding site for RNA polymerase is near the band-interband junction, and transcription proceeds into the band region from the initiation site. The binding of a destabilising molecule, possibly a non-histone protein is postulated to cause a local relaxation in supercoiling of the DNA allowing the approach of RNA polymerase to the initiation site. Transcription of

RNA could cause further unwinding of the adjacent compact nucleohistone region by competing with the DNA for some of the positive charge on the histones.

Paul's model is attractive as he describes how large transcripts could arise from gene reduplication, the non-transcribed spacer RNA in the HnRNA molecule corresponding to "ancestral genes". Such transcripts would contain structural genes separated by spacer regions similar to ribosomal precursor RNA (Miller and Beatty, 1969). There is some evidence that histone mRNA genes may be similarly clustered and have spacer regions (Kedes and Birnstiel, 1971).

4.2. Models for Gene Control and the Transcriptional Unit

There are several models to explain transcriptional and post-transcriptional control in eukaryotes. However the first model for gene control was the model of Jacob and Monod (1961).

Jacob and Monod's model for gene control in prokaryotes was based on the "operon" - a length of DNA containing the information for one or more mRNA molecules, plus the adjacent DNA which contained control sequences. When a protein repressor molecule (synthesised from a regulator gene) is bound to the operator, RNA polymerase is prevented from transcribing the structural genes. Transcription can be induced by the binding of an activator molecule to the repressor protein. This prevents the binding of the repressor molecule to the operator region of the operon and thus allows transcription.

Experiments to elucidate control mechanisms in eukaryotic systems are complicated by several factors. One is the size of the

eukaryotic genome which is 1,000 times larger than the prokaryotic genome. Another is the fact that although genes coding for enzymes on the same biosynthetic pathway form one operon in bacteria, they are scattered throughout the genome in eukaryotes (Kuff and Roberts, 1967). Furthermore the feasibility of genetic manipulation is limited compared to the prokaryotic genome. Despite these difficulties, several models for eukaryotic gene control do exist. These are based mainly on studies of HnRNA and will be briefly described.

The "Cascade Model" (Scherrer and Marcaud, 1968) is so called because it involved transcriptional regulation, post-transcriptional regulation and translational regulation. In this model, the heterogeneous transcriptional unit consists of polycistronic mRNA sequences and non-functional sequences. Its main features are post-transcriptional modification (e.g. polyadenylation) in the processing mechanism and selective retention of particular segments of the transcriptional unit.

The "Gene Battery Model" of Britten and Davidson (1969) was based on several pieces of experimental evidence. These are as follows, 1) differentiation in higher cells can be mediated by external signals such as hormones, 2) a given state of differentiation tends to require the integrated activation of many non-contiguous genes, 3) there are many genomic sequences which are transcribed in the nuclei of higher cell types but appear to be absent from the cytoplasmic RNAs, 4) the eukaryotic genome is much larger than the prokaryotic genome and contains a large fraction of repetitive sequences which are scattered throughout the genome, 5) these repetitive sequences are transcribed in differentiated cells according to cell type - specific patterns.

A "gene battery" is defined as a set of producer genes (similar to the prokaryotic structural genes) which is activated when a particular sensor gene activates its set of integrator genes. The integrator genes would synthesise RNA molecules which could bind to receptor genes thus co-ordinating the activity of a number (or battery) of producer genes.

Of special interest in this model is the possible involvement of RNA as well as protein for the activation of receptor genes.

The "Eukaryotic Operon Model" is the name commonly used to describe the model of Georgiev (1969). Based on the prokaryotic model of Jacob and Monod (1961), the transcriptional unit consists of two parts, 1. an acceptor, non-informative zone adjacent to the promotor region, and 2. a structural informative zone at the end of the transcriptional unit which may consist of more than one structural gene. The acceptor region would be able to bind many regulatory proteins which would be specific for one or several operon acceptor sites. By this mechanism, the switch on and off of large numbers of genes noticed in cell differentiation could be explained.

Acceptor proteins would be of two types, either repressor or derepressor. The repressor protein would bind to DNA inhibiting transcription and the derepressor protein would remove some inhibitory protein, say histone, and thus allow transcription to start.

Transcription of DNA via the "eukaryotic operon model" could give rise to RNA molecules which include transcripts of the acceptor genes as well as the structural genes. This could in part explain the existence of very large RNA molecules, like HnRNA. The non-informative

acceptor gene transcripts would be attached to the 5' end of the structural RNA and after transcription would be degraded by nuclease action to leave the structural 3' end of the HnRNA molecule. This agrees with the experimental evidence which suggests that the 3' end of the HnRNA molecule corresponds to mRNA.

This theory has been checked against experimental data and found in good agreement (Georgiev et al., 1972).

A model of transcriptional and post-transcriptional controls which include detailed mechanisms of HnRNA metabolism in relation to mRNA synthesis and polyadenylation has been proposed by Darnell et al. (1973). It suggests that the regulation of mRNA production in prokaryotes and eukaryotes could be similar, even though the steps in the manufacture of mRNA in mammalian cells are different from those in bacteria. Whereas most models have regulation at or before the initiation site, two of the four models which Darnell et al. propose have both initiation site and termination site regulation.

5. RIBONUCLEOPROTEIN PARTICLES

High molecular weight RNA in eukaryotes is found in the form of ribonucleoprotein particles (RNP). This association with protein could be a reflection of the complex control mechanisms used to regulate transcription and post-transcriptional processing of RNA in eukaryotes.

Ribosomal RNA-protein particles (ribosomes) have been well characterised for their role in the translational (protein synthesising) process. However, three other types of RNP, distinct from ribosomes, are thought to exist in eukaryotic cells. These are, 1. nuclear HnRNA containing particles called informofers (Samarina et al., 1965), 2. messenger RNP released from polysomes after treatment with EDTA (Henshaw, 1968; Perry and Kelly, 1968) and 3. free cytoplasmic messenger RNP or informosomes (Spirin et al., 1964).

Both mRNA and HnRNA have been isolated from many cell types in the form of RNP. However it has been shown that RNA can form artificial complexes with cellular proteins in some instances (Girard and Baltimore, 1966; Baltimore and Huang, 1970). Pederson (1974) has examined proteins associated with HeLa cell HnRNA. Using RNP particles ranging in size from 40 - 250S he prepared a complex mixture of proteins with sizes between 39,000 to 180,000 daltons. The isoelectric point of these proteins ranged between 4.9 and 8.3 as measured by analytical isoelectric focusing. Nevertheless they were distinguishable from proteins of other cell fractions, including chromatin, when examined by polyacrylamide gel electrophoresis. When the possible formation of

non-specific complexes between HnRNA and nuclear proteins was tested directly by mixing experiments the outcome was negative. In these experiments, radioactive deproteinised HnRNA was added to nuclei just prior to sonication (Pederson, 1974). This work agrees with similar experiments carried out on sea urchin embryos (Wilt et al., 1973) and slime mould (Firtel and Pederson, 1975). Furthermore it suggests that the RNA binding proteins which have been found in the cytoplasm (Girrard and Baltimore, 1966; Schweiger and Hennig, 1971; Baltimore and Huang, 1970) are absent from the nucleus.

Nuclear acidic proteins are popularly thought to be positive effectors in the control of transcription. Their synthesis has been observed to increase during experimental gene activation (Stein and Baserga, 1970; Rovera and Baserga, 1971; Rovera et al., 1972; Rovera and Baserga, 1973). Pederson (1974) has examined this hypothesis by analysing nuclear acidic proteins from chromatin and HnRNA containing RNP. When he stimulated the synthesis of HnRNA in rat liver by administration of hydrocortisone, there was a parallel increase in the labelling of acidic protein particles. However Pederson did not find any detectable effect on the labelling of chromatin acidic proteins or histones. Thus he argues that the increase in nuclear acidic proteins may be the effect of gene activation rather than the cause.

Evidence that the protein component of nuclear HnRNA containing particles and messenger RNP from dissociated polysomes of rat liver are different has been found by immunological experiments (Ludanidin et al., 1972). That the messenger RNP from L cells was distinct from ribosomes was shown by Perry and Kelley (1968) in their analysis of RNP

released from ribosomes by EDTA. However, messenger RNP and mRNA from rabbit reticulocytes have been compared for protein synthesising activity (Ernst and Arnstein, 1975) and no difference found. Thus they concluded that proteins associated with polysomal mRNA do not alter the specificity of translation of α or β globin mRNA or the requirement for initiation factors.

Studies on both nuclear and cytoplasmic RNP have revealed the presence of poly (A) binding proteins. In the cellular slime mould Hn-RNP particles are apparently only 55S in size. Firtel and Pederson (1975) have shown that most of the proteins associated with these particles are lost when RNP particles are washed with 0.35 M NaCl. However a single protein is left (73,000 daltons) which is specifically bound to the poly (A) region of the HnRNA.

Studies of proteins bound to mRNA associated with polysomes in mouse L cells and rat hepatocytes have shown the presence of two polypeptides. The smaller polypeptide is 52,000 daltons. The larger is 78,000 daltons in size and is found to be tightly associated with the poly (A) region of mRNA (Blobel, 1973). Similar results have been obtained for globin messenger RNP particles (Blobel, 1972). Blanchard et al. (1974) have isolated a tetrameric poly (A) binding protein from HeLa cells. This protein formed from four 38,000 molecular weight subunits, does not appear to be the same protein as that studied by Blobel (1972, 1973).

Proteins which may regulate the release of mRNA from nuclei have been reported (Schum, 1973). However it is not known whether those proteins are related to the proteins found in association with HnRNA or mRNA.

6. AIMS OF THE PRESENT WORK

The above introduction has attempted to introduce the field of high molecular weight RNA; how it is transcribed, its structural features, the possible role of HnRNA as a precursor to mRNA, and models of control at transcriptional and post-transcriptional level. This work aims to shed some further light on these problems mainly by the use of nucleotide sequencing techniques. Experiments were chiefly concerned with the possible relationship of some of the HnRNA to mRNA.

MATERIALS and METHODS

1. MATERIALS

All chemicals were, wherever possible, AnalaR reagents supplied by B.D.H. Chemicals Ltd., Poole, Dorset, except for the following:

Triton X-114 (Scintillation grade)	Koch-Light Laboratories Ltd., Colnbrook, England.
Tween 40	"
Toluene (AR grade)	"
Methylene Bisacrylamide, Acrylamide	"
2,5 diphenyloxazole (PPO) (Scintillation grade)	"
Poly (A)	P-L Chemicals Inc., Wisconsin, U.S.A.
Calf Serum	Bio-Cult Laboratories Ltd., Paisley, Scotland.
Amino Acids	"
Vitamins	"
Penicillin	Glaxo Pharmaceuticals, London.
Streptomycin	"
Trypsin	Difco Laboratories, Michigan, U.S.A.
p-Bis(o-methyl-styryl)benzene (Bis-MSB)(Scintillation grade)	Kodak Ltd.
Kodirex KD 54T (35 x 43 cm) X-ray film	"
DX-80 developer	"
FX-40 X-ray liquid fixer	"
Sephadex G25 (medium)	Pharmacia, Upsala, Sweden.

AG 501-X8 analytical grade mixed bed ion exchange resin	Bio Rad Laboratories, Richmond, California.
Hydroxylapatite (Biogel HTP) - DNA grade	"
Nitrocellulose Filters, 2.5 cm diameter, 0.45 μ pore size	Millipore (U.K.) Ltd., Middlesex, England.
Oligo (dT) Cellulose	Collaborations Research Inc., 1365 Main Street, Waltham, Massachusetts, U.S.A.
Cellulose Acetate Electrophoresis strips (25 x 95 cm)	Oxoid Ltd., London.
Pre-coated DEAE cellulose sheets - 40 x 20 cm,	Machery-Nagel and Co., Camlab, Cambridge, CB4 1TH.
Polygram cel 300 DEAE	"
Whatman DE81 paper (46cm x 50 m)	H. Reeve-Angel & Co. Ltd., London.
Whatman No. 52 and 3MM paper	"
2.5 cm paper discs	"
Whatman G/FC 2.5 cm glass fiber discs	"
Deoxyribonuclease (electrophoretically purified free of RNase) DN-EP grade	Sigma Chemical Co. Ltd., London.
Actinomycin D (200 μg/vial)	Calbiochem Ltd., Hereford, England.
T_1 Ribonuclease (crystalline) (made by Sankyo Co. Ltd., Tokyo, Japan)	"
Pancreatic Ribonuclease (chromatographically homogeneous)	"

Bacterial Alkaline Phosphatase (electrophoretically purified)	Worthington Biochemical Corporation via Cambrian Chemicals Ltd., London.
Snake Venom Phosphodiesterase	"
Radiochemicals:	
^{32}P-orthophosphate (^{32}Pi; 10 mC/ml) carrier free (supplied in a solution of dilute HCl)	Radiochemical Centre, Amersham.
L-[methyl-^{14}C]methionine (58 C/mol) (supplied as a solid)	"
^{3}H Poly (A) (11.1 µg/µC)	Miles Laboratories Inc., Stoke Poges, England.

2. STANDARD SOLUTIONS

2.1. Scintillation Spectrometry Solutions

A) For most experiments a toluene based scintillation fluid was used. This consisted of 0.5 g of 2,5 diphenyloxazole (PPO) per litre of toluene.

B) Triton/Toluene scintillation fluid consisted of 5 g of PPO plus 0.5 g of p-Bis (o-methyl-styryl) benzene (Bis-MSB) in 350 ml of triton X-114 and 650 ml of toluene.

2.2. Fingerprinting and Sequencing Solutions

A) Marker dye for all electrophoresis was 1% xylene cyanol F.F. (blue); 2% orange G (yellow); 1% acid fuschin (pink).

B) Buffer for the 1st Dimension electrophoresis on Cellulose Acetate at pH 3.5 in 7M urea was prepared by dissolving 210.2 g of urea and 25 ml of glacial acetic acid in distilled water and making up to 500 ml after buffering to pH 3.5 by the addition of a small quantity of Pyridine.

C) 2nd Dimension electrophoresis on DEAE Paper, 7% Formic acid was used for this purpose.

D) Homomix B.

10 g of yeast RNA were dissolved in 200 ml of 7M urea and the pH adjusted to 7.5 with 10N KOH. The mixture was then dialysed against 7M urea for 2-3 hr at $4^{\circ}C$.

E) Homomix C

10 g of yeast RNA were dissolved in 100 ml of 1M KOH and hydrolysed for 15 min at room temperature. The solution was then neutralised to pH 7.5 with concentrated HCl and dialysed against distilled water for 2-4 hr. 84 g of urea were then added and the final volume made up to 200 ml with distilled water.

F) 30% Triethylamine Carbonate Solution (v/v)

30 ml of triethylamine were mixed with 70 ml of distilled water. CO_2 gas was bubbled through the two phase mixture till it became a homogeneous solution.

G) T_1 RNase Buffer

0.01M tris-HCl, 0.001M EDTA, pH 7.4 was prepared as a X10 stock solution and stored at -10°C.

H) Alkaline Phosphatase Buffer

0.02M tris-HCl, pH 8.0 was prepared as a X10 stock solution and stored at -10°C.

I) T_1 RNase (EC 2.7.7.26)

A stock solution of 2 mg/ml in distilled water was kept at -10°C.

J) Alkaline Phosphatase

The bacterial alkaline phosphatase as supplied was used as stock.

K) Pancreatic RNase (EC 2.7.7.16)

A stock solution of 2 mg/ml in distilled water was kept at -10°C.

TABLE A

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

MEM amino acids	mg/litre	MEM vitamins	mg/litre
L-arginine	126.4	D-calcium pantothenate	2.0
L-cystine	24.0	choline chloride	2.0
L-glutamine	292.0	folic acid	2.0
L-histidine HCl	38.3	i-inositol	4.0
L-isoleucine	52.5	nicotinamide	2.0
L-leucine	52.5	pyridoxal HCl	2.0
L-lysine	73.1	riboflavin	0.2
L-methionine	14.9	thiamine HCl	2.0
L-phenylalanine	33.0		
L-threonine	47.6		
L-tryptophan	10.2		
L-tyrosine	36.2		
L-valine	46.9		

Inorganic salts and other components

	mg/litre
$CaCl_2 \cdot 6H_2O$	393.0
KCl	400.0
$MgSO_4 \cdot 7H_2O$	200.0
NaCl	6800.0
$NaH_2PO_4 \cdot 2H_2O$	140.0
glucose	4500.0
$NaHCO_3$	2240.0
phenol red	15.3
streptomycin	100.0
penicillin	100,000 units/litre

2.3. Cell Culture Solutions

A) Buffered Saline Solution (BSS)

BSS consisted of 116 mM NaCl, 5.4 mM KCl, 1 mM $MgSO_4$, 1 mM NaH_2PO_4, 1.8 mM $CaCl_2$ and 0.002% (w/v) phenol red. This mixture was adjusted to pH 7.0 by the addition of 8.4% (w/v) $NaHCO_3$.

B) Minimal Essential Medium (MEM)

As used in the Dept. of Biochemistry, University of Glasgow, this consists of the amino acids, vitamins and other components shown in Table A.

C) Versene

0.6 M Na_2 EDTA dissolved in 0.17 M NaCl, 3.4 mM KCl, 10 mM Na_2HPO_4 and 2.4 M KH_2PO_4, pH 7.4 - 0.002% (w/v) phenol red was added to this solution.

2.4. Cell Fractionation Solutions

A) Reticulocyte Standard Buffer (RSB)

10 mM NaCl, 3 mM $MgCl_2$, 10 mM tris-HCl, pH 7.4.

B) Detergent Mixture (Magik)

6.6% v/v Tween 80 and 3.3% w/v Sodium Deoxycholate were dissolved in sterile water and stored at -10°C.

C) High Salt Buffer (HSB)

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

D) DNase (RNase free)

1 mg/ml in 1 mM $MgCl_2$. This solution was stored in 0.1 ml aliquots in small plastic tubes at -10°C.

E) Phenol Solution

0.55 g of hydroxyquinoline and 130 ml of distilled water were added to a 500 g bottle of Phenol.

2.5. RNA Fractionation Solutions

A) Lithium - EDTA - Tris - SDS Buffer (LETS)

0.1 M LiCl, 0.01 M EDTA, 0.1 M tris-HCl, 0.2% SDS, pH 7.4.

B) High Salt Digestion Buffer

0.3 M NaCl, 0.01 M EDTA and 0.01 M Tris-HCl, pH 7.4.

C) High Salt Digestion Enzyme Mixture

50 mg/ml pancreatic RNase, 50 mg/ml T_1 RNase dissolved in High Salt Digestion Buffer.

D) Millipore Binding Buffer

0.5 M KCl, 1 mM $MgCl_2$, 10 mM Tris, pH 7.6.

E) Millipore Eluting Buffer

0.5% SDS, 0.1 M Tris, pH 9.0.

2.6. Sterilisation Procedures

Buffered sucrose solutions, used to prepare density gradients, were autoclaved at 5 P.S.I. for 50 min in 100 ml batches.

All other solutions were autoclaved for 20 min at 15 P.S.I.

Glassware was either flamed over a bunsen burner, heated to 180-200°C in an oven, or rinsed with a solution saturated in diethyl-pyrocarbonate before use.

These procedures were used to destroy ribonuclease activity in solutions and on glassware.

3. METHODS

3.1. Cell Culture System

A HeLa cell line (Gey et al., 1952) was used for all experiments. Cells were cultured as monolayers in rotating 80 oz winchester bottles according to the technique of House and Wildy (1965). They were grown on Eagle's Minimal Essential Medium (modified to contain double concentrations of all vitamins) to which calf serum 10% (v/v) was added.

The stock cultures were routinely examined for contamination by fungi and yeasts using Sabouraud's medium. Bacterial contamination was checked with blood agar plates and contamination by pleuropneumonia-like organisms (PPLO) was monitored by use of PPLO agar plates.

Generally cells were seeded at either 20×10^6 or 25×10^6 cells per bottle and grown for two days before labelling. Occasionally cells were grown for one day before labelling. The difference in growth curves shown in figure A indicates that HeLa cells did not grow reproducably throughout the course of this work. For this reason bottles of cells were always examined before use to ensure that they were not confluent. This was performed using an inverted microscope.

3.1.1. Labelling of Cells

A. With $^{32}PO_4$

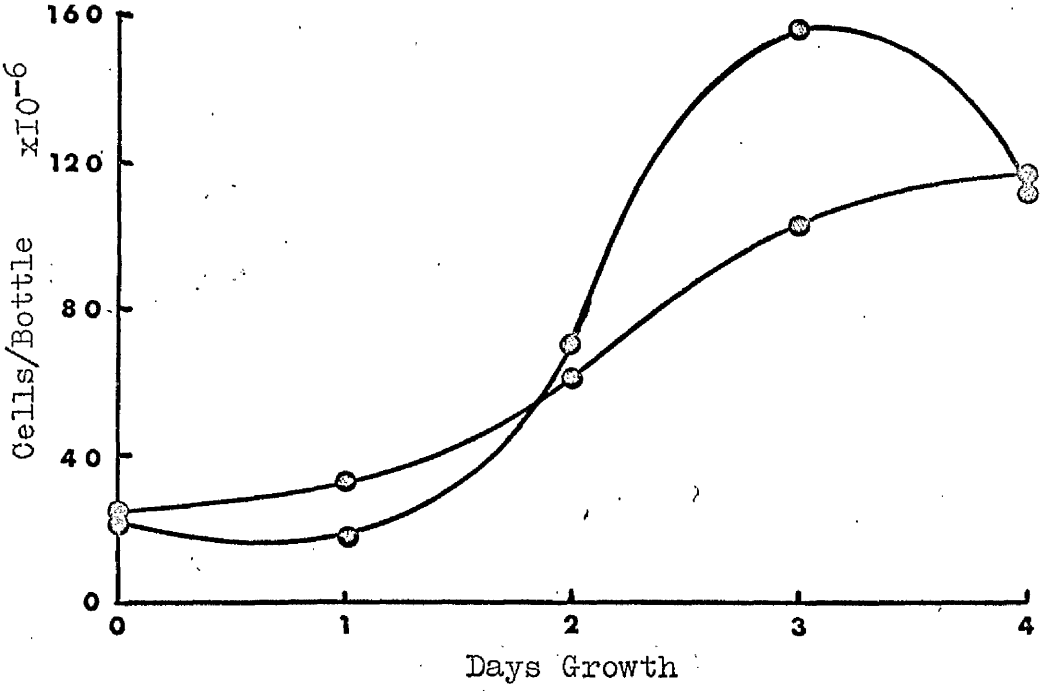
Cells in 80 oz bottles were labelled with $^{32}PO_4$ when they were growing exponentially. The normal medium was poured off and the bottles were washed twice with 25 ml of low phosphate medium (1/10 normal phosphate concentration). 50 ml of low phosphate medium and $^{32}PO_4$ (usually 10 mCi,

FIGURE A

Growth of HeLa Cells in 80 oz Bottles

22×10^6 HeLa cells were seeded into 80 oz bottles containing 180 ml of medium at 37°C. Cells were grown to confluence. At one day intervals during this growth duplicate bottles of cells were harvested by the trypsin-versene technique and the number of cells measured using a Coulter counter.

The experiment was repeated after a period of approximately 1 year.


160
120
80
40
0
Cells/Bottle xI0-6
0
1
2
3
4
Days Growth

carrier free) were then added to the bottles and the cells incubated at 37°C for the requisite time.

B. With L-[methyl-^{14}C] methionine

Basically the technique of Maden, Salim and Summers (1972) was used. Eagle's medium without methionine (methionine-minus medium) was used to prepare the labelling medium which contained 10^{-2} M sodium formate and 2×10^{-5} M adenosine and guanosine. These additions were made to prevent labelling of purine rings via "C_1" metabolism. 125 μC of L-[methyl-^{14}C] methionine (60 C/mol) was added to give a final methionine concentration of 5×10^{-5}M (½ of the normal methionine concentration).

Cells growing in 80 oz bottles were labelled with L-[methyl-^{14}C] methionine when they were growing exponentially. To do this the normal medium was poured off and the cells washed twice with 25 ml of methionine-minus medium. The cells were then left in 50 ml of labelling medium for the requisite time.

3.1.2. Harvesting of Cells

A. Trypsin-Versene Technique

Trypsin-versene (0.05% w/v) solution was used to harvest cells when polysomes were not required. After the cells had been labelled with radioactivity, the medium was poured off. The monolayer of cells were then washed twice with approximately 25 ml of BSS before the addition of 20 ml of trypsin-versene solution. After 30 seconds this solution was poured off and the 80 oz bottles were rotated at 37°C until the cells appeared to be coming off the glass (approximately 1-2 min). At this point the trypsin-versene solution was neutralised by the addition of

20 ml of BSS containing a small amount of serum. The cells were then shaken off the glass and poured out of the 80 oz bottle into a 50 ml centrifuge tube. The bottle was washed once with 10 ml of BSS and the washings added to the centrifuge tube.

Cells were pelleted by centrifuging for 2 min at 450 g.

B. Scraping Technique

A good yield of polysomes could not be obtained using the trypsin-versene technique; presumably because EDTA, present in the trypsin-versene solution, disaggregated them. Thus scraping of cells from the glass was the technique of choice when polysomes were to be isolated.

After the cells had been labelled radioactively, the medium was decanted. Cells were washed with ice-cold BSS (2 x 50 ml) before 20 ml of ice-cold BSS were added to the 80 oz bottle and the cells scraped from the glass using a rubber wiper mounted on a stainless steel frame.

The cell suspension was poured into a 50 ml centrifuge tube and the 80 oz bottle washed with 10 ml of BSS. These washings were added to the centrifuge tube and the cells pelleted by centrifugation at 450 g for 2 min.

3.2. Preparation of Subcellular Fractions

The techniques were basically those of Penman (1969).

3.2.1. Nuclei and Cytoplasm

All operations were carried out at 4°C. The cell pellets

obtained from harvesting the 80 oz bottles were washed once with 4 ml BSS and once with 4 ml RSB, pelleting the cells each time by centrifuging at 450 g for 2 min. The cells were then resuspended in 4 ml of RSB and left to swell. After 5 min the cells were broken by 15-20 strokes in a stainless steel ball homogeniser (Dounce Homogeniser) with a clearance of 0.003 inches diameter.

The homogenate was centrifuged at 800 g for 2 min and the supernatant called cytoplasm. The pellet was resuspended in 4 ml of RSB and 0.6 ml of 'magik' detergent mixture added. After vortex mixing for 15 seconds the mixture was centrifuged at 800 g for 2 min. This procedure removes the outer nuclear membrane, complete with cytoplasmic tags, and causes the clean nuclei to clump. The supernatant was discarded and the pellet called nuclei.

3.2.2. Nucleoli and Nucleoplasm

Nuclei were suspended in 4 ml of HSB and incubated at 37°C with 200 µg of DNase for 2-5 min (until the solution had lost its viscosity). 1.2 ml of 0.1 M EDTA, pH 7 were then added to the solution.

This solution was then layered onto a 15%-30% sucrose/HSB gradient and centrifuged for 20 min at 22,000 rpm in a SW27 rotor of a Spinco ultracentrifuge at 20°C. The gradient was then decanted and called nucleoplasm and the pellet called nucleoli (Penman, 1969). Generally nucleoplasm was precipitated with two volumes of ethanol and dissolved in a small volume of LETS buffer before phenol extraction of the RNA.

3.2.3. Polysomes

These could only be prepared from the cytoplasm of cells that had been harvested by scraping.

1 ml samples of cytoplasm were layered onto 15-30% w/w sucrose/RSB gradients and spun in a Spinco SW27 rotor at 25,000 rev/min for 90 min. The gradients were then pumped through the flow cell of a Gilford recording spectrophotometer and approximately 1.4 ml fractions were collected. The fractions corresponding to polysomes as shown in figure B were pooled and sedimented in a Spinco Ti 50 rotor at 50,000 rev/min for 150 min. All steps were carried out at 4°C.

3.3. RNA Isolation

3.3.1. Hot Phenol Extraction

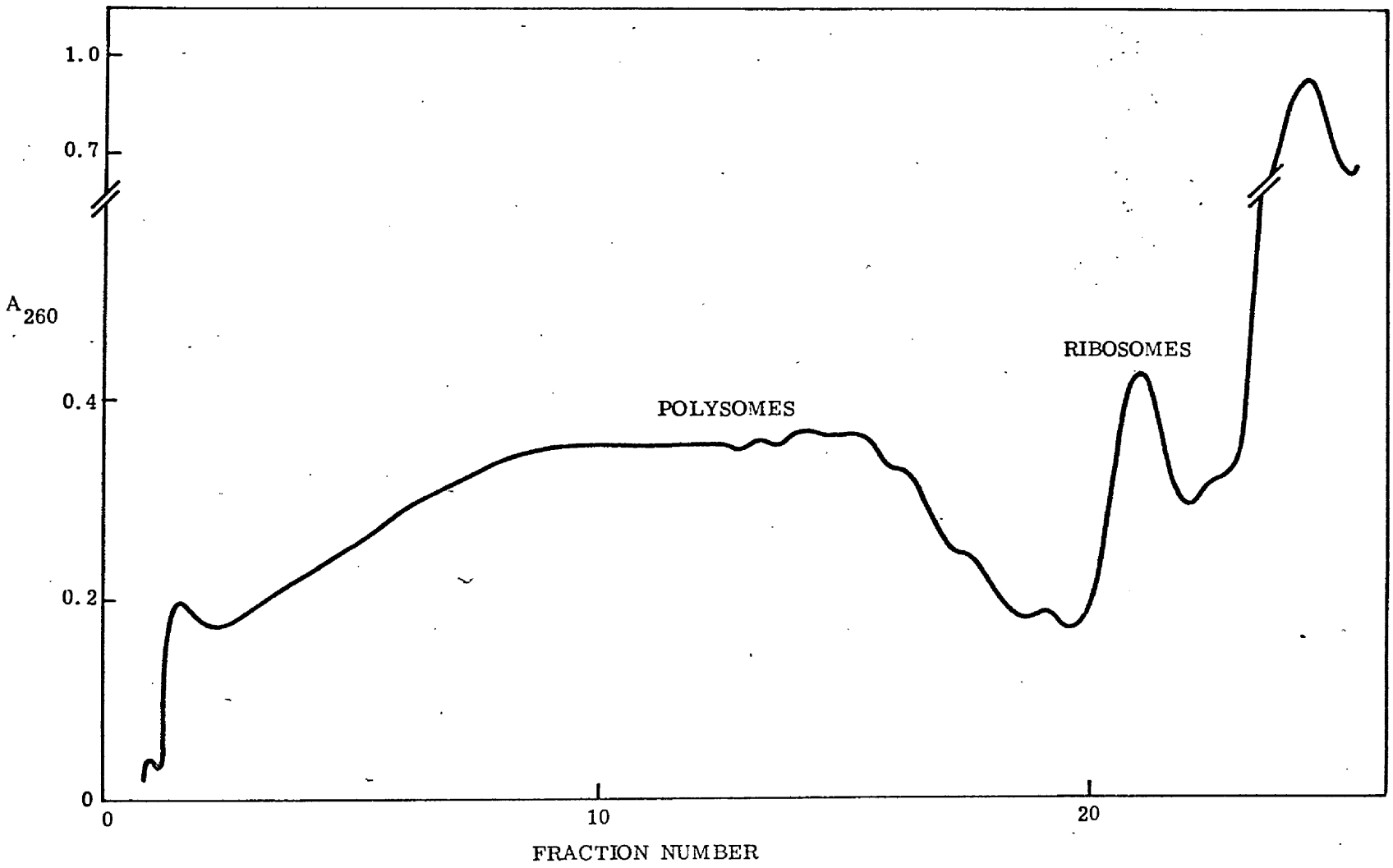
Basically the hot phenol - SDS technique of Scherrer and Darnell (1962) was used. Phosphodiester bonds are relatively stable to heat, but hydrolysis of RNA will inevitably occur after prolonged heating (Gordon et al., 1963). The combined effects of heat and of a strong detergent immediately dissolve the nucleoprotein complexes of chromatin and ribosomes.

Pelleted material was dissolved in a 4 ml LETS buffer, and an equal volume of phenol was added. This was mixed on a vortex mixer and left in a 66°C water-bath for 5-10 min with occasional vortexing. Centrifugation at 10,000 RPM on a MSE18 centrifuge separated the two phases after which the phenol (bottom) layer was removed and fresh phenol added. After vortexing and incubating at 66°C for a further 5-10 min with occasional mixing, the mixture was centrifuged as before. This time the aqueous (top) layer was pipetted off and 4 ml of fresh LETS buffer added to the phenol (bottom) phase. The vortex-incubate-centrifuge

FIGURE B

Sucrose Gradient Analysis of Cytoplasm

Cytoplasm was sedimented through 15 - 30% sucrose/RSB gradients as described in the text (section 3.2.3). The gradient was then pumped, from the bottom of the centrifuge tube through the flow cell of a Gilford recording spectrophotometer. Approximately 1.4 ml fractions were collected. Generally fractions 6 to 19 were pooled and called polysomes.


1.0
0.7
0.4
0.2
0
A_{260}
POLYSOMES
RIBOSOMES
0
10
20
FRACTION NUMBER

procedure was then performed once more and the aqueous (upper) layer added to the first aqueous phase. The combined aqueous layers were then ethanol precipitated. Occasionally when there was a large amount of material at the interphase after removal of the first phenol phase, an equal volume of chloroform was added to the second phenol phase after the incubation step. This helped to separate the aqueous and phenol layer and reduced the interfacial material to a thin band.

3.3.2. Cold Phenol Extraction

Cold phenol extraction was mainly used to prepare 28S rRNA from cytoplasm. Cytoplasm and an equal volume of phenol solution were mixed on a vortex mixer. The mixture was left at 20°C (room temperature) for 10 min with occasional vortexing before centrifugation at 10,000 RPM on an MSE18 centrifuge. The aqueous layer was removed and the procedure repeated with 4 ml of LETS buffer. The resulting aqueous layers were pooled and precipitated with 2 volumes of absolute ethanol.

3.4. RNA Fractionation

3.4.1. Oligo (dT) Cellulose Chromatography

Oligo deoxynucleotides bound to celluloses have properties which make them particularly suitable for the isolation of RNA molecules containing homopolymeric sequences. These properties include the covalent nature of the binding, and the resistance of the polydeoxynucleotide to RNase or alkaline hydrolysis. Oligo (dT) cellulose, prepared by the technique of Gilham (1968) can be purchased commercially.

1 g of oligo (dT) cellulose suspended in high salt buffer (0.5 M NaCl, 0.01 M Tris-HCl, pH 7.5) was packed into a 1.5 cm diameter

column. After washing the column with 10 ml of this buffer, the RNA sample was applied. The column was run slowly (approximately 1.0 ml min^{-1}) in order to ensure binding of all of the poly (A) containing RNA. The column was then washed with high salt buffer until no more radioactivity was washed off and the eluting buffer changed to 0.1 M NaCl, 0.01 M Tris-HCl, pH 7.5. After washing the column with this buffer until no more radioactivity was recovered, the eluting buffer was changed to 0.01 M Tris-HCl, pH 7.5 with no salt present. This buffer released the poly (A) containing RNA from the column.

All buffers contained 0.1% SDS and radioactivity was measured directly by use of a recording Geiger-Muller counter, the probe of which was placed in contact with the delivery tube between the column and the fraction collector. Approximately 1 ml fractions were collected and small aliquots further counted in triton/toluene scintillation fluid.

3.4.2. Millipore Filtration

In addition to its capacity to form complementary base-paired structures with materials such as poly (U) sepharose and oligo (dT) cellulose, the poly (A) segment contained in certain RNAs is capable of binding to cellulose nitrate membrane filters (Millipore filters) at high ionic strength (Lee et al., 1971).

The RNA solution was diluted at least 10 fold with binding buffer (0.5 M KCl, 1 mM $MgCl_2$ and 10 mM Tris-HCl, pH 7.6). This solution was then passed through a Millipore filter that had been pre-soaked for 10 min in binding buffer. The rate of filtration was approximately 1 ml min^{-1} and two Millipore filters were used to ensure a slow flow rate and complete absorption of all poly (A) containing RNA. The second filter

generally had about 5% of the counts present on the first filter and was usually discarded. The binding procedure was always carried out at 4°C.

The elution of the absorbed material was by the use of SDS and high pH (Lee et al., 1971; Brawerman et al., 1972). The Millipore filters containing bound RNA were placed in glass scintillation vials and 1 ml of eluting buffer added (0.5% SDS in 0.1 M Tris, pH 9.0). After approximately 1 hour with occasional shaking at 4°C the eluting buffer was pipetted off and precipitated with 2 vol of absolute ethanol. This procedure was repeated with a further 1 ml of eluting buffer which was then added to the first ml with a further 2 ml of absolute ethanol. During the first elution a precipitate of potassium dodecyl sulphate crystals appears because of KCl present on the filter. However this does not interfere with the elution (Brawerman et al., 1972).

It should be noted that millipore filters are reported to bind only poly (A) regions greater than 50-60 nucleotides in length (Gorski et al., 1974).

3.4.3. Formamide-Polyacrylamide Gel Electrophoresis

The electrophoretic mobility of single-stranded RNA in polyacrylamide gels has been reported to vary in inverse relation to molecular size (Bishop et al., 1967; Peacock and Dingman, 1968; Loening, 1969). The electrophoretic mobility of RNA in polyacrylamide gel depends on its Stoke's radius and thus on its conformation. Fisher and Dingman (1971) reported a change in apparent molecular weight from 2×10^6 to 1×10^6, due to changes in conformation, on varying the

temperature of polyacrylamide gel electrophoresis from 9°C to 40°C. In fact, by determining the way in which the electrophoretic mobility of a nucleic acid varies when analysed across a range of acrylamide gel concentrations, Harley et al. (1973) showed that it is possible to decide whether nucleic acids are single stranded or double stranded.

An approach to the elimination of conformational effects is the disruption of all base-pairing in the experimental conditions. Stanov et al. (1972) have developed a completely non-aqueous system using formamide as the solvent. This has a very high dielectric constant and thus causes complete loss of polynucleotide structure (Helmkamp and Ts'O, 1961; Ts'O et al., 1962; Helmkamp et al., 1962). A 4% gel in formamide is mechanically similar to a 2.5% gel in aqueous media but the relative mobilities of RNA species are changed. Thus a 4% gel is satisfactory for very high molecular weight RNA (Stanov et al., 1972). Basically, the method of Stanov et al. (1972) as modified by Duesberg and Voght (1973) was used during this work.

Formamide was deionised by stirring with 5% wt/vol mixed-bed ion-exchange resin (AG 501-X8) for 1 hour, and filtering through a Whatman No. 1 filter paper on a Buchner filter funnel. To prepare a batch of 6-8 cm standard size disc gels, 0.7 g of acrylamide and 0.122 g of bisacrylamide were dissolved in 19.75 ml of 2 mM (pH 7.0) phosphate buffered formamide. 250 µl of ammonium persulphate solution (0.1 g/ml H_2O) and 40 µl of T.E.M.E.D. (tetramethylethylenediamine) were then added. The solution was then poured into plastic tubes (0.6 cm x 12 cm), closed at the bottom by dialysis membrane and overlaid with 70% formamide in water. It was difficult to form a sharp interface between the gel solution and

the overlay solution. However this could be improved by inserting a syringe needle to just below the interface and carefully sucking off the mixed liquid until a sharp interface was visible.

When the gels had set (about 1 hour) the overlay was replaced by buffered formamide and both ends of the gel tube were sealed with parafilm and stored until needed at 4°C.

Samples were lyophilised and then dissolved in buffered formamide with a small amount of sucrose (about 5%) and bromophenol blue. Usually 10-20 µl samples were applied to the gels underneath 1 cm layer of buffered formamide. This was overlaid with 10 mM sodium phosphate buffer (pH 6) which was the buffer used in the lower and upper reservoirs. Thus we have a stack consisting of gel-sample-buffered formamide-reservoir buffer.

Electrophoresis was carried out at 5 mA per tube constant current, until the bromophenol blue marker dye had reached the bottom. The gels were then frozen and chopped to 1 mM slices using a Mickle Gel Slicer.

Two slices were placed in each scintillation vial and 0.1 ml of hydrogen peroxide added. Samples were digested overnight and often required a further 0.1 ml to complete digestion of the gel. Triton/ toluene scintillation fluid to ten times the volume of hydrogen peroxide used, was then added. The vials were shaken and left ten minutes to clear before counting. This method of assay for radioactivity in polyacrylamide gel slices is based on that of Tischler and Epstein (1968).

3.4.4. Formamide Sucrose Gradients

By using formamide-sucrose gradients MacNaughton et al., (1974) were able to overcome the problem of aggregation in nuclear RNA.

Gradients were 2-10% sucrose in 85% formamide, 1 mM EDTA, 10 mM tris (pH 7.5). They were centrifuged for 40 hours at 24,000 rev/min in a Spinco SW40 rotor. 0.5 ml samples were layered on top of 12 ml gradients. Lyophilised RNA samples were first dissolved in 10 µl of water and then made to 0.5 ml with buffered 85% formamide. The sucrose-formamide solutions were prepared by dissolving the sucrose in a small volume of water and then adding formamide to 85%. These solutions were stored for several weeks at 4°C. However the optical density of the solution increased over this time. 0.8 ml fractions were collected from the gradients. Samples (usually 0.1 ml) from these gradient fractions were placed on 2.5 cm Whatman 3 MM filters. These were then washed in ice-cold 10% trichloro-acetic acid, 5% trichloro-acetic acid, ether and ether again, in that order, before drying and counting in a scintillation counter with 5 ml of toluene scintillation fluid.

3.4.5. LETS/Sucrose Gradients

LETS/sucrose gradients were used mainly to separate nuclear RNA species by size. 15% to 30% (w/w) sucrose dissolved in LETS buffer (see standard solutions) were used to pour 36 ml gradients in a Spinco SW27 bucket. Usually 2 ml RNA samples in LETS buffer were layered on top of the gradient which was spun for 16 hours at 16,000 rev/min and 20°C.

1.6 ml fractions were collected from the gradient after passage through the flow cell of a Gilford recording spectrophotometer.

A recording of the absorbance at 260 nm was made and 10 µl samples of each fraction were pipetted onto aluminium planchettes. After drying under an infra-red lamp, these planchettes were counted for 1 min in a Nuclear Chicago gas-flow counter.

3.4.6. Hydroxylapatite Chromatography

The chromatography of RNA and polyribonucleotides on columns of hydroxylapatite has been investigated by Bernardi (1969). Absorption of polynucleotides on hydroxylapatite takes place essentially because of the interaction between the negative phosphate groups of the polynucleotides and the positive calcium ions on the surface of the hydroxylapatite crystals, with no direct intervention of the bases and the sugars. Thus flexible, randomly coiled polynucleotides are eluted from hydroxylapatite columns by lower phosphate molarities than rigid, helical polynucleotides because the phosphate groups which are available for the interaction with absorbing sites on hydroxylapatite in the rigid ordered structures, greatly decrease in number on the 'outer surface' of randomly coiled, denatured nucleic acids (Bernardi, 1969).

1 g of hydroxylapatite was suspended in 5 ml of 50 mM potassium phosphate buffer (pH 6.8) and packed into a 1 cm diameter column with a No. 3 porus fitted disc at the bottom after the fines had been decanted. After washing the columns with 10 ml of 50 mM phosphate buffer the sample was applied. RNA samples, usually 1.0 ml from a 'high salt digestion' were diluted five times with ice-cold 50 mM phosphate buffer and loaded onto the column. The column was run under the influence of gravity and approximately 1 ml fractions were collected. 50 mM phosphate buffer was used to wash all the unbound RNA off the column leaving the

double stranded, helical pieces bound to the hydroxylapatite. 500 mM potassium phosphate was then used to elute the double stranded RNA.

At this point a problem was encountered as the high concentration of phosphate caused two phases to appear on ethanol extraction and if the RNA was precipitated by 5% trichloro-acetic acid it was found to be impossible to redissolve more than about 40% of it. Therefore the double stranded RNA fractions from the hydroxylapatite column were passed through a small column of Sephadex G-25 (2.5 cm x 10 cm) to de-salt it. The void volume fractions from this column, which contained most of the radioactivity were then lyophilised for further digestion and fingerprinting.

All operations were carried out at $4^{\circ}C$.

3.5. Digestion of RNA

3.5.1. T_1 Ribonuclease Digestion

Samples of RNA, usually 10-20 µg were lyophilised in small siliconised test tubes. Digestion was done in 2.5 or 5 µl volumes of enzyme mixture which was added to the test tube just prior to incubation.

An enzyme to substrate ratio of 0.1 was used, and the enzyme mixture was prepared by mixing the correct amounts of sterile water T_1 RNase (from a 2 mg/ml stock solution) and buffer (from a X10 stock solution). On addition of the enzyme mixture a small glass capillary attached by some thin tubing to a Pasteur pipette was used to ensure all of the lyophilised RNA sample was dissolved. This was done by carefully blowing the mixture out of the capillary tube onto the $^{32}PO_4$ labelled RNA in the test-tube, and allowing it to return to the tube by capillary

action. Digestion itself was carried out in the tip of the capillary at 37°C for 30 min in a humidified oven.

3.5.2. T_1 Ribonuclease plus Alkaline Phosphatase Digestion

These digestions were basically by the same technique as for T_1 RNase. They were generally carried out in 5 µl samples at an enzyme to substrate ratio of 0.2 for alkaline phosphatase at 0.1 for T_1 RNase. The digestion time was 60 min.

A problem often encountered with this type of digestion was that which is often referred to as the shadow graticule effect. T_1 RNase plus phosphatase fingerprints were often shown to contain numerous extra spots displaced to the left and slightly above the expected graticules of spots. These 'shadow' spots were not only noticed in our laboratory but also by workers in other laboratories (J. Klootwijk - personal communication). It was believed that this effect was due to some impurity in the commercial enzyme preparation. However re-purification with diethyl pyrocarbonate by the technique of Wimmer (1972) did not seem to improve the digestion.

3.5.3. Pancreatic Ribonuclease Digestion

These digestions were done at enzyme to substrate ratio of 0.1 per 30 min. The enzyme mixture was prepared from a 2 mg/ml stock enzyme solution using stock T_1 RNase buffer.

3.5.4. Combined T_1 Ribonuclease plus Pancreatic Ribonuclease Digestion

Digestion was for 30 minutes using an enzyme to substrate ratio of 0.1 for T_1 RNase, and 0.1 for pancreatic RNase.

3.5.5. Ribonuclease Digestion in High Salt Buffer

Pancreatic RNase hydrolysis of the phosphodiester linkage of poly (A). Above pH 6 degradation is random, below pH 6 pancreatic RNase behaves as an endonuclease. Beers (1960) showed that 0.3 M KCl gave almost 100% protection to poly (A) while still cleaving after C and U nucleotides. Thus to prepare poly (A) or double stranded regions of RNA molecules, a mixture of T_1 RNase plus pancreatic RNase in 0.3 M salt solution was used.

The lyophilised RNA sample was dissolved in 0.5 ml of 'high salt digestion buffer' and 50 µl of high salt digestion enzyme mixture added. After incubation for 30 min at 37°C the mixture was rapidly chilled in an ice bath.

3.5.6. Complete Digestion with Snake Venom Phosphodiesterase

The oligonucleotides were first treated with bacterial alkaline phosphatase to remove the terminal 3'-phosphate group as follows. The material was dissolved in 10 µl of 0.02 M tris buffer (pH 8.5) containing 1 mg/ml phosphatase. After incubation for 1 hour at 37°C, the products were separated on Whatman No. 52 paper at pH 3.5. The bands were located by autoradiography, cut out and eluted with water.

The dephosphorylated oligonucleotides were then treated with a mixture containing 0.1 mg/ml Venom phosphodiesterase in 25 mM tris buffer (pH 8.5), 10 mM magnesium acetate. After incubation for two hours at 37°C the mononucleotides were separated by electrophoresis at pH 3.5 on Whatman No. 52 paper (as for base analysis).

3.5.7. Partial Digestion with Snake Venom Phosphodiesterase

Oligonucleotides were first dephosphorylated as in section 3.5.6.

The dephosphorylated oligonucleotides were then dissolved in a mixture of 0.02 mg enzyme per ml in 20 mM tris buffer (pH 8.5) - 0.01 M $MgCl_2$. This was incubated at 37°C and the material was spotted on to DEAE paper after 10, 20 and 30 min. The products were then electrophoresed at pH 3.5.

3.5.8. Base Analysis of RNA

$^{32}PO_4$ labelled RNA was hydrolysed for 24 hours in 0.2 M sodium hydroxide by dissolving the RNA in 5 µl of 0.2 M sodium hydroxide and incubating it in a sealed capillary tube for 24 hours at 37°C. The resulting hydrolysates were subjected to ionophoresis on Whatman No. 52 paper for 40 min at 4.5 Kv. The No. 52 paper was then dried and autoradiographed. The radioactivity present in each spot was measured as described in section 3.6.4. and the percentage of radioactivity present in each base calculated.

It should be noted that nucleotide bonds adjacent to 2-O-methyl ribonucleotides are not cleaved by alkali.

3.5.9. Analysis of RNA for Pseudouridine

Analysis for pseudouridine was carried out as follows. $^{32}PO_4$ labelled mRNA was hydrolysed for 24 hrs in 0.2 M NaOH and the resulting hydrolysate subjected to electrophoresis on Whatman No. 52 paper for 40 min at 4.5 Kv. After autoradiography the Up spot (as judged by the behaviour of markers) was cut out, eluted with water and applied to a sheet of Whatman No.1 paper. This was subjected to descending chromatography in an isopropanol-HCl-H_2O system, 68: 17.6: 14.4 v/v (Wyatt, 1951; Felner, 1969). Samples of $^{32}PO_4$ labelled χ_p were also subjected to this procedure as controls. Spots were located by autoradiography.

3.6. Fingerprinting

3.6.1. Two-Dimensional Electrophoresis

The RNA digestion products were separated by electrophoresis in the first dimension on cellulose acetate at pH 3.5. 2.5 x 95 cm strips of cellulose acetate were soaked with 7 M urea, pH 3.5 buffer and excess buffer removed from the application area by blotting with pads of tissue paper. The RNA digestion mixture was applied as a spot about 10-15 cm from one end of the strip. A spot of marker dye was applied at either side of the digest spot. The rest of the cellulose acetate strip was wiped clean of excess buffer before the strip was placed into position in the electrophoresis tank. Electrophoresis was carried out at 4.8 Kv for about 3.5 hrs.

Generally for a pancreatic RNase or T_1 RNase digest the first dimension was run until the distance between the slowest pink dye and the blue marker was approximately 35 cm. The section of the cellulose acetate strip from 8 cm behind the blue marker dye and 2 cm in front of the slowest pink marker was transferred to the second dimension.

Before laying onto the second dimension sheet of DEAE paper, excess white spirit was allowed to drip from the first dimension cellulose acetate strip. After placing the first dimension strip about 10 cm from one end of the sheet of DEAE paper (Whatman DE81, 43 x 95 cm), five strips of Whatman 3 MM paper, one inch wide and soaked in distilled water, were laid over the cellulose acetate strip. Pressure was then applied to blot the oligonucleotides from the first dimension onto the second dimension where they were bound to the DEAE groups of the paper. This was achieved by placing a glass plate over the strips of Whatman 3 MM paper. After

half an hour most of the radioactivity was transferred to the DEAE paper which was then washed in methylated spirit to remove urea.

After drying, marker dye was applied to each end of the origin and half of the sheet of DEAE paper was sprayed with 7% formic acid. The sheet was then carefully placed over a perspex electrophoresis rack and the other half sprayed. The electrophoresis rack was carefully lowered into an electrophoresis tank and run at 1-1.4 Kv for approximately 16 hours for T_1 RNase or pancreatic RNase digests (blue marker travelled almost to the top of the rack). For the T_1 RNase plus pancreatic RNase digests this is reduced so that the blue dye is about 75% of the distance to the top of the rack. T_1 RNase plus phosphatase digests required longer runs of approximately 36-40 hours (blue marker 75% of the distance down the far side of the paper).

Cooling coils at the top of the tank dissipate the large amount of heat generated by passage of current through the paper.

After electrophoresis the DEAE paper was dried on the electrophoresis rack as the paper has little mechanical strength when wet. Only when the paper was completely dry and free from the smell of formic acid was it subjected to autoradiography. Small amounts of formic acid left on the DEAE paper can cause fogging of the X-ray film.

3.6.2. Homochromatography

Using the technique of two dimensional electrophoresis, most long oligonucleotides remain at the origin of the second dimension although they are well separated in the first dimension. This is because long oligonucleotides are bound so tightly to DEAE paper that a very high

concentration of anion is required to remove them. This is not possible with ionophoretic systems as it increases the conductivity too much, resulting in overheating.

Brownlee and Sanger (1969) found that chromatography on DEAE cellulose thin layer sheets developed by a mixture of oligonucleotides was the best technique for separating long oligonucleotides. In this system the radioactive oligonucleotides are displaced by a series of anions of different affinity for the DEAE groups.

RNA was digested and separated in the first dimension by electrophoresis as described in the preceding section (3.6.1.). However electrophoresis was terminated after 1.5 hours and the first dimension blotted onto a thin layer sheet of DEAE cellulose (40 x 20 cm). The transfer was done 4 cm from one end using the technique described in section 3.6.1.

If a concentrated mixture of oligonucleotides is applied to the end of the DEAE thin layer chromatography sheet, oligonucleotides saturate the DEAE groups and displace one another producing a series of fronts. The smaller oligonucleotides with low affinity are displaced by the larger oligonucleotides and therefore move faster. This technique is called homochromatography because a mixture of oligonucleotides (a homomix) is used to separate the radioactive nucleotides.

The DEAE thin layer sheet was briefly chromatographed with distilled water till the solvent front had travelled about 5 cm up the plate. It was then transferred to a chromatography tank equilibrated with 100 ml of homomix at 60°C. Sheets were chromatographed overnight,

and to soak up the ascending solvent front a wad of 1 inch strips of Whatman 3 MM strips were attached to the top of the chromatography sheet.

At a temperature of 60°C oligonucleotides of up to 50 units in length can be separated. By diluting the concentration of DEAE groups and by using higher temperatures, better fractionation with higher mobility of large oligonucleotides was obtained. In addition material appeared in more compact spots resulting in improved resolution.

Brownlee and Sanger (1969) suggest three homomixtures which may be used. Homomix A separates oligonucleotides in the size range 15-50 nucleotides, homomix B separates 10-25 nucleotides and homomix C separates 1-15 nucleotides approximately. Figure C shows the difference in separation between homomix B and homomix C.

3.6.3. Autoradiography

When dry, the DEAE paper was marked for identification with ^{35}S -sulphate containing ink. It was then cut to X-ray film size and placed on top of a sheet of X-ray film in a lead-lined folder. These folders were stored, stacked on top of each other in a dark cupboard for 10-14 days before development. Generally a 6 minute development was used, this being altered according to the rate of appearance of spots on the X-ray film. After a quick wash in water the developed film was fixed for twice the length of time required to clear the film. X-ray film processing was performed in a Kodak P3 X-ray film processing unit.

3.6.4. Estimation of Radioactivity

After autoradiography fingerprints were placed over X-ray films on an X-ray film viewer and the outline of the spots to be examined

marked in pencil. These spots were cut out and placed in scintillation vials with 10 ml of toluene scintillation fluid. Scintillation spectrometry for ^{32}P was done mainly on a Packard Tri-Carb scintillation spectrometer using a window setting of 100-1000 and 1% gain. Samples were normally counted for 10 minutes.

^{14}C radioactivity was assessed on a Beckman LS-333 Liquid Scintillation System using a preset ^{14}C window. Samples containing ^{14}C were prepared by cutting out spots, adding 0.1 ml of hymine hydroxide (just enough to soak the spot) and incubating at 60°C for 10 minutes prior to the addition of 5 ml of toluene scintillation fluid.

3.6.5. Calculation of Relative Molar Oligonucleotide Frequency

The relative oligonucleotide frequency was obtained by dividing the radioactivity in each spot by the radioactivity present in the spot for Gp (plus any radioactivity present as cyclic Gp). This is converted to relative molar oligonucleotide frequency by dividing the relative oligonucleotide frequency by the number of nucleotides present in its sequence.

3.6.6. Calculation of Theoretical Relative Molar Oligonucleotide Frequency

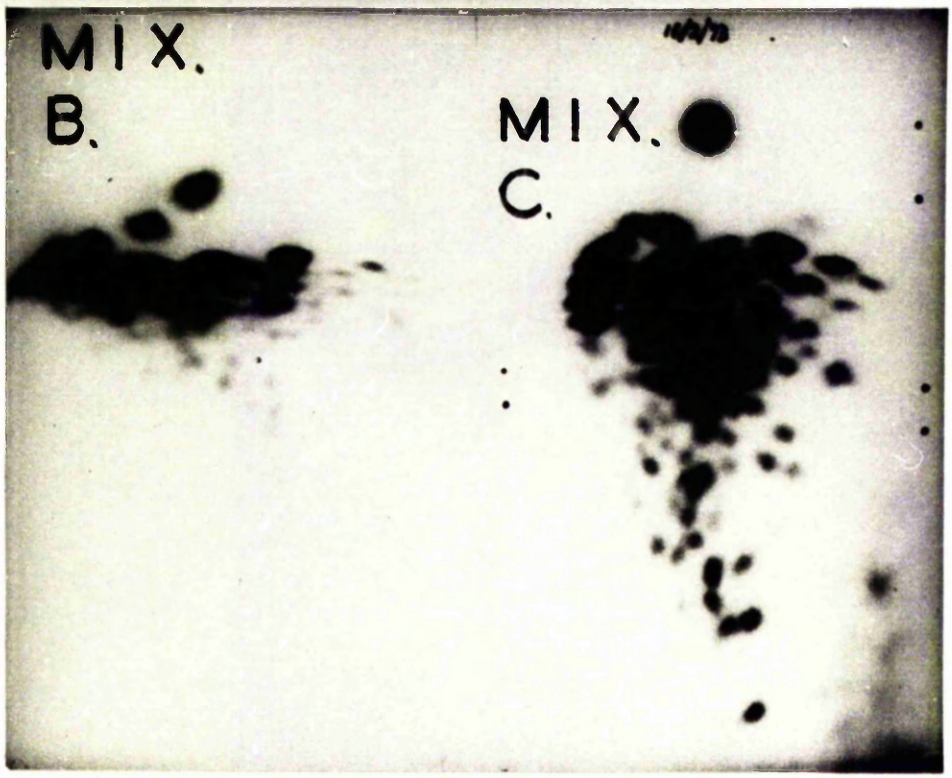
Theoretical relative molar oligonucleotide frequencies can be calculated from the base composition of an RNA species. Assuming random nearest neighbour relationships between nucleotides within the limits imposed by the base composition, the proportion of radioactivity present in various nucleotides is given by the formula, (length of sequence) x (product of base compositions) x (proportion of base as G).

FIGURE C

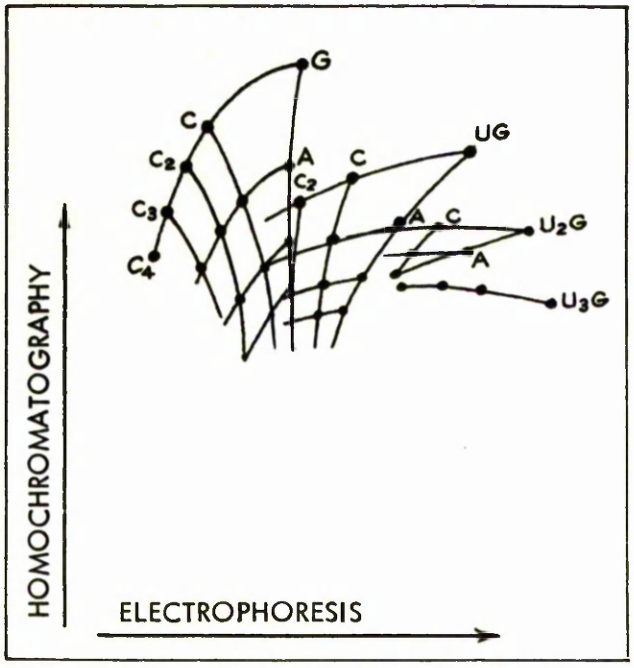
Homochromatography of T_1 Ribonuclease Digests of 28S rRNA

RNA was prepared and fingerprinted as described in the Methods section. First dimension left to right, cellulose acetate, pH 3.5 (5% acetic acid, 7 M urea). Second dimension upwards, homochromatography on DEAE thin layer chromatography sheets at 60°C overnight.

The homochromatography key indicates how the graticules of spots observed in the standard two-dimensional electrophoresis fingerprints of a T_1 RNase digest appear in a homochromatogram.


MIX.
B.
MIX.
C.

KEY


G
C
C2
C3
C4
A
C2
C
UG
A
C
U2G
A
U3G
HOMOCHROMATOGRAPHY
ELECTROPHORESIS

sheet of Whatman No. 1 paper. This was subjected to descending chromatography in an isopropanol-HCl-H_2O system, 68:17.6:14.4 v/v (Wyatt, 1951; Felner, 1969). Samples of $^{32}PO_4$ labelled Xp were also subjected to this procedure as controls. Spots were located by autoradiography.

RESULTS

1. COMPARISON OF HnRNA and 28S rRNA

Studies on mammalian cell HnRNA have revealed several interesting chemical features such as the occurrence of polyadenylic acid, oligouridylate regions, and double stranded regions. In this study, to further investigate chemical features present in HnRNA, the two-dimensional "fingerprinting" technique of Sanger et al. (1965) was employed. Using this method it was hoped to gain some general information on the oligonucleotide composition of HnRNA.

1.1. Two-Dimensional Electrophoresis of HnRNA and 28S rRNA T_1RNase Digests

Ribosomal RNA and its precursors are the most likely sources of contamination of HnRNA preparations. However rRNA and its precursors do have characteristic methylated sequences by which they can easily be identified. Therefore to study the nucleotide composition of HnRNA by fingerprinting it is useful to prepare fingerprints of rRNA for comparison.

Fingerprints of rRNA and its precursors prepared by the technique of Sanger et al. (1965) have been well characterised (Maden et al., 1972; Robertson and Maden, 1973; Maden and Salim, 1974).

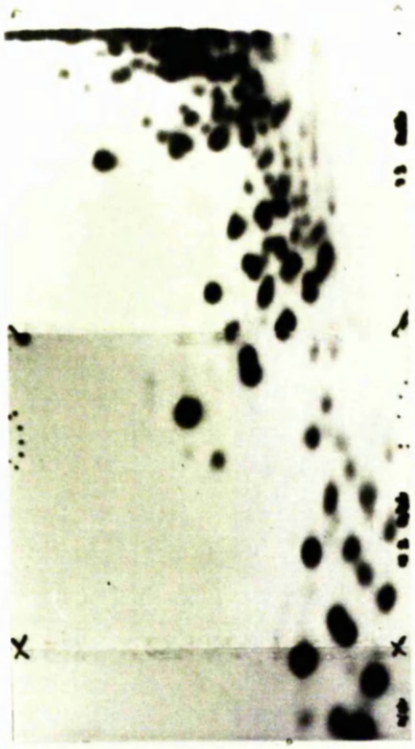
Figure 1 shows T_1RNase fingerprints of 28S rRNA and HnRNA separated by two-dimensional electrophoresis as described in the Methods section. It will be noted that there are three main differences between the 28S rRNA fingerprint and that of HnRNA. Firstly 28S rRNA yields a complex but finite array of discrete products, as might be expected for a homogeneous RNA molecule. By contrast, the HnRNA pattern reveals diffuse regions (shaded in the HnRNA key) which indicate an extremely compl...

FIGURE 1

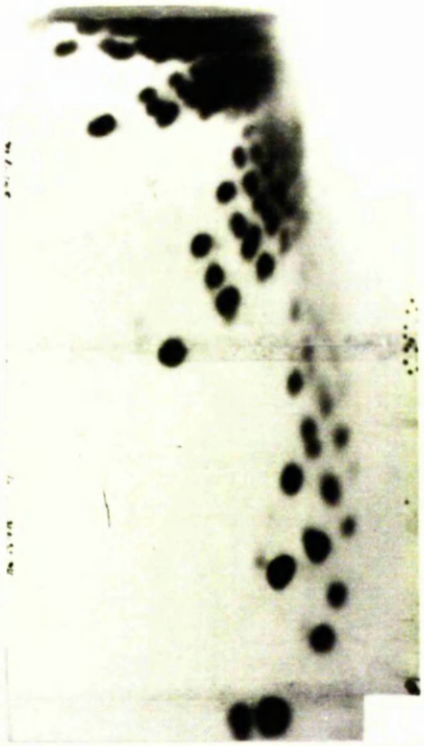
T_1 Ribonuclease Fingerprints of HnRNA and 28S rRNA

RNA was prepared and fingerprinted as described in the Methods section. First dimension, right to left, cellulose acetate, pH 3.5 (5% acetic acid 7M urea). Second dimension, downwards, DEAE paper, 7% formic acid. Several 28S rRNA methylated products are numbered and marked in black in the 28S rRNA key. The sequences of products have been determined by Maden *et al.* (1972). Spot m21 corresponds to the sequence Gm-G_p and is the strongest methylated spot in the fingerprint.

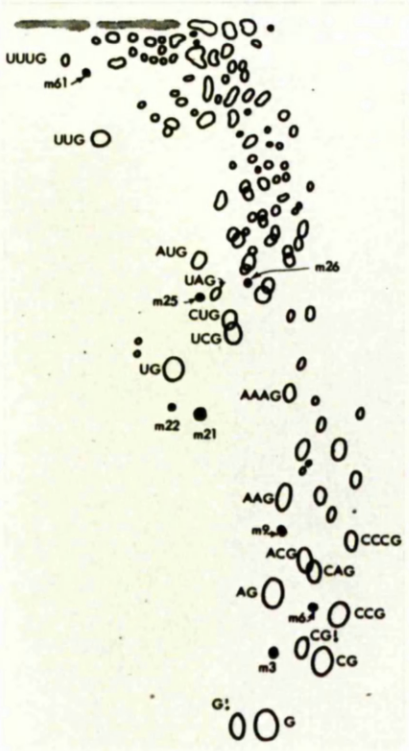
G_p has run off the end of this particular 28S rRNA fingerprint but was obtained in fingerprints actually used for subsequent quantitation.



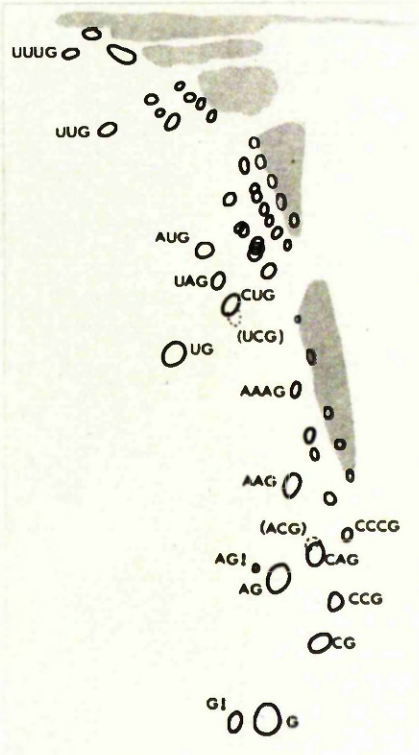
28S RNA



Hn RNA



28S RNA Key



Hn RNA Key

mixture of larger T_1 RNase digestion products. Secondly the HnRNA pattern clearly lacks the several weakly labelled but distinctive spots seen in the 28S rRNA fingerprint. These are methylated sequences and they are also present in precursor rRNA (Maden and Salim, 1974). Their absence from HnRNA serves as useful evidence that the latter is not contaminated by any precursor rRNA, such as 45S or 32S pre rRNA, as these RNAs contain similar methylated sequences to those seen in 28S rRNA. A third difference is as follows - to the right of the 28S rRNA product U-Gp is a pair of isomers, C-U-Gp (upper) and U-C-Gp (lower). By contrast, the HnRNA pattern contains only one strongly labelled spot (C-U-Gp) in this position with a very weakly labelled satellite spot just below. Similarly in the A-C-Gp, C-A-Gp positions, HnRNA yields a single strongly labelled spot, C-A-Gp, with only very weak labelling of A-C-Gp.

1.2. Comparison of HnRNA and 28S rRNA by Combined T_1 plus Pancreatic Ribonuclease Digestion

Combined T_1 plus pancreatic ribonuclease digestion of RNA yields products which are of the type $(A-)_nNp$ (where n = integer and N = nucleotide other than A). Thus combined T_1 plus pancreatic ribonuclease digestions give rise to simpler patterns than T_1 ribonuclease digests.

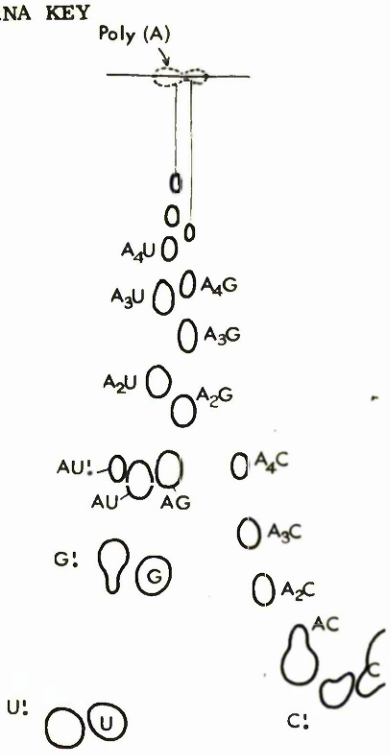
Figure 2 shows combined T_1 plus pancreatic ribonuclease digests of HnRNA and 28S rRNA separated by Sanger's two dimensional electrophoresis fingerprinting technique as described in the Methods section. From these fingerprints it can be seen that HnRNA has very long series of spots compared with 28S rRNA. These spots have sequences of the type $(A-)_nNp$ (where n = integer and N = nucleotide other than A). Furthermore the 28S rRNA fingerprint shows many weak spots due to the

FIGURE 2

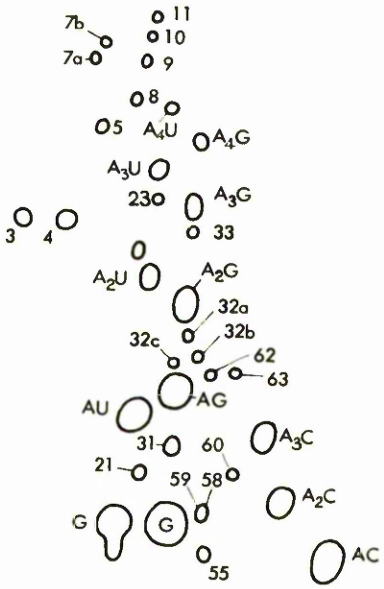
Combined T_1 plus Pancreatic Ribonuclease Fingerprints of HnRNA and 28S rRNA

RNA was prepared and fingerprinted as described in the Methods section. First dimension, right to left, cellulose acetate, pH 3.5 (5% acetic acid, 7M urea). Second dimension, downward, DEAE paper, 7% formic acid. Several 28S rRNA methylated products are numbered in the 28S rRNA key. The sequences of these spots have been determined by Maden and Salim (1974). Spot 4 corresponds to the sequence Gm-G_p. The weak spots to the left of the $(A-)_nCp$ series of spots in the HnRNA fingerprint, are probably breakdown products of poly (A) sequences. The weak spots above and to the right of the A-G_p spot were not identified. Neither was the strong spot to the left of the $(A-)_4Up$ spot which varied in intensity from fingerprint to fingerprint.

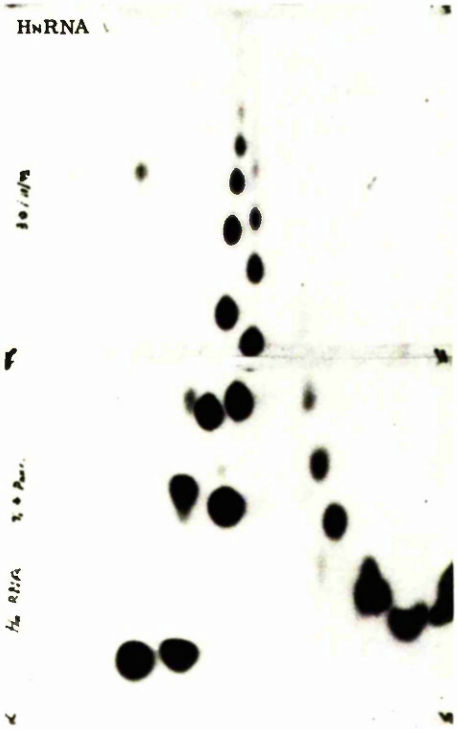
HnRNA KEY



28S rRNA KEY



HnRNA



28S rRNA

numerous methylated oligonucleotides present. These methylated sequences do not seem to be present in the HnRNA fingerprint. This confirms the observed lack of methylated products in T_1 RNase digests of HnRNA as seen in Figure 1.

1.3. Homochromatography of HnRNA and 28S rRNA T_1 Ribonuclease Digests

An alternative technique to two dimensional electrophoresis is homochromatography (Brownlee and Sanger, 1969). Homochromatography differs from the classical two dimensional electrophoresis technique of Sanger et al. (1965) at one stage only. Instead of electrophoresis in the second dimension, chromatography is performed on thin layers of DEAE cellulose. The chromatography is performed at 60°C, the developing agent being an alkaline digest of commercial yeast RNA. Hence the name homochromatography.

It is advantageous to use homochromatography when longer sequences are to be separated. Figure 3 shows the result of homochromatography on T_1 ribonuclease digests of HnRNA and 28S rRNA. The difference between 28S rRNA and HnRNA is striking. Whereas 28S rRNA has a few discrete spots corresponding to large molecular weight sequences, HnRNA has so many that individual spots are not resolved. Instead there is a general darkening of the area of the chromatogram occupied by long sequences. This indicates the immense sequence complexity of HnRNA.

1.4. Quantitation of Oligonucleotides from T_1 Ribonuclease Fingerprints

When quantitating the molar yields of oligonucleotides in fingerprints of a discrete RNA molecule, such as 28S rRNA, it is possible to exploit the fact that certain oligonucleotide sequences occur only

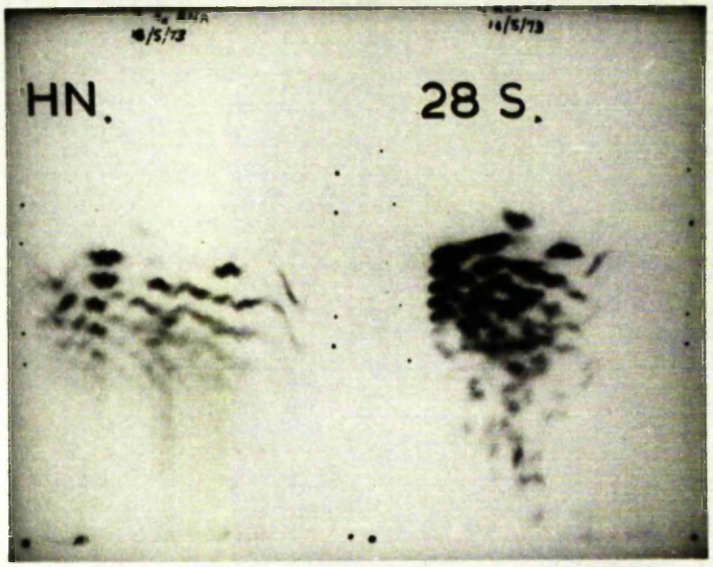
FIGURE 3

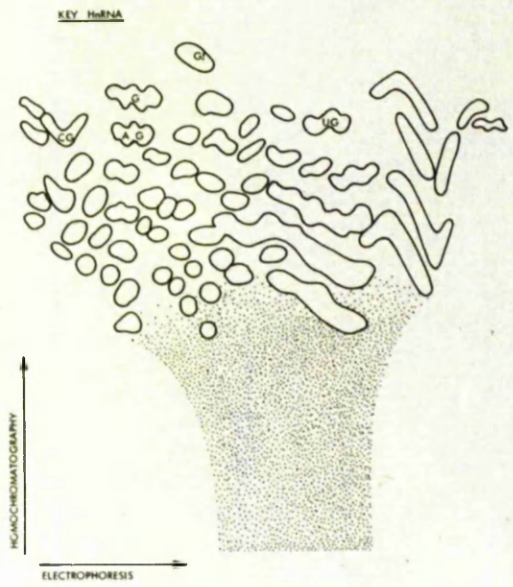
Homochromatography of HnRNA and 28S rRNA

T_1 Ribonuclease Digests

RNA was prepared, and (as described in the Methods section 3.6.2) subjected to electrophoresis in the first dimension left to right, cellulose acetate, pH 3.5 (5% acetic acid, 7M urea). The second dimension, upwards was homochromatography on DEAE thin layer chromatography sheets at 60°C using homomix C for about 16 hours.

It will be noted that there are small black "dots" at the side of the HnRNA and the 28S rRNA homochromatograms. The lowest spots mark the position of the first dimension after transfer to the thin layer sheet. Marker dye was applied in this position and the orange dye moved to a position between the two dark spots which are $\frac{1}{3}$ of the distance up the chromatogram, while the blue dye moved to a position between the two spots $\frac{2}{3}$ of the distance up the chromatogram.


HN.
28 S.


KEY HnRNA
HOMOCHROMATOGRAPHY
ELECTROPHORESIS

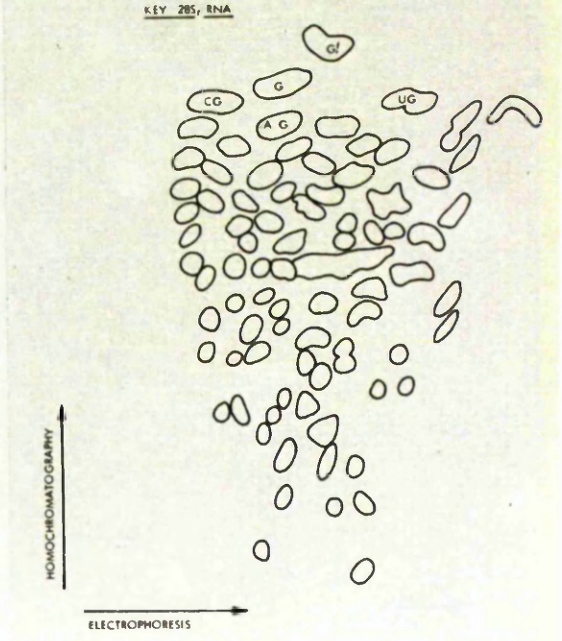

KEY 28S, RNA
CG
G
UG
A G
HOMOCHROMATOGRAPHY
ELECTROPHORESIS

TABLE 1

Relative Molar Oligonucleotide Frequencies in T_1 Ribonuclease Digests of HnRNA and 28S rRNA

RNA	Oligonucleotide			
	G	CG	CCG	CCCG
Hn	100(100)	3.90(24.30)	1.26(5.90)	0.47(1.43)
28 S	100(100)	39.0 (34.3)	11.5(11.76)	3.00(4.03)
		AG	AAG	AAAG
Hn		31.5 (27.80)	7.46(7.73)	2.65(2.15)
28 S		13.4 (15.2)	4.5 (2.31)	0.99(0.35)
		UG	UUG	UUUG
Hn		23.9 (28.40)	8.06(8.06)	4.25(2.29)
28 S		15.4 (17.1)	2.52(2.92)	0.14(0.50)
	UCG	CUG	ACG	CAG
Hn	0.33(6.90)	8.07(6.90)	1.26(6.75)	6.53(6.75)
28 S	2.30(5.86)	1.80(5.86)	3.26(5.21)	2.98(5.21)

Oligonucleotides were assayed for radioactivity as described in Methods, and their respective molar yields calculated and expressed relative to an arbitrary value of 100 for G plus cyclic G. The values in parentheses are theoretical molar yields, also relative to 100 for G. These theoretical values were calculated from the base compositions of HnRNA and 28S rRNA, assuming random nearest neighbour relationships between nucleotides within the limits imposed by these base compositions. The latter were for HnRNA:- A, 27.8%; U, 28.5%; G, 19.3%; C, 24.3%. For 28S rRNA:- A, 15.2%; U, 17.1%; G, 33.3%; C, 34.3%.

once per molecule. For heterogeneous populations of molecules, such as mRNA or HnRNA, this cannot be done. Nevertheless relative molar oligonucleotide frequencies can be determined as described in the Methods section (3.6.5.). In addition, theoretical relative molar oligonucleotide frequencies can also be calculated from the base composition of the RNA as described in the Methods section (3.6.6).

Table 1 presents the relative molar oligonucleotide frequencies and theoretical relative molar oligonucleotide frequencies for certain sequences from T_1RNase fingerprints such as those shown in Figure 1. From the data, it will be seen that sequences ending in the dinucleotide C-Gp are present in lower amounts than would be expected from the theoretical relative molar oligonucleotide frequency calculations for HnRNA but not for 28S rRNA.

1.5. Pancreatic Ribonuclease Digestion of HnRNA

Pancreatic RNase cleaves RNA after pyrimidine nucleotides to give 3' nucleoside monophosphates. Therefore we can examine poly (A) and poly (G) runs in RNA using this type of digestion as all oligonucleotides will end in either C or U. Figure 4 shows a pancreatic RNase digest of HnRNA separated by two dimensional electrophoresis. It is interesting to note that the intensity of the spot corresponding to the sequence G-Cp is very strong. This contrasts with its isomer, C-Gp, seen in T_1 RNase digest fingerprints of the same RNA, which is fairly weak. Furthermore if we look at the two isomers G-A-Cp and A-G-Cp we can see that they are of similar intensity, unlike the A-C-Gp, C-A-Gp pair, in T_1 RNase digest fingerprints.

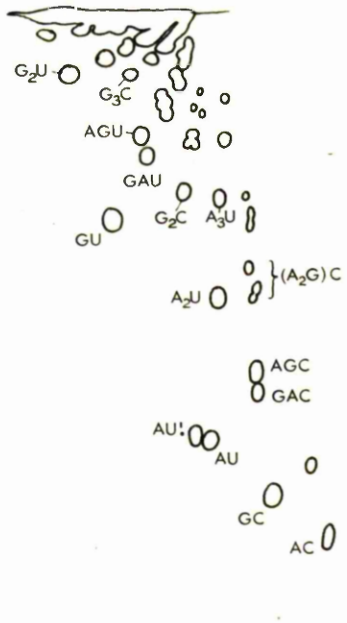
FIGURE 4

Pancreatic Ribonuclease Fingerprint of HnRNA

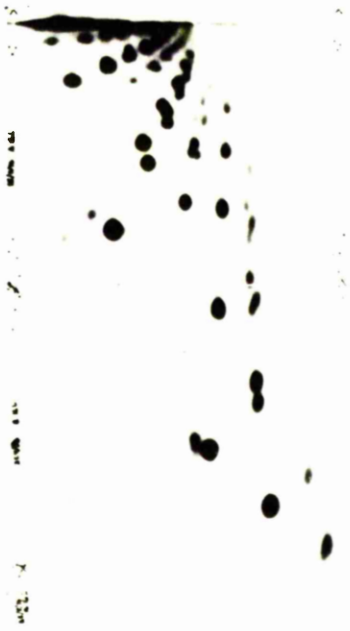
RNA was prepared and fingerprinted as described in the Methods section. First dimension, right to left, cellulose acetate, pH 3.5 (5% acetic acid, 7M urea). Second dimension, downwards, DEAE paper, 7% formic acid.

Sequences shown in the "HnRNA Key" were inferred from similar fingerprints of 16S rRNA from E. coli (Beck et al., 1970), and 5S rRNA (Brownlee, 1972).

HnRNA KEY



HnRNA



Thus it seems clear that although the sequence C-Gp is deficient in HnRNA the reverse sequence G-Cp is not deficient.

2. FINGERPRINTING OF mRNA

Several unusual features of HnRNA fingerprints have been shown in Section 1. Since a fraction of HnRNA is widely believed to be the precursor of mRNA, it is now of interest to fingerprint mRNA. In order to do this, one must first find the most suitable technique for mRNA preparation.

The first stage in this process was to isolate polysomes.

2.1. Polysome Preparation

In early experiments to prepare nuclear RNA cells were harvested by the trypsin-versene technique (see Methods section 3.1.2). This proved to be an unsatisfactory method for the harvesting of cells when polysomes were required from the cell cytoplasm. Figure 5A shows the absorbance profile obtained after sucrose gradient analysis of cytoplasm prepared from cells harvested in this way. Figure 5B shows the absorbance profile obtained after sucrose gradient analysis of cytoplasm prepared from cells harvested by the "scraping" technique (see Methods section 3.1.2). In figure 5B, to the left of the ribosome peak there is a broad peak. This is the polysome peak and it consists of many individual peaks for different sizes of polysomes. The small polysomes, from one to five ribosomes per mRNA strand, are seen to be partially resolved. Figure 5A, on the other hand, does not show any polysome peak to the left of the ribosome peak.

Thus when preparing polysomes, cells were always harvested by the "scraping technique".

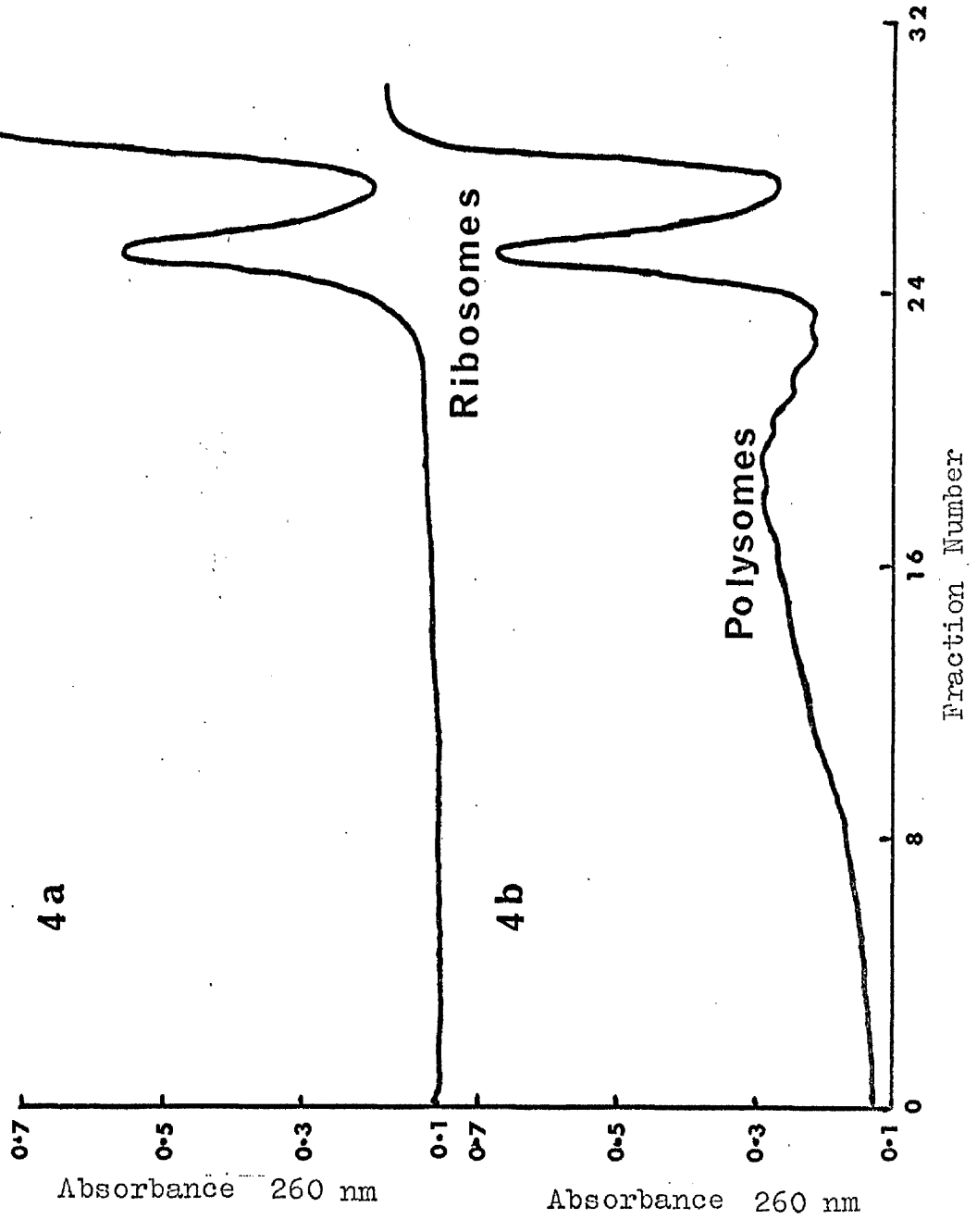
FIGURE 5

Sucrose Density Gradient Analysis of HeLa Cell Cytoplasm

Cytoplasm was prepared from cells as described in the Methods section. It was layered onto 15% - 30% sucrose/RSB gradients and centrifuged for 1.5 hours at 25,000 RPM in a Spinco SW27 centrifuge rotor at 4°C. Gradients were pumped from the bottom through the flow-cell of a Gilford recording spectrophotometer.

A) Cells harvested by "trypsin-versene" technique.

B) Cells harvested by "scraping" technique.


4a
4b
Ribosomes
Polysomes
0·7
0·5
0·3
0·1
0·7
0·5
0·3
0·1
Absorbance 260 nm
Absorbance 260 nm
0
8
16
24
32
Fraction Number

2.2. Comparison of mRNA Purification Techniques

Polysomes prepared as described above were pelleted by centrifuging at 50,000 rpm for 150 min in a Spinco Ti 50 rotor at 4°C. Messenger RNA was prepared from the pelleted polysomes by hot phenol extraction (as described in the Methods section 3.3.1). After three hours of labelling with $^{32}PO_4$ in the presence of low levels of actinomycin D (Methods section 3.1.1) 650 µg of polysomal RNA were recovered with a specific activity of 400 CPM/µg.

This specific activity is not high enough to be useful for fingerprinting studies. Moreover the RNA is not pure enough to fingerprint, there probably being $^{32}PO_4$ labelled tRNA present. This is possible because actinomycin D, although inhibiting rRNA, does not affect tRNA synthesis. Several techniques were therefore examined as possible further steps in the purification of mRNA from polysomal RNA. These were sucrose gradient centrifugation, oligo (dT) cellulose chromatography, and millipore filtration.

2.2.1. Sucrose Gradient Analysis of Polysomal RNA

It is known that large amounts of polysomal RNA are present as homogeneous species. These are 28S rRNA, 18S rRNA, 5S rRNA and 4S tRNA. One possible method of purifying mRNA is to remove these species of RNA after sucrose density gradient analysis of polysomal RNA.

Polysomal RNA was prepared, as described in the Methods section from cells labelled with $^{32}PO_4$ for 16 hours in the presence of low levels of actinomycin D. This RNA was layered onto 10% - 25% sucrose/LETS gradients and centrifuged for 16 hours at 23,000 rpm in a Spinco SW27

centrifuge rotor at 20°C. Fractions sedimenting from about 32S to about 7S were pooled (except for those corresponding to the peak 28S and 18S rRNA fractions which were discarded) and precipitated with 2 volumes of ethanol. This fraction was designated "Gradient Fraction" mRNA and was compared to mRNA purified by the "Millipore Filtration" technique by fingerprinting studies (see section 2.5.2.).

2.2.2. Oligo (dT) Cellulose Chromatography

Unlike other species of RNA found in polysomes, mRNA has been shown to have a polyadenylated tail (see Introduction 2.3.1). This feature of mRNA facilitates its separation by the technique of affinity chromatography using deoxythymidilate oligonucleotides (oligo (dT)) bound to cellulose; a technique which was developed by Gilham (1968).

Using small columns of commercially prepared oligo (dT) cellulose, attempts were made to prepare poly (A) containing mRNA and HnRNA. Figure 6A shows the elution profile of commercial ^{3}H poly (A). It will be noted that all of the sample is bound in 0.5 M NaCl/Tris buffer. When washed with 0.1 M NaCl/Tris buffer, used to remove non-specifically bound material, 25% of the commercial ^{3}H poly (A) was recovered. Finally when washed with Tris buffer alone approximately 75% was recovered.

Unfortunately when 28S rRNA, which does not appear to contain poly (A) regions was subjected to oligo (dT) cellulose chromatography under similar conditions to the commercial poly (A) sample, 25% was found to bind. Thus a single passage of total polysomal RNA through an oligo (dT) cellulose column would be insufficient to remove all 28S rRNA (in fact, only 75% of 28S rRNA would be removed). Additionally, on this same passage 25% of the poly (A) containing RNA may also be lost.

FIGURE 6

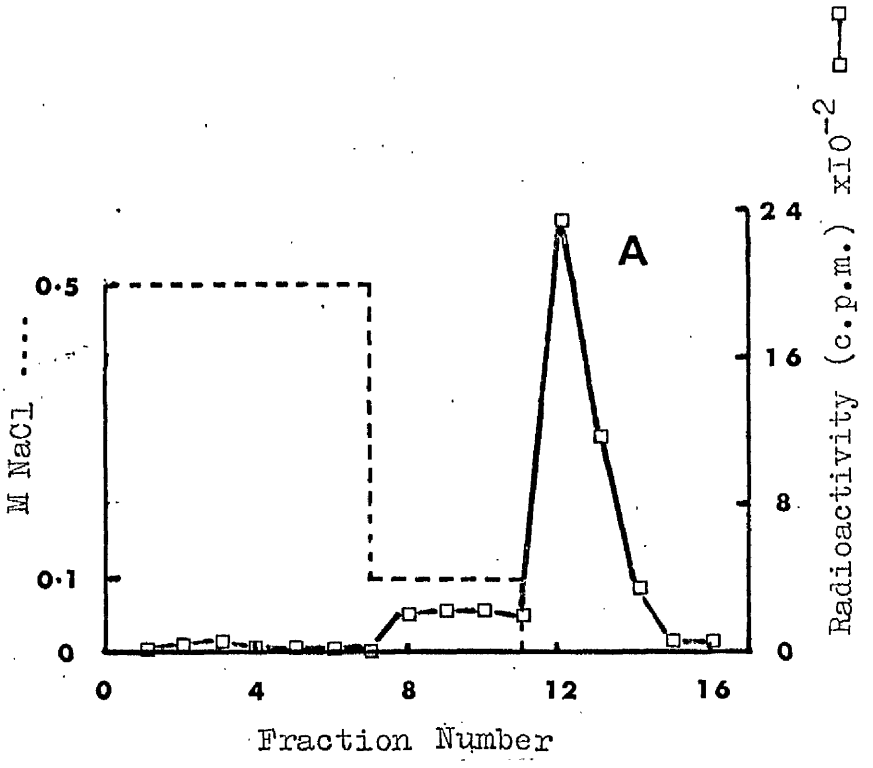
Oligo (dT) Cellulose Column Chromatography

1 g of oligo (dT) cellulose powder was suspended in 0.5 M NaCl, 0.01 M Tris-HCl (pH 7.5), 0.1% SDS and poured into a Whatman 1.5 cm diameter glass column. After washing with 0.01 M Tris-HCl (pH 7.5), 0.1% SDS and 0.5 M NaCl, the column was used repeatedly.

RNA samples were applied to the column in 0.5 M NaCl, 0.01 M Tris-HCl (pH 7.5), 0.1% SDS. Non-specifically bound RNA was eluted with 0.1 M NaCl, 0.01 M Tris-HCl (pH 7.5), 0.1% SDS, and the poly (A) containing RNA with 0.01 M Tris-HCl (pH 7.5), 0.1% SDS. Approximately 1 ml fractions were collected.

A) Chromatography of commercial ^{3}H poly (A)

B) Chromatography of polysomal RNA


A
M NaCl
0·5
0·1
0
0
4
8
12
16
Fraction Number
24
16
8
0
Radioactivity (c.p.m.) xIO^{-2}

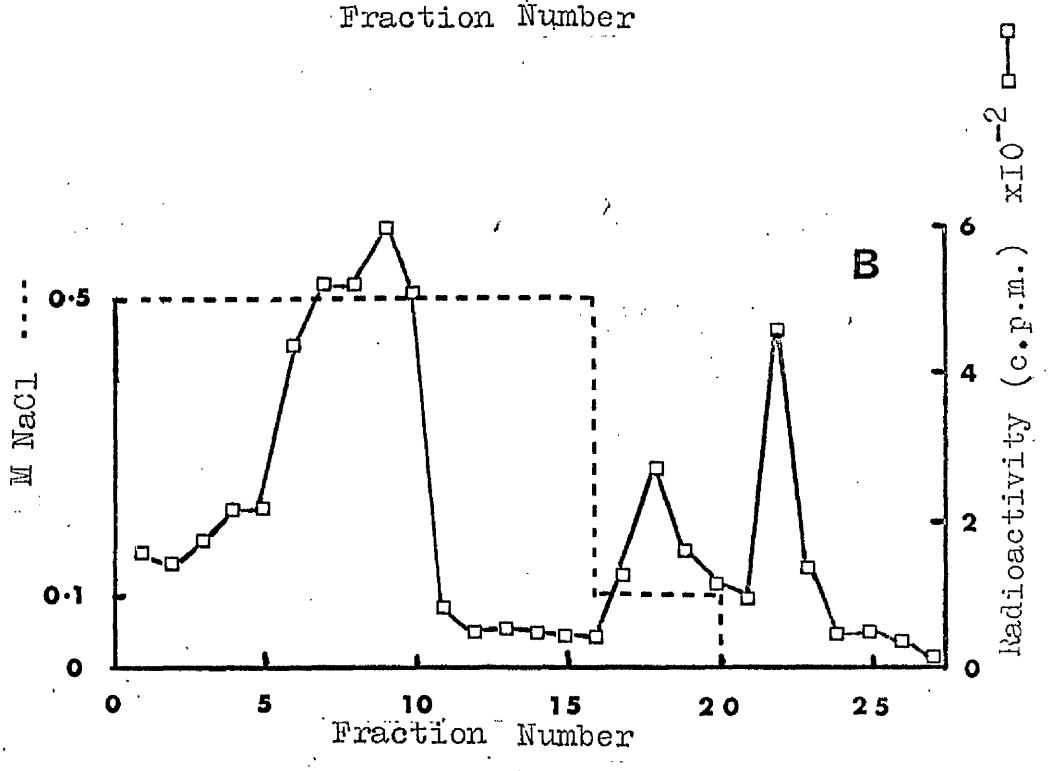
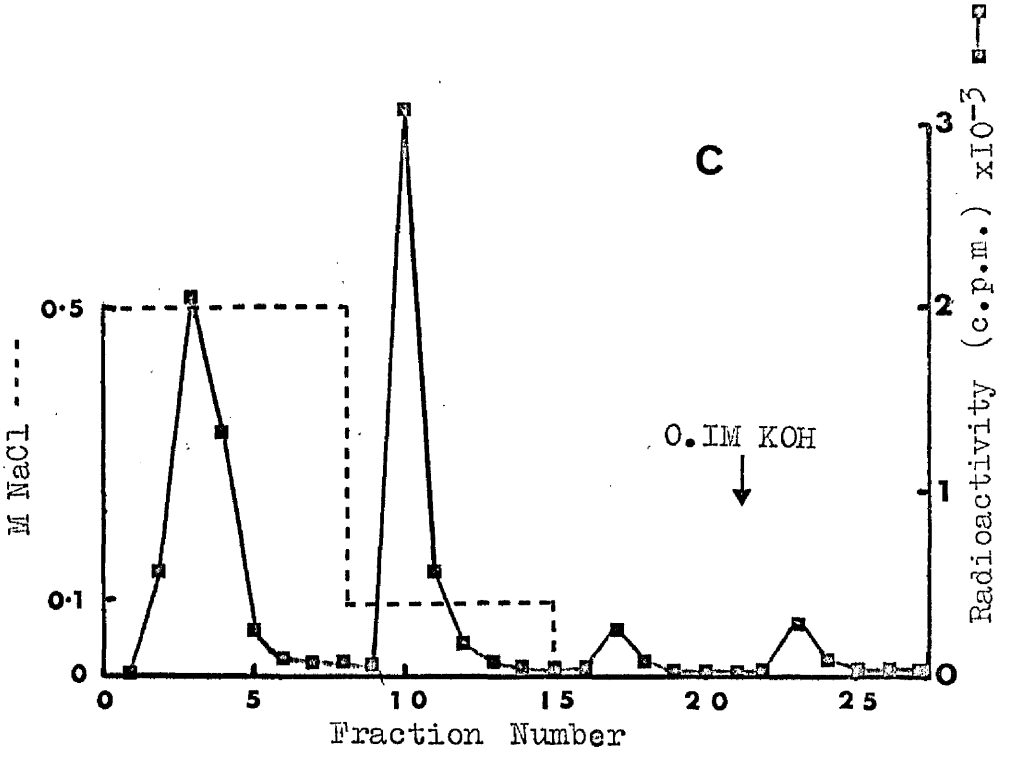

B
M NaCl
0·5
0·1
0
0
5
10
15
20
25
Fraction Number
6
4
2
0
Radioactivity (c.p.m.) xIO^{-2}

FIGURE 6 (CONT.)

C) Chromatography of HnRNA

D) % of RNA Fractions from Oligo (dT) Cellulose Chromatography which bind to Millipore Filters

Fractions from figure 6C were combined, ethanol precipitated and dissolved in 5 ml of millipore "binding buffer" (0.5 M KCl, 1 mM MgCl, and 10 mM Tris-HCl, pH 7.6). A small aliquot of this solution was pipetted directly onto a millipore filter and dried for radioactive determination. The remainder was subjected to millipore filtration as described in the Methods section. The radioactivity on the filters was measured in toluene/PPO scintillation fluid and from the counts, the % of ethanol precipitable radioactivity which binds to millipore filters in each oligo (dT) cellulose chromatography fraction was calculated.


C
M NaCl
0·5
0·1
0
0.IM KOH
Radioactivity (c.p.m.) xIO⁻³
3
2
1
0
0 5 10 15 20 25
Fraction Number

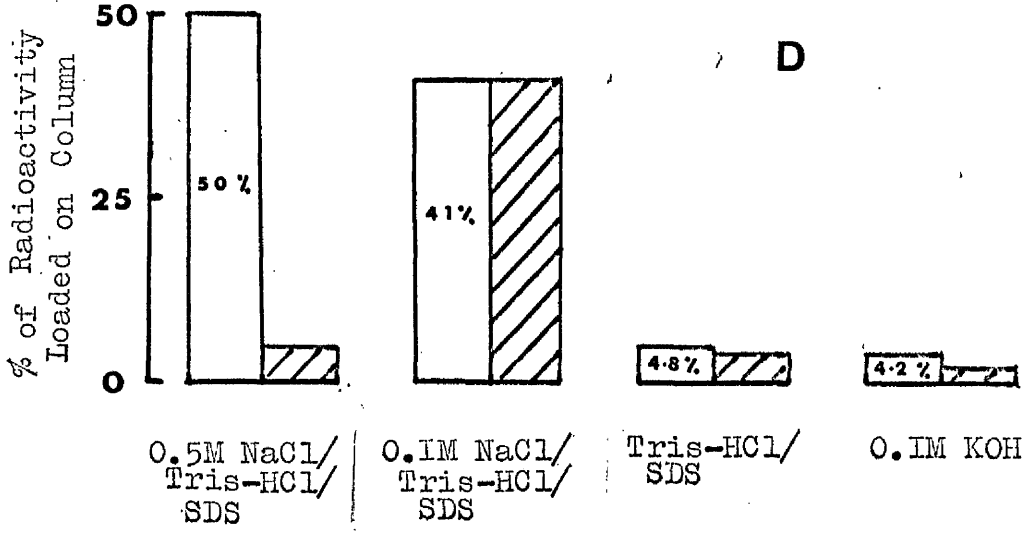

D
% of Radioactivity Loaded on Column
50
25
0
50 %
41%
4·8%
4·2 %
0.5M NaCl/ Tris-HCl/ SDS
0.IM NaCl/ Tris-HCl/ SDS
Tris-HCl/ SDS
0.IM KOH

Figure 6B shows the result obtained from oligo (dT) cellulose chromatography of polysomal RNA. It was not possible to recover sufficient poly (A) containing RNA from oligo (dT) cellulose chromatography of polysomal RNA isolated from a 3 hour labelling experiment in the presence of low levels of actinomycin D.

Figure 6C shows the result of oligo (dT) cellulose chromatography on HnRNA. Darnell's laboratory has reported that 30 - 40% of large HnRNA molecules (20,000 nucleotides) in HeLa cells have poly (A) regions while 10 - 20% of the smaller (5,000 nucleotides) HnRNA molecules have poly (A) regions (Jelinek et al., 1973). In mouse L cells 20% of total HnRNA appears to contain poly (A) regions (Greenberg and Perry, 1972; Latorre and Perry, 1973). However it will be noted that only 5% approximately of the radioactive RNA is recovered with 0.01 M Tris-HCl (pH 7.5) buffer during this work. Furthermore when the oligo (dT) cellulose column is washed with 0.1 M KOH a further 4% of RNA is recovered! Thus this technique, although working relatively well with commercial poly (A), does seem to have disadvantages for purifying poly (A) containing RNA molecules, especially large molecules such as HnRNA.

2.2.3. Millipore Filtration

The fractions of RNA recovered from figure 6C were tested for poly (A) regions by another technique: "Millipore filtration". Figure 6D shows the result of binding the various fractions from figure 6C to millipore filters. The technique is that of Brawerman et al. (1962) as described in the Methods section 3.5.2. It will be noted that although the 0.01 M Tris-HCl (pH 7.5), 0.1% SDS buffer fraction from the oligo (dT) cellulose chromatography (the poly (A) containing HnRNA fraction) does

contain RNA exhibiting a high affinity for millipore filters, so also does the 0.1 M NaCl, 0.01 M Tris-HCl (pH 7.5), 0.1% SDS buffer fraction.

Thus although the oligo (dT) cellulose column chromatography works relatively well with pure poly (A) samples, it seems that when presented with a heterogeneous sample of RNA it does not select for poly (A) containing RNA so efficiently as the "millipore filter" technique. Therefore "millipore filtration" of polysomal RNA gives higher recoveries of radioactive RNA thus favouring fingerprinting experiments.

The recovery of RNA from millipore filters was routinely greater than 95% after extraction as described in the Methods section 3.4.2.

2.3. Two Dimensional Electrophoresis of mRNA T_1 Ribonuclease Digests

HeLa cells, labelled with $^{32}PO_4$ for 3 hours in the presence of low levels of actinomycin D were fractionated into cytoplasm and nuclei as described in the Methods section 3.2.1. Polysomal RNA was prepared from the cytoplasm (Methods section 3.2.3). The millipore filtration technique was then used to isolate the poly (A) containing mRNA from the polysomal RNA.

The nuclei were phenol extracted to give nuclear RNA and HnRNA was prepared from the nuclear RNA by sucrose density gradient analysis as described in the methods section.

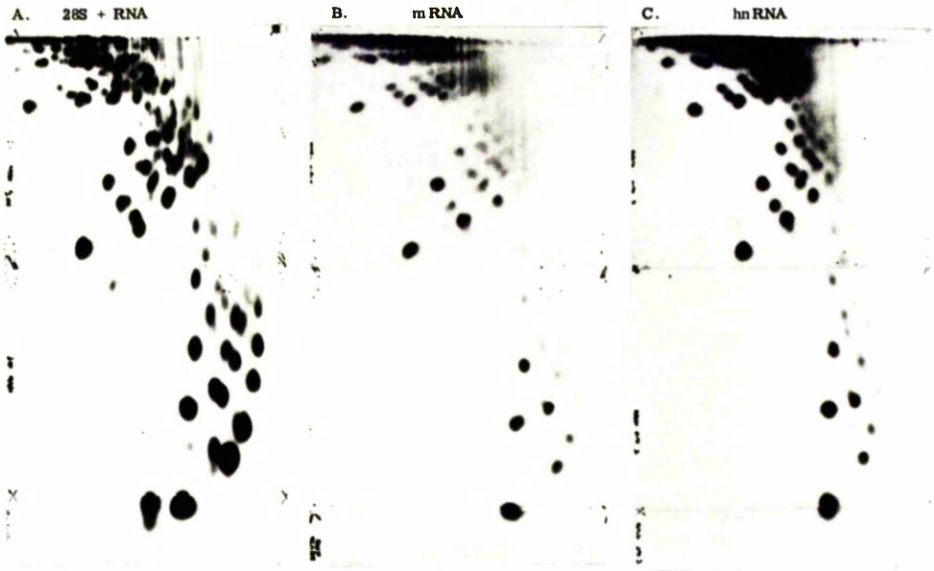
Figure 7 shows T_1 RNase digests of HnRNA, mRNA and 28S rRNA after two dimensional electrophoresis. On inspection it will be seen that the mRNA fingerprint shows the same complexity of larger sequences seen in HnRNA. Furthermore it shows the same lack of apparent methylated sequences and appears to be deficient in oligonucleotides

FIGURE 7

T_1 Ribonuclease Fingerprints of HnRNA, mRNA and 28S rRNA

RNA was prepared and fingerprinted as described in the Methods section. First dimension right to left, cellulose acetate, pH 3.5 (5% acetic acid, 7 M urea). Second dimension, downwards DEAE paper, 7% formic acid. The second dimension was run for approximately 18 hours.

Several 28S rRNA methylated products are numbered and marked black in D, the 28S rRNA Key. The sequences of these products have been determined previously (Maden et al., 1972).


A. 28S + RNA
B. m RNA
C. hn RNA


D. 28S + RNA Key
UUUG
m61
UUG
AUG
UAG
m25
CUG
UCG
UG
AAAG
m22
m21
AAG
m2
ACG
CCCG
CAG
AG
m6
CCG
CG1
m3
CG
G:
G


E. hn RNA Key
UUUG
UUG
AUG
UAG
CUG
(UCG)
UG
AAAG
AAG
(ACG)
CCCG
AG1
CAG
AG
CCG
CG
G1
G

FIGURE 8

Analysis of mRNA for Pseudouridine

mRNA was alkali digested and electrophoresed to separate the mononucleotides as described in the Methods section 3.5.8.

The region of the paper corresponding to Up was cut out, eluted with water and subjected to descending chromatography in an isopropanol-HCl-H_2O system, 68:17.6:14.4 v/v.

Sample 1, 9 = marker Up

Sample 2, 8 = marker Ψp

Sample 3 - 7 = mRNA


U
Ψ
U
Ψ
1 2 3 4 5 6 7 8 9

containing the sequence C-G. Quantitative data on this point are presented in section 2.5.

2.4. Analysis of mRNA for Pseudouridine

The synthesis of tRNA (transfer RNA) is not affected by low levels of actinomycin D. Therefore tRNA is a possible source of contamination of the mRNA preparations. As well as containing methylated oligonucleotides tRNA contains unusual nucleotides, one of which is pseudouridylic acid. Analysis for pseudouridine was carried out as described in the Methods section 3.6.9. The results showed no apparent pseudouridine in the mRNA preparations. Thus the mRNA was judged free of tRNA and rRNA contamination.

2.5. Quantitation of Oligonucleotides in T_1 Ribonuclease Fingerprints of mRNA

Although a qualitative difference in C-G sequence content is observed between mRNA and HnRNA on one hand, and 28S rRNA on the other, it is not possible to say whether mRNA and HnRNA are the same or different without quantitating the data accurately.

2.5.1. Quantitative Comparison of Sequences Occurring in mRNA and HnRNA

Autoradiography is useful for positioning oligonucleotides on two dimensional fingerprint. It is also useful for estimating the relative amounts of various oligonucleotides in a particular fingerprint. However it is of limited use for comparing the relative amounts of oligonucleotides from different fingerprints as the length of exposure and development of each X-ray film will affect the intensity of the spots. To compare different fingerprints accurately, the amount of radioactivity

TABLE 2

Relative Molar Oligonucleotide Frequences in T_1 Ribonuclease Digests of mRNA and hnRNA

	G	CG	C_2G	C_3G
mRNA	100(100)	12.4±3.3(21.6)	4.0±1.4(4.7)	1.2±0.5(1.0)
hnRNA	100(100)	4.6±1.6(20.5)	1.2±0.4(4.2)	0.7±0.7(0.9)
		AG	A_2G	A_3G
mRNA		37.0±10.6(32.4)	12.5±1.1(10.5)	2.7±0.5(3.4)
hnRNA		29.8±5.4(27.3)	7.2±1.4(7.4)	1.9±0.4(2.0)
		UG	U_2G	U_3G
mRNA		24.3±3.0(24.8)	6.5±0.6(6.2)	2.4±0.5(1.5)
hnRNA		25.3±1.8(30.1)	8.4±0.2(9.1)	3.0±0.5(2.7)
		UCG	ACG	VAG
mRNA		1.8±0.8(5.4)	2.4±0.9(7.0)	2.6±0.5(8.0)
HnRNA		0.9±0.5(6.2)	0.9±0.2(5.6)	5.2±2.2(8.2)
		CUG	CAG	AUG
mRNA		10.6±2.1(5.4)	8.1±3.4(7.1)	10.6±2.1(8.0)
HnRNA		9.6±2.8(6.2)	7.4±0.9(5.6)	8.4±2.2(8.2)
	C	A	G	U
mRNA	21.6±1.4	32.5±4.0	21.0±1.4	24.8±3.4
hnRNA	20.5±1.6	27.3±1.7	21.8±3.1	30.2±1.6

For method of determination see Methods section. These results are the average of five independent experiments ± standard deviations. The figures in brackets are theoretical values calculated from the base composition.

present in selected spots of the two-dimensional electrophoretograms must be cut out and measured for radioactivity.

mRNA was prepared from the polysomal RNA of cells labelled for 3 hours with $^{32}PO_4$ in the presence of actinomycin D by the "millipore filtration" technique. HnRNA was prepared as described in the Methods section.

Table 2 shows relative molar oligo-nucleotide frequencies of T_1 RNase digests of mRNA and HnRNA calculated as described in the Methods section 3.6.5. From this data it can be seen that although there is a large deficiency in oligonucleotides containing the sequence C-G in HnRNA, there is a less noticeable deficiency in mRNA. More precisely, the C-G deficiency seen in the C-Gp, C-C-Gp, ... $(C-)_n$ Gp series of oligonucleotides in T_1 ribonuclease digests of HnRNA is much less marked for C-Gp itself, and there is no deficiency in C-C-Gp or C-C-C-Gp. There is however deficiency in U-C-Gp and A-C-Gp, though again, not as marked as in HnRNA.

A further difference between mRNA and HnRNA can be seen in the sequence U-A-Gp. This sequence occurs less frequently than its isomer A-U-Gp in mRNA and HnRNA. However the relative molar oligonucleotide frequency of U-A-Gp in mRNA is half that of HnRNA. Thus it would seem that HnRNA, destined to become mRNA, is more deficient in U-A-Gp than total HnRNA.

2.5.2. Comparison of mRNA prepared by "Millipore Filtration" and Gradient Fraction Selection

Messenger RNA was prepared from the polysomal RNA of cells

TABLE 3

Relative Molar Oligonucleotide Frequences in T_1 Ribonuclease Digests of mRNA, Prepared by Different Techniques

	G	CG	C_2G	C_3G
Millip.	100(100)	11.2±2.2(17.4)	4.0±0.9	0.8±0.3(0.5)
Grad.	100(100)	11.19±3.5(24.7)	5.9±0.5	1.6±0.4(1.5)
		AG	A_2G	A_3G
Millip.		39.6±11.5(40.6)	14.3±5.0(16.5)	3.9±1.9(6.7)
Grad.		36.9±0.6(25.1)	13.9±0.8(6.3)	2.6±0.2(1.6)
		UG	U_2G	U_3G
Millip.		27.7±9.6(21.9)	6.5±2.8(4.8)	2.5±1.1(1.1)
Grad.		23.8±1.5(24.1)	6.8±1.2(5.8)	1.9±0.6(1.4)
	UCG	CUG	ACG	CAG
Millip.	1.1±0.6(3.8)	12.1±4.5(3.8)	2.2±0.4(7.1)	7.0±2.4(7.1)
Grad.	1.9±0.4(5.9)	12.0±3.4(5.9)	2.1±0.7(6.2)	9.3±1.2(6.2)

Base Composition

	C	A	G	U
Millip.	17.4±1.6	40.6±8.6	19.9±4.5	21.9±3.8
Grad.	24.7±0.2	25.1±0.9	26.1±0.7	24.1±0.6

These results are the average of 3 fingerprints ± standard deviations from one 16 hr labelling experiment for "Gradient" prepared mRNA, and 5 fingerprints ± standard deviations from three 16 hr labelling experiments for "Millipore" prepared mRNA.

labelled with $^{32}PO_4$ for 16 hours in the presence of low levels of Actinomycin D. Either the "millipore filtration" technique or the sucrose density gradient fractionation technique was used to purify the mRNA. Comparison of RNA prepared by the two techniques should reveal if there is any difference between the poly (A) containing mRNA, as prepared by "millipore filtration", and total mRNA (poly (A) containing and poly (A) minus) as prepared by sucrose density gradient analysis in terms of oligonucleotide composition.

Table 3 shows the relative molar oligonucleotide frequencies of T_1 RNase digests of mRNA prepared by both techniques.

Apart from the increase in oligo (A) containing sequences in the mRNA prepared by "millipore filtration" the two RNA preparations appear to be very similar with regard to lack of C-Gp containing oligonucleotides. Thus we may conclude that the different preparations of mRNA are similar.

3. SEPARATION OF LONGER SEQUENCES

From T_1 RNase digestion and two dimensional electrophoresis of RNA, it is only possible to separate short oligonucleotides. To show that the apparent deficiency of oligonucleotides containing the sequence C-G is a general trend and not simply confined to small oligonucleotides, RNA was subjected to both T_1 RNase and alkaline phosphatase digestion prior to fingerprinting. Alkaline phosphatase removes the terminal phosphate group of each oligonucleotide and those dephosphorylated nucleotides migrate electrophoretically much faster on DEAE paper. Also the second dimension of electrophoresis was run for 32 hours instead of the normal 16 hours used for a T_1 RNase digest. This technique achieves a greater separation of longer oligonucleotides and thus allows us to study tetra and pentanucleotides such as U-U-C-G and C-U-U-G. Figure 9 shows T_1 RNase plus phosphatase digests of HnRNA, mRNA and 28S rRNA after two dimensional electrophoresis. It will be noted that the sequences ending in C-G always run further in the second dimension of electrophoresis than their corresponding isomers. Furthermore from Table 4 it will be seen that as with T_1 RNase digestion fingerprints, the C-G containing oligonucleotides in T_1 + Phosphatase fingerprints are deficient, compared with their corresponding isomers, in both HnRNA and mRNA but not 28S rRNA.

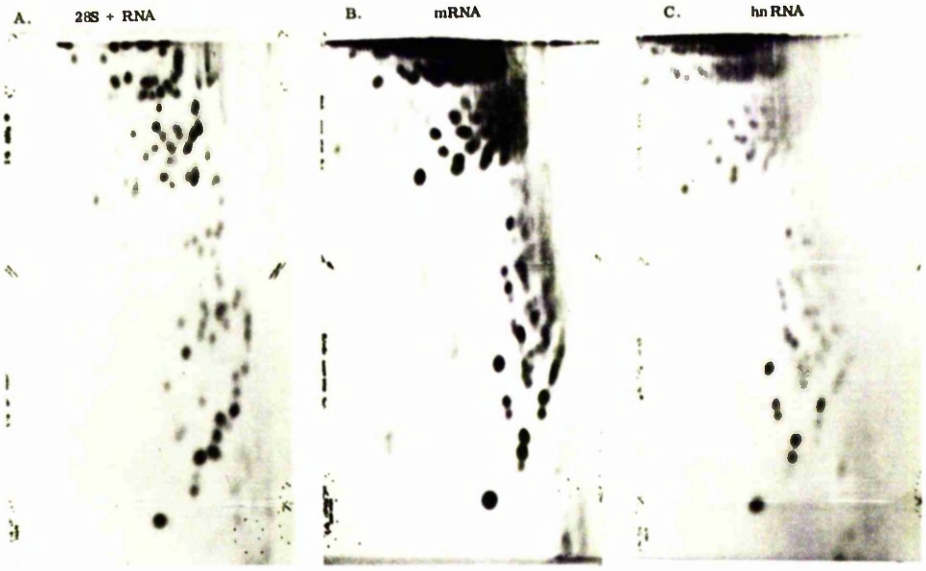
In summary, the results so far presented show that 1.) HnRNA is very deficient in C-Gp terminated sequences; 2.) mRNA has also a similar deficiency, though much less marked; 3.) mRNA also differs from HnRNA in that it has a greater deficiency of U-A-Gp as compared with A-U-Gp. On the basis of this, further experiments were directed towards analysing the effect of labelling time and size class of HnRNA on these results.

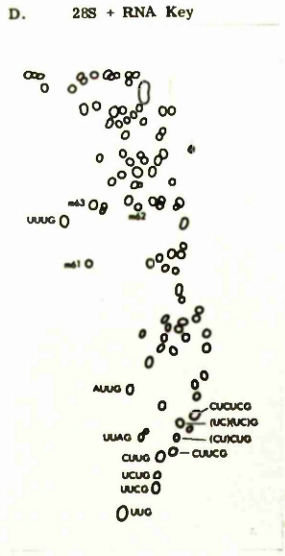
FIGURE 9

"T_1 + Phosphatase" Fingerprints of HnRNA, mRNA and 28S rRNA

RNA was prepared and fingerprinted as described in the Methods section. First dimension, right to left, cellulose acetate, pH 3.5 (5% acetic acid, 7 M urea). Second dimension, downwards, DEAE paper, 7% formic acid. The second dimension was run for 36 hours approximately.

Sequences shown in the "Keys" were deduced from partial venom digestion and comparison to published sequences in "T_1 + phosphatase" fingerprints (Felner *et al.*, 1970). The three partially sequenced pentanucleotide spots are probably impure.


A. 28S + RNA
B. mRNA
C. hn RNA


D. 28S + RNA Key
m43
m42
m41
UUUG
AUUG
UUAG
CUUG
UCUG
UUCG
UUG
CUCUCG
(UC)(UC)CG
(CU)CUG
CUUCG

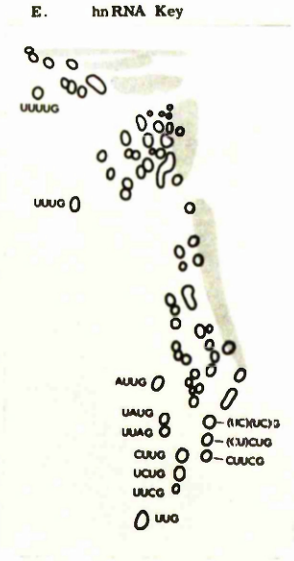

E. hn RNA Key
UUUUG
UUUG
AUUG
UAUG
UUAG
CUUG
UCUG
UUCG
UUG
(UC)(UC)3
(CU)CUG
CUUCG

TABLE 4

Relative Molar Oligonucleotide Frequences in T_1 + Phosphatase Digests of HnRNA, mRNA and 28S rRNA

Sequence	Relative Molar Oligonucleotide Frequency		
	HnRNA	mRNA	28S rRNA
U-U-G	6.8	5.8	2.9
U-U-C-G	0.22	0.19	0.23
U-C-U-G	0.76	0.81	0.10
C-U-U-G	0.73	0.69	0.67
U-U-A-G	0.54	0.30	0.51
U-A-U-G	0.49	0.44	0.29
A-U-U-G	0.59	0.63	0.33
C-U-U-C-G	0.14	0.11	0.08
(UC)-C-U-G	0.29	0.38	0.06
(UC)-(UC)-G	0.56	0.52	0.11
U-U-U-G	0.82	0.94	0.21
U-U-U-U-G	0.43	0.38	0.19

Base Composition

RNA	C	A	G	U
HnRNA	22.5	27.0	21.8	28.6
mRNA	20.9	37.7	19.6	21.8
28S rRNA	34.3	15.2	33.3	17.1

These results are the average of two fingerprints. Relative molar oligonucleotide frequences were calculated relative to U-U-G = 100 and then converted relative to G = 100 using the value of U-U-Gp from T_1 RNase fingerprints of the same RNA.

4. EFFECT OF LABELLING TIME AND ACTINOMYCIN D ON RELATIVE MOLAR OLIGONUCLEOTIDE FREQUENCIES

4.1. Effect of Labelling Time

As was mentioned in section 2 of the Introduction, mRNA has a half-life which is much longer than 3 hours. Therefore to prepare the highest possible specific activity of mRNA, cells should be incubated with $^{32}PO_4$ for longer periods of time than 3 hours. Table 5 shows the specific activity obtained after labelling HnRNA and mRNA with $^{32}PO_4$ (as described in the Methods section).

TABLE 5

Specific Activity of RNA Labelled with $^{32}PO_4$ for Differing Times

Time of Incubation	cpm/µg	
	HnRNA	mRNA
3 hr	71×10^3	5×10^3
16 hr	47×10^3	39×10^3

It will be noted that although the specific activity of the mRNA increases 8 fold on increasing the labelling time from 3 hours to 16 hours, the specific activity of HnRNA drops by 34%. These results reflect the fact that HnRNA turns over much faster than mRNA.

Tables 6 and 7 show the effect of labelling for various lengths of time on the relative molar oligonucleotide frequencies of mRNA and HnRNA respectively. Little if any difference in the relative molar oligonucleotide frequencies of mRNA at these different times is shown in table 6. This implies that the $^{32}PO_4$ label has had time to equilibrate between the four nucleotide precursors of mRNA. Furthermore it suggests

TABLE 6

mRNA-Incorporation of ^{32}P for various lengths of time

RNA	G	CG	C_2G	C_3G
3 hr	100(100)	12.4±3.3(21.6)	4.0±1.4(4.7)	1.2±0.5(1.0)
9 hr	100(100)	13.9±1.8(20.9)	4.9±0.5(4.3)	1.3±0.3(0.9)
16 hr	100(100)	11.2±2.2(17.4)	4.0±0.9(3.0)	0.9±0.3(0.5)
		AG	A_2G	A_3G
3 hr		37.0±10.6(32.4)	12.5±1.1(10.5)	2.7±0.5(3.4)
9 hr		31.6±4.3(37.7)	10.5±2.9(14.2)	2.1±0.4(5.3)
16 hr		39.6±11.5(40.6)	14.3±5.0(16.5)	3.9±1.9(6.7)
		UG	U_2G	U_3G
3 hr		24.3±3.0(24.8)	6.5±0.6(6.2)	2.4±0.5(1.5)
9 hr		23.3±10.4(21.8)	5.8±0.4(4.7)	2.0±0.4(1.0)
16 hr		27.7±9.6(21.9)	6.5±2.8(4.8)	2.5±1.1(1.1)
		UCG	ACG	UAG
3 hr		1.8±0.8(5.4)	2.4±0.9(7.0)	2.6±0.5(8.0)
9 hr		1.3±0.1(4.6)	2.7±0.7(7.9)	2.5±0.2(8.2)
16 hr		1.1±0.6(3.8)	2.2±0.4(7.1)	2.7±0.5(8.9)
		CUG	CAG	AUG
3 hr		10.6±2.1(5.4)	8.1±3.4(7.0)	10.6±2.1(8.0)
9 hr		10.7±3.9(4.6)	10.1±0.4(7.9)	7.4±0.4(8.2)
16 hr		12.1±4.5(3.8)	7.0±2.4(7.1)	11.5±4.5(8.9)

Base Analysis

	C	A	G	U
3 hr	21.6±1.4	32.5±4.0	21.0±1.6	24.8±3.4
9 hr	20.9±0.6	37.7±0.9	19.6±0.1	21.8±1.7
16 hr	17.4±1.6	40.6±8.6	19.9±4.5	21.9±3.8

The 3 hour results are the average of 5 experiments, the 9 hour results are the average of 2 fingerprints, and the 16 hour results are the average of 5 fingerprints from 3 experiments. The figures in brackets are theoretical values calculated from the base composition.

TABLE 7

HnRNA-Incorporation of ^{32}P for various lengths of time

	G	CG	C_2G	C_3G
3 hr	100(100)	4.6±1.6(20.5)	1.2±0.4(4.2)	0.7±0.7(0.9)
9 hr	100(100)	6.8 (29.0)	2.8 (8.4)	0.9 (2.4)
16 hr	100(100)	8.7±2.5(19.1)	2.9±0.8(3.6)	1.0±0.3(0.7)
		AG	A_2G	A_3G
3 hr		29.8±5.4(27.3)	7.2±1.4(7.4)	1.9±0.4(2.0)
9 hr		33.2 (30.3)	6.5 (9.2)	1.4 (2.8)
16 hr		38.4±4.5(26.6)	10.6±1.4(7.1)	2.9±0.8(1.9)
		UG	U_2G	U_3G
3 hr		25.3±1.8(30.1)	8.4±0.2(9.1)	3.0±0.5(2.7)
9 hr		32.3 (22.6)	6.8 (5.1)	1.7 (1.2)
16 hr		27.7±2.6(29.6)	7.1±1.0(8.8)	2.2±0.6(2.6)
		UCG	ACG	UAG
3 hr		0.9±0.5(6.2)	0.9±0.2(5.6)	5.2±2.2(8.2)
9 hr		1.2 (6.5)	1.5 (8.8)	5.9 (7.7)
16 hr		1.1±0.5(5.6)	1.8±0.4(5.1)	4.3±0.7(7.9)
		CUG	CAG	AUC
3 hr		9.6±2.8(6.2)	7.4±0.9(5.6)	8.4±2.2(8.2)
9 hr		9.7 (6.5)	9.2 (8.8	10.5 (7.7)
16 hr		10.9±0.5(5.6)	6.8±2.0(5.1)	9.3±3.0(7.9)

Base Composition

	C	A	G	U
3 hr	20.5±1.6	27.3±1.7	21.8±3.1	30.2±1.6
9 hr	29.0	30.3	18.1	22.6
16 hr	19.1±2.9	26.6±2.5	22.7±1.4	29.6±4.0

The 3 hour results are the average of 5 experiments, the 9 hour result is from one fingerprint only, and the 16 hour result is the average of 5 fingerprints from 3 experiments. The figures in brackets are theoretical values calculated from the base composition.

that any differences between the HnRNA and mRNA frequency patterns is unlikely to be due to variations in the labelling of the nucleotide precursors. Table 7 shows the equivalent results for HnRNA as those shown in table 6 for mRNA. It will be noted that although the relative molar oligonucleotide frequency for mRNA oligonucleotides ending in the sequence C-Gp are fairly constant at various labelling times, the HnRNA oligonucleotides of the $(C-)_n$ Gp series, especially C-Gp itself, is not constant. In fact the relative molar oligonucleotide frequency of oligonucleotides of the $(C-)_n$ Gp series in HnRNA seem to increase towards the level of those of mRNA when labelled for long periods of time. Figure 10 summarises the apparent change in deficiency of C-G containing oligonucleotides.

The increase in value of certain oligonucleotides containing the sequences C-G in HnRNA on increasing the labelling time of HnRNA could be explained in several ways. One explanation is the possible contamination of HnRNA by small amounts of mRNA. At short labelling times the specific activity of the mRNA would not be high enough to affect the result, but after longer labelling times the specific activity of such a contamination might rise to a significant level. Nevertheless it would require large amounts of this contaminating mRNA to give values for C-Gp containing sequences which approach those gained in HnRNA after 16 hours of labelling. For example, in terms of the relative molar oligonucleotide frequency of the sequence C-Gp itself, it would require approximately 50% contamination with mRNA. However this value of contamination would not give the observed value for any other C-Gp containing sequence. Therefore contamination of HnRNA by mRNA does not appear to be the correct answer.

FIGURE 10

Variation of C-Gp Sequence Deficiency with Time

From the data given in tables 6 and 7, the value of the relative molar oligonucleotide frequency divided by the theoretical molar oligonucleotide frequency was calculated. These values were plotted against time of incubation with $^{32}PO_4$.

A = HnRNA

B = mRNA

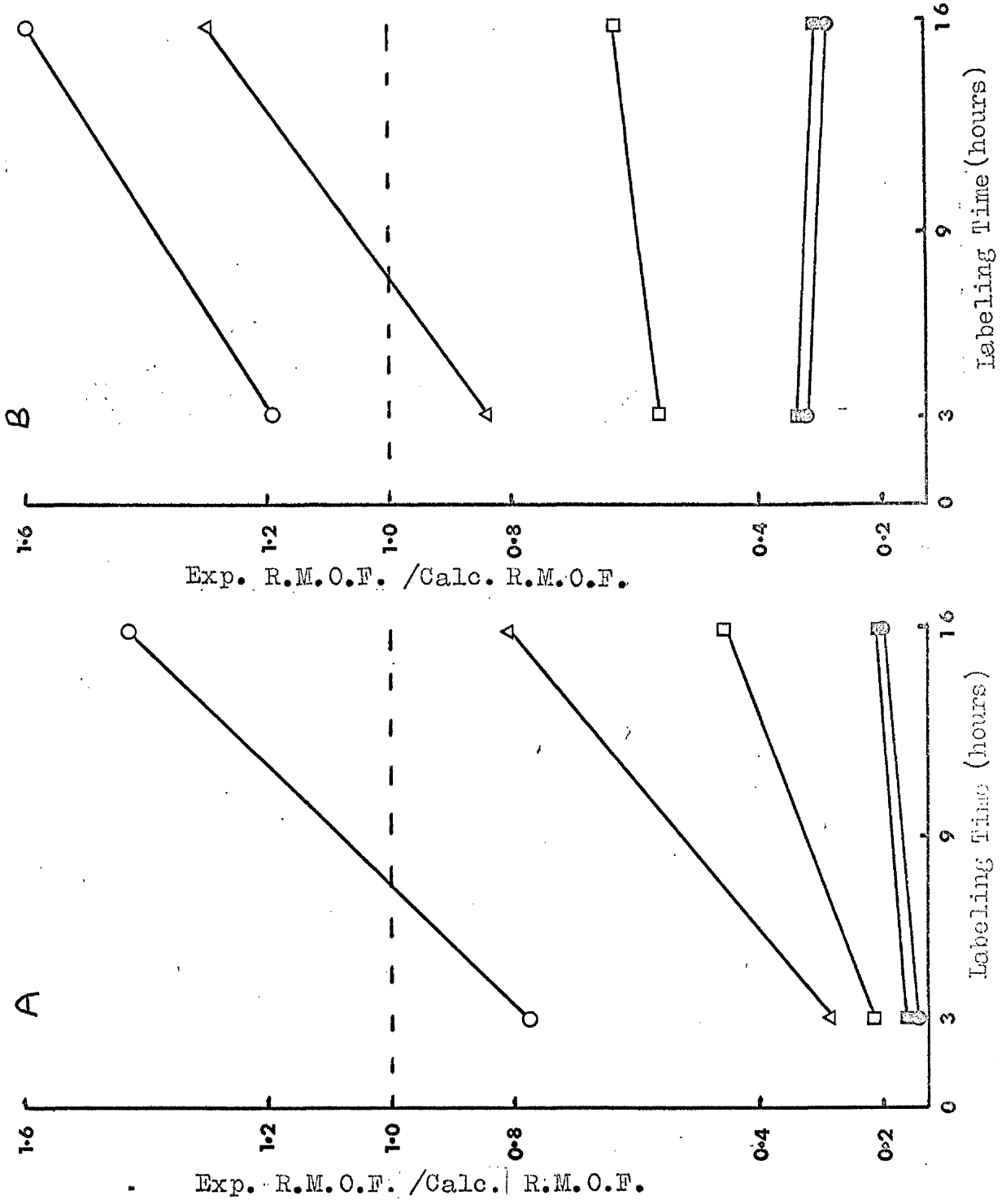
○ = U-C-Gp

□ = A-C-Gp

□ = C-Gp

△ = C-C-Gp

○ = C-C-C-Gp


A
B
1·6
1·2
1·0
0·8
0·4
0·2
0
3
9
16
Exp. R.M.O.F. /Calc. R.M.O.F.
Labeling Time (hours)

From formamide/sucrose gradient analysis (section 5.3) it will be seen that although HnRNA prepared from cells labelled for 16 hours with $^{32}PO_4$ in the presence of actinomycin D does contain some large molecules, of similar size to those produced in cells labelled for 3 hours, substantially more of the molecules are of lower molecular weight. From experiments in which cells are treated with low levels of actinomycin D for 13 hours before being labelled for 3 hours with $^{32}PO_4$, we can specifically study the RNA produced towards the end of a 16 hour labelling experiment. Formamide/sucrose gradient analysis of this RNA (section 5.3) indicates that the HnRNA produced towards the end of long labelling experiments is substantially small in size (figure 14). In summary with increasing labelling time, an increasing proportion of relatively small HnRNA molecules appear to be produced. However, a simple contamination of the nuclear material with mRNA is ruled out as C-Gp containing sequences do not all change in the manner in which one would expect if this were the case.

A second possibility is that after prolonged labelling of the cells in the presence of low levels of actinomycin D (0.04 µg/ml) a low level of rRNA synthesis becomes noticeable. The production of only a s: proportion of rRNA could account for the increase in C-Gp containing sequences. However as there is 1.) little increase in the A-C-Gp and U-C-Gp relative molar oligonucleotide levels to match that in the $(C-)_nGp$ series of oligonucleotides and 2.) no sign of Gm-Gp the strongest rRNA methylated spot, it seems unlikely that this is the correct reason.

A third possibility is that the increase in $(C-)_nGp$ sequence frequency is due to the effect of ^{32}P radioactivity, or low phosphate

levels in the culture medium, which are disrupting the normal cell metabolism in some unknown manner after long incubation times. However mRNA relative molar oligonucleotide frequencies do not change therefore this possibility is less likely.

The fourth possibility is that we are observing an effect of actinomycin D which is causing selective cessation of the synthesis of some HnRNA species. These species would have a low C-Gp sequence content and thus an overall increase in C-Gp sequence content would be observed with time. Penman et al. (1960) has shown 30% reduction of HnRNA production in similar concentrations of actinomycin D as used in this work.

Alternatively we could be observing the build up of some relatively stable species of HnRNA (perhaps a mRNA precursor as it has a high C-Gp sequence content) while the bulk of HnRNA is rapidly turning over in the nucleus.

4.2. Effect of Actinomycin D

That HnRNA synthesised in the presence of actinomycin D is similar to that produced in its absence was difficult to demonstrate. All attempts to obtain pure HnRNA from cells labelled in the absence of actinomycin D led to the appearance of faint methylated spots (especially Gm-Gp). Accurate correction for the percentage contamination of HnRNA by rRNA would have required the quantitation of several methylated spots. This was not possible as only Gm-Gp had sufficient radioactivity to be measured. Furthermore the radioactivity present in Gm-Gp was often at the limits of detection.

FIGURE 11

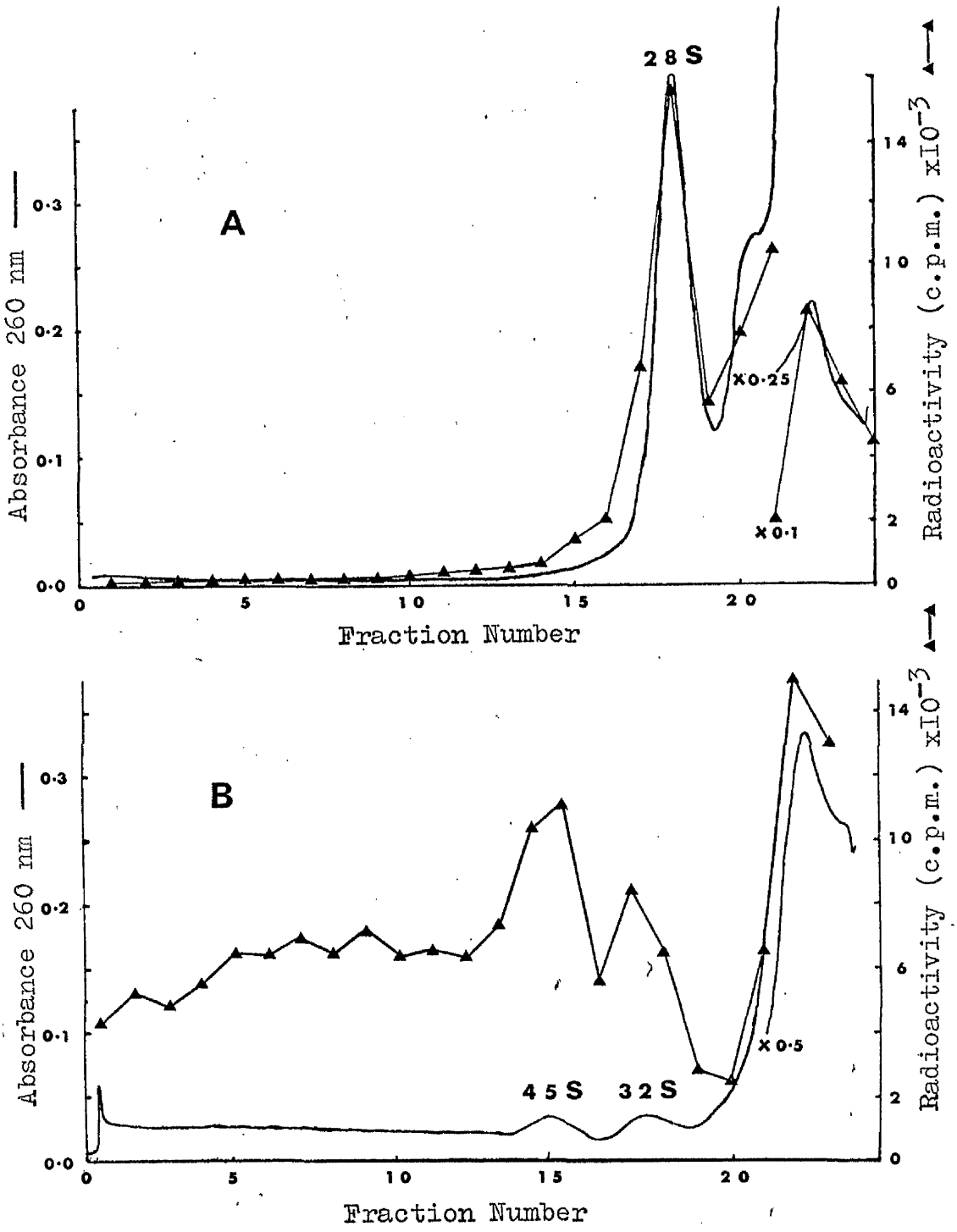
Sucrose Density Gradient Analysis of Differential Thermal Phenol Extracts

Nuclei were prepared from cells grown with $^{32}PO_4$ for 3 hours without the presence of any actinomycin D. Nuclear RNA was then prepared from the nuclei by DNase treatment, as described in the Methods (section 3.2.2) followed by cold phenol extraction and then by hot phenol extraction, in that order.

The two phenol extracts were ethanol precipitated, dissolved in LETS buffer, and loaded onto 15% - 30% sucrose/LETS density gradients. These were centrifuged for 16 hours at 16,000 RPM in a Spinco SW27 centrifuge rotor at 20°C. Gradients were pumped, from the bottom, through the flow-cell of a Gilford recording spectrophotometer and approximately 1.6 ml fractions collected. 10 µl samples were counted for radioactivity as described in the Methods section 3.4.5.

A = 20°C extract

B = 65°C extract


A
28S
×0·25
×0·1
Absorbance 260 nm
Radioactivity (c.p.m.) x10⁻³
Fraction Number
B
45S
32S
×0·5
Absorbance 260 nm
Radioactivity (c.p.m.) x10⁻³
Fraction Number

An attempt at picking out poly (A) containing RNA by "millipore filtration" failed to produce HnRNA free from ribosomal contamination even after two cycles of "millipore filtration".

In 1962, Georgiev and Mantieva developed the technique of differential thermal extraction of nuclear RNAs. In 1973 Markov and Arion successfully used this procedure to obtain different types of nuclear RNA without preliminary isolation of nuclei and nucleoli. Therefore it seemed that this technique could be useful in purifying HnRNA labelled in the absence of actinomycin D. Figure 11 shows the result of this type of extraction procedure on HeLa nuclear RNA. Figure 11A shows the RNA extracted at room temperature and appears to be mainly low molecular weight RNA. Figure 11B shows the effect of elution at 65°C after the room temperature extraction had been performed. It shows that high molecular weight nuclear RNA is being extracted, however it also shows that nucleolar precursor rRNA is also being extracted at this temperature. Therefore to make use of this technique some further work must be done to find an intermediate temperature at which nucleolar, but not nucleoplasmic RNA will be extracted.

An alternative method used to study the effect of actinomycin D was to incubate cells for different lengths of time with actinomycin D and then label for a standard time (3 hr) with $^{32}PO_4$. The results of this type of experiment are shown in table 8. Table 8 seems to suggest that HnRNA produced after prolonged exposure to actinomycin D could be of higher C-G-sequence content than that produced with short exposure to actinomycin D. These results would support the hypothesis that the effect of increased C-G-sequence content is due either to selective cessation of HnRNA synthesis or to the build up of a long half-life precursor to mRNA.

TABLE 8

Effect of Actinomycin D on HnRNA

Time of incubation with actinomycin D.	G	CG	C_2G	C_3G
3½ hr	100(100)	4.6±1.6(20.5)	1.2±0.4(4.2)	0.7±0.7(0.9)
16½ hr	100(100)	6.7±0.2(19.3)	2.1±0.4(3.7)	1.0±0.1(0.7)
		AG	A_2G	A_3G
3½ hr		29.8±5.4(27.3)	7.2±1.4(7.4)	1.9±0.4(2.0)
16½ hr		43.0±4.6(28.8)	12.7±1.4(8.3)	3.6±0.4(2.4)
		UG	U_2G	U_3G
3½ hr		25.3±1.6(30.1)	7.5±0.5(9.1)	3.0±0.5(2.7)
16½ hr		38.1±4.3(31.5)	12.5±1.7(9.9)	9.9±1.0(3.1)
		UCG	ACG	UAC
3½ hr		0.9±0.5(6.2)	0.9±0.2(5.6)	5.2±2.2(8.2)
16½ hr		1.6±0.3(6.1)	1.7±0.3(5.6)	11.5±0.9(9.1)
		CUG	CAG	AUG
3½ hr		9.6±2.8(6.2)	7.4±0.9(5.6)	8.4±2.2(8.2)
16½ hr		11.5±0.9(6.1)	8.8±0.7(5.6)	7.2±0.1(9.1)

Base Compositions

	C	A	G	U
3½ hr	20.5±1.6	27.3±1.7	21.8±3.1	30.2±1.6
16½ hr	19.3±0.4	28.8±0.2	20.2±0.3	31.5±0.4

The 16½ hour incubation with actinomycin D results are the average of 3 fingerprints from two experiments. The figures in brackets are theoretical values calculated from the base composition.

5. SIZE OF HnRNA

5.1. Preparation of Size Classes of HnRNA

Since mRNA is a heterodisperse collection of molecules, relatively small compared to HnRNA, it is a widely held belief that, perhaps, the large HnRNA molecules are progressively cleaved by nucleases to form smaller molecules such as mRNA. For this reason, size fractions of HnRNA were prepared from 15% - 30% sucrose/LETS gradients and tested to see if there was any trend towards mRNA-like relative molar oligonucleotid frequency on progressing from large HnRNA to small HnRNA molecules.

Figure 12 shows a sucrose/LETS density gradient profile of HnRNA. Three size fractions of HnRNA were collected. These were called heavy, medium and light HnRNA and approximated to the following sizes: > 55S, 55S - 35S and 35S - 18S.

5.2. Comparison of HnRNA Size Classes

When subjected to digestion with T_1 RNase and two dimensional electrophoresis, the data shown in Table 9 was obtained. From this data it seems that there is little noticeable difference in relative molar oligonucleotide frequency between the size classes of HnRNA.

It is possible that, because of the great heterogeneity in size of a population of HnRNA molecules at any one stage of maturation to mRNA, the size fractions of HnRNA did not represent populations of molecules at sufficiently different states of maturation to detect any difference in sequence composition. However the C-G-containing sequences in the smallest size fraction of HnRNA are marginally closer to the mRNA figures.

FIGURE 12

Sucrose Density Gradient Analysis of HnRNA

Nuclear RNA prepared as described in the Methods section was dissolved in LETS buffer. 1 ml was loaded onto a 15% - 30% sucrose/LETS density gradient and centrifuged for 16 hours at 15,000 RPM in a Spinco SW27 centrifuge rotor at 20°C. Gradients were pumped through the flow-cell of a Gilford recording spectrophotometer and approximately 1.6 ml fractions collected. 10 µl samples of each fraction were counted for radioactivity as described in the Methods section 3.4.5.

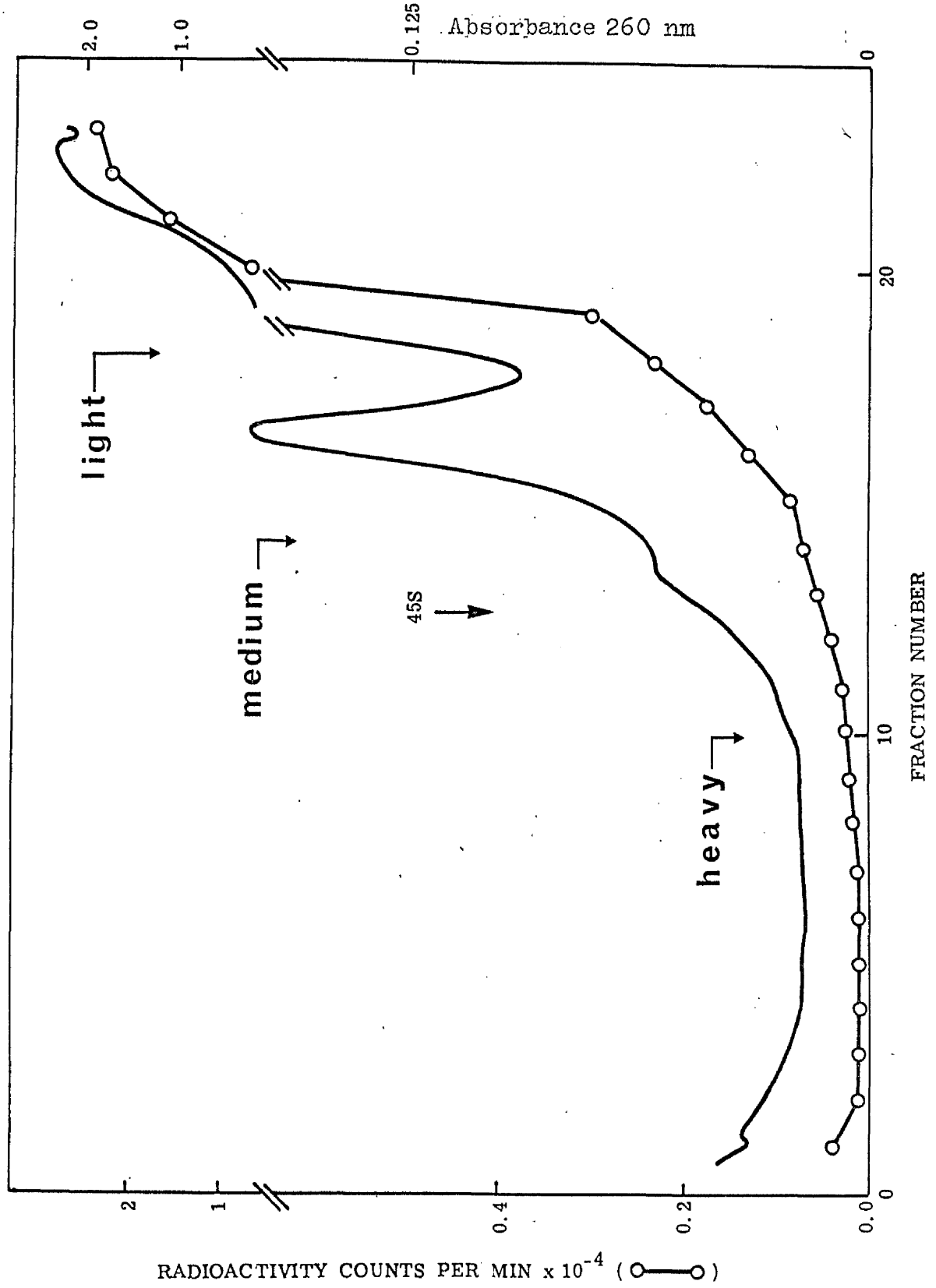

Absorbance 260 nm
2.0
1.0
0.125
0
light
medium
45S
heavy
20
10
0
FRACTION NUMBER
2
1
0.4
0.2
0.0
RADIOACTIVITY COUNTS PER MIN x 10^{-4} (O—O)

TABLE 9

Size classes of HnRNA

RNA	G	CG	C_2G	C_3G
Heavy	100(100)	4.6±1.6(20.5)	1.2±0.4(4.2)	0.7±0.7(0.9)
Medium	100(100)	5.1±0.9(21.2)	1.6±0.2(4.5)	0.5±0.2(1.0)
Light	100(100)	5.8±0.9(21.9)	2.0±0.1(4.8)	0.5±0.2(1.0)
		AG	A_2G	A_3G
Heavy		29.8±5.4(27.3)	7.2±1.4(7.4)	1.9±0.4(2.0)
Medium		37.0±10.6(25.5)	8.4±1.1(6.5)	2.4±0.5(1.6)
Light		26.4±9.7(24.6)	8.9±1.7(6.0)	2.2±0.2(1.5)
		UG	U_2G	U_3G
Heavy		25.3±1.8(30.1)	7.5±0.5(9.1)	3.0±0.5(2.7)
Medium		28.1±4.7(30.6)	7.6±2.9(9.4)	3.6±0.7(2.9)
Light		26.0±2.0(29.5)	7.1±0.7(8.7)	2.5±1.0(2.6)
		UCG	ACG	UAG
Heavy		0.9±0.2(6.2)	0.9±0.2(5.6)	5.2±2.2(8.2)
Medium		0.7±0.2(6.5)	0.9±0.4(5.4)	6.3±1.4(7.8)
Light		1.1±0.2(6.5)	0.9±0.2(5.4)	4.6±0.5(7.2)
		CUG	CAG	AUG
Heavy		9.6±2.8(6.2)	7.4±0.9(5.6)	8.4±2.2(8.2)
Medium		7.8±1.6(6.5)	7.8±1.6(5.4)	8.5±0.6(7.8)
Light		9.4±3.7(6.5)	9.1±13.8(5.4)	6.5±0.4(7.2)

Base Composition

	C	A	G	U	(Ave)
Heavy	20.5±1.6	27.3±1.7	21.8±3.1	30.2±1.6	(4)
Medium	21.2±0.6	25.5±3.0	20.6±0.9	30.6±3.2	(5)
Light	21.9±2.7	24.6±4.0	19.5±1.2	29.5±3.1	(4)

These results are the average of 5 independent experiments for the heavy and medium size fractions and 4 independent experiments for the light size fraction. The figures in brackets are theoretical values calculated from the base composition.

This small change is in agreement with the hypothesis that only a fraction of HnRNA acts as a precursor to mRNA.

Aggregation between HnRNA molecules of different sizes and at different stages of maturation would interfere with the results of sucrose density gradient analysis experiments such as those just described. To examine the possibility of aggregation the technique of formamide - sucrose density gradient analysis was used.

5.3. Formamide-Sucrose Density Analysis of RNA

Formamide has a very high dielectric constant and thus causes complete loss of polynucleotide structure (Helmkamp and Ts'O, 1961; Ts'O et al., 1962; Helmkamp et al., 1962). Thus by using formamide as a solvent for sucrose gradients we can be sure that no aggregation occurs.

5.3.1. HnRNA, mRNA and 28S rRNA

Figure 13 shows the results of formamide-sucrose density gradient analysis.

The HnRNA and mRNA were prepared from cells labelled for 3 hours with $^{32}PO_4$ in the presence of actinomycin D. It will be noted that a considerable proportion of the Hn (heavy) fraction is of very large size, much greater than the 28S rRNA, the remainder being a small amount of lower molecular weight material. However the Hn (medium) fraction appears to form a peak of radioactivity at approximately 28S or smaller size. This indicates a change in size from that observed in the ordinary sucrose gradients (55S - 35S). Thus it seems probable that although many of the HnRNA (heavy) size fraction molecules are large, most of the HnRNA (medium) size fraction consists of aggregate HnRNA molecules.

FIGURE 13

Formamide-Sucrose Density Gradient Analysis of HnRNA, mRNA and 28S rRNA

Radioactive RNA samples were lyophilised in small siliconised tubes. Samples were dissolved in 10 µl water and made to 0.5 ml with buffered formamide. After carefully layering onto the top of a 2 - 10% sucrose formamide gradient in a Spinco SW41 rotor at 25°C these samples were centrifuged at 24,000 RPM for 40 hours.

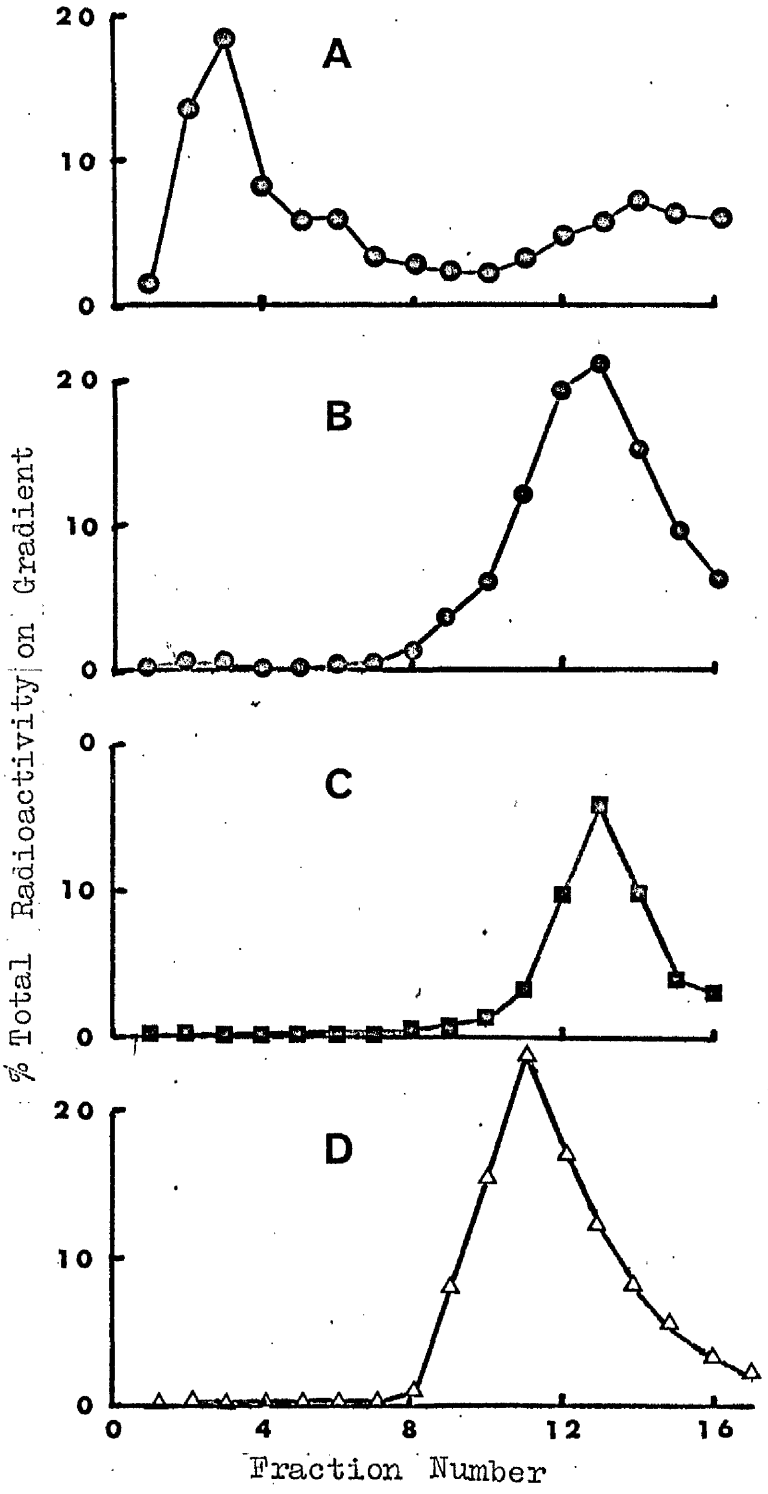
Gradients were pumped through the flow-cell of a Gilford recording spectrophotometer and approximately 0.8 ml fractions collected. 0.1 ml samples of each fraction were measured for radioactivity as described in the Methods (section 3.4.4.).

A = HnRNA (large size fraction)

B = HnRNA (medium size fraction)

C = mRNA

D = 28S rRNA


A
B
C
D
20
10
0
0
4
8
12
16
% Total Radioactivity on Gradient
Fraction Number

Always when HnRNA was prepared by centrifuging through 15% - 30% sucrose/LETS gradients some radioactivity was found to be pelleted at the bottom of the centrifuge tube. This RNA, when subjected to formamide-sucrose density gradient analysis was shown to be mostly very large. Thus it seems likely that there are very large molecules of RNA transcribed from the DNA of HeLa cells.

5.3.2. HnRNA and mRNA Radioactively Labelled for Different Time Intervals

In the previous section we have seen that very large molecules of HnRNA can be isolated from cells labelled with $^{32}PO_4$ for 3 hours. In this experiment cells were labelled for 16 hours with $^{32}PO_4$ in the presence of actinomycin D or were labelled for 3 hours with $^{32}PO_4$ after 13 hours pre-incubation with actinomycin D. HnRNA and mRNA were isolated from these cells and subjected to formamide-sucrose density gradient centrifugation.

From the results shown in figure 14 it will be noted that the HnRNA (heavy size fraction) produced after 13 hours pre-incubation with actinomycin D appears to contain a considerable proportion of lower molecular weight RNA molecules (figure 14A). This would seem to suggest that HnRNA (heavy size fraction) produced towards the end of a 16 hour period in the presence of actinomycin D, consists of aggregates of small molecules.

HnRNA (heavy size fraction) was also prepared from sucrose gradient analysis of nuclear RNA isolated from cells labelled with $^{32}PO_4$ for 16 hours in the presence of actinomycin D. This fraction when subjected to formamide-sucrose density gradient analysis reveals the presence of large, medium and small RNA molecules (figure 14B). Thus it seems that although large HnRNA molecules are produced during the early

FIGURE 14

Formamide-Sucrose Density Gradient Analysis of HnRNA and mRNA Radioactively Labelled for Different Time Intervals

Radioactive RNA samples were lyophilised in small siliconised tubes. They were then dissolved in 10 µl of water and made to 0.5 ml with buffered formamide. After carefully layering onto the top of 2% - 10% sucrose formamide gradients, these samples were centrifuged at 24,000 RPM in a Spinco SW41 centrifuge rotor at 25°C for 40 hours.

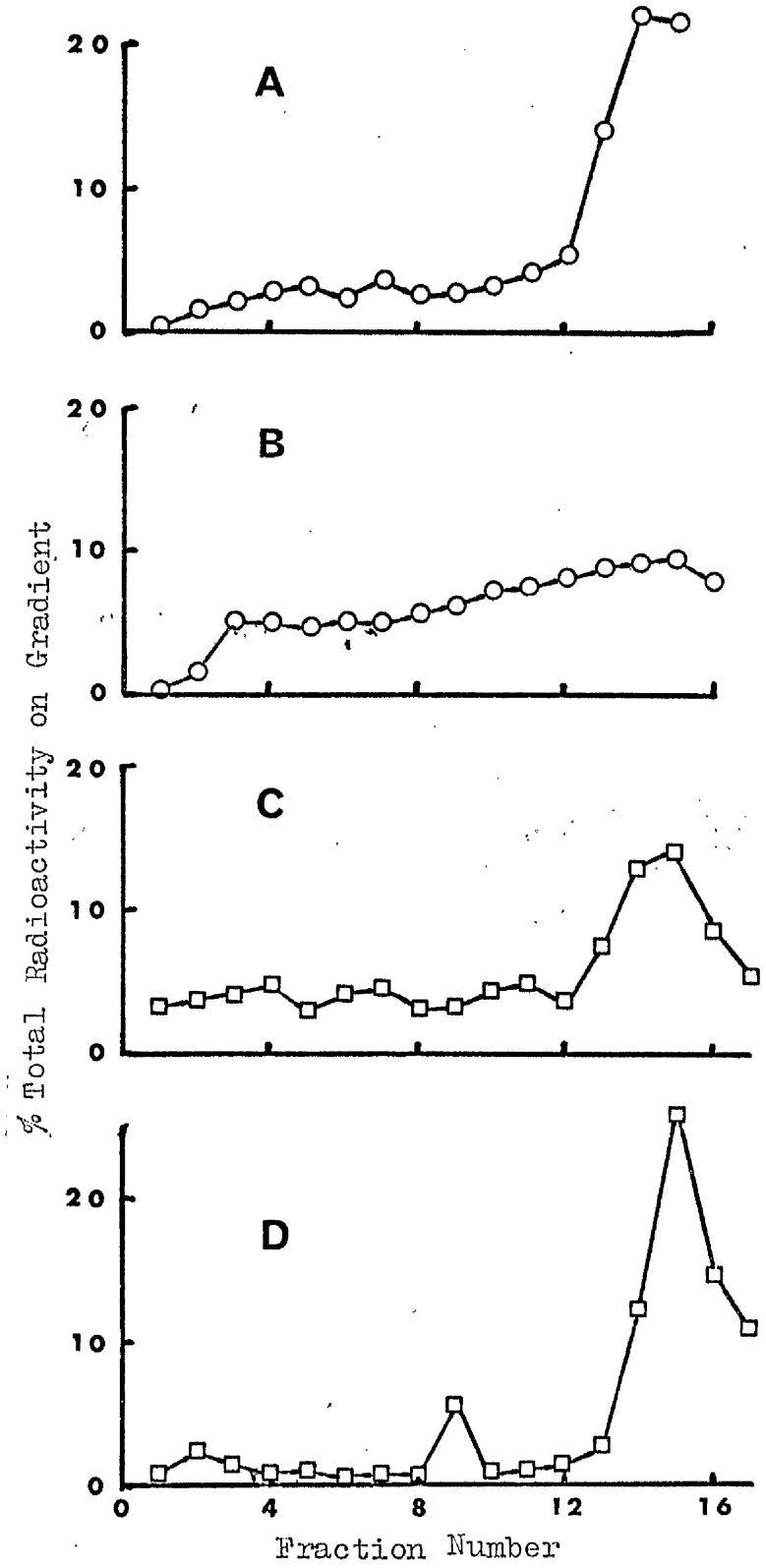
Gradients were pumped through the flow-cell of a Gilford recording spectrophotometer and approximately 0.8 ml fractions were collected. 0.1 ml samples of each fraction were measured for radioactivity as described in the Methods (section 3.4.4.).

A = HnRNA (heavy size fraction) - labelled for 3 hours with $^{32}PO_4$ after 13 hours incubation with low levels of actinomycin D.

B = HnRNA (heavy size fraction) - labelled for 16 hours with $^{32}PO_4$ in the presence of low levels of actinomycin D.

C = mRNA - labelled for 3 hours with $^{32}PO_4$ after 13 hours incubation with low levels of actinomycin D.

D = mRNA - labelled for 16 hours with $^{32}PO_4$ in the presence of low levels of actinomycin D.


A
B
C
D
20
10
0
% Total Radioactivity on Gradient
0
4
8
12
16
Fraction Number

stages of a 16 hour labelling period, they are not produced later on.

The mRNA formamide-sucrose gradients show that the mRNA produced from cells after a long pre-incubation with actinomycin D contains a greater proportion of large mRNA molecules than one would normally expect (figure 14C). However mRNA from cells labelled for 16 hours shows a distribution in size centred at approximately 18S with few molecules of really large size (figure 14D).

In summary it would seem that actinomycin D does affect the production and maturation of HnRNA molecules. On increasing the period of incubation with actinomycin D, increasingly fewer large HnRNA molecules are produced. This effect is coupled to a change in the relative molar oligonucleotide frequencies pattern obtained for HnRNA towards that for mRNA (as described in section 4).

6. FRACTIONATION ACCORDING TO HOMOPOLYMERIC SEQUENCE CONTENT

As described in the Introduction, although most mRNA and some HnRNA contain poly (A) regions, only HnRNA has been shown to contain oligo (U) regions. It has been proposed that some of the poly (A) containing HnRNA acts as a precursor to mRNA. Thus the fingerprinting of fractions of HnRNA containing poly (A) as well as those which lack poly (A) termini or have U-rich regions could throw some light on the relationship of these species to mRNA.

Poly (A) containing HnRNA was selected from total HnRNA by the "millipore filtration" technique. The oligo (U) containing HnRNA was then selected from the HnRNA which did not contain poly (A). This was accomplished by complexing the HnRNA with unlabelled poly (A) and selecting the complexed material by "millipore filtration" (Burdon and Shenkin, 1973). In this way various fractions of HnRNA were obtained. These fractions were a) poly (A) containing HnRNA b) oligo (U) containing HnRNA and c) HnRNA containing neither poly (A) nor oligo (U) regions.

Jelinek *et al.* (1973) found that 10 to 20% of small HnRNA molecules (< 32S) and about 20 to 40% of large HnRNA molecules (> 32S) bound to poly (U) sepharose. In this work 26% of HnRNA (medium size fraction) and 12% HnRNA (light size fraction) were typical results for "millipore filtration" of HnRNA. As these species of RNA only occur in small quantities, only a limited number of fingerprints were performed. When the oligo (U) containing HnRNA was isolated, the amount obtained varied from a few per cent to 23% of the amount of poly (A) containing RNA previously extracted. The variation in this figure may be due to the labile nature of oligo (U) containing RNA (Burdon *et al.*, 1974).

TABLE 10

Relative Molar Oligonucleotide Frequencies in T_1 Ribonuclease Digests of HnRNA fractionated According to Homopolymeric Sequence Content

	G	CG	C_2G	C_3G
HnRNA	100(100)	4.6±1.6(20.5)	1.2±0.4(4.2)	0.7±0.7(0.9)
HnRNA(+A)	100(100)	3.7±0.5(21.5)	1.3±0.4(4.6)	0.4±0.1(1.0)
HnRNA(+U)	100(100)	5.7±0.1(19.0)	2.4±0.7(3.6)	0.8±0.4(0.7)
HnRNA(-AU)	100(100)	7.4±1.1(18.6)	2.3±0.6(3.4)	0.8±0.1(0.6)
		AG	A_2G	A_3G
HnRNA		29.8±5.4(27.3)	7.2±1.4(7.4)	1.3±0.4(2.0)
HnRNA(+A)		26.4±4.0(27.4)	11.1±3.2(7.5)	2.6±0.4(2.1)
HnRNA(+U)		30.1±1.1(29.0)	9.2±0.3(8.4)	2.5±0.9(2.4)
HnRNA(-AU)		33.8±7.5(26.4)	8.1±0.2(7.0)	2.0±0.0(1.8)
		UG	U_2G	U_3G
HnRNA		25.3±1.8(30.1)	7.5±0.5(9.1)	3.0±0.5(2.7)
HnRNA(+A)		27.0±4.3(29.6)	8.4±0.2(9.1)	3.3±0.1(2.7)
HnRNA(+U)		29.4±0.2(25.8)	8.6±0.4(6.6)	3.9±0.8(1.7)
HnRNA(-AU)		25.9±3.9(31.2)	8.4±3.0(9.7)	2.8±1.2(3.0)
		UCG	ACG	UAG
HnRNA		0.9±0.5(6.2)	0.9±0.2(5.6)	5.2±2.2(8.2)
HnRNA(+A)		0.8±0.1(6.3)	1.1±0.4(5.7)	4.7±0.7(8.3)
HnRNA(+U)		1.8±0.7(4.9)	1.3 (5.5)	4.6±1.4(7.5)
HnRNA(-AU)		1.4±0.3(5.8)	1.4±0.7(4.9)	5.6±1.7(8.2)
		CUG	CAG	AUG
HnRNA		9.6±0.2(5.6)	7.4±0.9(5.6)	8.4±2.2(8.2)
HnRNA(+A)		6.4±0.1(6.3)	8.2±0.8(5.7)	5.9±1.0(8.3)
HnRNA(+U)		10.0±1.0(4.9)	6.8±0.9(5.5)	8.1±2.4(7.5)
HnRNA(-AU)		10.1±1.4(5.8)	7.4±2.2(4.9)	9.5±1.7(8.2)

Base Composition of RNA

RNA	C	A	G	U
HnRNA	20.5±1.6	27.3±1.7	21.8±3.1	30.2±1.6
HnRNA(+A)	20.8±1.4	27.4±2.1	21.7±0.7	30.2±1.4
HnRNA(+U)	19.0±10.5	29.0±0.9	26.1±9.1	25.8±2.3
HnRNA(-AU)	18.6	26.4	23.6	31.2

These results are the average of 5 experiments for HnRNA and 2 fingerprints from 2 experiments for the other RNA species. The figures in brackets are theoretical values calculated from the base composition.

Table 10 shows relative molar oligonucleotide frequencies for HnRNA fractionated according to homopolymeric sequence content. Although HnRNA containing a poly (A) region is believed to be a precursor to mRNA it will be seen that poly (A) containing HnRNA is similar to total HnRNA rather than to poly (A) containing mRNA. In fact none of the HnRNA fractions studied after 3 hours of labelling with $^{32}PO_4$ in the presence of actinomycin D appeared to be similar to mRNA.

On the other hand preliminary experiments (1 preparation only) with poly (A) containing HnRNA from cells labelled for 9 hours with $^{32}PO_4$ in the presence of actinomycin D, suggest that this RNA may have higher frequencies of C-Gp containing oligonucleotides.

RNA containing U-rich regions have also been isolated from polysomal RNA. This was done by complexing the millipore filtered polysomal RNA with non-radioactive poly (A) and passing through a millipore filter once more. However only very small quantities of this RNA were isolated. Table 11 shows the relative molar oligonucleotide frequency of two T_1 RNase fingerprints of this type of RNA. One is from a 3 hour $^{32}PO_4$ labelling experiment and one from a 9 hour $^{32}PO_4$ labelling experiment. It will be noted that this type of polysomal RNA does not have the relatively high frequency of oligonucleotides containing the C-Gp sequence that poly (A) containing polysomal RNA (mRNA) contains.

Attempts to characterise mRNA without poly (A) were unsuccessful because of the very small yields so far obtained. Experiments to prepare this type of mRNA were of the type used to prepare HnRNA containing neither poly (A) nor oligo (U) regions, only after the poly (A)

TABLE 11

Relative Molar Oligonucleotide Frequency of T_1 Ribonuclease Digests of Oligo(U) containing Polysomal RNA

	G	CG	C_2G	C_3G
3 hour	100(100)	5.8 (22.4)	2.9(5.0)	0.5(1.1)
9 hour	100(100)	7.5(17.8)	2.0(3.2)	0.4(0.6)
		AG	A_2G	A_3G
3 hour		31.7(30.1)	7.4(9.1)	1.0(2.7)
9 hour		30.2(35.0)	8.6(12.2)	1.8(4.3)
		UG	U_2G	U_3G
3 hour		26.4(26.3)	5.1(6.9)	1.2(1.8)
9 hour		23.7(26.8)	6.6(7.2)	2.0(1.9)
		UCG	ACG	UAG
3 hour		2.2(5.9)	2.6(6.7)	2.9(7.9)
9 hour		1.1(4.8)	1.7(7.7)	3.3(9.4)
		CUG	CAG	AUG
3 hour		11.2(5.9)	5.5(6.7)	8.6(7.9)
9 hour		8.9(4.8)	7.6(6.2)	6.9(9.4)

Base Composition

	C	A	G	U
3 hour	22.4	30.1	21.1	26.3
9 hour	17.8	35.0	20.4	26.8

The figures in brackets are theoretical values calculated from the base composition.

and oligo (U) containing RNA had been extracted the remaining radioactive RNA was subjected to sucrose density gradient analysis as outlined in section 2.2.1 (Sucrose Gradient Analysis of polysomal RNA). This step was performed to remove low molecular weight RNA.

Messenger RNA without poly (A) has been studied by Milçarek et al. (1974) working in the laboratories of Penman. She found that approximately 30% of the total mRNA labelled for a short time (90 min) or long (20 hr) period did not contain poly (A) regions. Furthermore cordycepin which inhibited 95% of the poly (A) containing mRNA only inhibited 60% of the mRNA without poly (A). Messenger RNA without poly (A) behaved like normal poly (A) containing mRNA in its sensitivity to EDTA and puromycin release from polysomes (Milcarek et al., 1974).

7. CHROMATIN ASSOCIATED HnRNA

As mentioned in the Introduction HnRNA occurs in association with proteins in the cell nucleus (see Introduction: 5. Ribonucleoprotein Particles). Because of these proteins HnRNA molecules appear to have a strong affinity for the chromatin of the cell nucleus. However Penman (1966) showed that if chromatin was digested with DNase this renders the HnRNA-protein particles soluble. Price et al. (1974) have isolated a small sub-fraction of the HnRNA-protein particles from total chromatin associated HnRNA by exposure of the chromatin to high salt concentrations.

The amount of HnRNA released (about 10%) was quite reproducible and it appeared to have the characteristics expected of a precursor to mRNA. The major reason for assigning significance to this subfraction of HnRNA was its large poly (A) content (40% of pulse labelled poly (A) of the nucleus) and the fact that this poly (A) containing fraction may leave the nucleus quickly in cordycepin chase experiments. This subfraction of HnRNA was called S-HnRNA by Price because of its relatively small size (18S - 28S). As this subfraction had been suggested as a possible precursor to mRNA it seemed of great interest to analyse it by the fingerprinting technique.

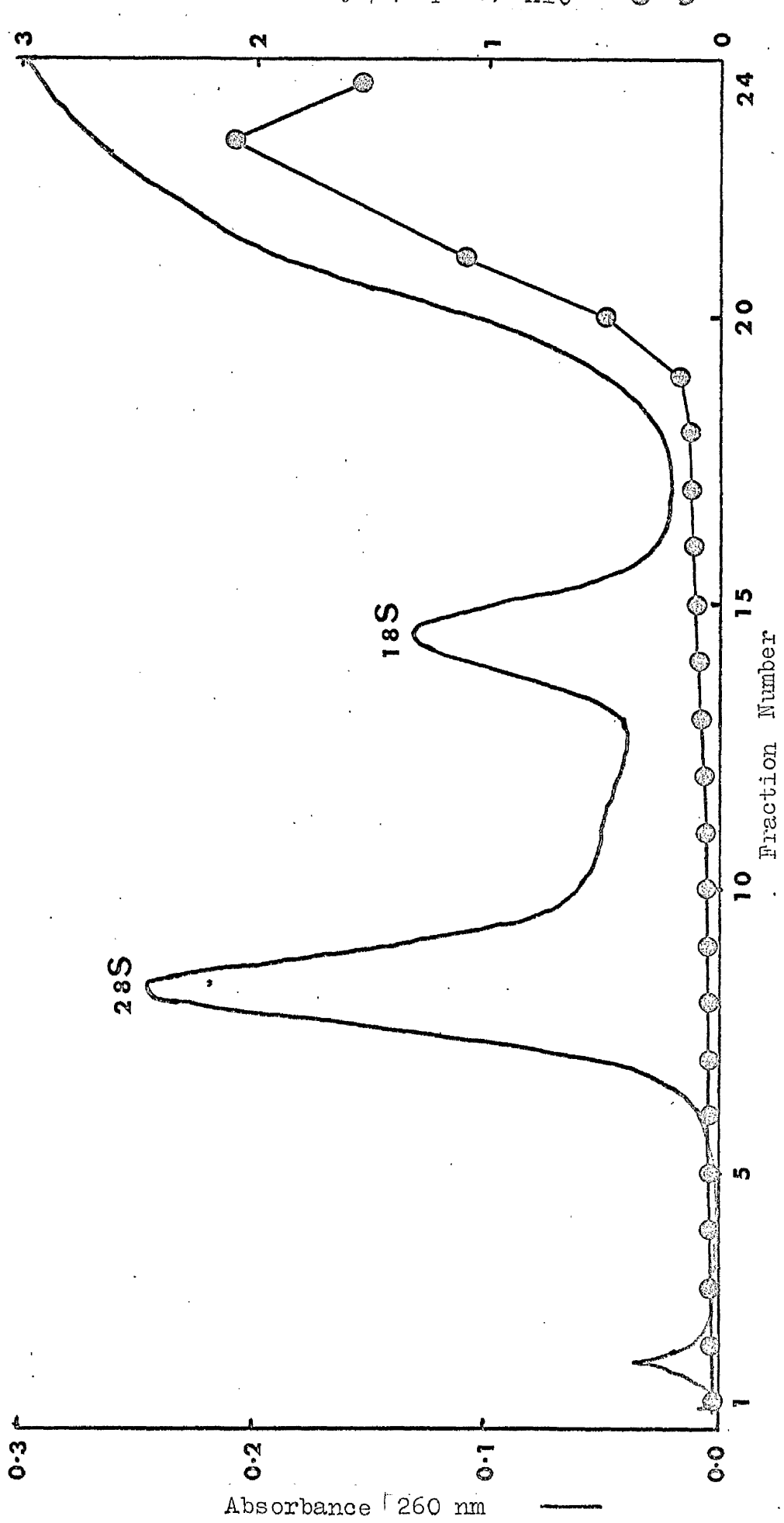
Price labelled his cells for short time intervals (15 - 20 min). However to prepare HnRNA for fingerprinting HeLa cells were grown for 3 hours in the presence of actinomycin D. The cells were then harvested by scraping and nuclei prepared as detailed in the Methods section. Nuclei were suspended in 0.4 M ammonium sulphate, 0.03 M Tris pH 7.4 and

FIGURE 15

Sucrose Density Gradient Analysis of S-HnRNA

S-HnRNA was dissolved in LETS buffer and 2 ml samples loaded onto 15% - 30% sucrose/LETS density gradients. These were centrifuged for 16 hours at 25,000 RPM in a Spinco SW27 centrifuge rotor at 20°C.

Gradients were pumped through the flow-cell of a Gilford recording spectrophotometer and approximately 1.6 ml fractions collected. 10 µl samples of each fraction were counted for radioactivity as described in the Methods section 3.4.5.


Radioactivity (c.p.m.) xIO⁻⁵
3
2
1
0
24
20
15
10
5
1
18S
28S
Fraction Number
0·3
0·2
0·1
0·0
Absorbance 260 nm

gently agitated. The nuclear structure disappears in the high ionic strength solution resulting in a viscous solution of chromatin and other nuclear components. The chromatin was spun down at 20,000 g for 15 min and the S-HnRNA left in the supernatant. S-HnRNA was precipitated by diluting the supernatant 4 times with water and then ethanol precipitating. RNA was then hot phenol extracted.

Because of the relatively long labelling time the precipitated RNA was fractionated on a sucrose gradient to remove the large amount of radioactivity present in low molecular weight RNA (see figure 15). Fractions 6 to 17 were pooled and ethanol precipitated (because they corresponded to the size of Price's S-HnRNA).

Table 12 shows the relative molar oligonucleotide frequencies in T_1 RNase digests of S-HnRNA. HnRNA and mRNA figures are also given for comparison.

The frequencies for oligonucleotides containing the sequence C-Gp in S-HnRNA appear to be much higher than those in total HnRNA, in fact they seemed to be similar to those of mRNA. This would seem to support Price's suggestion that S-HnRNA is a precursor to mRNA. However the sequence U-A-Gp is not identical to that in mRNA.

TABLE 12

Comparison of T_1 Ribonuclease Digests of S-HnRNA with HnRNA and mRNA

	G	CG	C_2G	C_3G
HnRNA	100(100)	4.6±1.6(20.5)	1.2±0.4(4.2)	0.7±0.7(0.9)
S-HnRNA	100(100)	10.2±2.9(23.2)	3.6±1.4(5.4)	1.9±1.4(1.2)
mRNA	100(100)	12.4±3.3(21.6)	4.0±1.4(4.7)	1.2±0.5(1.0)
		AG	A_2G	A_3G
HnRNA		29.8±5.4(27.3)	7.2±1.4(7.4)	1.9±0.4(2.0)
S-HnRNA		35.6±12.0(26.8)	7.9±1.2(7.2)	2.2±0.5(1.9)
mRNA		37.0±10.5(32.4)	12.5±1.1(10.6)	2.7±0.5(3.4)
		UG	U_2G	U_3G
HnRNA		25.3±1.8(30.1)	8.4±0.2(9.1)	3.0±0.5(2.7)
S-HnRNA		25.7±1.3(28.0)	7.2±0.1(7.8)	2.6±0.1(2.2)
mRNA		24.3±3.0(24.8)	6.5±0.6(6.2)	2.4±0.5(1.5)
		UCG	ACG	UAG
HnRNA		0.9±0.5(6.2)	0.9±0.2(5.6)	5.2±2.2(8.2)
S-HnRNA		2.1±1.0(6.5)	2.1±1.6(6.2)	4.7±2.3(7.5)
mRNA		1.8±0.8(5.4)	2.4±0.9(7.0)	2.6±0.4(8.0)
		CUG	CAG	AUG
HnRNA		9.6±2.8(6.2)	7.4±0.9(5.6)	8.4±2.2(8.2)
S-HnRNA		9.9±1.8(6.5)	6.7±3.8(6.2)	10.2±3.6(7.5)
mRNA		10.6±2.1(5.4)	8.1±3.4(7.0)	10.6±2.1(8.0)

Base Analysis

	C	A	G	U
HnRNA	20.5±1.6	27.3±1.7	21.8±3.1	30.2±1.6
S-HnRNA	23.2±2.3	26.8±1.4	22.0±0.4	28.0±3.3
mRNA	17.4±1.6	40.6±8.6	19.9±4.5	21.2±3.8

These results are the average of five independent experiments for HnRNA and mRNA, and 3 fingerprints from two experiments for S-HnRNA. The figures in brackets are theoretical values calculated from the base composition.

8. DOUBLE STRANDED REGIONS OF HnRNA

While studying the poly (A) regions of mRNA and HnRNA Darnell et al. (1971) noted the existence of other ribonuclease-resistant sequences in HnRNA. Later Jelinek and Darnell (1972) went on to study these ribonuclease resistant regions of HnRNA. They concluded that HnRNA contained double-stranded regions that arise from the base pairing of complementary sequences within the same molecule (intramolecular base pairing). 2% - 3% of total HnRNA which resists ribonuclease treatment appeared to have the characteristics of double stranded RNA. Furthermore mRNA did not seem to contain the same amount or type of double-stranded RNA sequence as found in HnRNA. Thus double stranded regions of HnRNA seem a likely source of non-conserved sequences in the processing of HnRNA to mRNA.

8.1. Measurement of Ribonuclease Resistant RNA

Various types of RNA were subjected to digestion with T_1 RNase and pancreatic RNase as described in the Methods section. The buffer used contained high levels of salt which protects the double-stranded regions of RNA. It also prevents the digestion of poly (A) by pancreatic RNase which can occur under normal conditions (Beers, 1960).

Figure 16 shows the time-course for digestion of HnRNA and 28S rRNA under high salt conditions.

Most RNA is digested within 10 minutes, however digestion was normally carried out for 30 minutes to ensure complete digestion of RNA to double-stranded pieces and poly (A) sequences. Table 13 indicates the resistance shown by various RNA species to RNase digestion in high salt

FIGURE 16

Time Course of High Salt Ribonuclease Digestion

Lyophilised RNA samples in small siliconised test-tubes were dissolved in 0.95 ml of "high salt digestion" buffer. These solutions were incubated at 37°C for 5 minutes before 50 µl of "high salt" enzyme mixture (T_1 RNase plus pancreatic RNase in 0.3 M salt solution) was added. After mixing, duplicate 50 µl samples were removed and precipitated by adding to 1 ml of ice-cold 5% trichloroacetic acid solution along with 5 µg of carrier RNA. The precipitates were collected on millipore filters and washed with a further 15 ml of 5% trichloroacetic acid before drying and counting in toluene(PPO scintillation fluid.

○ = HnRNA

□ = 28S rRNA

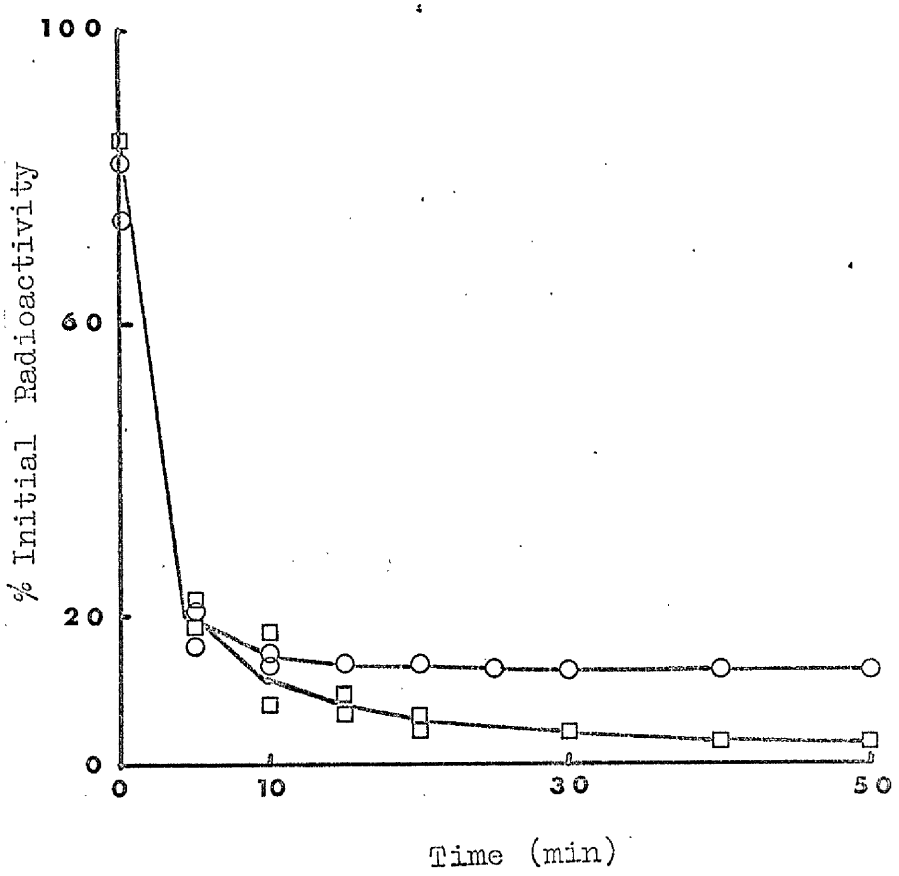

100
60
20
0
% Initial Radioactivity
0
10
30
50
Time (min)

TABLE 13

Resistance of RNA to Ribonuclease in High Salt Digestion Buffer

RNA	% Resistance
28S rRNA	3.5%
3 hour-labelled HnRNA	12.4%
16 hour-labelled HnRNA	24.5%
3 hour labelled HnRNA (13 hour preincubation with actinomycin D)	11.3%

These results are the average of 2 determinations, performed as described in the legend to figure 17. In each case they were from one preparation of RNA only.

conditions. It will be noted that HnRNA labelled for 3 hours is significantly less ribonuclease resistant than that at 16 hours. Furthermore from the similar low % resistance of HnRNA, labelled for 3 hours with $^{32}PO_4$ after 13 hours pre-incubation with actinomycin D, we can say that the ribonuclease resistant RNA content of HnRNA produced after 16 hours of labelling with $^{32}PO_4$ is not due to an effect of actinomycin D. This suggests the possibility of accumulation of ribonuclease resistant sequences in HnRNA on labelling for longer periods of time (16 hours).

8.2. Fingerprinting of Double Stranded HnRNA

To fingerprint double stranded sequences of HnRNA as distinct from single stranded RNase-resistant material (such as poly (A)) hydroxylapatite column chromatography was used (Bernardi, 1969). The double stranded RNA fraction can be eluted from hydroxylapatite columns with 0.5 M phosphate buffer whereas the poly (A) sequences and small single stranded oligonucleotides are washed straight through.

However double stranded RNA fractions, eluted at 0.5 M phosphate concentration formed a biphasic mixture when 2 volumes of ethanol were added to effect ethanol precipitation. To overcome this problem, the pooled double stranded RNA fractions were desalted by passage through a short column of sephadex G-25. After this treatment the double stranded RNA could be ethanol precipitated as normal. 5.0% of HnRNA labelled for 3 hours with $^{32}PO_4$ in the presence of actinomycin D could be recovered as double stranded RNA by this technique.

To produce a T_1 RNase digestion fingerprint from which

quantitation of radioactivity present in various oligonucleotide spots can be made, at least 1.5×10^5 CPM of RNA are required. Thus if only 5% of RNA is double stranded, at least 3×10^6 CPM of radioactive RNA must be digested under high salt conditions to fingerprint double stranded regions of HnRNA. This meant that only one or perhaps two fingerprints of the double stranded regions of HnRNA could be prepared from each cell labelling experiment. HnRNA was prepared twice for studying double stranded RNA and both times the experiment was a partial failure. On the first occasion the base analysis of the putative double stranded RNA did not reveal quite the expected base composition (i.e. A = U, G = C). It is possibly still contaminated with poly (A). However these results of fingerprinting this material are included as they probably represent a fraction of HnRNA enriched for double stranded RNA. These are shown in table 14 along with the figures for a normal T_1 RNase digestion fingerprint before the procedure for selection of the putative double stranded regions.

The second attempt to fingerprint double stranded regions from HnRNA was unsuccessful. This appeared to be due to a residual contamination of the putative double stranded material with pancreatic RNase which interferes with the T_1 digestion pattern. It is known that pancreatic RNase may associate with RNA and in this way survive purification steps such as the phenol extraction (Robins and McNutt, 1974) whereas T_1 RNase does not. Nevertheless the base composition the resistant RNA before T_1 RNase digestion was found to be symmetrical (A = U, G = C) and is presented in table 14. It will be seen that this material is richer in G + C than the original HnRNA, possibly because G ≡ C base pairs are thermodynamically stronger than A = U and therefore produce more stable double stranded RNA sequences.

TABLE 14

Double Stranded RNA

	G	CG	C_2G	C_3G
HnRNA (medium)	100(100)	6.4(20.7)	1.9(4.3)	0.6
DS.HnRNA (medium)	100(100)	8.8(26.1)	2.9(6.8)	0.9
		AG	A_2G	A_3G
HnRNA (medium)		27.5(26.8)	8.7(7.2)	2.5
DS.HnRNA (medium)		37.9(31.3)	3.2(9.8)	0.6
		UG	U_2G	U_3G
HnRNA (medium)		27.9(30.5)	4.0(9.3)	-
DS.HnRNA (medium)		21.0(18.8)	4.7(3.5)	1.3
		UCG	ACG	UAG
HnRNA (medium)		0.5(6.3)	0.3(5.5)	8.6(8.2)
DS.HnRNA (medium)		1.4(5.9)	2.2(8.2)	4.9(5.9)
		CUG	CAG	AUG
HnRNA (medium)		5.9(6.3)	9.7(5.5)	7.5(8.2)
DS.HnRNA (medium)		11.9(5.9)	6.2(8.2)	4.1(5.9)

Base Analysis

	C	A	G	U
HnRNA (medium)	20.7	26.8	21.9	30.9
DS.HnRNA (medium)	26.1 ± 0.1	31.3 ± 0.5	23.8 ± 0.1	18.8 ± 0.5
2nd Prep. DS.RNA (medium)	27.1 ± 0.3	21.5 ± 0.6	29.1 ± 0.4	22.2 ± 0.5

These are the results of 1 fingerprint from before and after "high salt digestion". The figures in brackets are theoretical values calculated from the base compositions.

9. SEARCH FOR METHYLATED NUCLEOTIDES OF HnRNA

Until recently methylated bases such as those in tRNA or rRNA had not been reported to occur in mRNA or HnRNA. This situation changed when Perry and Kelley (1974) reported low levels of methylated sequences in mouse L cell HnRNA (1 in 2,500 bases approximately) and mRNA (2.2 per 1,000 bases). They suggested that poly (A) containing HnRNA was probably more methylated than HnRNA lacking poly (A). Furthermore they suggested that methylation occurred on both base and ribose moieties.

Since Perry and Kelley's paper there have been many reports on methylation in mRNA (see Discussion). However, the present work was done before publication of these papers.

Cells were labelled with ^{14}C-methyl methionine in the presence of actinomycin D (as described in the Methods section, 3.1.1). After 11 hours of labelling 44% of the radioactivity present in the cell culture medium had been taken up by the HeLa cells. After 16 hours this value was 83%.

From an 11 hour incorporation experiment HnRNA was prepared as described in the Methods section. Approximately 700 CPM were incorporated into heavy size fraction HnRNA and 5000 CPM into light size fraction HnRNA (HnRNA size fractions were as shown in Figure 12). Both fractions of HnRNA and some 28S rRNA labelled with $^{32}PO_4$ were subjected to T_1 RNase plus pancreatic RNase digestion and fingerprinted in the standard manner.

After 11 months of autoradiography the X-ray films were developed. Figure 17 shows the HnRNA (medium) size and 28S rRNA

FIGURE 17

Combined T_1 + Pancreatic Ribonuclease Fingerprints of Methyl Labelled HnRNA

RNA was prepared and fingerprinted as described in the Methods section. First dimension right to left, cellulose acetate, pH 3.5 (5% acetic acid, 7 M urea). Second dimension, downward, DEAE paper, 7% formic acid.

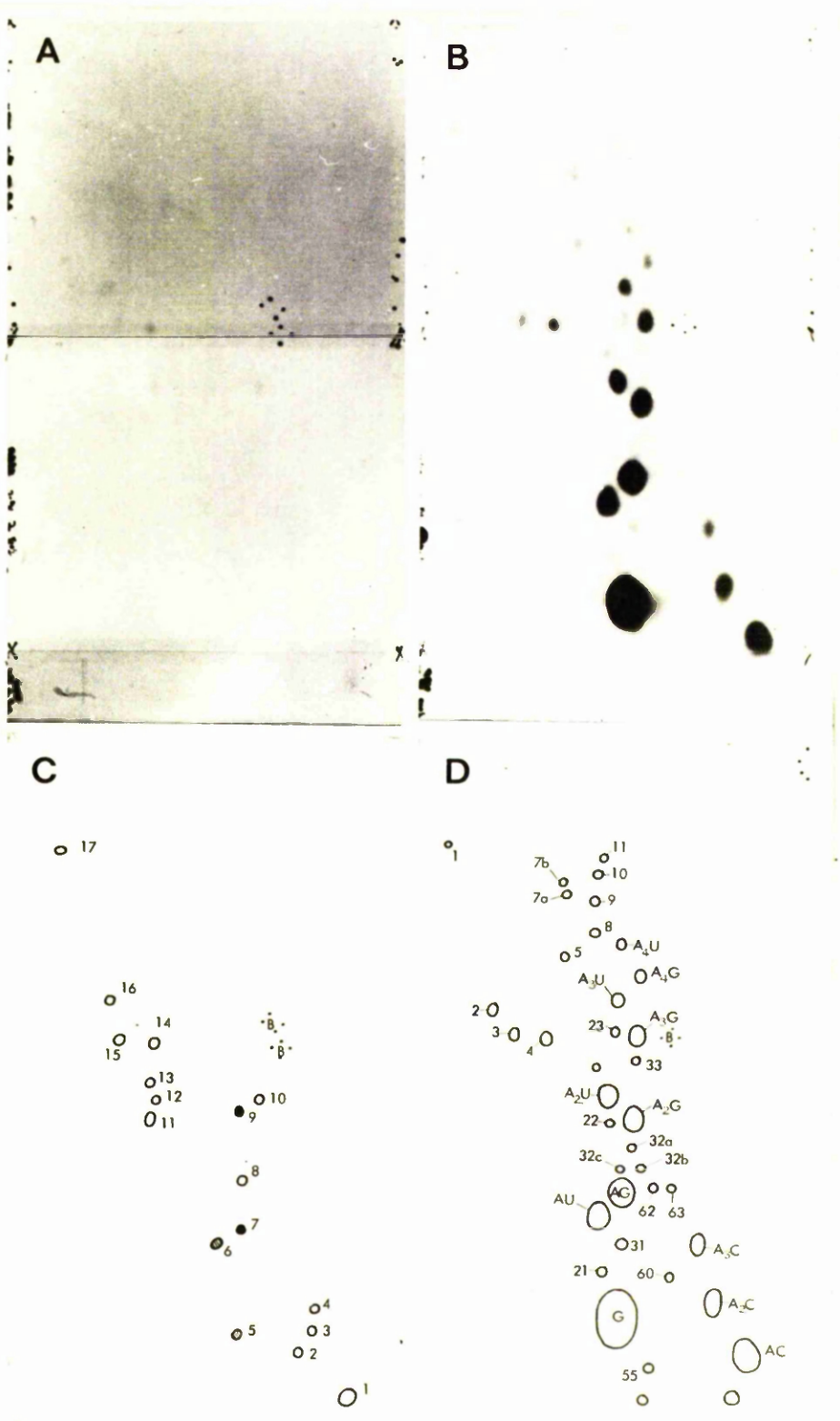
The $^{32}PO_4$ labelled 28S rRNA fingerprint was run at the same time and under the same conditions as the ^{14}C -methyl methionine labelled HnRNA fingerprint to act as a reference. In the Key to the HnRNA fingerprint, the cross-hatched spots occurred only in the HnRNA (heavy size fraction) fingerprint (which is not shown), the filled-in spots occurred in both HnRNA fingerprints and the open-circles only in HnRNA (light size fraction), which is shown above.

A = ^{14}C-methyl methionine labelled HnRNA

B = $^{32}PO_4$ labelled 28S rRNA

C = Key to HnRNA

D = Key to 28S rRNA


A
B
C
D
17
16
14
15
13
12
11
10
9
8
7
6
5
4
3
2
1
7b
7a
11
10
9
8
5
A4U
A3U
A4G
2
3
4
23
A3G
33
A2U
A2G
22
32a
32c
32b
AG
AU
62
63
31
A3C
21
60
A2C
G
AC
55

fingerprints. It can be seen that some of the spots in the HnRNA fingerprint correspond to some of the methylated spots in the 28S rRNA fingerprint - for example, the spot (No. 14) corresponding to the sequence Gm-Gp. It is possible that there has been a significant proportion of radioactivity incorporated into rRNA, due to incomplete inhibition of rRNA synthesis. A further possibility is that a significant amount of radioactivity is being incorporated into purine rings via "C_1" metabolisim despite the additions made to reduce this (formate, guanosine, adenosine).

When subjected to alkaline hydrolysis, the ^{14}C radioactivity present in the HnRNA was found in the position expected for non-methylated nucleotides. Of the radioactivity present in these spots for HnRNA (heavy) 45% corresponded to C, 26% to A, 21% to G and 8% to U. For HnRNA (medium) the values were: 26% C, 32% A, 20% G, 22% U. However there was always a small but variable amount of radioactivity remaining at the origin.

To show that the radioactivity at the origin was not due to contaminating DNA the origin spots from electrophoresis of alkali digests were cut out and soaked with 0.03 µg of pancreatic DNase (RNase free) in 10 µl of 0.01 M tris buffer pH 6.9. After incubating for 2 hours in a humidified oven the spots were dried, sewn onto another sheet of Whatman No. 52 paper and re-electrophoresed as before. This treatment should have reduced some of the DNA to 5' mononucleotides, however all of the radioactivity was still at the origin. Therefore it seems unlikely that the radioactivity at the origin was DNA.

The spots corresponding to the four normal bases of which RNA is composed were cut out and analysed for minor bases (see Methods section 3.5.9).

After chromatography the chromatogram was cut into small squares and counted. Unfortunately the amount of radioactivity was so low that no peaks of radioactivity could be detected even after counting for 50 minutes on a scintillation counter.

This work was limited by the low levels of activity which could be incorporated into the HnRNA.

DISCUSSION

Though much work has been performed on the possible role of some HnRNA as a precursor to mRNA in eukaryotic cells, there is as yet no final conclusion to the relationship. In this work, a study of sequences present in HnRNA and mRNA has been undertaken in order to throw further light on this problem.

1. STRUCTURE OF HnRNA

As mentioned in the introduction, HnRNA is a very large molecule. It is heterogeneous in size and contains poly (A), oligo (A), oligo (U) and double stranded sequences. The pattern of short sequence present in HnRNA has been examined by the two dimensional fingerprinting technique of Sanger et al. (1965) and the results compared with those obtained from 28S rRNA.

T_1 ribonuclease fingerprints, such as those shown in figure 1, clearly show the difference in sequence complexity between HnRNA and 28S rRNA. 28S rRNA has only certain nucleotide sequences present, there being particularly few longer sequences, whereas the opposite appears to be true for HnRNA.

Possibly the most interesting difference observed between HnRNA and 28S rRNA is the frequency with which C-Gp containing oligonucleotides occur. From T_1 ribonuclease fingerprints of HnRNA (figure 1) it can be seen that sequences ending in the dinucleotide C-Gp are deficient compared with their isomeric sequences.

From the T_1 ribonuclease fingerprints shown in figure 1, it can also be seen that there is no apparent methylation of HnRNA. There

are no methylated sequence spots characteristic of rRNA fingerprints (Maden and Salim, 1974) nor are there any other minor spots, which do not fit into the pattern of normal (unmethylated) sequence spots, on the fingerprints of HnRNA.

A more useful system for studying methylated sequences is combined T_1 ribonuclease plus pancreatic ribonuclease digestion fingerprints. An inspection of this type of fingerprint (figure 2) revealed no apparent methylation of HnRNA. Furthermore the presence of a spot corresponding to Gm-Gp (the most prominent methylated sequence in rRNA fingerprints) is not seen. This indicates that HnRNA, as prepared for this work, was free of rRNA contamination.

These results do not rule out the possibility that HeLa cell HnRNA is methylated, as it is possible for methylated product spots to coincide with non-methylated product spots. Furthermore it is possible that there is such a low level of radioactivity present in the methylated spots that they are not detectable on fingerprints produced with the relatively small amounts of radioactivity used in this work.

To test these possibilities, cells were labelled with L-[methyl-^{14}C] methionine. Any fingerprint of RNA labelled with this compound as described in the Methods section 3.1.1 should contain radioactivity in methylated nucleotides only. After 11 hours of labelling there were just sufficient counts incorporated into the HnRNA to prepare a combined T_1 ribonuclease plus pancreatic ribonuclease fingerprint and perform a base analysis. Autoradiography of this fingerprint however took 11 months and revealed the presence of some methylated spots though the activity present in these was too small to warrant further examination

with regard to sequence. Most of these spots appeared close to methylated spots present in a similar digest of ^{32}P labelled 28S rRNA which was fingerprinted at the same time and used as a control. Nevertheless there were several spots which did not coincide with rRNA methylated products and which may have been due to methylation of HnRNA. The base analysis of [methyl-^{14}C] labelled HnRNA showed radioactivity apparently in all four nucleotides. However there was insufficient radioactivity present in the four spots to subject them to chromatography for methylated nucleotides.

Studies of mouse L cells by Perry and Kelly (1974) have revealed methylated sequences in HnRNA and mRNA. Since then many reports have appeared of methylation in viral mRNA (Furuichi and Mura, 1975; Furuichi et al., 1975A; Abraham et al., 1975). It would seem that the 5' terminus of viral mRNA consists of sequences of the type $m^7G(5')ppp(5')Nm-Np$. This terminal sequence contains 7 methylguanosine in a 5'-5' pyrophosphate linkage to a 2'-O-methyl nucleotide and has been called a "cap" sequence (Rottman et al., 1974). Recently similar sequences along with 6-methyladenine have been found in eukaryotic mRNA (Adams and Cory, 1975; Furuichi et al., 1975B). The "cap" sequence is presumably added after cleavage of mRNA from precursor mRNA.

Muthukrishnan et al. (1975) have shown that reovirus and vesicular stomatitis virus (VSV) require "cap" sequences before they are translated. Wheat germ and mouse L cell protein synthesising extracts have the ability to add "caps" to mRNA. Rabbit reticulocyte mRNA contains m^7G and its removal has been shown to cause the loss of translocational activity in vitro (Muthukrishnan et al., 1975).

Another feature of HnRNA structure is the occurrence of double stranded (hairpin) regions. These do not appear to the same extent in mRNA (see Introduction, section 2.2.5). Thus double stranded regions are a source of non-conserved sequences in the processing of HnRNA to mRNA.

The products from a combined T_1 ribonuclease plus pancreatic ribonuclease digestion of RNA in High Salt Digestion Buffer consists of the expected oligonucleotides from such a digestion of single stranded RNA plus double stranded regions of RNA and intact poly (A) regions. From table 13, the amount of ribonuclease resistant material (poly (A) plus double stranded RNA) appears to rise with increasing labelling time. However this appears to be due to an accumulation of ribonuclease resistant material during the longer labelling period rather than the production of more ribonuclease resistant RNA towards the end of this time (see figure 13).

Double stranded RNA was prepared by subjecting ribonuclease resistant material to hydroxylapatite chromatography. Fingerprints of this portion of the HnRNA molecule should have revealed whether relative molar oligonucleotide frequencies present in hairpin structures were similar to those of total HnRNA, and if there were any special sequences present. However technical difficulties, which were not completely overcome (Results section 8.2), were encountered in preparing these fingerprints.

Base analysis of the double stranded fragments of HnRNA revealed 27% C, 22% A, 29% G, 22% U. These figures are in good agreement with those of Jelinek and Darnell (1972) who found a base composition of

28.6% C, 22.1% A, 28.7% G and 21.2% U. It is interesting to note that the level of G + C in double stranded HnRNA are much higher than that of total HnRNA (56% compared with 42%). A possible explanation is that G ≡ C base pairs are thermodynamically more stable than A = T base pairs.

As mentioned already, a notable feature of HnRNA is a deficiency of C-Gp sequences. The question arises as to how this relates to the nucleotide sequences present in the human genome.

In 1961 the new technique of "nearest neighbour base sequence analysis" was reported (Josse et al., 1961). This technique allows some characterisation of DNA in terms of the distribution of dinucleotide sequences in it. One of the DNA precursor nucleotides is labelled with radioactive $^{32}PO_4$ and this 5' nucleoside triphosphate incorporated into DNA. The DNA is then degraded by micrococcal nuclease and spleen diesterase. Both of these enzymes cleave so as to give 3' nucleoside monophosphates. Thus the neighbour to the original $^{32}PO_4$ labelled nucleotide is now labelled with radioactive $^{32}PO_4$.

Studies by Swartz et al. (1962) revealed that the occurrence of the base doublet C-G was very rare in vertebrate DNAs. It was only present in 25% of the expected frequency of doublets calculated from base composition assuming random neighbours.

The predicted relative molar frequency of oligonucleotides a T_1 ribonuclease digest of a hypothetical random transcript of the human nuclear DNA have been calculated (Burdon et al., 1974). The prediction was carried out from the known "doublet" frequencies from DNA (Swartz et al., 1962), assuming that each base depends only on its nearest neighbour. These calculated results are shown in table

TABLE B

THE PREDICTED RELATIVE MOLAR FREQUENCIES OF OLIGONUCLEOTIDES IN A T_1 RIBONUCLEASE DIGEST OF A HYPOTHETICAL RANDOM TRANSCRIPT OF TOTAL HUMAN NUCLEAR DNA

Oligonucleotide	% molar yield relative to G	
	[x] predicted	[xx] observed
CG	4.26	4.6
CCG	0.99	1.2
CCCG	0.23	0.7
AG	28.28	29.8
AAG	9.08	7.2
AAAG	2.92	1.9
UG	24.58	25.3
UUG	8.08	8.4
UUUG	2.66	3.0
UCG	0.94	0.9
CUG	7.58	9.6
ACG	1.08	0.9
CAG	7.30	7.4

[x] The prediction is carried out by taking the known 'doublet' frequencies of human DNA (Swartz et al., 1962) and assuming that each base depends only on its nearest neighbours calculating the frequency of any given oligonucleotide $X_1\ X_2 \text{----} X_n$ (where X_1 represents bases) by $P(X_1)P(X_2/X_1)P(X_3/X_2)\text{----}P(X_n/X_{n-1})$, where P(Y/X) is the probability that base Y follows base X.

[xx] These are the observed frequencies from Table 2 presented for comparison.

with the actual values found from T_1 ribonuclease digests of HnRNA. It will be noted that the predicted and observed values bear a good deal of similarity. This suggests that HnRNA is a general transcript of the total DNA and not from a portion of the DNA which is unusual in sequence content.

2. STUDY OF mRNA

2.1. Preparation of mRNA for Fingerprinting

Polysomes consist of single mRNA molecules with a number of ribosomes attached. These structures form part of the translational machinery of the cell and it is from these structures that mRNA was prepared. Ribonuclease can quickly degrade polysome structure by cleaving the mRNA strand. Thus the preparation of polysomes from cells can be difficult.

HeLa cells were chosen for this project because their RNA has been studied extensively by other workers, and it is known that the preparation of polysomes from these cells is relatively straightforward. There appear to be two types of RNase activity present in the cytoplasm of HeLa cells (Penman et al., 1969). The first is a particulate enzyme associated with lysosomes. It has a pronounced pH dependence, and if operations are carried out at pH 7.4, the activity of this enzyme is minimal. However if care is taken not to destroy the lysosomes there is little problem with this enzyme anyway. The second ribonuclease activity is a soluble nuclease with a pH optimum of 8. However the activity of this enzyme is extremely sensitive to temperature, and the activity is negligible at 4°C. Penman et al. (1969) showed that when

cytoplasmic extracts of HeLa cells are kept cold, undegraded polysomes can be prepared.

The specific activity of polysomal RNA, from cells labelled with $^{32}PO_4$ as described in the Methods section 3.1.1, was insufficient to permit fingerprinting of polysomal RNA. A further purification step had to be performed.

Many methods have been used for the purification of mRNA. These include:- sucrose gradient centrifugation (Lingrel et al., 1971), gel electrophoresis (Williamson et al., 1971; Lockard and Lingrel, 1972), gel filtration (Means et al., 1974), immunoprecipitation (Palacios et al., 1972; Stevens and Williamson, 1973), binding to millipore filters (Lee et al., 1971), and absorption to oligo (dT) cellulose (Avia and Leder, 1972). Recently gel isoelectric focussing has been used to separate mRNA species.

Many of these procedures have been used to prepare particular mRNA species. However in this study the object was to study total cell mRNA, and use was made of the fact that oligo (dT) chains have the potential to hybridise to poly (A) regions of mRNA. However only a small fraction of polysomal RNA was recovered as poly (A) containing mRNA by this method. It was suspected that this was due to the particular batch of oligo (dT) cellulose purchased. The "millipore" filtration technique of Lee et al. (1971) was found to provide a convenient procedure for the routine purification of poly (A) containing mRNA. Nevertheless the method by which (Millipore) nitrocellulose filters select for the poly (A) containing RNA is not well understood.

It has been reported that "millipore" filters do not bind poly (A) regions of less than approximately 50 residues (Gorski et al., 1974). Nevertheless mammalian cells have been shown to contain poly (A) regions of 100-200 residues in length which bind efficiently to millipore filters. Brawerman (1972) has bound rabbit haemoglobin mRNA to millipore filters though this mRNA has a poly (A) region which is shorter than 100-200 nucleotides.

Though it is known that poly (A) regions are attached to most mammalian mRNA, some mRNA species do not contain poly (A) regions. These mRNA will not be selected for by techniques specific for the 3' terminal poly (A) region. For example mRNAs coding for histone proteins do not contain a poly (A) region. Attempts to prepare mRNA without poly (A) regions, by subjecting a "millipore" filtrate of polysomal RNA to sucrose gradient analysis and selecting mRNA size fractions, did not provide sufficient radioactivity to fingerprint. However total mRNA (containing poly (A) and without poly (A)) was prepared by sucrose gradient analysis of polysomal RNA and selection of mRNA size fractions.

2.2. Fingerprinting of mRNA

Sequencing work has already begun on some eukaryotic mRNA e.g. immunoglobulin (Brownlee et al., 1973; Milstein et al., 1974) and globin (Proudfoot and Brownlee, 1974B; Marotta, 1974). Particular attention has been paid to the non-translated 3' end nucleotides of these mRNAs. Proudfoot and Brownlee (1974B) have sequenced 52 nucleotides adjacent to the poly (A) tail of rabbit β globin mRNA. They have shown striking sequence and structural homologies between it and mouse immuno-

globulin light chain mRNA. Furthermore they suggest that these sequence homologies at the 3' end of the mRNA molecules may be common to all mRNA and may represent binding sites for specific cytoplasmic proteins involved in general functions such as transport of mRNA from the nucleus to the ribosome, or the control of degradation of mRNA. This present work has not attempted to look at specific mRNA species. However it has examined the mRNA of HeLa cells and drawn some general conclusions from the oligonucleotide frequency data obtained.

Since we are dealing with a population of mRNA molecules, quantitation of absolute sequence frequencies cannot be achieved. Nevertheless we can calculate the frequency of sequences relative to a reference sequence. This method suffers from the criticism that it depends on the fraction of G present as G-Gp in an RNA molecule and this may vary from RNA to RNA. However for the simple sequence G-Gp this variation is likely to be at a minimum compared to the variation of other larger sequences which are represented less often. The effect of variation of G-Gp content between two RNA species would be a proportional variation in all relative sequence frequencies. This effect is not observed.

T_1 ribonuclease fingerprints of mRNA reveal similar features to those of HnRNA - high sequence complexity compared to 28S rRNA, apparent lack of methylated products, and a C-Gp sequence deficiency. However the C-Gp sequence deficiency is not so large as that observed in HnRNA. It is interesting to note that the C-G deficiency seen in the series C-Gp, C-C-Gp, ... $(Cp)_nGp$ series of oligonucleotides from T_1 ribonuclease digestion fingerprints of HnRNA does not seem to be so

marked in mRNA. Thus if HnRNA is the precursor of mRNA, there appears to be selection of C-Gp rich regions on its processing to mRNA. Alternatively only a C-Gp rich fraction of the HnRNA molecule population may act as precursors to mRNA.

The observation that C-Gp levels in mRNA are higher than in HnRNA would seem to argue against the possibility that the radioactive $^{32}PO_4$ has not had sufficient time to equilibrate with the four RNA precursor nucleotide pools of the cells. In fact when mRNA was labelled for different lengths of time, from 3 to 16 hours, the relative molar oligonucleotide frequencies of mRNA remained fairly constant. On the other hand, the HnRNA relative molar oligonucleotide frequencies varied significantly.

There is always the possibility that the C-G deficiency observed in short T_1 ribonuclease digest sequences of HnRNA and mRNA is not present in longer sequences. Nevertheless T_1 ribonuclease plus alkaline phosphatase digest fingerprints of RNA, which allow the separation of longer oligonucleotides (tetramers and pentamers) seem to confirm that the deficiency is present in sequences of 4 and 5 nucleotide length. An alternative digestion of the RNA would be with U_2 ribonuclease which cleaves after G and A residues (T_1 ribonuclease cleaves after G only). The complexity of the resulting pattern would be much less than that of a T_1 ribonuclease fingerprint, probably similar to that of a pancreatic ribonuclease fingerprint. Using this system it may be possible to study other C-Gp containing sequences.

Because mRNA was routinely prepared from polysomal RNA by selection for poly (A) containing RNA it is possible that it was not

representative of total mRNA in relative molar oligonucleotide frequencies. To check this point mRNA was prepared from polysomal RNA by sucrose gradient analysis and the selection of mRNA size fractions. Fingerprints of this mRNA showed no difference from that selected by "millipore filtration". Attempts to prepare only mRNA without poly (A) regions were unsuccessful. This was disappointing as it is known that histone proteins, which are coded for in mRNA without poly (A), are rich in arginine and arginine is coded by C-G containing triplet codons.

A further interesting deviation from predicted frequency is seen in the sequence U-A-Gp. This sequence, which is read as a chain terminating codon by the translational machinery of the cell, appears to be deficient in HnRNA. In mRNA, the deficiency is more marked. This may reflect the use of U-A-Gp as a termination codon in mRNA.

It was observed that a small fraction of the polysomal RNA appeared to be oligo (U) containing RNA. This RNA had a relative molar oligonucleotide frequency typical of HnRNA rather than poly (A) containing mRNA. Work on nearest neighbour frequencies of mitochondrial DNA from Physarum polycephalum (Cummins et al., 1967) has shown this eukaryotic DNA to be similar to procaryotic DNA in that neither exhibit a deficiency in the C-G doublet. Thus it seems unlikely that the oligo (U) containing RNA extracted from HeLa cell polysomal RNA, which exhibits a C-G deficiency equal to that of HnRNA, is a contaminant of mitochondrial origin.

2.3. mRNA Sequence Relationship to Genome

Bullock and Elton (1972) examined the dipeptide frequencies

present in proteins. They found evidence for a C-G containing codon shortage in mRNA reflected in the nearest neighbour amino acid pairs of vertebrate, but not bacterial, protein sequences.

When the relative molar oligonucleotide frequencies of mRNA were compared with those of HnRNA it was seen that mRNA differs markedly from HnRNA especially with regard to sequences of the type $(C-)_nGp$ (where n = integer). Although there is a marked decrease in the deficiency of these sequences in mRNA there is still a sizeable deficiency of other sequences terminating in C-Gp (see table 2). These results may signify that mRNA is not a typical transcript of DNA.

From the work of Warner and de Mol (1973) it is possible to calculate relative molar oligonucleotide frequencies for SV-40 specified RNA. These are shown in table C and do not represent a typical transcript of SV-40 DNA (R.A. Elton - personal communication). Both the 19S viral RNA which is produced early and late, and the 16S, which is only produced late after infection contain poly (A) tracts of about 130-150 residues long (Warnaar and de Mol, 1973). It will be noted that the 16S viral RNA is deficient in C-Gp, C-C-Gp and A-C-Gp whereas the 19S viral RNA is only notably deficient in the sequence A-C-Gp. It is interesting to speculate whether this difference in C-G sequence deficiency is related to a functional difference of the RNA, as the 16S viral RNA codes for virion structural proteins and the 19S viral RNA for viral antigens.

Recently Hughes and Maden (1975) have shown that T_1 ribonuclease fingerprints of Polio and Encephalomyocarditis (EMC) virus mRNA are deficient in C-G containing sequences. These mRNAs, similar to HeLa

TABLE C

Relative Molar Oligonucleotide Frequency of Two SV-40 RNA Molecules

	G	CG	C_2G	C_3G
19S	100(100)	21.9(23.0)	7.2(5.3)	1.7(1.2)
16S	100(100)	8.8(19.3)	3.0(3.7)	0.7(0.7)
		AG	A_2G	A_3G
19S		22.1(28.3)	5.9(8.0)	
16S		28.5(34.0)	9.8(11.6)	
		UG	U_2G	U_3G
19S		20.2(24.8)	4.6(6.1)	
16S		31.5(24.7)	6.0(6.1)	
		UCG	ACG	UAG
19S			2.7(6.5)	2.9(7.0)
16S			1.7(6.6)	3.6(8.4)
		CUG	CAG	AUG
19S			5.9(6.5)	6.1(7.0)
16S			8.2(6.6)	9.1(8.4)

Base Composition

	C	A	G	U
19S	23.0	28.3	23.9	24.8
16S	19.3	34.0	22.0	24.7

These figures were calculated from the results of Warnaar and de Mol (1973)

cell mRNA, are less deficient in certain C-Gp containing sequences than HeLa cell HnRNA.

3. RELATIONSHIP BETWEEN HnRNA AND mRNA

Kinetics experiments involving the use of actinomycin D and cordycepin suggest the possibility that a poly (A) containing fraction of HnRNA functions as the precursor to mRNA. Competition hybridisation experiments of mRNA and HnRNA with DNA indicate that there is some sequence homology between mRNA and HnRNA. Moreover experiments with complementary DNA (prepared from an mRNA template using the reverse transcriptase enzyme) confirm that some HnRNA is complementary to mRNA. HnRNA molecules are on average larger than mRNA molecules by a factor of 10. However experiments with cDNA suggest that HnRNA which functions as a precursor to haemoglobin mRNA is only 3 times larger than haemoglobin mRNA (Macnaughton et al., 1974). As these experiments were done on a steady state population of RNA, they do not completely rule out the possibility of there being a large primary transcript which is quickly reduced to a size about 3 times that of mRNA. Alternatively only a fraction of the HnRNA molecules may act as mRNA precursors, the rest of the molecules serving some unknown function.

We have seen from the present work that although HnRNA is a typical transcript of DNA, mRNA seems to be transcribed from portions of the DNA which are relatively more abundant in C-G sequences. This difference in frequency of C-Gp containing sequences allows us to test various fractions of HnRNA for mRNA like sequence composition. Size fractions of HnRNA were studied first.

If large HnRNA molecules are the precursor of mRNA then one would expect to observe a change to C-G deficiency in HnRNA on examination of HnRNA of different size classes. One would expect the smallest HnRNA size fraction (equivalent in size to mRNA) to be most messenger like in terms of relative molar oligonucleotide frequency. However when HnRNA was divided into three size classes by sucrose gradient analysis only a small change in C-G containing sequence content was observed. This small change could be explained if only a percentage of the small HnRNA molecules were precursors to mRNA. Possibly the other molecules could have a regulatory role.

A major problem of experiments to size mRNA is that of aggregation. Recent work by Macnaughton et al. (1974) has shown that haemoglobin mRNA and HnRNA, from immature duck red blood cells, both aggregate on phenol extraction. However these aggregates where shown to disperse when dissolved in formamide. Aggregation has also been observed in mouse ascites tumor RNA (de Kloet et al., 1970; Mayo and de Kloet, 1971). Nevertheless Holmes and Bonner (1973), working on Novikoff ascites tumors in rats, have shown the existence of very large molecules of HnRNA under disaggregating conditions. Derman and Darnell (1974) have shown a similar result for HeLa cell RNA.

Formamide is recognised as being one of the most useful disaggregating solvents for RNA (Pinder et al., 1974). Thus formamide-polyacrylamide gel electrophoresis and formamide-sucrose gradient analysis were used to study the size classes of HnRNA, prepared from density gradients for aggregation. The results of these experiments confirmed that aggregation did occur, however large molecules of HnRNA were also evident.

Formamide-sucrose density gradient analysis of HnRNA labelled for 16 hours with actinomycin D showed that this HnRNA was more aggregated than that produced by 3 hours of labelling. Moreover HnRNA labelled for 3 hours after 13 hours preincubation with actinomycin D was mainly aggregated. This suggests that with increasing labelling time in the presence of actinomycin D, either smaller molecules are produced or the large molecules are "nicked" more quickly.

WE HAVE looked at different size classes of HnRNA from truly giant SIZE to mRNA dimensions and seen little difference in C-G sequence content. A possible explanation of this observation is that only a fraction, such as that which carries poly (A) regions of the total population of HnRNA molecules are precursors to mRNA.

To test this hypothesis, HnRNA was fractionated into poly (A) containing, oligo (U) containing and neither poly (A) nor oligo (U) containing HnRNA. None of these fractions appear to differ from the bulk HnRNA in C-G sequence content after 3 hours of labelling with $^{32}PO_4$. This would appear to suggest that only a fraction of poly (A) containing HnRNA is destined to become mRNA and agrees with the work of Perry et al. (1974) who show intra nuclear turnover of poly (A).

Only very small amounts of oligo (U) containing HnRNA were found in HeLa cell nuclei. Molloy et al. (1974) have shown the occurrence of oligo (U) regions near the 5' terminus of poly (A) containing HnRNA. This oligo (U) containing RNA may represent pieces which are cleaved from large HnRNA molecules when they are processed to mRNA. However there are other possible explanations for this RNA.

There is evidence from pulse labelling experiments that both strands of the mitochondrial genome in HeLa cells are completely transcribed (Aloni and Attardi, 1971A). However one strand appears to be quickly degraded leaving almost all of the steady state RNA transcribed from one strand (Aloni and Attardi, 1971B). It is possible that symmetrical transcription also occurs in the nucleus. This would give rise to large amounts of HnRNA, containing oligo (U) regions which would be quickly turned over in the nucleus without maturation to mRNA. The amount of HnRNA containing oligo (U) regions (corresponding to oligo (A) regions in the fraction of HnRNA molecules destined to become mRNA) was found to be very small. This is to be expected if oligo (U) containing HnRNA is quickly degraded. Apparently this RNA does have a fast turnover rate (R.H. Burdon - personal communication).

It seems from the experiments on size and homopolymeric sequence containing fractions of HnRNA that it is unlikely that these fractions as such function as precursor mRNA.

Price et al. (1974) have reported the isolation of a small size fraction of HnRNA from chromatin which is rich in poly (A) and is rapidly labelled (S-HnRNA). This subfraction of HnRNA is rendered soluble from chromatin by treatment with 0.4M ammonium sulphate, 0.03M Tris, pH 7.4. It is about 18S - 28S in size and comprises 10% of the total HnRNA. S-HnRNA contains about 40% of the pulse labelled poly (A) in HeLa cell nuclei and this poly (A) can be chased from the nucleus more rapidly and completely than the poly (A) which remains tightly bound to the chromatin when the cells are treated with actinomycin D and cordycepin.

Fingerprinting studies of S-HnRNA showed that this fraction of HnRNA was messenger like in its C-G containing sequence content. Thus relative molar oligonucleotide frequency data from T_1 ribonuclease digest fingerprints of S-HnRNA would seem to support the suggestion that S-HnRNA is a precursor of mRNA. No other fraction of HnRNA, fingerprinted during this work gave data which was similar to mRNA except perhaps the HnRNA prepared from cells labelled in the presence of low levels of actinomycin D for long periods of time.

4. EFFECT OF ACTINOMYCIN D ON HnRNA SYNTHESIS

It is known that actinomycin D binds to certain G-C sequences of DNA (Sobel, 1974). Thus actinomycin D might be expected to be most effective in inhibiting RNA synthesis from genes with a high G + C content, such as rRNA genes.

Nucleoplasmic RNA synthesis has been shown to be relatively unaffected by doses of actinomycin D which completely inhibit rRNA synthesis (Penman et al., 1968). At a concentration of 0.04 µg/ml actinomycin D a 30% decrease in the incorporation of radioactivity into nucleoplasmic RNA and a corresponding 100% decrease in nucleolar RNA synthesis has been observed (Penman et al., 1968). All of the present experiments have been performed in the presence of 0.04 µg/ml actinomycin D. Hence it is important to attempt to establish whether the 30% of nucleoplasmic RNA synthesis inhibited under these conditions has any effect on the fingerprinting results presented in this thesis.

Results from the labelling of cells for different lengths of time show that the relative molar oligonucleotide frequencies of HnRNA

approach those of mRNA when cells are labelled for longer periods of time (16 hours instead of 3 hours). On the other hand mRNA relative molar oligonucleotide frequencies remain the same whether cells are labelled for 3 hours or 16 hours. There are several possible explanations of these results (Results section 4.1).

It seems unlikely that the HnRNA is contaminated with mRNA which has a significantly high level of radioactive labelling after incubation for long periods of time with $^{32}PO_4$, or that HnRNA is contaminated with radioactive rRNA synthesized slowly in spite of the presence of actinomycin D, because high levels of contamination would be required to give the observed C-G sequence content (75%) yet the base composition of the HnRNA does not change accordingly. The possibility that low levels of phosphate or large amounts of radioactivity in the culture medium are causing disruption of the normal cell metabolism in some unknown manner cannot be ruled out. However it seems most likely that we are observing an effect of actinomycin D on HnRNA synthesis.

When cells were incubated with actinomycin D for different periods of time and then labelled with $^{32}PO_4$ for 3 hours, the resulting relative molar oligonucleotide frequencies suggest that HnRNA produced after long incubations with actinomycin D had an increased C-G sequence content. Thus it seems possible that actinomycin D selectively inhibits the synthesis of HnRNA with a low C-G content.

A further possibility is that on labelling for longer periods of time we are observing the build-up of some relatively stable species of HnRNA which may act as a precursor to mRNA as it has a high C-G sequence content while the bulk of HnRNA is rapidly turning over in the

nucleus. This possibility gains support from recent work by Spohr et al. (1974) who have shown the presence of three metabolically distinct size fractions of HnRNA in avian erythroblasts. These are termed 1) nacent precursor mRNA with a half-life of 30 minutes and a size range from 5-20 x 10^6 mol. wt., 2) intermediate size precursor mRNA with a half-life of 3 hours and a size range from 1-5 x 10^6 mol. wt., and 3) small precursor mRNA with a half-life of 15 hours and a size range from 0.5-1.5 x 10^6 mol. wt. It is interesting to compare these size classes of HnRNA with the sizes of HnRNA found on sucrose-formamide gradients during the present work. HnRNA labelled for 3 hours shows predominantly "nacent precursor mRNA" size molecules whereas 16 hour labelled HnRNA shows predominantly "small precursor mRNA" size molecules.

Thus it seems most likely that although actinomycin D causes a reduction in the synthesis of HnRNA, the increase in C-G sequence content of HnRNA produced after long periods of labelling is due to the build-up of a relatively stable, small size class subfraction of HnRNA.

CONCLUSION

When HeLa cells have been radioactively labelled with $^{32}PO_4$ for 3 hours in the presence of low levels of actinomycin D (0.04 μg/ml), the HnRNA differs from the mRNA with respect to the relative molar oligonucleotide frequency of certain short sequences. This HnRNA can be shown to be a typical transcript of cellular DNA in terms of oligonucleotide frequency of selected short sequences.

Of fractions of HnRNA tested for mRNA like oligonucleotide frequency pattern, only S-HnRNA, a fraction of HnRNA loosely bound to chromatin (Price et al., 1974), approximated to that of mRNA. Other fractions tested included various size and homopolymeric sequence containing fractions of HnRNA.

Actinomycin D reduces the level of HnRNA synthesis in HeLa cells (Penman et al., 1968). However it probably does not alter its composition sufficiently to explain the increase in C-G sequence content observed on labelling HnRNA for 16 hours in the presence of low levels of actinomycin D. The most probable explanation for the relative molar oligonucleotide frequency of HnRNA approaching that of mRNA under these conditions is the accumulation of a relatively stable, small size, subfraction of HnRNA.

It seems probable that only a fraction of HnRNA functions as a precursor to mRNA and that there are other functions, perhaps of a regulatory nature, for HnRNA. Britten and Davidson (1969) have suggested that RNA could bind to a complementary region of DNA and thus activate a gene (see Introduction section 4.2). Monahan and Hall (1974) have suggested that RNA may displace tightly bound proteins and thus allow transcription. Recently a novel role for RNA fragments in influencing

gene expression by serving as primers for the transcription of additional RNA molecules has been proposed (Robertson and Dickson, 1975). In this model HnRNA fragments could control gene expression by priming the transcription of related genes. Maybe theories of this type explain the relationship of HnRNA to mRNA.

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