INTRACELLULAR COMMUNICATION OF GENETIC INFORMATION IN MAMMALIAN CELLS

by

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To my parents

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ABBREVIATIONS

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

AMV	Avian myeloblastosis virus
BHK/21	Baby Hamster Kidney
BSS	Balanced salt solution
cDNA	complementary DNA
CHO	Chinese Hamster Ovary
cpm	counts per minute
DEP	Diethypyrocarbonate
d-s	double-stranded
DMSO	Dimethylsulphoxide
DTT	Dithiothreitol
Hepes	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
HnRNA	Heterogeneous nuclear RNA
MES	Morfolino ethane sulfonic acid
NP-40	Nonidet P-40 (Non-ionic detergent)
poly(A)$^+$mRNA	RNA species containing poly(A) sequences at their 3' termini
poly(A)$^-$mRNA	RNA species lacking poly(A)
poly(A)$^-$u$^+$RNA	RNA species lacking poly(A) but having high affinity for poly(A)
PPLO	Pleuropneumonia like organism
PPO	2,5 diphenyloxazole
rDNA	DNA sequences that are transcribed into rRNA
SDS	Sodium dodecyl sulphate

ABBREVIATIONS (contd.)

SSC	0.15M NaCl, 0.015M Sodium citrate pH7.0
TEMED	NNN'N' tetramethylethynediamine
t-DNA	DNA sequences that are transcribed into t-RNA

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SUMMARY

Utilising the technique of poly(A)-sepharose affinity chromatography, it has proven possible to isolate a class of RNA from polysomes of Friend leukaemia cells, clone M_2, which does not appear to be of ribosomal origin since its synthesis is unaffected by concentrations of actinomycin D (0.04μg/ml) which inhibit rRNA synthesis. Although this class displays messenger-like behaviour in being released from polysomes on treatment with EDTA and in being able to direct polypeptide synthesis in a cell-free system it is nonetheless distinct from the well-known polyadenylated messenger RNAs (poly(A)$^+$mRNAs) as judged by the following properties -

(a) A complete lack of poly(A) tracts

(b) A different size distribution on denaturing gradients, displaying a mean size of 20s compared to 18s for poly(A)$^+$ RNAs.

(c) The presence, close to the 3' terminus, of an "uridylate-rich" region in contrast to the 3'-poly(A) tracts of the poly(A)$^+$RNA.

(d) Little sequence homology with poly(A)$^+$RNAs as judged from molecular hybridisation data.

(e) A different base composition compared to poly(A)$^+$RNA.

(f) In contrast to poly(A)$^+$RNA which is transcribed from both unique and middle-repetitive DNA sequences, this new class of RNA seems to be transcribed solely from unique sequences.

(g) A difference in metabolic stability between this class and the poly(A)$^+$RNA class was found.

(h) The size distribution of polypeptide products produced when this RNA is used as template in a cell-free protein synthesising system appears slightly different to that directed by poly(A)$^+$mRNA.

The lack of poly(A) tracts coupled with the presence of "U-rich" region(s) has led to the designation of this class of RNA as poly(A)$^-$u$^+$RNA. In addition to detecting these poly(A)$^-$u$^+$RNAs in polysomes, a similar class of RNAs have also been detected in nuclei. The nuclear poly(A)$^-$u$^+$RNAs elute from poly(A)-sepharose with increasing formamide concentration in a similar fashion to that of the polysomal poly(A)$^-$u$^+$RNAs. Indeed, the "U-rich" region(s) of nuclear and polysomal poly(A)$^-$u$^+$RNAs appear similar. In a further examining of the total nuclear RNA, two other RNA classes having either poly(A) tracts (poly(A)$^+$RNA) or oligo(A) tracts (poly(A)$^-$a$^+$RNA) were also detected. These three RNA classes display distinct size distribution and content of double-stranded regions. When the stability of these nuclear RNA classes were examined, using a "pulse-chase" approach, it was found that the poly(A)$^-$u$^+$RNA class was relatively labile compared to the poly(A)$^+$RNA. Interestingly, the poly(A)$^+$RNA appeared to have at least two distinct metabolic components. The "pulse-chase" approach used, allowed an estimate to be made of the conversion of nuclear poly(A)$^+$ and poly(A)$^-$u$^+$ RNAs into cytoplasmic poly(A)$^+$ and poly(A)$^-$u$^+$RNAs. Finally the metabolic behaviour of both the nuclear and cytoplasmic classes of RNA was found to be relatively unaffected by induction of Friend cells using Dimethylsulphoxide.

INTRODUCTION

1. Transcription

It is generally considered that the genetic information, which is ultimately responsible for the development and functioning of an organism, resides in the nucleotide sequence organisation of an organism's DNA.

Transcription may be viewed as the process by which the genetic information in DNA nucleotide sequences is converted into complementary RNA nucleotide sequences and is the first step in the expression of genetic information. By expressing information firstly through RNA transcripts, an organism obtains a "ready amplification" of genetic information and also a greater control over its ultimate expression. In vivo, this process occurs by reference to only one strand of the DNA molecule at specific regions within the DNA. It is catalysed by enzymes known as RNA polymerases.

For the most part, this study is concerned with the products of transcription. However, to provide a perspective for subsequent discussion, the nature of the DNA template and RNA polymerases will be briefly described.

2. Nature of eukaryotic DNA

In eukaryotes the DNA is distributed in a discrete fashion amongst the chromosomes of the cell. The nucleotide sequences that make up eukaryotic DNA have various degrees of repetition. This was first demonstrated by the work of Britten and Kohne (1968), who distinguished a highly repetitive fraction, an intermediate repetitive fraction and

a unique fraction, the precise proportions of these three classes being species dependent (see Table 1).

Further, molecular hybridization studies have allowed the examination and characterisation of specific eukaryotic DNA sequences, such as DNA sequences that are transcribed into ribosomal RNA, 5S RNA, transfer RNA and a few specific messenger RNAs. Some of the results of these studies are summarised in Table 1. The arrangement of these sequences relative to one another in the genome was investigated by Davidson et al (1973) and Graham et al (1974), who concluded that the unique sequences are interspersed with repetitive sequences. This interspersion of repetitive sequences has subsequently been shown in most eukaryotes, although exceptions have been found, interestingly, in a few insect species (Davidson et al 1975).

This pattern of interspersion may be summarised as below:

(i) The majority of DNA is in single copy sequences

(ii) A broad spectrum of repetition frequencies is present ranging from a few copies to a few thousands copies per genome

(iii) Repetitive and single copy sequences are interspersed, and single copy lengths range from less than a thousand nucleotides to several thousands nucleotides

(iv) The interspersed repetitive sequences are short ranging from 300 to 600 nucleotides.

That some functional significance underlies this pattern of interspersion is suggested by the observations of

Table 1

Frequency classes of DNA sequences found in eukaryotes

Frequency class of DNA	Percentage of the haploid genome	Number of copies per haploid genome	Examples
Unique	10-80	1	DNA sequences for haemoglobin, ovalbumin and silk fibroin mRNAs
Middle-repetitive	10-40	10^1-10^5	DNA sequences for rRNA, tRNA and histone mRNA
Highly-repetitive	0-50	$> 10^5$	*Satellite DNA sequences of 5-300 nucleotides

*Highly repeated, clustered, simple sequences of the genome that differ in buoyant density from the bulk of the DNA

(Adopted from Hood et al 1975)

Davidson et al (1975) who found that most sea urchin polysomal mRNAs are transcribed from single-copy genome regions adjacent to regions of repetitive DNA. This situation also seems to hold for haemoglobin coding sequences in duck DNA (Bishop and Freeman 1973).

The general model of interspersion of single-copy DNA and repetitive DNA has now also to accommodate the recent discovery of so-called gene "inserts". Gene "inserts" are sequences of DNA occurring actually within sequences of DNA corresponding to the mature RNA molecules. So far, their presence has been confirmed in a number of DNA sequences giving rise to mouse and rabbit β-globin, avian ovalbumin, murine immunoglobin light chain mRNAs, tRNA in yeasts as well as for portions of DNA sequences for ribosomal RNA in Drosophila melanog aster. In terms of length these insertion(s) range from 10-6000 base pairs (Wellauer and Dawid 1977, Goodman et al 1977, Jeffrey and Flavell 1977, Brack and Tonegawa 1977, Tilghman et al 1978, Dugaiczyk et al 1978, Gaparin et al 1978).

3. Structure of chromatin

In considering the possible interaction between RNA polymerase and DNA during transcription a complication arises in that all eukaryotes have their genomic DNA associated with both histone and non-histone proteins in a complex known as chromatin. Studies using biochemical and biophysical methods have shown considerable order in chromatin structure. Essentially an array of basic repeating units (nucleosomes) is found. Each nucleosome

consists of 140 base pairs of polymeric DNA arranged around the outside of an octameric histone core containing two copies of H_{2A}, H_{2B}, H_3 and H_4 histones. Each subunit is separated by a similar length of internucleosomal DNA in the range 20-40 base pairs long, whose length is dependent on cell type (Compton et al 1976) and transcriptional status (Lohr et al 1977). This intranucleosomal is associated with one histone, H_1 (Kornberg 1977, Felsenfeld 1978). It is thought that the histone H_1 may be responsible for the transnucleosomal and internucleosomal crosslinking necessary for the integrity of the higher orders of chromatin structure found in the nucleus (Felsenfeld 1978). Two levels of higher organisation of chromatin structure have been reported. The first appears as a thin chromatin filament (100A^{o}) and the second as a thicker fibre (300A^{o}). The thin filament is a linear array of connected nucleosome cores. The thick fibre seems to be generated by coiling of the thin filament (Felsenfeld 1978).

Such a subunit structure is a characteristic feature of both transcriptionally active and non-active regions of the chromatin (Gottesfeld and Melton 1978). Thus, if the chromatin template is being changed either as a result of transcription, or to facilitate it, then some quite subtle alterations are apparently occurring.

Using both in vitro transcription from chromatin and nuclease sensitivity as probes, considerable evidence concerning the structure of transcriptionally active chromatin has been obtained. The observation that

transcriptionally active regions of the genome are preferentially attacked by either pancreatic DNA ase I (Garel and Axel 1976) or spleen DNA ase II (Gottesfeld et al 1974) suggests that the DNA within these regions is less protected. This is consistent with the work of Axel et al (1973), Gilmour et al (1973) who described preferential transcription of globin genes from erythroid chromatin by bacterial RNA polymerase, since the globin DNA might be less protected and allow preferential binding of polymerase.

That the non-histone component of chromatin may influence transcription was suggested from early work utilising in vitro transcription from reconstituted chromatin, again using bacterial RNA polymerase (Barrett et al 1974, Gilmour and MacGilliveray 1976). These observations, however, were questioned by the work of Zasloff and Felsenfeld (1977 a, b) and Konkel and Ingram (1978). In these studies, the availability of mercuronucleotides permitted newly synthesised RNA to be purified. In this way, it was shown that under the conditions of chromatin transcription generally employed, E. coli RNA polymerase can utilise endogeneous RNA as templates for the synthesis of complementary RNA (antisense strand) (Zasloff and Felsenfeld 1977 a, b). Thus, when transcripts of duck reticulocyte chromatin were prepared in the presence of mercurinucleotides and purified by sulfhydryl-Sepharose affinity chromatography without prior heat denaturation, globin sequences were readily detected in the mercurated RNA.

This apparent synthesis of globin sequences, however, was shown to be artefactual as the newly synthesised mercurated-RNA was in fact present as a duplex with endogeneous globin mRNA. Mercurated transcripts prepared by affinity chromatography after heat denaturation were essentially devoid of globin sequences.

Nevertheless, Konkel and Ingram (1978) using appropriate disaggregation procedures concluded that a detectable level of selective transcription occurs from globin genes in chicken reticulocyte chromatin, whereas dissociation of erythroid chromatin in salt and urea, followed by reconstitution using standard methods, destroyed even this low degree of specificity. As a consequence of this work the previous results obtained with in vitro systems should be viewed with some caution, especially in their use of bacterial polymerase.

A preferable technique would be the use of an endogeneous polymerase. This has been attempted for Xenopus 5S RNA transcription using purified Xenopus polymerase III. Here, Parker and Roeder (1977) found a preferential and asymmetric transcription from Xenopus chromatin although no reconstitution experiments were attempted.

4. RNA polymerase

Transcription is mediated by DNA-dependent RNA polymerase(s) which have been isolated from a wide variety of sources both eukaryotic and prokaryotic.

In terms of their characterisation and properties these enzymes are probably best discussed under two headings, the prokaryotic enzymes and the eukaryotic enzymes.

4.1 Prokaryotic

Bacterial RNA polymerase is a complex oligonermic enzyme with capacity to catalyse the transcription of DNA. This enzyme consists of a catalytically competent core of four subunits $\beta\beta'a_2$ (core enzyme) and a fifth dissociable subunit (σ) responsible for the selection of specific sites for chain initiation. The core enzyme plus the subunit (σ) is known as the holoenzyme.

The functional role(s) of the various subunits is under investigation, nevertheless some evidence suggests that the β' subunit is involved in template binding and the β subunit contain at least part of the catalytic centre, but the function of a subunits are not known (Zilling et al 1977).

4.2 Eukaryotic

In eukaryotes, synthesis of RNA is mediated by several DNA-dependent RNA polymerases. These enzymes are tightly bound to the nuclear chromatin complex, but can be solubilised and characterised into three main classes I, II and III based on their elution order from DEAE-Sephadex (Roeder and Rutter 1969). These classes of polymerase can be further resolved into sub-classes, depending on the cell type, by chromatographic and electrophoretic techniques. Each of the sub-classes is composed of a number of polypeptides, most of which seem to be common

to sub-classes in the same enzyme class. In contrast, only a few common polypeptides have been identified between different polymerase classes (Roeder et al 1976, Roeder 1977).

Interestingly, it has been possible to ascribe a nuclear localisation to the different RNA polymerase classes. Class I activity appears restricted in the nucleolar fraction whilst class II and class III appears only in the nucleoplasm (Roeder and Rutter 1970). Consistent with the different polypeptide composition of the polymerase classes are their properties in relation to ionic strength optima, stimulation by divalent anions, inhibition by various drugs and template requirements. The class I enzymes have optimal activity at low ionic strength, show maximum stimulation by Mn^{2+} and Mg^{2+} and are strongly resistant to the fungal agent α-amanitin. The class II and III enzymes exhibit optimal activity under high Mg^{2+} concentration, high ionic strength, and are inhibited by α-amanitin. In addition, the enzymes shows markedly different activities depending upon the composition and physical state of the DNA template (Roeder 1977).

The sensitivity to α-amanitin has been used as a tool to elucidate the transcriptional function of the various polymerase classes. Under different α-amanitin conditions, the RNA transcribed can be characterised using both electrophoretic analysis and molecular hybridisation techniques. In this way, using a number of in vitro systems, class I enzymes can be shown to transcribe 18S

and 28S ribosomal RNA, whilst class II seems to transcribe hnRNA and mRNA, class III transcribing t-RNA and 5S RNA (Susuki and Giza 1976, Parker and Roeder 1977, Matsui et al 1977, Roeder 1977).

Since differential alterations in the rate of synthesis of the major classes of RNA (rRNA, tRNA, hnRNA) have been observed during a number of physiological changes and the major RNA species are transcribed by distinct enzymes, it is possible that some modulations in gene activity are affected in part by the changes in RNA polymerase levels. Supporting evidence for this possibility, in which rates of RNA synthesis could be correlated to amounts of endogeneous RNA polymerase, has been reported in a variety of systems, notably those involving the stimulation of differentiated cell types into proliferation as in regenerating liver (Roeder 1977).

In contrast to these observations, it has been shown that during early development of Xenopus laevis, although qualitative and quantitative changes in gene expression occur, the absolute amounts and relative proportions of the nuclear RNA polymerases do not change (Roeder et al 1974). This suggests that some additional factor(s) must also regulate the activity and/or selectivity of various RNA polymerases (Roeder 1977).

4.3 Mechanism of transcription

The mechanism(s) of transcription in eukaryotes is still, however, largely unknown. The analogous reaction in

bacteria, especially E. coli, on the other hand is more fully understood, and an outline of the mechanism(s) of transcription in bacteria follows.

The mechanism by which the bacterial holoenzyme synthesises RNA can be viewed as a four step process (Chamberlin 1977).
(i) The first involves random binding of the holoenzyme to the DNA leading to an extremely stable binding at the promoter regions.
(ii) Once this has occurred RNA chains are initiated by the catalytic coupling of a purine triphosphate with a second ribonucleoside triphosphate to generate a dinucleotide tetraphosphate of the structure pppPupX.
(iii) This dinucleotide tetraphosphate is elongated by sequential addition of nucleoside triphosphates to the 3'-OH terminus of the nascent RNA chain by a single RNA polymerase molecule, the selection of nucleotide triphosphates almost certainly being dictated by Watson-Crick base pairing with the template DNA strand. Thus, for example, a guanine residue in the template DNA will cause insertion of a complementary cytosine residue in the RNA transcript and so on. Evidence for the complementary nature of the RNA has been obtained from many sources. As early as 1961, Geidushek et al (1961) demonstrated that the RNA synthesised in vitro by micrococcus RNA polymerase has a high complementarity for the primer DNA. Other techniques have also been used to establish this point. Thus the base ratios of RNAs synthesised by E. coli RNA polymerase, using DNA primers from various

sources, were shown to be complementary to the base ratios 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.

5. Products of transcription and their relationship to the DNA template

The relationship between the DNA template and its RNA transcripts has been the subject of a number of investigations in a variety of systems. A distinction must be made, however, between the primary products of transcription and the mature transcripts, since it is now well established that the initial RNA products of transcription differ in a number of respects from the mature RNA species (Perry 1976), the sequential events producing the mature form being described as post-transcriptional processing.

The section that follows is intended to provide a summary of the current evidence, concerning the production of a number of eukaryotic primary transcripts and their post-transcriptional processing.

5.1 Ribosomal RNA precursor

The DNA sequences that are transcribed into ribosomal RNA (rDNA) form part of the intermediate repeated fraction and may be repeated from hundreds to thousands of times per genome depending on the cell type considered. All these copies are clustered into a few regions of certain

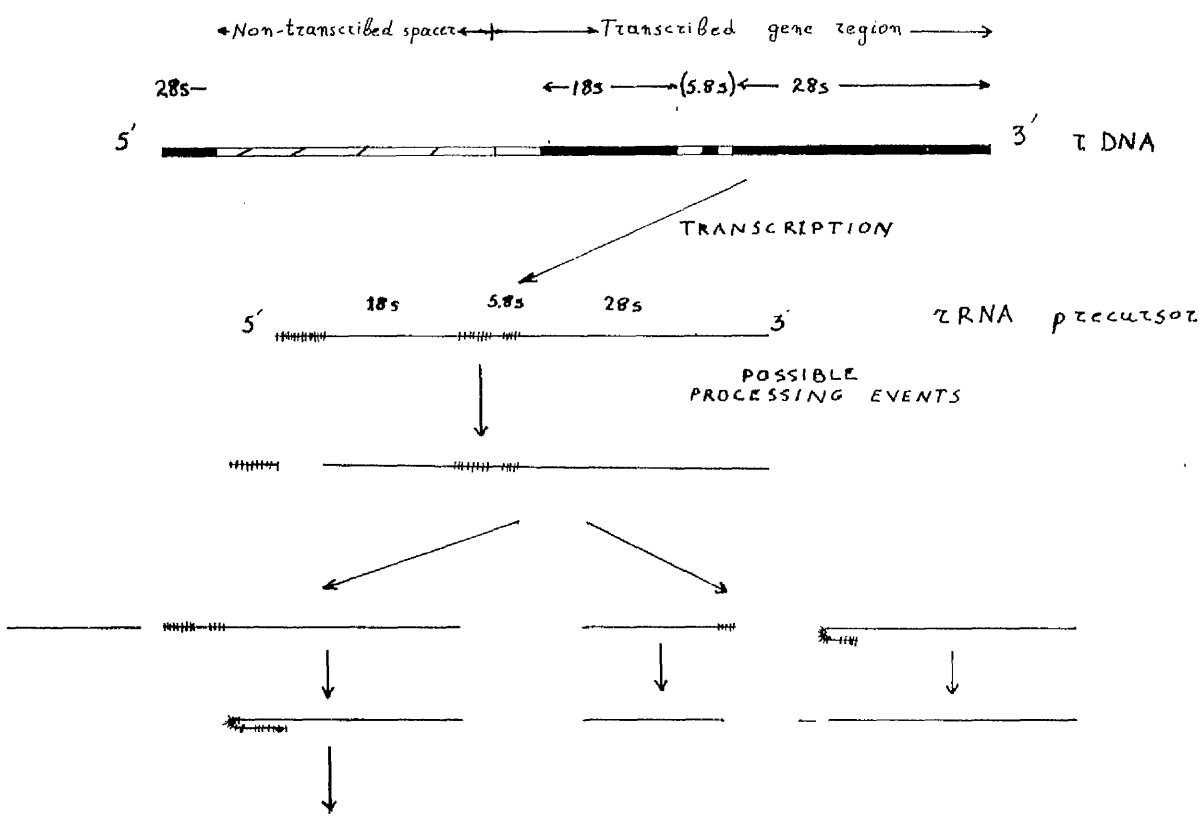
chromosomes, each region forming a nucleolar organiser (Tartof 1975).

The basic organisation of rDNA takes the form of alternating transcribed regions and non-transcribed spacer regions (see Fig 1). The transcribed regions consist of the coding sequences for the 18S, 5.8S and 28S rRNAs separated by transcribed regions that are degraded during ribosome maturation (Dawid and Wellauer 1976, Maden 1978). Recent data also indicate that in Drosophila some of the DNA sequences that are transcribed into 28S rRNA appear to contain an additional segment of DNA inserted within the coding sequences (Wellauer and Dawid 1977). Transcription of rDNA gives rise to a large precursor RNA molecule, which varies in size between species, due to differences in length of transcribed spacer region, and, to a lesser extent, the coding sequences (Hadjiolov 1977). The arrangement of the ribosomal and spacer RNA sequences in this precursor is shown in Fig 1.

In all eukaryotes, processing of ribosomal RNA precursors occurs almost entirely within the nucleolus involving post-transcriptional modifications and cleavage of specific sites by processing enzymes. The modifications include ribose methylation and other base modification (e.g. methylation, pseudouridylation) which occur at the primary transcript level, these being preferentially confined to the regions of transcripts that are ultimately processed into mature RNA. On the other hand the transcribed spacer regions are eliminated (Maden 1976, Perry 1976).

Figure 1

The current model relating the DNA template, primary transcript and processing events leading to the formation of mature mammalian ribosomal RNAs



▭ represent transcribed spacer in rDNA

⋯ represent transcribed spacer in rRNA precursor

— represent transcribed regions in rRNA precursor

(Adopted from Perry 1976, Maden 1978)

This elimination can be represented as in Fig 1. The processing enzymes involved in the maturation of the ribosomal RNA have not yet been isolated and characterised (Perry 1976).

5.2 Transfer RNA precursor

The DNA sequences that are transcribed into eacht-RNA species (t-DNA) ase present in multiple copies in the order of ten to a few hundreds repeats (Tartof 1975). Unlike the rDNA, the t-DNA sequences appears to be distributed over many chromosomes (Aloni et al 1971). In terms of the arrangement of the DNA sequences that are transcribed into different t-RNAs considerable controversy still exists. In Xenopus, early work suggested that the sequences coding for the same tRNAs (i.e. t-RNA^{Met}) are clustered but are separated from the clusters of sequences coding for other t-RNAs (i.e. t-RNA^{Val})(Clarkson and Brinstiel 1973). Quite recently, however, it has been possible to demonstrate that DNA fragments containing the t-RNA^{Met}, seem to contain at least one other kind of t-RNA species (Clarkson and Kurer1976). The work on Drosophila suggests that sequences coding for t-RNA^{Glu} are not clustered in one site, but distributed over three sites of Drosophila genome (Kubli and Schmidt 1978). Further in yeast the DNA sequences that are transcribed into t-RNA^{Tyr} or t-RNA^{Phe} is interupted by a small intervening DNA sequences (Goodman et al 1977, Valenzaela et al 1978).

The primary transcript for most t-RNA species is a larger

molecule 10-20 nucleotides longer than the mature t-RNA, to which is converted by either "spligation" (endonucleolytic cleavage and removal of the transcribed intervening DNA sequences, and rejoining of the coding sequences)(Knapp et al 1978, O'Farrell et al 1978) or exonuclease (trimming) reactions (Burdon 1975) or possibly both. In addition, specific nucleotide sequences are modified by processing events which alter certain nucleotide residues (e.g. methylation, pseudouridylation)(Burdon 1975, Perry 1976). The timing of these processing events is, as yet, not fully investigated although the processing may take place in both nuclei and cytoplasm (Burdon 1975, Knapp et al 1978, O'Farrell et al 1978).

5.3 5S RNA precursor

Here again, the DNA sequences that are transcribed into 5S RNA appears to be present in multiple copies ranging from 100 to 24000. Interestingly the DNA sequences that are transcribed into 5S RNA are not physically linked to rDNA sequences in eukaryotes and may be clustered in a single site on one chromosome as in human cells or clustered as discrete sites over many chromosomes (Tartof 1975). The primary transcript of Drosophila 5S RNA has been shown to be a molecule 15-25 nucleotides longer than the mature form (Jacq et al 1977, Levis and Penman 1978). Like t-RNA, this molecule is transcribed outside the nucleolus and the processing may take place in both nuclei and cytoplasm (Jacq et al 1977).

5.4 Heterogeneous nuclear RNA

The nuclei of eukaryotic cells also contain a rapidly labelled high molecular "DNA-like" RNA fraction of heterogeneous size termed heterogeneous nuclear RNA. This contrasts with the large nucleolar-located precursor of rRNA and the products of its processing which are discrete homogeneous species (Scherrer et al 1963, Yoshikawa-Fukada et al 1965, Warner et al 1966, Attardi et al 1966, Seiro et al 1968, for review see Weinberg 1973).

5.4.1 DNA sequences that are transcribed into hnRNA

Most hnRNA molecules appear to be transcribed from DNA organised as interspersed middle repetitive and unique sequences. This was suggested by the observations that the majority of the rapidly labelled hnRNAs in sea urchin embryos (Smith et al 1974), Ehrlich ascites cells (Holmes and Bonner 1974) and HeLa cells (Molloy et al 1974) consisted largely of transcripts having both unique and middle repretitive sequence components within the same molecule, this repetitive component comprising about 10-20% (Smith et al 1974, Holmes and Bonner, 1974).

Unfortunately little is known of the nature of the repetitive DNA sequences which are transcribed into hnRNA (Davidson, 1976). On the other hand, considerable data relating to the transcribed unique DNA has been accumulated, particularly from molecular hybridisation studies using highly labelled unique DNA and total cellular or nuclear RNA. In this way it was found that in sea

urchin embryos 28.5% of the unique DNA sequences of the genome are asymmetrically transcribed into gastrula hnRNA (Hough et al 1975). Similarly, it has been shown that 20-30% of Drosophila single-copy DNA coding capacity is expressed as nuclear RNA (Levy et al 1977). Different tissues in the same organism have been shown to transcribe different proportions of their unique DNA. For example, in rodent spleen 6-12% of the unique DNA is transcribed whilst in brain this figure is 14-42% (Brown and Church 1972, Bantle and Hahn 1976, Chitarashi et al 1978). Also, it has been reported that the same tissue at different developmental stages of the organism transcribe different proportions of unique DNA (Brown and Church 1972).

5.4.2 Structural features of hnRNA molecules

To date a number of structural features have been shown in hnRNA, although not every feature is necessarily present in any particular hnRNA molecule.

They can be listed as below:

(i) A long (180-230 nucleotides) poly(A) segment which seems to be added post-transcriptionally to the 3' end of some hnRNA molecules by sequential addition of adenylate residues (Darnell et al 1971, Lee et al 1971, Mendecki et al 1972, Molloy et al 1972, Nakazato et al 1973, Dubroff et al 1975). Subsequently, hnRNA molecules possessing such a segment will be referred to as poly(A)$^+$ hnRNA.

(ii) A short (20-40 nucleotides) internally located

oligo(A) segment which is produced by transcription (Nakazato et al 1973, 1974). This is not found in mammalian mRNA, however, Dyctiostelium mRNA does contain internally an oligo(A) segment having a size of 20-30 nucleotides (Lodish et al 1974).

(iii) A short (20-30 nucleotides) oligo(U) segment which is transcribed from repetitive region of the genome (Molloy et al 1972). Clustering of such segments may occur and this has been reported in the 5' half of at least some HeLa poly(A)$^+$ hnRNA species (Molloy et al 1974), and Ehrlich carcinoma cells hnRNA species (Bajszar et al 1976). Additionally, the occurrence of either oligo(U) or "U-rich" regions in non-polyadenylated hnRNA species (poly(A)$^-$ hnRNA) has been reported in a variety of cell types including HeLa cells (Korwek et al 1976), BHK/21 cells (Burdon et al 1976) and sea urchin embryos (Dubroff 1977).

(iv) Mammalian hnRNA also contain a non-poly(A) fraction resistant to the action of pancreatic and T_1 ribonuclease at high ionic strength (Jelinek and Darnell 1972, Ryscov et al 1972). These regions correspond to at least 3-5% of the tritiated uridine labelled hnRNA, and appear to be double-stranded RNA as was judged by their ability to bind to hydroxyapatite, their buoyant density in Cs_2SO_4, their symmetrical base composition, and their ability to self-anneal after denaturation (Jelinek and Darnel 1972, Ryscov et al 1972, Jelinek et al 1974). That double-stranded regions observed in hnRNA could not be artifacts generated by rapid intermolecular reassociation during deproteinisation, as in the case of intermolecular duplexes formed by

complementary sequences in separate hnRNAs (Fedoroff et al 1977), was suggested by the work of Calvet and Pederson (1977), where double-stranded (d-s RNA) was found on native hnRNP particles. Furthermore, the fact that some of these d-s RNA regions have no tissue specificity (Ryscov et al 1976), as well as their ability to hybridise with mRNA (Ryscov et al 1976b) suggests that d-s RNA might possibly serve as cleavage sites for endonucleolytic processing involved in the biogenesis of mRNA.

(v) Large hnRNA molecules seem to possess a 5' terminal triphosphate which is almost always a purine (Ryscov and Georgiev 1970, Georgiev et al 1972, Schibler and Perry 1976, Bajart et al 1976). In addition, 5' terminal triphosphates have also been detected in relatively small ($\leq$28S) poly(A)$^+$ hnRNA from Ehrlich ascites cells (Schmincke et al 1976).

(vi) The presence of post-transcriptionally modified bases found in hnRNA molecules. Such modifications include internal methylated adenines (m^6A) and also frequently found "block 5' terminus" which has the general structure $m^7G(5')pppX^mpYp$ (Perry et al 1976, Salditt-Georgief et al 1976). Such structures are also found in mRNAs (see section 6.1) and are referred to as "cap" structures.

5.4.3 Metabolism of hnRNA molecules

The majority of the rapidly labelled hnRNA molecules remain in the nucleus only a small minority entering the cytoplasm (Harris 1963, Georgiev and Lerman 1964, Attardi et al 1966). More accurate measurements suggest that

2-6% of hnRNA synthesised in HeLa or L cells enters the cytoplasm (Brandhorst and McConkey 1974, Herman and Penman 1977). In addition, hybridisation and kinetic analysis experiments suggest that a large proportion of nuclear poly(A)$^+$hnRNA sequences as well as the poly(A) itself are confined in the nucleus (Perry et al 1974, Ryffel 1976, Herman et al 1976, Levy and McCarthy 1976, Minty et al 1977). These data suggest that the hnRNA may be turning over quite rapidly. Evidence supporting this came from a study using the transcriptional inhibitor actinomycin D, in which HeLa hnRNA was found to have a half-life at 30 minutes (Penman et al 1968). The accuracy of such measurements is open to question, however, since evidence has been obtained suggesting that actinomycin D can alter the normal processing of hnRNA (Levis and Penman 1977, Herman and Penman 1977, Bastos and Aviv 1977). In addition, Brandhorst and McConkey (1974), and Williamson and Tobin (1977) using data from the molar accumulation of radioactive precursors into hnRNA subjected to first order analysis concluded that half-lives of L-cells hnRNA and chicken erythroblast hnRNA were about 23 and 18 minutes respectively. Of this magnitude, but somewhat greater is the hnRNA half-life estimate for HeLa hnRNA of 70 minutes, suggested by Herman and Penman (1977) using a very efficient pulse-chase procedure. However, Spohr et al (1974) report that avian erythroblast hnRNA can be resolved into three metabolically distinct size-fractions, with half-lives ranging from 30 minutes to 15 hours. Similarly

it has been shown that Xenopus hnRNA can be resolved into at least two metabolically distinct size-fractions (Anderson and Smith 1977). Furthermore, poly(A)$^+$hnRNA from both Drosophila and rat liver cells was shown to have at least two kinetically distinct components. In contrast, the poly(A)$^-$hnRNA exhibited a single kinetic component (Levis and Penman 1977, Chemovskaya and Lerman 1977).

5.4.4. The possible relationship between hnRNA and mRNA

That sequences occurring in mRNA also occur in much larger RNA units was first suggested by work examining viral messengers in HeLa cells productively infected with Adenovirus-2 (Wall et al 1972).

The occurrence of poly(A) sequences at the 3' end of some messengers (see section 6.1) led to the development of a technique in which oligo(dT) hybridised to this poly(A) and the resultant hybrid used as a substrate for viral reverse transcriptase (Verma et al 1972). This enables a labelled DNA strand to be synthesised complementary to the messenger RNA. A DNA strand produced in this way is termed complementary DNA (cDNA). This DNA could be used as a probe for complementary messenger RNA sequences in hnRNA, using molecular hybridisation techniques.

In this way, the presence of globin messenger sequences was shown in high molecular weight hnRNA isolated from a variety of erythroid cells (Imaizumi et al 1973, Spohr et al 1974, MacNaughton et al 1974, Ross 1976, Kwan et al 1977). The work of several investigators also revealed

globin mRNA sequences in hnRNA isolated from immature red blood cells. These hnRNA molecules sedimenting faster than 28S ribosomal RNA (Imaizumi et al 1973, Spohr et al 1974, Spohr et al 1976). Smaller values for the size of the large nuclear molecules were obtained by MacNaughton et al (1974).

More recently Strair et al (1977) have reported that under conditions which fully denatured RNA, duck erythrocyte nuclear RNA molecules containing globin mRNA sequences can be detected in three distinct size classes, of 28S, 16.5S and 10S, respectively. Similar sized molecules containing globin mRNAs sequences have also been described in pulse label hnRNA prepared from dimethylsulfoxide-induced Friend leukaemia cells (Bastos et al 1977), although Curtis et al (1976) could detect only two distinct classes of about 14-15S and 10S, respectively. In addition, Ross (1976) and Kwan et al (1977) have also detected a labelled 15S RNA, which hybridised to globin cDNA from mouse fetal liver and spleen cells of anemic mice. A number of other messenger sequences, for example histone, albumin, ovalbumin and immunoglobin light chain messengers, have also been successfully detected in much larger hnRNA molecules (Melli et al 1977, Stair et al 1978, Roop et al 1978, Gilmore-Hebert and Wall, 1978).

Furthermore, Sippel et al (1977), using cDNA prepared from the most abundant class of rat liver poly(A)$^+$mRNA, was able to identify messenger sequences in large nuclear molecules. Similarly, Hames and Perry (1977) prepared

a DNA probe complementary to L cell poly(A)$^+$RNA by hybridising single-copy L-cell DNA to excess poly(A)$^+$RNA. After purifying the hybrid by a multi step process (i.e. hydroxyapatate chromatography followed by Cs_2SO_4 buoyant density gradient centrifugation) they were able to detect messenger sequences in large hnRNA molecules.

In contrast to the preceding observations, McKnight and Schimke (1974) could only detect ovalbumin messenger RNA sequences in steady-state nuclear RNA molecules of the same size as the mature messenger. This situation was also reported for a murine leukaemia virus specific messenger where messenger sequences could be found in hnRNA molecules of the same size as the mature mRNA (Fan 1976).

These differences may reflect purely technical problems, such as those encountered in the sizing of RNA molecules and in the detection of the messenger sequences. Alternatively, such differences may have biological significance and may arise due to a very rapid processing of some large precursors, or could, indeed, signify that no large precursors exist.

Apart from the above RNA sequences studies, a variety of other evidence suggests that hnRNA and mRNA have a precursor-produce relationship. This evidence includes kinetic studies with or without inhibitors, and striking similarities in the type of post-transcriptional modification. In the former case, drug treatment that inhibits hnRNA synthesis or prevents the addition of poly(A) also inhibits

the production of poly(A)$^+$mRNA (Darnell et al 1971 Adesnik et al 1972, Latorre and Perry 1973, Jelinek et al 1973, Johnson et al 1975). Also pulse-chase experiments have revealed a relationship between the breakdown of poly(A)$^+$ hnRNA and the appearance of poly(A)$^+$mRNA (Levis and Penman 1977, Herman and Penman 1977).

Post-transcriptional additions, including the presence of a poly(A) segment at the 3' end as well as terminally methylated nucleotides shared by hnRNA and mRNA, have suggested that some mRNA might be derived from larger hnRNA molecules (Perry et al 1976).

5.4.5 The possible location of mRNA sequences in hnRNA

If at least some hnRNA is a precurosr to mRNA, the question arises as to the location of the messenger sequences within the larger molecule.

Several models have been proposed on the following experimental grounds:

(i) The detection of a poly(A) segment at the 3' end of some hnRNA as well as mRNA molecules (Darnell et al 1973).

(ii) Hybridisation studies that have revealed a large portion of mRNA sequences to be located near to the 3' end of the poly(A) containing hnRNA molecules (Herman et al 1976, Ryffel 1976, Minty et al 1977).

(iii) The remarkably similar composition of 5' terminal methylated nucleotides and the conservation of methyl-labelled 'caps' (see section 6.1) in pulse-chase

experiments indicating that mRNA might be derived from the 5' terminal portion of hnRNA molecules (Perry et al 1976). This was particularly striking in the resemblance of cap-containing oligonucleotides derived from hnRNA and mRNA after exhaustive digestion with T1 ribonuclease (Schibler et al 1977).

(iv) An interesting recent finding in relation to the question of possible mRNA precursors and the location of mRNA sequence in hnRNA has been the discovery of so-called gene inserts. These have been found in a number of genes (e.g. mouse and rabbit β-globin, avian ovalbumin) and are typified by the presence of an intervening DNA sequence(s) within the messenger coding region itself (Jeffrey and Flavel 1977, Tilghman et al 1978a, Garapin et al 1978). Recent evidence suggests that these intervening sequences are transcribed although they do not occur in the final messenger. For example, the 16S hnRNA containing the β-globin gene transcriptalso contains sequences complementary to the inserted sequences found in the β-globin sequence at the genomic level (Tilghman et al 1978b). Thus messenger RNA sequences may be arranged in the precursor molecules in a discontinuous way reflecting the genome organisation.

5.4.6 Possible mechanism of processing of hnRNA to mRNA

The results mentioned above provide strong evidence for the occurrence of mRNA sequences in hnRNA although direct

evidence for the various steps in processing of hnRNA to mRNA is still lacking.

Processing of hnRNA seems to be confined to the nucleus and takes about 5-120 minutes as judged by the time taken for the appearance of globin messenger sequences or pulse labelled poly(A)$^+$mRNA in the cytoplasm of Friend or HeLa cells respectively (Bastos et al 1977, Herman and Penman 1977). On the other hand, histone mRNA enters the cytoplasm faster than poly(A)$^+$mRNA (Schochetman and Perry 1972).

Three general mechanisms might be involved.

(i) Cleavage of the coding region(s) from the large hnRNA molecules. Georgiev et al (1973), Naora and Whitelam (1975), and Ryscov et al (1976) have obtained evidence that hair-pins and loops in hnRNA molecules may be sites for the cleavage events. In addition, recent data suggest that in some eukaryotic messenger RNA precursors, the messenger sequences are arranged in a discontinuous way (Tilghman et al 1978). Therefore, it has been proposed that mRNA precursors are cleaved by endocleolytic actions separating the messenger sequences from the inserted sequences, and the required messenger sequences are joined to give the mature messenger by some splicing mechanism (Marx 1978).

(ii) Capping of the 5' terminus would seem to be the other event in the processing of at least some hnRNAs as judged by the similarities in caps in some hnRNAs and most messengers (Perry et al 1976).

(iii) Addition of poly(A) is also a processing event for some hnRNAs and involves post-transcriptional addition of a tract of poly(A) which is up to 200 nucleotides long (Darnell et al 1973).

The timing of these various events relative to each other may be quite different between different hnRNA molecules, suggesting that processing may be under some form of control. For example, poly(A) addition may occur immediately after transcription (Derman and Darnell, 1974) or after transcript cleavage (Derman and Darnell 1974, Bastos et al 1977). That some control on processing may occur is also suggested by observations on sea urchin embryos where the occurrence of histone messenger in precursor may be dependent on the developmental state (Kunkel et al 1978). The nature of processing is very complex and emphasised by the observation that a wide variation in stability is exhibited by the different subfractions of the putative messenger RNA precursors (Perry et al 1976, Levis and Penman 1977, Chemovskaya and Lerman 1977).

It seems likely that hnRNA processing and transport occur at the level of ribonucleoprotein particles. In fact, poly(A)$^+$ messenger RNA sequences have been detected in association with proteins in the nucleus, in polysomes and in free cytoplasmic particles (Williamson 1973, Brawerman 1975, Kinniburg et al 1976). Whether the proteins of these different ribonucleoproteins are totally or partially conserved either during transport into the

cytoplasm or during the activation of inactive messenger ribonucleoproteins is under investigation (Liautard et al 1976, Van Venesolj and Janseen 1978). Further studies on the release and composition of ribonucleoproteins from isolated nuclei may cast some light on the processing of hnRNA.

6. Messenger RNA

In eukaryotes the radioactively labelled RNA present in polysomes after a short pulse label period consists primarily of an heterogeneous population of "DNA-like" RNA molecules, with sedimentation value ranging from 10S to 30S (Penman et al 1963, Girard et al 1965, Perry and Kelly 1968, Penman et al 1968, Darnell 1968).

The existence of mRNAs which are not associated with polyribosomes, however, has also been reported (Cander et al 1973, Macleod 1975), and there is evidence suggesting that certain poly(A)$^+$mRNAs extractable from total cytoplasm are absent or undetectable in polysomal poly(A)$^+$ mRNA (Levy and Rizzino 1977).

Whilst a definite proof that an RNA can function as a message is its ability to direct the synthesis of specific protein in a cell-free protein synthesing system, other criteria have been used to identify mRNAs. For example, their heterogeneous size, their distinctive base composition and their release from polysomes by EDTA or puromycin in the form of slowly sedimenting ribonucleoproteins (Darnell 1968, Penman et al 1968, Perry and Kelly 1968,

Mathews 1973).

6.1 Messenger RNA structure

A number of structural features have been described in mRNA, although not every feature is necessarily present in any particular mRNA.

They can be listed as follows:

(a) Evidence for the occurrence of poly(A) sequences in cytoplasmic and viral mRNA was first provided by Lim and Canellakis (1970), Kates et al (1970). The location of the poly(A) segment in mammalian and viral mRNA is known to be the 3'-OH terminus. This conclusion has been reached by using a variety of techniques such as:

(i) End group analysis (Kates 1970)

(ii) Digestion with exonuclease (Molloy et al 1972)

(iii) Periodate oxidation (Yogo and Wimmer 1972).

The occurrence of messenger RNAs with a poly(A) segment covalently bound to the 3' end has been demonstrated in organisms throughout the phylogenenic scale (Kates 1970, Lee et al 1971, Darnell et al 1971, Firtel et al 1972, Mclaughlin et al 1973). Furthermore poly(A) segments have also been found in mRNA molecules from mitochondria (Perlman et al 1973), and viruses (Kates 1970).

The poly(A) segment of mammalian mRNAs have been shown to undergo a process of gradual size decrease after appearance in the cytoplasm (Sheiness and Darnell,1973) reaching a final size which is still, however, quite large

(Greenberg and Perry 1972, Sheiness and Darnell 1973). This behaviour suggests that the poly(A) segment in functional mRNAs is the target of a specific poly(A) cleaving agent(s) (Bergman and Brawerman 1977). The limited nature of this degradation may also reflect the association of the poly(A) segment with one or more proteins (Kwan and Brawerman 1972, Blobel 1973, Soreq et al 1974).

(b) Recent studies indicate that after transcription both eukaryotic and viral poly(A)$^+$mRNA sequences are modified by methylation, which may occur both internally and at the 5' terminus (Perry 1976, Shatkin 1976). The terminal structures, known as caps, are of three general types: Cap I ($m^7G(5)pppXp$), Cap II ($m^7G(5)pppX^mpYp$ or $m^7G(5)ppp\ m^6ApYp$), and Cap III ($m^7G(5)pppX^mpY^mp$) (Shatkin 1976). Internal methylation produces N^6 methyladenylic acid (m^6A) and smaller amounts of N^7 methylcytidilic acid (m^7C) (Shatkin 1976). The location of m^6A in the poly(A)$^+$mRNA was suggested to be in both 3' and 5' portions of mouse cell poly(A)$^+$mRNA (Perry et al 1975) but only in the 5' portion of HeLa cell poly(A)$^+$mRNA (Salditt-Georgiev et al 1976). This is not to say, however, that all poly(A)$^+$mRNA contain m^6A, viral and cellular poly(A)$^+$mRNA have been shown to lack m^6A (Rottman 1976). Poly(A)$^-$mRNA also have both caps and m^6A, except histone mRNAs, which only have methylated 5' terminal sequences (Surrey and Nemer 1976).

The widespread occurrence of caps in both eukaryotic and viral mRNAs suggests a role in protein synthesis. Evidence supporting this conjecture was the observation

that removal of the m^7G residue from the native mRNA resulted in a decrease in both protein synthesis and ribosome binding ability (Shatkin 1976). However, this role for 5' terminal m^7G has been recently questioned in a critical review by Griffin (1976). Also, in contradiction to a role in protein synthesis, is the recent evidence that a variety of viral mRNAs lack 5' caps and yet function normally as templates for protein synthesis Griffin 1976, Shatkin 1976).

(c) Careful size measurements have indicated that mRNA is longer than required for coding of polypeptides, but not long enough to code for more than one protein (Davidson and Britten 1973, Lewin 1975b). For instance, rabbit globin mRNA is about 660 nucleotides long (Williamson et al 1971). Comparing this length with that needed to code for the a or b polypeptide (430-450 nucleotide) and making allowance for the length of poly(A) (50-100 nucleotides) there appears to be up to 180 untranslated base in rabbit globin message. This suggestion is supported by the sequence data of Proodfoot (1977) who concluded that a total of 95 and 134 nucleotides at 3' end (adjacent to poly(A)) of rabbit and human globin mRNA, respectively remained untranslated. Further, Barrale (1977) found no initiator codon within 53 nucleotides from the 5' end of the rabbit β globin mRNA.

Additionally, the terminal non-coding regions appears to be highly conserved, for example the hexanucleotide AAUAAA is present in the non-coding region of every mRNA

so far sequenced (Proodfoot et al 1976).

6.2 Relation of mRNA to the genome

The extreme complexity of the eukaryotic genome raises the question of the extent to which the DNA functions as template for synthesis of mRNA.

Two approaches have been used to measure the complexity of mRNA transcripts. The first approach is based on saturation hybridisation in which trace amounts of highly radioactive unique DNA are annealed to saturation with an excess unlabelled RNA. The amounts of DNA driven into DNA-RNA hybrids give a direct measurement of the proportion of unique DNA transcribed (Galau et al 1974, Bantle and Hahn 1976).

The second approach is based on kinetic measurements of the rate of annealing between DNA complementary to poly(A)$^+$ mRNA (cDNA), made using reverse transcriptase, and the poly(A)$^+$mRNA itself (Birnie et al 1974, Bishop et al 1974).

Although some reports show similar levels of complexity using both techniques (Bishop et al 1974, Axel et al 1976, Hereford and Rosbash 1977), others find a wide disparity in values for the same tissue (Ryffel and McCarthy 1975, Bantle and Hahn 1976, Kleiman et al 1977).

In general, cDNA measurements tend to underestimate complexity because of the difficulty in estimating the high complexity low abundance class of RNAs. An additional, related problem is the validity of the assumption that

cDNA accurately reflects the number and distribution of mRNA sequences. Thirdly, cDNA hybridisation is usually restricted to poly(A)$^+$ containing RNA sequences so any additional sequences present in poly(A)$^-$ messengers are not detected.

On the other hand, the single-copy method gives a much more sensitive measure of total base sequence complexity of RNA, but has two major drawbacks. Firstly, it overlooks mRNA species of low complexity, and secondly, the obtained values for the proportion of the genome transcribed into mRNA is quite small ($\leqslant 4\%$) making any small variation quite significant. Thus for example, contamination of the polysomal RNA with complex nuclear RNA species should cause the measured base complexity to be overestimated.

Using the experimental approaches mentioned above, it was found that about 1-4% of the unique DNA is transcribed into poly(A)$^+$mRNA (Birnie et al 1974, Bishop et al 1974, Galau et al 1974, Ryffel and McCarthy 1975, Bantle and Hahn 1976, Kleiman et al 1977). A much larger value 5-8% was obtained when mouse fibroblast's polysomal RNA was used (Grady and Campell 1975). Generally, such methods indicate that between 10^4-10^5 genes are expressed during the life span of a higher eukaryote (Galau et al 1974, Bishop et al 1974, Bantle and Hahn 1976, Grady et al 1978). Most expressed genes are present as only a few transcripts per cell. Some gene transcripts, however, occur in a relatively large number per cell. Which

genes make up each class is a variable dependent upon the developmental state of the cell (Birnie et al 1974, Bishop et al 1974, Galau et al 1974, Galau et al 1976, Hastie and Bishop 1976, Young et al 1976, Paterson and Bishop 1977).

The same methods have also been applied to a number of specific messengers to evaluate whether or not these messengers are transcribed from genes present as multiple copies. Thus, histone genes in sea urchin embryos and HeLa cells exist as multiple copies of between 400-1000 and 30-40 repeats, respectively (Weinberg et al 1972, Tartof 1975, Wilson and Melli 1976). Furthermore, chicken keratin genes also occur in multiple copies of between 100-200 (Kemp 1975). More generally, results presented by Klein et al (1974) also suggest that a small proportion of HeLa cell poly(A)$^+$mRNAs are transcribed from repetitious DNA. Other results from a wide variety of eukaryotic cells are also consistent with this suggestion, values as high as 20% of total poly(A)$^+$mRNA being claimed to have been transcribed from repetitious DNA (Campo and Bishop 1974, Spradling et al 1974, Levy et al 1975, Ryffel and McCarthy 1975). Conversely, globin silk moth fibroin and ovalbumin could only be detected as 1-2 copies per genome (Lewin 1975a).

Interestingly, in view of the apparent generality of repetitious DNA transcripts, Goldberg et al 1973 have failed to detect repetitious messenger transcripts in sea

urchin embryos, although a low level (≤3%) of such transcripts were found in another study (McColl and Aronson 1974). On the other hand, it has been suggested that even poly(A)$^+$mRNA transcripts from so-called single-copy DNA do actually contain a region derived from repetitive DNA at their 5' ends (Dina et al 1973). This conclusion was drawn from studies using Xenopus but has not been supported in other systems. Whilst poly(A)$^+$mRNA derived from single-copy DNA have not been generally shown to contain repetitive sequences, there is evidence that regions of DNA adjacent to at least some coding sequences are repetitious in nature (Bishop and Freeman 1973, Davidson et al 1975)

A further insight into the structure of the coding sequences for a number of specific genes has recently been obtained using either DNA fragments (Jeffrey and Flavell 1977) or cloned DNA fragments (Tilghman et al 1978, Brack and Tonegowa 1977, Dugaiczyk et al 1978) containing the gene in question. Utilising the techniques of electron miscroscopy (R-looping) or restriction endonuclease mapping or both, it has been found that within the structural gene sequences coding for rabbit and mouse β globin mRNA (Jeffrey and Flavell 1977, Tilghman et al 1978), mouse immunoglobin light chain mRNA (Brack and Tonegawa 1977) and ovian ovalbumin mRNA (Dugaiczyk et al 1978, Gaparin et al 1978), there are DNA sequences which are not represented in the mature mRNA product.

6.3 Messenger RNA stability

The metabolic behaviour of mRNA may be dependent upon the differentiation state of a particular cell. For example the stability of myosin mRNA was found to increase throughout differentiation (Buckingham et al 1974). In contrast, however, no change in the stability of globin messenger could be detected between reticulocytes and erythroid precursor cells from spleen of anaemic mice (Bastos et al 1977). Furthermore there is some evidence suggesting that stimulation of resting cells to division may influence the stability of mRNA species, although many results are still contradictory (Abelson et al 1974, Chermovskaya et al 1976).

Evidence based on the use of transcriptional inhibitor actinomycin D, as well as continuous labelling and pulse-chase experiments, indicate that mRNA species of a widely varying stability exist in eukaryotes. In early experiments Brandhorst and Humphrey (1972), Perry and Kelley (1973) using a continuous labelling approach reported that mRNA in sea urchin embryos and L cells decay as a single component with a half-life of about 60-90 min and 10-15 hours, respectively. Singer and Penman (1973), using both pulse-chase and actinomycin D techniques, have obtained data concerning the poly(A)$^+$mRNA turnover in HeLa cells, which they interpreted in terms of two components with half-lives of 6-7 and 21-24 hours, respectively. Two classes of poly(A)$^+$mRNA of different half-lives have also been found in Friend cells (Aviv et al

1976), Spleen cells (Bastos et al 1977), mouse kidney cells (Quellete et al 1976), resting lyphocytes (Berger and Cooper 1975) and insect cells (Spradling et al 1975). In addition, Perry et al (1976), in a more accurate study, obtained data for poly(A)$^+$mRNA turnover in L cells which can be described in terms of two mRNA classes with half-lives of 3.5 and 18 hours.

Although analysis of poly(A)$^+$mRNA stability in eukaryotic cells has revealed stable, or relatively stable, classes of mRNA with half-lives ranging from 3 to 24 hours, poly(A)$^+$mRNA from Aedes cells was found to exhibit bi-phasic decay kinetics with half-lives of 20 and 1.2 hours (Spradling et al 1975). The presence of labile poly(A)$^+$ mRNA classes has also been found in kidney cells (Quellete et al 1976) using a label of [^{3}H]-methionine, the pools of which are decreased very rapidly, allowing the short-lived mRNA to be detected. Similarly, a rapid chase following a [^{3}H]-guanosine label allowed Puckett et al (1975) to demonstrate poly(A)$^+$mRNA with a half-life of 1-2 hours in HeLa cells. This compares with Drosophila cells, where the rapid chase of incorporation of [^{3}H]-uridine by a vast excess of unlabelled uridine and cytidine has allowed the detection of three classes of poly(A)$^+$mRNA with half-lives ranging from 0.7 to 100 hours (Lengyel and Penman 1977). Furthermore, extremely short lived poly(A)$^+$mRNA have been reported in resting lymphocytes (Berger and Cooper 1975) poly(A)$^+$mRNA species and for interferon mRNA (Cavelieri et al 1977). These estimates of mRNA half-lives apply

only to poly(A)$^+$mRNAs.

In addition to this class, an appreciable number of messengers lacking poly(A) have been detected in eukaryotes. The first members of this class to be characterised were the histone messengers, but others, non-histone messengers also occur which lack poly(A) (Adesnik and Darnell 1972, Nemer et al 1974, Milcarek et al 1974, Rosen et al 1975, Grady et al 1978). The stability of poly(A)$^-$ histone messengers, although significantly shorter than that of the long-lived poly(A)$^+$mRNA, is quite long compared to the short-lived poly(A)$^+$mRNA (Perry and Kelley 1973). On the other hand, the metabolic behaviour of non-histone poly(A)$^-$ mRNA from HeLa and Aedes cells appears to be similar to that of poly(A)$^+$mRNA (Milcarek et al 1974, Spradling et al 1975). Consistent with this result are the similar half-lives of poly(A)$^+$ and non-histone poly(A)$^-$mRNAs in sea urchin embryos (Nemer et al 1975).

Despite this similar metabolic behaviour of non-histone poly(A)$^-$ and poly(A)$^+$ messengers, it has been suggested that poly(A) may nonetheless play a role in stabilising messengers. Evidence in support to this came initially from the in vitro translation of globin messengers in an Xenopus oocyte system. Here, enzymic removal of poly(A) resulted in a destabilisation of globin mRNA after injection into the oocyte. This conclusion was supported when Huez et al (1975) showed that re-synthesis of a new 3'-OH poly(A) segment on a previously deadenylated globin mRNA restored its stability. Interestingly, it has also been

shown that the poly(A) stretch must contain a minimum number of adenylate residues to ensure its protective function (Nudel *et al* 1976). More conclusive evidence that poly(A) may provide a means of poly(A)$^+$mRNA stabilisation is provided by the work of Huez et al (1978), where artificially polyadenylated histone mRNA were shown to be more stable compared to the native histone mRNAs.

AIMS

The above introduction is an attempt to outline some of the initial processes in eukaryotic gene expression as it is currently understood.

It is clear, however, that our knowledge in this area is far from complete, especially when consideration is given to the extensive occurrence of non-polyadenylated mRNAs and their possible relation to primary nuclear transcripts also lacking poly(A) termini. The aim of this work is to examine the structure, origin and possible role of a class of non-polyadenylated polysomal RNAs which can be isolated from a number of cultured mammalian cells by virtue of their ability to bind to poly(A)-Sepharose columns.

MATERIALS AND METHODS

MATERIALS

1. Biological

Friend murine leukaemia cells, clone M_2, were gifted from Dr. G.D. Birnie, The Beatson Institute for Cancer Research.

Wheat germ is a product of Bar Rav Mill, Tel Aviv, Israel, and was a gift from Ms. H. Singer of this Department.

2. Radiochemicals

All isotopically labelled compounds were obtained from the Radiochemical Centre, Amersham, England, unless otherwise stated.

(5,6-[^{3}H]) Uridine	38-43 Ci/mmol	
L-(4,5-[^{3}H]) leucine	57 Ci/mmol	
L-(1-[^{14}C])leucine	58 mCi/mmole	
L-(methyl-[^{3}H]) methionine	5,2-14 Ci/mmol	
L-[^{35}S] methionine	820-1020 Ci/mmol	
[^{3}H]-CTP	10 Ci/mmol	
Ortho [^{32}P] phosphate	20 mCi/ml	
[^{3}H]-poly(U)	40 µCi/µmolP	Miles
[^{3}H]-poly(A)	26.5 µCi/µmolP	Miles

3. Chemicals

All other chemicals were, where possible, Analar reagents supplied by B.D.H. Chemicals Ltd., Poole, Dorset, except for the following

Triton	Koch-Light Laboratories Ltd. Colnbrook, England

2,5 diphenyloxazole (PPO)	Koch Light Laboratories Ltd. Colnbrook, England
Toluene (AR grade)	"
Trichloroacetic acid	"
2-mercaptoethanol	"
Sephadex G_{25} (medium)	Pharmacie Uppsala, Sweden
Sephadex G_{50}	"
Dextran Sulfate	"
Poly(U)-Sepharose 4B	Pharmacia Ltd., G.B.
Poly(A)-Sepharose 4B	"
Cn-Br activated Sepharose 4B	"
Hydroxyapatite	Bio-Rand Lab., Richmond, California
Chelex-100	"
Horse serum	Bio-cult Lab. Ltd., Paisley, Scotland
Amino acids	"
Vitamins	"
Penicillin	Glaxo Pharmaceutical, London
Streptomycin	"
Actinomycin D	Calbiochem, California
Cytidine	"
RNAase A	Sigma (U.K.), London, England
RNAase T_1	"
S. nuclease	"
Pancreatic DNAase I	"

GTP	Sigma (U.K.), London, England
ATP	"
Hepes	"
P-Bis(o-methyl-styzyl benzene (Bis-MSB)(Scintillation grade)	Kodak Ltd., London
Koredex KD 54T (35 x 43 cm) X-ray film	" "
Fix-40 X-ray liquid fixer	" "
Dx-80 developer	" "
dTTP	Boehringer Corporation (London) Ltd., Sussex, England
dGTP	"
dATP	"
Creatine kinase	"
Creatine phosphokinase	"
Proteinase K	Boehringer, Mannheim, W. Germany
Heparin	Evans Medical Ltd., Speke, Liverpool
Whatman DE81 Paper (46 x 50 cm)	H. Reeve-Angel and Co. Ltd., London
Whatman No. 52 and 3mm Paper	"
2.5cm paper disc	
Oligo $(dT)_{17}$	P.L. Biochemicals Inc., Milwaukee, Wisconsin
Oligo $(dT)_{10}$	
Poly(A)	"
Repelcote	Hopkin and Williams Ltd., Chadwell Heath, England

Spermidine	Hopkin and William Ltd., Chadwell Heath, England
Spermine	"
Dithiothreitol	"
Adenosine	"
Polynucleotide phosphorylase	"
E. coli DNA	"
Calf Thymus DNA	"
Glutamine	"
Formamide	Fluka
Ampholines	LKB
Kodak X-Omat-R film	Kodak (Canada) Ltd.

METHODS

1. Growth and radioactive labelling of tissue culture cells

1.1 Routine maintenance of cells

Friend murine leukaemia cells, clone M_2, were used in all experiments. This is a line derived from the 707 clone, as described by Gilmour *et al* (1974). The culture medium used was made up from the Glasgow modification of Eagle's Minimal Essential medium (Table 2) plus 2x glutamine (584mg/l), non-essential amino acids (Table 3) and 15% (v/v) horse serum. Growth was initiated by inoculating cells, as a suspension, into either Roux flasks (total final volume 50ml), or into stirrer cultures vessels, containing 1-1.5 l of medium. The inoculum was chosen to give an initial cell density of 0.5-0.6 x 10^5 cells per ml. Both types of culture were maintained at a temperature of 37°C, and in an atmosphere of 5% (v/v) CO_2 for 3 days before harvesting, at which time both types of culture were found to be in mid-log phase having a density of 0.6-0.8 x 10^6 cells per ml (Birnie *et al* 1974). When appropriate, Friend cells (grown in Roux flasks) were induced by the addition of dimethylsulphoxide (1.5% (v/v)) (BDH, Spect.) when the cells were at a density of 2-3 x 10^5 cells per ml. Under these conditions the Friend cells, clone M_2 began to synthesise haemoglobin after about 3 days (Gilmour *et al* 1974). When the cells had grown to mid-log phase, typically after 3 days, the culture was harvested by gentle centrifugation (800g, 5 min).

Table 2

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

Amino acids	mg/litre	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	41.9	i-inositol	4.0
L-isoleucine	52.5	nicotinomide	2.0
L-leucine	52.5	pyridoxal HCl	2.0
L-lycine	73.1	riboflavin	0.2
L-methionine	14.9	thiamin HCl	2.0
L-phenylalanine	33.0		
L-threomine	47.6		
L-tryptophan	10.2		
L-tyrosine	36.2		
L-valine	46.9		

Inorganic salts and other components

	mg/litre		
$CaCl_2$ $6H_2O$	393.0	penicillin	100,000 units/ml
KCl	400.0		
$MgSO_4$ $7H_2O$	200.0		
NaCl	6800.0		
NaH_2PO_4 $2H_2O$	140.0		
D-glucose	4500.0		
$NaHCO_3$	2240.0		
Phenol red	15.3		
Streptomycin	100.0		

Table 3

Non-Essential Amino Acid Mixture for Minimum Essential Medium Eagle

Amino acids	mg/lt
L-alaline	8.90
L-asparagine H_2O	15.00
L-aspartic acid	13.30
Glycine	7.50
L-glutamic acid	14.70
L-proline	11.50
L-serine	10.50

Successful induction gave a distinctly red cell pellet.

All sterile media and passaged cells were checked regularly for bacterial, fungal or mycoplasma infection as follows:

(a) Bacterial contamination

Aliquots were grown on blood agar plates and brain-heart infusion broth at 37°C. Results were considered negative if no growth was seen within 7 days.

(b) Fungal contamination

Aliquots were added to Sabouraud's medium and grown at 32°C. Again, no growth after 7 days was assumed to indicate the absence of fungal contamination.

(c) Mycoplasma (PPLO pleuropneumonia like organisms) infection

PPLO agar plates were seeded with passaged cells by piercing the agar surface with a charged Pasteur pipette. The plates were grown in an atmosphere of 5% (v/v) CO_2 in N_2 at 37°C for 7 days, and examined microscopically for the characteristic "fried egg" appearance of PPLO colonies.

1.2 Radioactive labelling

Radioactive labelling was generally performed on mid-log phase cells which had been concentrated by centrifugation at room temperature at 800g for 5 min followed by suspension to a density of 2-3 x 10^6 cells per ml in an appropriate medium, which was prewarmed at 37°C.

For labelling:-

(a) With [^{3}H] uridine

Cells were concentrated, as above, into normal medium and labelled by the addition of an appropriate quantity of [^{3}H] uridine (see Results).

(b) With ortho [^{32}P] phosphate

Cells were concentrated, as above, into low phosphate medium (1/10th the normal phosphate concentration) were labelled by addition of ortho [^{32}P] phosphate (usually 20mCi carrier free).

(c) With [^{3}H] methyl-methionine

Basically the technique of Maden et al (1972) was employed. Cells were concentrated, as above, into a medium comprising Eagle's medium but minus methionine, 15% (v/v) horse serum, sodium formate to a final concentration of 20mM and adenosine and guanosine to a final concentration of 20μM each, prior to labelling with 20μCi of [^{3}H] methyl methionine.

1.3 "Glucosamine-uridine" pulse-chase protocol

"Glucosamine-uridine" pulse-chase experiments essentially followed the method of Levis and Penman (1977). Cells were concentrated, as above, into normal medium and D-glucosamine, neutralised to pH7.4, was added to a final concentration of 20mM. This concentration of glucosamine is sufficient to reduce considerably the size of the intracellular UTP pools by "trapping" uridine in the form of UDP-N-acetylhexosamines (Scholtissek, 1971). The

culture was then incubated at 37°C for 60 min and then labelled for various periods of time with an excess of [^{3}H] uridine. To initiate the chase, four volumes of fresh medium containing both uridine and cytidine at concentrations of 6.25mM and D-glucosamine, neutralised at pH7.4, at a concentration of 31.25mM, was added.

2. Cell harvesting

Friend cells were centrifuged at 800g for 5 min at 4°C directly from the medium and washed twice with ice-cold Balanced Salt Solution (BSS) (0.116M NaCl, 5.4mM $MgSO_4$, 1mM NaH_2PO_4, 1.8 $CaCl_2$ and 0.002% (w/v) phenol red). The pH of the solution was adjusted to pH7.0 with 5.6% (w/v) $NaHCO_3$.

3. Cell fractionation

3.1 Nuclei and cytoplasm

All operations were carried out at 0-4°C. The washed cell pellet was resuspended in lysing buffer (0.14M NaCl, 1.5mM $MgSO_4$, 10mM tris-HCl, pH7.4) and NP-40 (BDH) was added to a final concentration of 0.5% (v/v) (Borun et al 1967). The cells were allowed to lyse for about three to six minutes, (lysis being monitored by phase contrast microscopy), then the lysed cells were centrifuged at 800g for 5 min in order to collect nuclei. The nuclei were resuspended in 2-3ml of 1.5% (w/v) citric-acid, 0.25M sucrose and homogenised with 10 strokes of a tight-fitting, motor driven, telfon homogeniser. The homogenate

was layered over an equal volume of 1.5% (w/v) citric-acid, 0.88M sucrose, and the nuclei were collected by centrifugation at 1,000g for 5 min. The procedure was repeated twice, or until nuclei were free of cytoplasmic tags, and cell debris, as judged by phase-contrast microscopy (Getz et al 1975). For some experiments, nuclei were stored at -20°C.

The supernatant remaining after the 800g spin was subsequently centrifuged at 10,000g for 10 min to remove mitochondria, the supernatant from this step (post-mitochondrial supernatant) will henceforth be referred as cytoplasm in this study.

3.2 Polysomes, sub-polysomes and cytosol

Polysomes were prepared from Friend cells, as described by Birnie et al (1974). Polysomes were pelleted from the cytoplasm (see Section 3.1) by centrifugation through 2M sucrose, in lysing buffer (see Section 3.1), either at $g_{(av)}$ 230,000g for 3 hrs at 4°C in a MSE 8 x 25ml titanium fixed-angle rotor, or for 2.5 hrs at 4°C in 60T_1 Beckman rotor. The supernatant fraction from this procedure is referred to, henceforth, as the cytosol. For analysis and fractionation the polysome pellet was resuspended either in buffer containing 10mM NaCl, 10mM tris-HCl (pH7.4), 2mM $MgSO_4$ (NTM), or the same solvent containing 10mM EDTA in place of $MgSO_4$ (NTE). Samples were layered over a 15-30% (w/v) sucrose/NTM or sucrose/NTE gradient

and centrifuged for 3.5 hrs at $g_{(av)}$ 96,000g at 4°C, in a Beckman SW27 rotor. The gradients were collected by descending displacement through the flow cell of a Gilford recording spectrophotometer. The fractions corresponding to the polysomal and sub-polysomal regions as defined in Fig 3 were pooled.

4. RNA isolation

4.1 Precautions against ribonuclease contamination

(a) Glassware

All glassware was washed in 0.1% (v/v) diethylpyrocarbonate (DEP), rinsed in sterile deionised water dried at 60°C and then baked at 300°C for several hours prior to use.

(b) Other equipment

All equipment (centrifuge tubes, caps, tubing and glass spectrophotometric glass etc.) was washed with a hot (60°C) solution SDS (10% (w/v)) and rinsed with 0.1% (v/v) DEP then extensively rinsed with sterile distilled water. Following washing, the equipment was dried at 60°C for about 4 hrs.

(c) Experimenter precautions

All operations were carried out wearing fresh disposable plastic gloves which were replaced if thought to be contaminated. Direct contact with solutions was avoided.

(d) Treatment of solution

In general solutions were autoclaved at 15 lb/in^2 pressure for 30 min. For sucrose solutions autoclaving was at 5 lb/in^2 pressure for 45 min.

4.2 Preparation of nuclear RNA

Nuclear RNA was isolated essentially as described by Penman (1969). Nuclei were resuspended in HSB buffer, (0.5M NaCl, 5mM $MgCl_2$, 10mM tris-HCl, pH7.4), at 10^8 nuclei/ml. Pancreatic DNAase I was added to 100µg/ml, and digestion was carried out for 3 min at 37°C. Two volumes of absolute alcohol were added to precipitate nucleic acids. The precipitate was spun down at 15,000g for 20 min and resuspended in NETS buffer, (0.1M NaCl, 10mM EDTA, 10mM tris-HCl, 0.5% (w/v) SDS, pH7.5). This was then extracted at room temperature with an equal volume of phenol-chloroform-isoamylalcohol as before. The aqueous phase was removed, and the organic phase and interphase re-extracted for 5 min at 60°C with 0.5 vol NETS. The combined aqueous phases were then exhaustively extracted with phenol-chloroform-isoamylalcohol at room temperature until the interphase appeared absolutely clear. The extraction was repeated once more with chloroform-isoamylalcohol (100:1 by volume) alone, and nucleic acids were precipitated from the aqueous phase by the addition of 0.1 vol at 2M NaCl, plus 2.5 vol absolute alcohol. After 20 hrs at -20°C RNAs were collected by centrifugation at 12,000g for 20 min at -10°C.

4.3 Preparation of cytoplasmic RNA

The cytoplasm (see Section 3.1) was made 0.5% (w/v) with respect to SDS and 10mM with respect to EDTA, and RNA was isolated by exhaustive extraction with equal volumes of phenol-chloroform-isoamylalcohol, followed by chloro-

form-isoamylalcohol alone (Perry et al 1972). RNA was precipitated from the aqueous phase by the addition of 0.1 vol of 2M NaCl, plus 2.5 vol absolute alcohol. After 20 hrs at -20°C RNAs were collected by centrifugation at 12,000g for 20 min at -10°C.

4.4 Preparation of polysomal, sub-polysomal and cytosol RNA

(a) Polysomal RNA

The polysomes were resuspended in NETS buffer (0.1M NaCl, 10mM EDTA, 10mM tris-HCl, 0.5% (w/v) SDS, pH7.5) and RNA extracted as described for cytoplasmic RNA (see Section 4.3).

(b) Sub-polysomal and cytosol RNA

Ethanol precipitates of sub-polysome and cytosol were resuspended in NETS buffer and RNAs were extracted as described for cytoplasmic RNA (see Section 4.3).

4.5 Preparation of total cellular RNA

Total cellular RNA was prepared essentially as described by Kwan et al (1977). The cell pellet was resuspended in NETSS buffer (0.1 NaCl, 5mM EDTA, 30mM tris-HCl, 2% (w/v) SDS, pH7.4) at 10^7 cell/ml, containing heparin (150µg/ml), dextran sulphate (100µg/ml) and proteinase K (300µg/ml). The proteinase K was allowed to auto digest in the above buffer for 30 min at 37°C prior to use. The cell suspension was incubated for 30 min at 37°C. Two and a half volumes of absolute alcohol were added to

precipitate total nucleic acids. The precipitate was spun down at 15,000g for 20 min, and total cellular RNA was extracted as described for nuclear RNA (see Section 4.1).

5. RNA fractionation

5.1 Affinity chromatography

5.1.1 Preparation of poly(A)-sepharose 4B

Poly(A)-sepharose was prepared essentially as described by Yogo and Wimmer (1973). One gr of CN-Br activated Sepharose 4B was allowed to swell in 1mM HCl and subsequently washed with 250ml 1mM HCl and with 200ml ice-cold H_2O. The gel was mixed in a universal, with 0.3M morfolinoethane sulphonic acid (MES), 0.15M NaCl, pH6.0, containing 1-2mg poly(A), and shaken for 20 hr at 4°C. The gel was washed with 30ml of 0.3M MES, 0.15 NaCl, pH6.0 to remove unbound polynucleotides. From the absorbance (260nM) it was estimated that 80-90% of the applied poly(A) had been linked to the gels. Any remaining active groups in the gel were blocked by reaction with 1-2 vol of 0.5M glycine, pH8.0, at 4°C for 2 hr. The gel was then washed alternately with 20ml of ice-cold 0.1M ammonium acetate, 1M NaCl, pH5.1, and 0.1M sodium citrate, 1M NaCl pH7.6. It was then resuspended in 0.1M NaCl, pH7.5, to give a final concentration of 1gr of dry gel per 4mls, and kept at 4°C. In later experiments, poly(A)-Sepharose 4B was obtained ready made from Pharmacia in the form of dry gel, and was resuspended in ice-cold 0.1M NaCl, pH7.5 for 15 min to give a final concentration of 1gr of dry gel per 4mls.

5.1.2 Preparation of poly(U)-Sepharose 4B

Poly(U)-sepharose was obtained from Pharmacia in the form of dry gel and was resuspended in ice-cold 0.1M NaCl, pH7.5, for 15 min to give a final concentration 1gr of dry gel per 4mls.

5.1.3 Isolation of RNA species with high affinity for poly(A)- or poly(U)-Sepharose 4B

Poly(U)- and poly(A)-sepharose affinity chromatography was performed essentially as described by Molloy et al (1974).

Poly(U)- or poly(A)-sepharose were packed in 5ml pipettes (1-3cm^3, column volume). These columns were washed with 3-4 column volumes of binding buffer (0.4M NaCl, 10mM EDTA, 10mM tris-HCl, pH7.4, 0.2% (w/v) SDS then with 90% (v/v) formamide in 10mM EDTA, 10mM tris-HCl, pH7.4, 0.2% (w/v) SDS, and finally with 10-20 vol of binding buffer (Adesnik et al 1972). Isolation of RNA species with a high affinity for poly(U)- or poly(A)-sepharose columns was then performed as follows:

RNA samples ($\leqslant$ 200μg) were resuspended in 0.5-1ml of binding buffer, and before loading were denatured by heating at 70^{o}C for 5 min (Rilley et al 1966) quench cooled in ice, and rapidly applied to the poly(U)- or poly(A)-sepharose columns. The columns were then washed with 5-10 column volumes of binding buffer. The adsorbed RNAs were either eluted with 90% (v/v) formamide in 1mM EDTA, 10mM tris-HCl, pH7.4, 0.2% (w/v)

SDS, or as is described in the Results Section.

5.1.4 Binding efficiencies of poly(U)- and poly(A)-sepharose columns

When poly(U)- and poly(A)-sepharose columns (0.5 x 1cm) were tested using the procedure outlined above (5.1.3), both proved capable of binding over 97% of the applied [^{3}H] poly(A) (0.1µg) or [^{3}H] poly(U) (0.1µg) respectively.

5.2 Hydroxyapatite chromatography

Hydroxyapatite chromatography was performed essentially as described by Bernardi (1969). One gr of hydroxyapatite was suspended in 25ml of 50mM sodium phosphate buffer (equimolar Na_2HPO_4 amd NaH_2PO_4, pH6.8). and packed into a 1cm diameter water-jacketed column. After washing the columns with 10ml of 50mM phosphate buffer, the RNA samples (0.1-0.2µg) were applied in 50mM phosphate buffer. The column was washed with 10ml of 50mM phosphate buffer to remove unbound RNA. Double-stranded RNA bound to hydroxyapatite was either eluted with 500mM phosphate buffer, or a stepwise elution was carried out by raising the column temperature in 10°C increasement from 30°C to 90°C. At each step the column was washed with 5 column volumes of 50mM phosphate buffer.

5.3 Polyacrylamide gel electrophoresis of RNA

RNA was analysed on 12% SDS-polyacrylamide gels, essentially as described by Loening (1969). RNA was dissolved in 50µl electrode buffer (30mM NaH_2PO_4, 1mM EDTA, 36mM tris-HCl, 0.2% (w/v) SDS, pH7.7), bromophenol

blue and glycerol were added to a final concentration of approximately 0.005% (w/v) and 20% (v/v) respectively. The RNA samples were applied to 80mm long, 6mm diameter gels, which has been pre-run for 30 min. Electrophoresis was carried out at a constant current of 5mA per tube until the bromophenol blue had travelled 15mm of the bottom of the tube. The gels were scanned for absorbance at 260nm in a Gilford 2,000 Gel Scanner recording spectrophotometer. For radioactivity determination the gels were frozen in dry ice and cut transversely into 1mm slices using a Mickle gel slicer. Two consecutive slices were then placed in a scintillation vial, and 0.3ml of hydrogen peroxide was added. Samples were digested at 60°C overnight, 3mls of Triton/toluene scintillation fluid (see Section 18) was then added and the vials counted, after shaking and allowing to stand for a few minutes.

5.3 Formamide-sucrose gradients

RNA was sedimented on formamide-sucrose gradients, essentially as described by Ross (1976). Four sucrose solutions were prepared by dissolving 8, 12, 16 and 20 gr sucrose in a final volume of 100ml, with 98% (v/v) formamide, 2mM EDTA and 10mM tris-HCl, pH7.5. Three hours before centrifugation the sucrose solutions were carefully layered in polyallomer tubes, using 0.7ml 20% sucrose at the bottom, followed by 1ml of 16% sucrose then 1ml of 12% sucrose, and finally 0.75ml of 8% sucrose.

The tubes were covered and set aside at room temperature. Samples for application to these gradients were prepared from ethanol precipitates which had been drained, dried in a gentle stream of air, and dissolved in 0.075ml of ET buffer (2mM EDTA, 10mM tris-HCl, pH7.5) and 0.15ml of 80% (v/v) formamide in ET buffer was added. The dissolved RNA was then heated at 45°C for 25 min and layered on the gradient. Conditions of centrifugation are given in the appropriate places in the Results Section.

6. Ribonuclease digestions of RNA

6.1 Digestion of RNA by a mixture of pancreatic and T_1 ribonucleases under high salt conditions

RNA samples (1-10μg) were dissolved in 2 x SSC (1 SSC, 0.15 NaCl, 0.015 sodium citrate, pH7.0) containing T_1 ribonuclease (100 units/ml) and pancreatic ribonuclease (50μg/ml) and digested at 37°C for 60 min. After digestion, the samples were adjusted to 10mM EDTA and 0.5% (w/v) SDS and with proteinase K (300μg/ml) for 15 min at 37°C to destroy ribonucleases. Yeast t-RNA (100μg/ml) was added as a carrier and the mixture was phenol-chloroform extracted (see Section 4.2). Nucleic acids were precipitated from the aqueous phase by the addition of 2.5 vol of absolute ethanol.

6.2 T1 ribonuclease digestion

RNA samples (0.3-0.8μg) were dissolved in 10mM EDTA, 100mM tris-HCl (pH7.4) and digested with T1 ribonuclease (100

units/ml) at 37°C for 60 min. Isolation of oligonucleotides from the digest was performed either by phenol-chloroform extractions, as described above (see Section 6.1), followed by redissolving the ethanol precipitate in binding buffer (0.4M NaCl, 10mM EDTA, 10mM tris-HCl, 0.2% (w/v) SDS, pH7.4) and passing through a poly(A)-sepharose column, or simply by adjusting the digest to 0.4M NaCl, 10mM EDTA, 10mM tris-HCl, (pH7.4) 0.2% (w/v) SDS directly, and passing over a poly(A)-sepharose. Bound RNAs were eluted with 90% (v/v) formamide in 10mM EDTA, 10mM tris HCl, 0.2% (w/v) SDS, (pH7.4).

7. Phosphorolysis of RNA from polynucleotide phosphorylase

RNA was phosphorolysed, essentially as described by Williamson et al (1974). Samples of RNA (100µg/ml) were incubated with M. lysodiekticus polynucleotide phosphorylase EC 2.7.7.8 (500µg/ml) in 5 $MgSO_4$, 10mM K_2HPO_4, 50mM tris-HCl (pH7.0) at 37°C for various times. The phosphorolysed RNA samples were extracted with phenol-chloroform, and ethanol precipitated as described previously (see Section 4.2). The ethanol precipitates were then redissolved in binding buffer (see Section 5.1.3) and chromatographed on poly(U)- or poly(A)-sepharose columns (see Section 5.1.3).

In some cases the digests were applied to a Whatman DE 81 paper. Descending chromatography was carried out in 0.75M ammonium acetate (pH8.6) Furlong (1965).

Following chromatography the DEAE-paper was thoroughly dried and cut transversely into 1cm wide strips. Each of these pieces was counted in toluene-based scintillation fluid (see Section 18.4.1).

8. Determination of base composition

Samples of [^{32}P] RNA (0.2-0.3µg) were hydrolysed for 24 hrs by dissolving the RNA in 40µl of 0.3M NaOH and incubating in a sealed capillary for 24 hrs at 37°C. The resulting hydrolysates were subjected to electrophoresis on Whatman paper No. 52 for 45 min at 4.7 KW, using a 5% (v/v) acetic acid-pyridine (pH3.5) buffer (Sebring and Salzman, 1964). After chromatography the areas of the chromatogram to be examined were cut out using the autoradiograph as a template. Each of these pieces was counted in a toluene-based fluid (Methods, Section 18.4).

9. Detection of poly(A) sequences in RNA molecules using [^{3}H] poly(U)

Hybridisations between [^{3}H] poly(U) and RNA fractions were essentially performed and analysed by pancreatic ribonuclease, as described by Macnaughton et al (1974). Various amounts of RNA samples (see Table 8) were hybridised in a buffer containing 2 x SSC (1 SSC, 0.15M NaCl, 0.015M Sodium citrate, pH7.0), with 10 fold excess of [^{3}H] poly(U) (calculated assuming a poly(A) content of poly(A)$^{+}$ RNA of about 7-9%). Approximately 50µl aliquots were incubated for 25 min at 45°C., chilled, diluted with 1ml

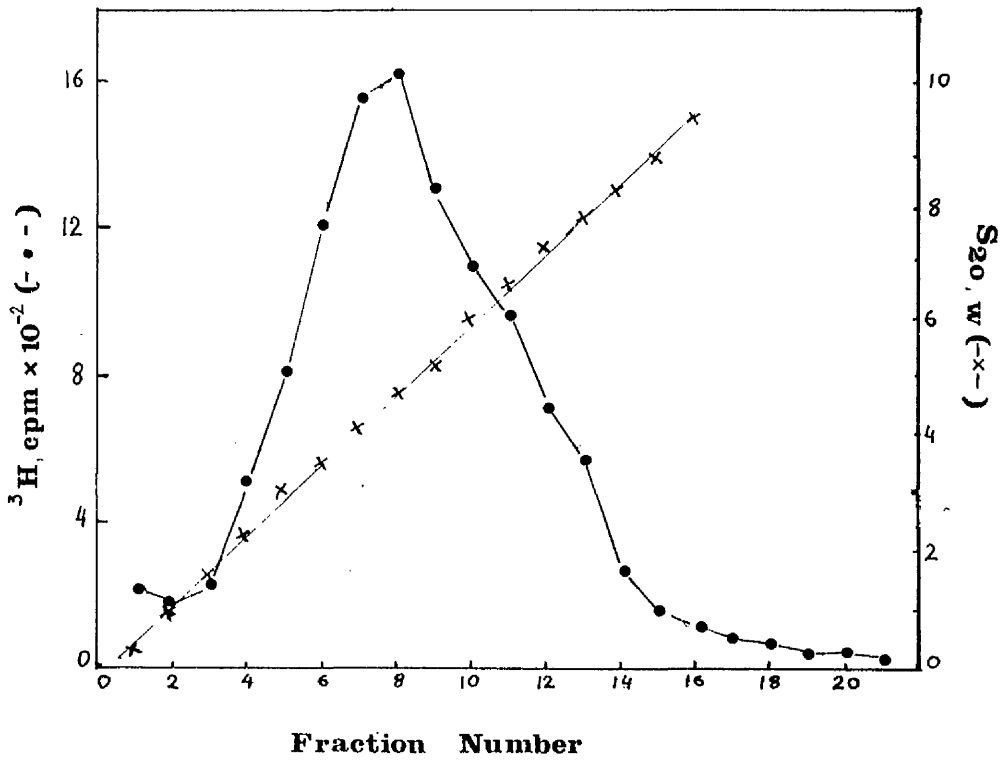
2 x SSC containing 25μg/ml of pancreatic ribonuclease, and held at 4°C for 20 min. The reactions were stopped with 10ml of ice-cold 10% (w/v) trichloro-acetic acid following the addition of 60μg/ml of unlabelled Friend cell cytoplasmic RNA as carrier. After 15 min on ice, the acid-insoluble material, composed of carrie· RNA and any hybrids produced between [^{3}H] poly(U) and the RNA fractions used, was collected as a precipitate, and radioactivity in this precipitate was determined as described in Methods (see Section 18.1).

10. Preparation and sizing of complementary DNA

Complementary DNA (cDNA) was prepared from Friend cell polysomal poly(A)$^+$ RNA (polysomal RNA with high affinity for poly(U)-sepharose, see Results Section 4.1.1), essentially as described by Birnie et al (1974). The template RNA (4-5μg) was incubated for 2 hrs at 37°C in a mixture (0.5ml) containing 2.5μg oligo(dT)$_{17}$, 0.2μmol of dGTP, 0.2μmol of dATP, 0.2μmol dTTP, 20nmole of [^{3}H]dCTP, 50μg of actinomycin D, 100mg of bovine serum albumin, 50mM tris-HCl, pH8.2, 50mM KCl, 100mM dithio-threitol (DTT), 5mM magnesium acetate and 100μl (25 units) of AMV reverse transcriptase in 0.15 potassium phosphate, pH8.0 containing 50% glycerol and 1mM dithiothreitol (gift of Dr. G.D. Birnie). The reaction was stopped by adding EDTA to 25mM, and carrier E. coli DNA was added (50-100μg). Deoxyribonucleosides were removed by passage through a G-50 sephadex column (fine, 15 x 1cm),

Figure 2

Sedimentation of cDNA on alkaline-sucrose gradients



with a pad of Chelex-100 (1 x 1cm)in 50mM NaCl, 10mM Hepes (pH7.0). The first excluded peak was lyophylised, adjusted to 0.9M NaCl, 0.1M NaOH, and layered onto a 4-11% (w/v) sucrose gradient in 0.9M NaCl, 0.1M NaOH. Gradients were spun at 74,000g, 20°C for 20 hrs in the MSE 3 x 25 SW rotor. Sedimentation coefficients were calculated by the methods of Funding and Steensgard (1973) using a computer program designed by Dr. B. Young, and molecular weights were calculated according to the equation $S_{20W} = 0.0528.M^{0.4}$ (Studier, 1965).

cDNA prepared by this method had a modal size of 4-5.5S as shown in Fig 2. The yields $\frac{\text{picomoles cDNA synthesised}}{\text{picomoles mRNA}}$ of cDNA obtained were about 3%. Fractions from the alkaline sucrose gradient sedimenting faster than 3S were pooled, 100µg of carrier E. coli DNA was added and the mixture was neutralised, then precipitated overnight with 2.5 vol of ethanol at 20°C. The precipitate was collected and dissolved in sterile water. The cDNA was desalted by gel filtration through G_{50} sephadex column (fine, 15 x 1cm) equilibrated with water.

11. RNA-cDNA hybridisation

Hybridisation in solution was essentially performed and analysed by S1 nuclease, as described by Birnie et al (1974). Appropriate amounts of RNA and cDNA (see Fig 5) dissolved in sterile distilled water, were mixed, lyophylised, and redissolved in hybridisation buffer

(0.5M NaCl, 25mM Hepes, 0.5mM EDTA, pH6.8, 50% (v/v) formamide). The salt solutions (before addition of formamide) were passed through a Chelex-100 resin to remove heavy metal ions treated with 0.1% (v/v) diethyl-pyrocarbonate (DEP) to destroy ribonuclease, and then autoclaved to remove any excess of DEP. Portions (0.5-1µl) of the hybridisation mixtures were sealed in siliconised capillaries which had been washed with 0.1% (v/v) DEP. The capillaries were heated at 70°C for 10 min, then incubated at 43°C for an appropriate period of time (see Fig 5). The contents of each capillary were flushed out with 250µl of a buffer comprising 70mM Sodium acetate (pH4.5), 2.8mM $ZnSO_4$, 140mM NaCl. Fourteen µg/ml of heat denatured calf-thymus DNA was added, and the mixture incubated with 100µl (20 units) S1 nuclease at 37°C for 2 hrs. The reaction vessels were chilled and 100µl of the incubation mixture was counted to determine total radioactivity (T). Two hundred µl of the reaction mixture was precipitated by the addition of 50µl carrier (150µg/ml E. coli DNA, 1mg/ml bovine serum albumin), and 50µl ice-cold 6N perchloric acid. After centrifugation of 1,000g for 15 min, 200µl of the supernatant was counted to determine the proportion of acid soluble counts (AS). The percentage of [^{3}H]cDNA in hybrids was calculated from the formula:-

$$\%[^3H]\text{cDNA in hybrid} = \frac{(T - 0.75AS)}{T} \times 100$$

12. Extraction and shearing of DNA

DNA was extracted and sheared, essentially as described

by Flint et al (1976). Nuclei were incubated with proteinase K (100μg/ml) at 37°C for 2 hrs in NETSS buffer (0.1M NaCl, 100mM EDTA, 10mM tris-HCl, pH7.4, 2% (w/v) SDS), and then extracted with phenol-chloroform-isoamyl-alcohol (24:24:1, by volume). The organic phase was extracted with further NETS buffer (0.1M NaCl, 10mM EDTA, 10mM tris-HCl, 0.5% (w/v) SDS, pH7.4), and nucleic acids were precipitated from the combined aqueous phases. The precipitates were collected by centrifugation (15,000g for 1 h) and dissolved in NET buffer (0.1M NaCl, 10mM EDTA, 10mM tris-HCl, pH7.4). Pancreatic and T1 ribonuclease (preheated at 80°C for 10 min) was added to 20μg/ml and 120 units/ml respectively, and the mixture incubated at 37°C for 1 h. SDS and proteinase K were added to final concentrations of 0.5% (w/v) and 50μg/ml respectively, and incubated for 30 min at 37°C to destroy ribonuclease. The solution was then phenol-chloroform extracted as before, and the DNA precipitated with 2.5 vol of absolute ethanol.

The DNA was sheared by incubation at 100°C for 15 min in 0.2M NaOH, and then neutralised. DNA fragments were desalted on a G_{50} sephadex column (fine, 20 x 1cm) with a pad (1 x 1cm) of Chelex-100. The sheared DNA exhibited a broad size distribution, with a mean length of about 0.3 Kilobase as determined from the formula of Studier (1965), using sedimentation data kindly obtained using the analytical centrifuge (Beckman, Model E) by Mr. J. Logan of this Department.

13. DNA.RNA hybridisation under condition of DNA excess

DNA excess hybridisation with RNA was performed essentially as described by Britten et al (1974). For these experiments, a DNA:RNA ratio of approximately 2,000:1 was employed. Appropriate amounts of sheared DNA, and either [^{3}H] uridine labelled polysomal poly(A)$^+$ or poly(A)$^-$u$^+$ RNAs (see Fig 11), each dissolved in sterile distilled water, were mixed, lyophilised and dissolved in 0.12M phosphate buffer (equimular NaH_2PO_4 and Na_2HPO_4, pH6.8, 0.1% (w/v) SDS). Aliquots, typically 3-5µl of these solutions were sealed in siliconised glass capillaries, denatured by heat at 100°C for 5 min then incubated at 63°C to various Cot values. The hybridisation reactions were terminated by flushing out with 200µl of 2 x SSC (1SSC, 0.15M NaCl, 0.015M Sodium citrate pH7.0), and the solutions stored frozen at -20°C. The amounts of [^{3}H] RNA which had formed stable hybrid was determined as follows: Each sample was divided into equal portions one being directly precipitated with 10% (w/v) trichloroacetic acid at 0°-4°C for 30 min to measure total acid precipitable radioactivity (T), the other being incubated for 40 min at 37°C with 10µg/ml of pancreatic ribonuclease and 20 units/ml of T1 ribonuclease (both ribonucleases were preheated for 60 min at 80°C) to determine acid-soluble radioactivity (AS). The percentage of [^{3}H] RNA in hybrids was calculated by the formula:-

$$\% \text{ of } [^3H] \text{ RNA in hybrid} = \frac{T - AS}{T} \times 100$$

14. In vitro protein synthesis system

14.1 Preparation of the wheat germ extracts

The wheat germ extracts were prepared, essentially as described by Marcus and Dudock (1974).. All steps were performed at 4°C. Generally 2grs of raw wheat germ were ground with a pestle and mortar for 60 sec, with an equal weight of powdered glass (1-2 pasteur pipettes). Four mls of extraction buffer (20mM Hepes, 100mM KCl, 2mM $CaCl_2$, 1mM magnesium acetate, 6mM 2-mercaptoethanol adjusted to pH7.5 with KOH), were then added, followed by gentle swirling for 15-30 sec. After centrifugation at 30,000g for 12 min in a Sorval SS-34 rotor, the supernatant fraction was removed (avoiding the layer of fat). The supernatant was then placed onto a G_{25} sephadex column (medium, 21 x 1cm), equilibrated with column buffer (20mM Hepes, 120mM KCl, 5mM magnesium acetate, 6mM 2-mercaptoethanol, adjusted to pH7.5 with KOH), and chromatographed. All initial fractions higher than 90 OD_{260}/ml were pooled and centrifuged for 20 min at 30,000g in a SS-34 rotor. The resultant supernatant was collected and stored in small aliquots at -70°C.

14.2 Translation of mRNA in a wheat germ cell-free protein synthesising system

Each assay mixture contained wheat germ extract (20µl), energy mixture (10µl) and RNA or distilled sterile H_2O (20µl) and was incubated at 25°C for 60 min.

The energy mixture was freshly made before use, and

consisted of GTP, ATP and creatine phosphate mixture (25µl), amino acid mixture (25µl), dithiothreitol (10µl), creatine kinase (5µl), salt mixture (10µl), labelled amino acids and water (25µl). The various constituents of the ATP, GTP, creatine phosphate mixture are given below:

(a) ATP, GTP, creatine phosphate mixture (20 times final assay concentration)

60mg of ATP was dissolved in 2ml of distilled sterilised water and neutralised with KOH. To this, 0.2gr of creatine phosphate and 1mg of GTP were added. The volume of the resultant solution was then adjusted to 5ml and stored at -70°C.

(b) Amino acid mixture (50 times final assay concentration)

A 400µM solution of 19 amino acids (leucine or methionine omitted as appropriate) was prepared, and the pH was adjusted to pH7.4 with KOH. The amino acid solution was stored at 70°C in small aliquots. In later experiments the amino acid mixture was omitted, the endogeneous levels of amino acids being found sufficient to support protein synthesis (personal communication from Ms H. Singer of this Department).

(c) Salt mixture (50 times final assay concentration)

This was 2ml of 1M Hepes pH7.5, 0.3gr KCl, 0.02gr magnesium acetate + 4.5mg Spermine, stored frozen in small aliquots at -70°C.

(d) Dithiothreitol

Nitrogen gas was bubbled through 0.12M solution of

dithiothreitol (DTT) for about 10 min. Immediately following this, the DTT solution was divided in small aliquots and stored at -70°C.

(e) Creatine kinase

A solution of 10mg/ml in 80% glycerol was initially used. In later experiments this was reduced 10 fold in order to reduce the endogeneous ribonuclease activity (personal communication from Ms H. Singer of this Department). This change was made concurrently with the omission of exogenously added amino acids.

15. Analysis of the cell-free products of translation

15.1 SDS-Gel electrophoresis in one dimension

The labelled products of *in vitro* translation were analysed by loading a 5-25µl aliquot of the cell-free reaction mixture onto SDS-polyacrylamide gels, prepared essentially as described by Laemmli (1970). The gels were prepared by layering 20mm of 4.5% stacker gel over 80mm 17.5% gel (30:0.2 acrylamide:bis-acrylamide) in 6mm diameter glass tubes. Before loading, the samples were adjusted to a final concentration of 0.05M tris-HCl, pH7.0, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol, 0.01% (w/v) bromophenol blue, and heated at 100°C for 2 min. This electrophoresis was run at constant current of 2-3mA per gel cylinder, using an electrode buffer consisted of 0.025M tris-HCl, 0.192M glycine, 0.1% (w/v) SDS, pH8.8. The run was terminated when the bromophenol blue tracker dye had migrated to a position close to the end of the gel. After freezing by dry ice,

the gels were sliced transversely into 1mm slices using a Mickle Gel slicer. Two consecutive gel slices were placed in a 5ml plastic insert vial (Sterlin), dried at 60°C, 0.3ml of hydrogen peroxide added and the gels solubilised by incubating at 60°C for 24 hrs. After the addition of 3ml of scintillator solution radioactivity was determined (see Section 18.3).

15.2 Two dimensional gel electrophoresis

Two dimensional gel electrophoresis was performed essentially according to the method of O'Farrel (1975).

Briefly, the first dimension involved electrofocusing in a 90 x 3mm tube gel containing 1.2% of pH3-10 and 0.2% of pH3-5, 5-7, 7-9, and 9-11 Ampholines (LKB). The gels were then extruded and equilibrated by immersion for 2 hrs in SDS sample buffer (10% (v/w) glycerol, 5% (v/v) 2-mercaptoethanol, 2.3% (w/v) SDS, 0.0625M tris-HCl, pH6.8). The second dimension was electrophoresed as above (15.1). The gels were fluorographed as described in Section 2.17.

16. Autoradiography

After electrophoresis of [^{32}P] nucleotides on DEAE-paper, the paper was dried and then placed on top of a sheet of X-ray film (Koredex KD 54T, 35 x 43cm) in a lead-lined folder. These folders were stored for two days at room temperature before development. Generally a 6 min development time using Dx-80 developer was employed. After a quick wash, in water, the developed

film was fixed using Fx-40 X-ray liquid fixer for twice the length of time required to clear the film. X-ray film processing was performed in a Kodak P_3 X-ray film processer.

17. Fluorography

Gels were processed for fluorography according to the method of Bonner and Laskey (1974). After electrophoresis, the gels were immersed in three successive baths of DMSO (Sigma), for a total period of 2.5 hrs. The gels were impregnated with PPO by immersion in 100mls of 20% (w/v) PPO in DMSO, with gentle shaking for 1 hr. The excess solution was decanted and PPO was precipitated in the gel by the addition of water. The gels were washed with several changes of water to remove any remaining DMSO. The gel was then dried under vacuum onto Whatman 3MM chromatography paper.

A fluorograph was obtained by placing a sheet of Kodak X-Omat-R film in contact with the gel, held in position between two glass plates. This was kept at -70°C for the necessary time of exposure (6 weeks). Films were developed as described for autoradiography (see Section 16).

18. Determination of radioactivity

1. Radioactivity in RNA molecules was determined by precipitation of RNA with ice-cold trichloroacetic acid (either 5% or 10% (w/v)), and collection of the

precipitates on a millipore filter (0.45μm pore size). Following drying (60 min at 60°C), radioactivity was determined by adding 5ml of toluene scintillator fluid composed of 0.5% PPO (w/v) in Toluene.

2. Radioactivity incorporated into proteins was determined as follows: From each cell-free assay, duplicate 5μl aliquots were spotted on Whatman 3MM 2.5mm paper discs and placed in ice-cold trichloroacetic acid (TCA) (10% (w/v)) containing 10^{-5}M leucine or methionine. These are then washed for 10 min in ice-cold 5% (w/v) TCA for 10 min followed by 10 min in 5% (w/v) TCA at 100°C, then in ice-cold ethanol-diethylether (1:1) and finally in ice-cold diethylether alone. The discs were dried under a heat lamp for 5 min prior to counting in toluene-PPO as above.

3. Gel slices containing either radioactive RNA or proteins were placed in 5ml plastic insert vials and dried for 1-2 hrs at 60°C, 0.3ml of hydrogen peroxide was then added, and the gels incubated at 60°C until they were completely solubilised. Three ml of Triton-toluene scintillation fluid 2 vol, Toluene, 1 vol Triton X-100, 5g PPO/1t, 0.5g Bis MSB/1t) were then added to each vial, the vials shaken well and counted. Labelled samples containing either [^{3}H] and [^{14}C] or [^{3}H] and [^{35}S] were counted in a Isocap-300 Liquid Scintillation counter using program 9. Program 9 relates the percentage counting efficiency to the external standard ratio. Data for the construction of % efficiency standardisation curves

for simultaneous assessment of two isotopes using program 9, were obtained with single isotope quench standards, either [^{3}H]or [^{14}C], which were counted in appropriately set windows (as factory set). Once these standard curves have been prepared, the dual labelled samples were counted in the presence or absence of the external standard. Thus the external standards channels ratio (ESCR) for each sample was determined, and is within the range 0.27-0.31. Knowing the ESCR, the efficiency of counting can be calculated, as can the spill-over using the standard quenching curves provided by the manufacture for program 9. For work involving [^{35}S], these calculations were performed using the curves provided for [^{14}C], since the energy spectrum of the β particles emmitted during the decay of both isotopes is remarkably similar.

4. (i) After autoradiography, the areas of the chromatogram to be examined, determined using the autoradiograph as a template, were placed in scintillation vials, along with 0.5ml of hyamine. These vials were incubated for 30 min at 60^{o}C, then 5ml of toluene-based fluid was added and they were counted.

(ii) The DEAE-paper chromatogram was thoroughly dried, and cut transversely into 1cm wide strips. Each of these pieces were treated as in (i).

RESULTS

RESULTS

1. Cytoplasmic RNAs with high affinity for poly(A) or poly(U) sepharose

Earlier work in this laboratory, using the baby hamster fibroblasts cell line BHK/21, demonstrated the co-existence of RNA species with a high affinity for poly(U) or poly(A) (Burdon et al 1976). These RNAs were at that time termed poly(A)-containing and poly(A)-binding RNAs and were detected in both nuclei and cytoplasm. Interestingly, both classes of RNA were found associated with polysomes suggesting that they may function as messenger, however, they were not fully characterised.

The detection of polysomal RNAs having ability to bind to poly(A)$^{s}_{\lambda}$ presumed to be due to nucleotide sequences rich in uridylate residues. Nemer et al (1974), also reported the presence of non-polyadenylated mRNA species (polyA^{-}mRNA), in sea urchin embryos, having a distinctively high content of uridylate residues.

The results to be presented in this section will show that such poly(A)-containing and poly(A)-binding RNAs, henceforth defined as poly(A)$^{+}$ and poly(A)$^{-}$u^{+}RNAs, respectively, can also be isolated from both nuclei and cytoplasm of Friend murine leukaemia cells, and whilst they have distinctive properties both cytoplasmic species can function as mRNAs.

1.1 Isolation of poly(A)$^+$ and poly(A)$^-$u$^+$ RNAs from the cytoplasm of Friend leukaemia cells

To investigate the location of the cytoplasmic poly(A)$^+$ and poly(A)$^-$u$^+$RNA species in Friend leukaemia cells, polysomes were prepared from these cells, which had been labelled with [^{3}H] uridine for 2 hrs, as described (see Methods Section 3.2). This procedure yields polysomes derived from both the free and membrane bound fractions (Borun et al 1967). The post-polysomal fraction (or cytosol) was also retained.

RNA was extracted from polysomes and cytosol using the phenol/chloroform method (Perry et al 1972), and characterised with regard to their ability to bind to poly(A)- or poly(U)- sepharose columns as follows:

RNA resuspended in binding buffer (0.4M NaCl, 10mM EDTA, 10mM tris-HCl, pH7.4, 0.2% (w/v) SDS), was denatured by heating at 70°C for 5 min (Rilley et al 1966) and rapidly chilled before being applied to a poly(U)-sepharose column, the material that failed to bind to this column was then applied to a poly(A)-sepharose column. Both columns were extensively washed with binding buffer and the bound RNAs were eluted with 90% (v/v) formamide in 10mM EDTA, 10mM tris-HCl, pH7.4, 0.2% (w/v) SDS. Yeast t-RNA was added to 50μg/ml as a carrier and RNA precipitated by the addition of 2.5 vol of ethanol.

The results obtained are summarised in Table 4. As can be seen about 60% of both the cytoplasmic poly(A)$^+$ and

Table 4

Cytoplasmic localisation of [^{3}H] uridine labelled poly(A)$^+$ and poly(A)$^-$u$^+$RNAs from Friend cells

RNA examined	% of total radioactive RNA bound on poly(U)-Sepharose	% of total radioactive RNA bound on poly(A)-Sepharose
Polysomal	11.9±0.9	0.78±0.09
Cytosol	8.4±0.7	0.65±0.08

Friend cells were labelled for 120 min with 20µci/ml of [^{3}H] uridine. Polysomal and cytosol RNAs were prepared (see Methods Section 4.3) and chromatographed on poly(U)- and poly(A)-Sepharose columns as described in the text. Bound RNAs were eluted with 90% (v/v) formamide in 10mM EDTA, 10mM tris-HCl, (pH7.4), 0.2% (w/v) SDS, and radioactivity was determined (see Methods Section 18.1).

Results are expressed as a percentage of total radioactivity in the applied RNA sample, together with the appropriate standard deviation. The quoted values were obtained from 10 separate experiments.

poly(A)$^-$u$^+$RNAs is found sedimenting with polysomes, the remainder being located in the post-polysomal fraction (or cytosol). This is consistent with the results of other workers (see Introduction Section 6).

1.2 Displacement of poly(A)$^+$ and poly(A)$^-$u$^+$RNAs from polysomes after EDTA treatment

The previous demonstration of the co-sedimentation of both poly(A)$^+$ and poly(A)$^-$u$^+$RNAs with polysomal fraction of BHK cells suggested a possible messenger function for these RNAs. Indeed, it is well established that most of the poly(A)$^+$RNA is functional messenger. To investigate this possible functional association of the poly(A)$^-$u$^+$RNA in Friend cells with polysomes, the behaviour of this RNA in response to EDTA treatment of polysomes was examined. This particular experiment also provides a means of assessing the presence of possible contaminating nuclear ribonucleoprotein particles. This was achieved by utilising an EDTA concentration which completely dissociates polysomes whilst having little effect on nuclear ribonucleoprotein particles (Penman et al 1968, Perry and Kelley 1968). Aliquots of [^{3}H] uridine labelled polysomes were either centrifuged in sucrose gradients containing 2mM $MgSO_4$ or 10mM EDTA (see Methods Section 3.2), and the fractions of the gradient corresponding to the region where polysomes would normally sediment were taken and examined for the presence of poly(A)$^+$ and poly(A)$^-$u$^+$RNA molecules. It has been established for HeLa and L cells that such conditions

(10mM EDTA) dissociate polysomes into ribosomal subunits and release the more slowly sedimenting messenger ribonucleoprotein particles whilst having little effect on the sedimentation properties of any contaminating nuclear ribonucleoprotein particles (Penman et al 1968, Perry and Kelley 1968).

Fig 3 shows that treatment of polysomes with EDTA converted the polysomes to subunits. Further the vast majority of poly(A)$^+$ and poly(A)$^-$u$^+$RNAs which prior to EDTA treatment sedimented with polysomes were found to sediment more slowly after EDTA treatment (Fig 3 inset).

These results suggest that poly(A)$^+$ and poly(A)$^-$u$^+$RNAs are associated with polysomes and the poly(A)$^-$u$^+$RNA are not artefacts arising as a result of nuclear ribonucleoprotein particles which might sediment in the polysome region of the gradient.

1.3 Specificity and efficiency of poly(U)- and poly(A)-sepharose columns for polysomal RNA molecules with high affinity for poly(A)- or poly(U)-sepharose columns

Before proceeding with the isolation of polysomal poly(A)$^-$u$^+$ RNA for future investigation, it was necessary to exclude the possibility that non-specific binding of the RNA molecules on the columns was occurring, and also to rule out the possible effects on intermolecular interactions influencing the RNA binding.

To determine the specificity of binding, the material

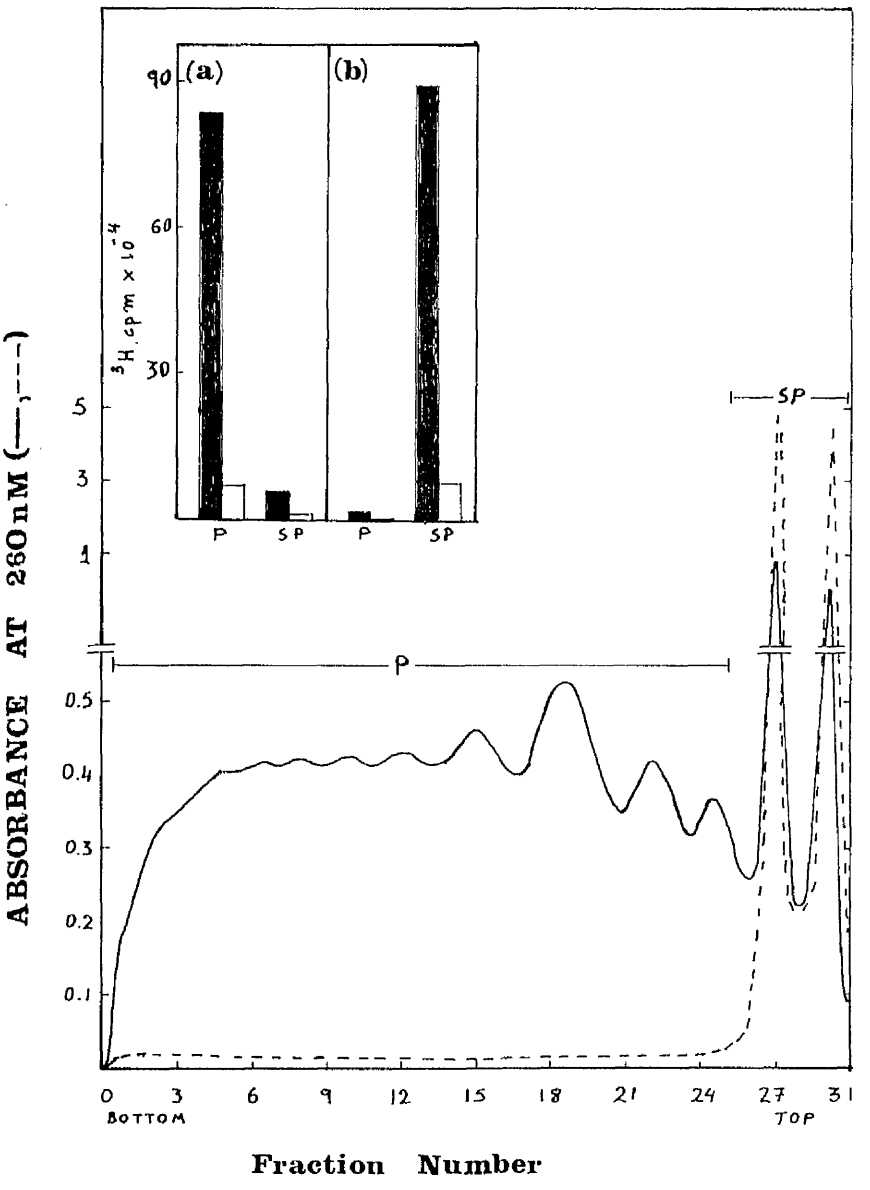
Figure 3

Effect of EDTA-treatment on the displacement of poly(A)$^+$ and poly(A)$^-$u$^+$RNAs from Friend cell polysomes

Polysomes from Friend cells labelled with 20μci/ml of [^{3}H] uridine for 2 hrs were centrifuged through sucrose gradients containing either 2mM $MgSO_4$ (——), or 10mM EDTA (- - -)(see Methods Section 3.2). Fractions corresponding to the "polysome" regions (P, >80s) and "sub-polysome" regions (SP <80s) were pooled (see diagram). RNA was extracted from these pooled fractions and analysed for content of labelled poly(A)$^+$ and poly(A)$^-$u$^+$RNAs as described in Table 4.

(Inset) [^{3}H]-cpm incorporated into poly(A)$^+$RNA (shaded histogram) or poly(A)$^-$u$^+$RNA (open histogram) in the polysomal (P) or subpolysomal (SP) fractions, after sedimentation through sucrose gradients in the absence (a) or presence (b) of EDTA. Radioactivity was determined as described in Methods Section 18.1.

Figure 3


(a)
(b)
90
60
30
^{3}H.cpm x 10^{-4}
P
SP
P
SP
SP
P
5
3
1
0.5
0.4
0.3
0.2
0.1
ABSORBANCE AT 260nM (—,- - -)
0
3
6
9
12
15
18
21
24
27
31
BOTTOM
TOP
Fraction Number

bound on the first passage through either a poly(A)- or poly(U)-sepharose column was eluted and tested for re-binding, and reciprocal binding, with poly(A)- and poly(U)-sepharose columns. The results summarised in Table 5 show that the poly(A)$^+$ and poly(A)$^-$u$^+$RNAs were efficiently recovered upon recycling. The percentage of poly(A)$^+$ and poly(A)$^-$u$^+$RNAs not rebound (Table 5) on the second passage, may reflect a lability of the poly(A) or poly(U) attached to the sepharose. Clearly, if some of the attached poly(A) becomes detached from the sepharose during the first elution, then a poly(A)$^-$u$^+$RNA.poly(A) hybrid molecule will be collected and this will not re-bind. However if this is the case it has been shown to occur to relatively low extent.

Since the poly(A)$^+$RNA is in excess, compared to poly(A)$^-$u$^+$RNA (see Table 4), there is a possibility that this excess of poly(A) may hybridise to the "U-rich" region(s) in the poly(A)$^-$u$^+$RNA. To assess the extent of such a possibility the binding behaviour of polysomal RNA was examined in its "native", and heat denatured state, it being assumed that heat denaturation leads to the disruption of such aggregates (Rilley et al 1966). The procedure adopted, and the results obtained, are described in Table 6. These results argue that any intermolecular aggregation which may occur on isolation, for example a "U-rich" region in one RNA molecule and a poly(A) segment of another, does not significantly affect fractionation.

Table 5

Binding of [^{3}H] uridine labelled polysomal poly(A)$^+$ and poly(A)$^-$u$^+$RNAs to poly(U)- and poly(A)-sepharose

RNA examined	% of applied radioactive RNA sample bound to poly(U)-Sepharose [^{3}H]	poly(U)-Sepharose [^{32}P]	poly(A)-Sepharose [^{3}H]	poly(A)-Sepharose [^{32}P]
(i) [^{3}H]-poly(A)$^+$RNA	95±3		⩽0.03	
(i) [^{3}H]-poly(A)$^-$u$^+$RNA	⩽0.01		86±3	
(ii) [^{3}H]-poly(A)$^+$RNA + 28s[^{32}P]-rRNA	95±3	⩽0.01	⩽0.04	⩽0.01
(ii) [^{3}H]-poly(A)$^-$u$^+$RNA + 28s[^{32}P]-rRNA	⩽0.05	⩽0.01	83±4	⩽0.01
(iii) [^{3}H]-poly(A)$^+$RNA	94±2		⩽0.05	
(iii) [^{3}H]-poly(A)$^-$u$^+$RNA	⩽0.05		84±3	

Polysomal poly(A)$^+$ and poly(A)$^-$u$^+$RNAs prepared from Friend cells labelled with 20μci/ml of [^{3}H] uridine for 2 hrs, were isolated as described in Table 4. These RNAs were ethanol precipitated then dissolved in binding buffer (0.4M NaCl, 10mM EDTA, 10mM tris-HCl, (pH7.4), 0.2% (w/v) SDS) and either (i) re-chromatographed on both poly(U)- and poly(A)-sepharose columns, or (ii) mixed with HeLa cell [^{32}P] labelled 28s rRNA and re-chromatographed on both column types, or (iii) mixed with unlabelled Friend cell cytoplasm, total RNA extracted and chromatographed on both types of column. Bound RNAs were eluted with 90% (v/v) formamide in 10mM EDTA, 10mM tris-HCl (pH7.4), 0.2% (w/v) SDS. Bound and unbound RNA fractions were assayed for 5% (w/v) trichloroacetic acid-precipitable [^{3}H] and [^{32}P] radioactivity.

Results are expressed as a percentage of total radioactivity in the applied radioactive RNA sample, together with the appropriate standard deviation. The quoted values were obtained from 10 separate experiments. Less than 0.01% [^{32}P] 28s ribosomal RNA [HeLa cells] bound to either poly(A)- or poly(U)-sepharose columns under these conditions.

Table 6

Effect of heat treatment on the binding properties of polysomal poly(A)$^+$ and poly(A)$^-$u$^+$RNAs to poly(U) or poly(A)-sepharose columns

Experiment	Treatment of polysomal RNA	RNA bound by (A) Poly(U)-Sepharose then Poly(A)-Sepharose cpm		(B) Poly(U)-Sepharose then Poly(A)-Sepharose	
1	- Heat	33880	2140	32750	2050
	+ Heat	33720	2060	32450	2005
2	- Heat	19870	1275	18675	1176
	+ Heat	19950	1305	18870	1210

Aliquots of [^{3}H] uridine labelled polysomal RNA dissolved in binding buffer (0.4M NaCl, 10mM EDTA, 10mM tris-HCl, pH7.4, 0.2% (v/v) SDS) were either heat denatured at 70°C for 5 min, or left untreated. They were then passed through columns of either poly(U)- or poly(A)-sepharose. The non-bound material was then tested for binding in the complementary column, for example the material not bound to a poly(A)-sepharose column was passed through a poly(U)-sepharose column. Bound RNA species were eluted with 90% (v/v) formamide in 10mM EDTA, 10mM tris-HCl, pH7.4, 0.2% (w/v) SDS. Radioactivity was determined as described in Methods (Section 18.1). Results are expressed as the amount of radioactive RNA (counts/min) retained on the columns.

(A) Polysomal RNA passed first through a poly(U)- and then through a poly(A)-sepharose column

(B) Polysomal RNA passed first through a poly(A)- and then through a poly(U)-sepharose column

For instance, in fractionating a non-polyadenylated, but poly(A)$^-$u$^+$RNA from the total RNA population, it is conceivable that poly(A)$^+$ RNAs could form partial hybrids between their poly(A) and the "U-rich" region(s) of the desired species and so be co-purified. Heating should, however, disrupt such aggregates. Therefore, on rapid application to the poly(A)-sepharose column the "U-rich" sequences of the desired RNA will bind to the column, since the poly(A) which is attached to the Sepharose will be in vast excess. Thus, if the levels of such aggregations are significant, a difference in bound radioactivity to the columns would be expected between heated and non-heated samples. As can be seen from Table 6, no such differences can be detected.

In addition, the effect of a possible aggregation on the estimation of poly(A)$^+$ and poly(A)$^-$u$^+$RNAs was determined by mixing [^{3}H] uridine labelled poly(A)$^+$ and poly(A)$^-$u$^+$ RNAs with total cytoplasm from Friend cells, and the mixture immediately phenol/chloroform extracted. Subsequent to extraction, RNA was chromatographed on poly(U)- and poly(A)-sepharose as described in Table 4. The results of this "reconstruction type" experiments appear in Table 5 (Section iii), and these results again argue against artefactual aggregations during the fractionation procedure used.

To ensure that the binding to the poly(A)-sepharose is most likely to be mediated by either uridylate homopolymeric sequences, or by sequences very "rich" in

uridylate residues, the binding of 28s ribosomal RNA was examined. This RNA species was used since it is known that rRNA lacks extensive "U-rich" regions (Burdon et al 1972, 1976), so no binding would be expected if the column is binding specifically. Using isolated HeLa cell [^{32}P] labelled 28s rRNA (gift from Dr. K. Vass of this Department), or a mixture of [^{32}P] rRNA, and either [^{3}H] uridine labelled poly(A)$^{+}$, or poly(A)$^{-}$u^{+}RNA, no binding of [^{32}P]radioactivity to both poly(U)- or poly(A)-sepharose columns could be observed (Table 5, legend).

In addition, polysomal RNA was extracted from Friend cells which had been labelled with [^{3}H] uridine either, following a 30 min pretreatment with actinomycin D (0.04μg/ml of medium), sufficient to abolish rRNA synthesis, (Penman et al 1968, Perry and Kelley 1970), or without actinomycin D pretreatment, and fractionated into poly(A)$^{+}$ and poly(A)$^{-}$u^{+}RNAs. If ribosomal RNA significantly contributes the poly(A)$^{-}$u^{+}RNA, it would be expected that the ratio of radioactivity in poly(A)$^{-}$u^{+} RNA to that in poly(A)$^{+}$ RNA would be less in cells pretreated with low levels of actinomycin D compared with non-treated controls. No such difference in ratio was detected (Table 7), so it appears that labelled RNA designated poly(A)$^{-}$u^{+}RNA is not ribosomal RNA.

Having demonstrated the occurrence of both poly(A) and poly(A)$^{-}$u^{+}RNAs associated with polysomes, a series of

Table 7

Effect of pretreatment of Friend cell with low levels of actinomycin D on the binding properties of [^{3}H] labelled polysomal poly(A)$^+$ and poly(A)$^-$u$^+$RNAs

Experiment	Cell treatment	RNA bound on Poly(U)-Sepharose (cpm)	RNA bound on Poly(A)-Sepharose (cpm)	Ratio of [^{3}H] poly(A)$^+$ RNA to [^{3}H] poly(A)$^-$u$^+$ RNA
1	-Act.D	62850	4110	15.3
	+Act.D	64610	4290	16
2	-Act.D	15750	980	16
	+Act.D	12950	874	14.8

A culture of Friend cells was halved one half was pretreated with actinomycin D (0.04µg/ml) for 30 min, the other half receiving no pretreatment. Both cultures were then labelled with 20µci/ml of [^{3}H] uridine for 120 min. Samples of polysomal RNA were chromatographed on poly(U)- and poly(A)-sepharose columns as described in Table 4. Radioactivity was determined as described in Methods (Section 18.1). Results are expressed as the amount of radioactive RNA (counts/min) retained on the columns

experiments were performed to further characterise these RNAs. In particular the following questions were investigated: Does poly(A)$^-$u$^+$RNA itself contain poly(A); Does the poly(A)$^+$RNA contain extensive "U-rich" regions; Does the poly(A)$^-$u$^+$RNA have sequences in common with poly(A)$^+$RNA, and more specifically, is the poly(A)$^-$u$^+$RNA some form of degradation product of the poly(A)$^+$RNA, for example, a poly(A)$^+$RNA with the 3' poly(A)tract removed; Does the poly(A)$^-$u$^+$RNA actually function as a messenger.

1.4 Attempts to detect poly(A) in polysomal poly(A)$^-$u$^+$RNA

It has been shown that poly(A)$^-$u$^+$RNA probably lacks poly(A) tracts, as judged by its chromatographic behaviour on poly(U)-sepharose (see Table 4 and Table 5).

A more sensitive hybridisation assay was devised to confirm this conclusion, based on the fact that poly(U).poly(A) hybrids are resistant to digestion by pancreatic ribonuclease whilst poly(U) itself is completely digested (Bishop et al 1974). In this way, [^{3}H] poly(U) was hybridised, in excess, to the various RNA fractions isolated, and the hybridisation mixture was subsequently digested with pancreatic ribonuclease. Thus radioactivity remaining acid insoluble is a measure of the amount of hybrid formed, and hence the poly(A) content of the isolated RNA. The method would also detect poly(A) already in an intermolecular hybrid with a "U-rich" tract, since poly(U) can react with poly(A).poly(U) to form a

triple helix hybrid structure poly(A):2 poly(U) (Stevens and Felsenfeld 1964). As Table 8 shows, the extent of hybridisation with [^{3}H] poly(U) is only about 2% that obtained with poly(A)$^+$RNA. In addition the data indicates that the isolation of poly(A)$^+$RNA is efficient, since the RNA fraction which failed to bind on either poly(U) or poly(A)-sepharose also demonstrates a very low ability to hybridise with poly(U).

1.5 Attempts to detect "U-rich" region(s) in Friend cell polysomal poly(A)$^+$RNA

The detection of oligo(U) sequences in HeLa cytoplasmic poly(A)$^+$RNA (Korwek *et al* 1976) has prompted the investigation of whether Friend polysomal poly(A)$^+$RNA contains such a "U-rich" region, which could remain undetected due to intramolecular hybridisation with the poly(A).

For this purpose [^{3}H] uridine labelled polysomal poly(A)$^+$ RNA was mixed with an excess (50-100 fold) of oligo(dT)$_{10}$ in the presence of 0.01M NaCl, 10mM EDTA, 10mM tris-HCl, (pH7.5). This level of oligo(dT)$_{10}$ has been shown to completely saturate any poly(A) tracts (Kish and Pederson 1977). The mixture was heated at 55^{o}C for 5 min, quickly cooled, and brought to 0.5M NaCl, 10mM EDTA, 10mM tris-HCl, (pH7.5). Incubation was carried out for 30 min at 4^{o}C, and the resultant poly(A)$^+$RNA.oligo(dT)$_{10}$ hybrid was purified from the remaining oligo(dT)$_{10}$ using a Sephadex G-25 column, (equilibrated with 0.5M NaCl, 10mM EDTA, 10mM tris-HCl, pH7.5) at 4^{o}C, and then ethanol precipitated.

Table 8

Hybridisation of [^{3}H]-poly(U) to different polysomal RNA fractions

RNA tested	Amounts (μg)	hybridised [^{3}H]-poly(U) radioactivity (cpm)
Poly(A)$^+$RNA	1.0	3850
Poly(A)$^-$u$^+$RNA	1.0	106
Poly(A)$^-$u$^+$RNA*	1.0	85
Non-bound RNA**	2.0	105
yeast t-RNA	2.0	35
no-RNA	-	31

Samples of polysomal RNA fractions were hybridised with a 10-fold excess of [^{3}H]-poly(U) in 2 x SSC (1SSC, 0.15 NaCl, 0.015 Sodium citrate, pH7.0) for 30 min at 45°C. Each reaction was terminated by dilution with 20 vol ice-cold 2 x SSC and the mixture digested with pancreatic ribonuclease (25μg/ml) at 4°C for 20 min. Trichloroacetic acid-precipitable radioactivity was determined as described in Methods (Section 18.1)

* Poly(A)$^-$u$^+$RNA in this case is taken as the material binding on passage of the polysomal RNA directly through a poly(A)-sepharose column without prior isolation of poly(A)$^+$ RNA using a poly(U)-sepharose

** The fraction of polysomal RNA which fails to bind to either poly(U)- or poly(A)-sepharose columns

The precipitates were dissolved in 0.5M NaCl, 10mM EDTA, 10mM tris-HCl (pH7.5) and chromatographed on a poly(A)-sepharose columns at 4°C. The levels of poly(A)$^+$RNA bound to the poly(A)-sepharose remained the same ($<$0.02%, see Table 5), suggesting that no "U-rich" region(s) occur in poly(A)$^+$RNA, at least from Friend cells. Additionally, no binding of the oligo(dT)$_{10}$ treated poly(A)$^+$RNA could be detected with poly(U)-sepharose, indicating that the oligo(dT)$_{10}$ has indeed "blocked" the poly(A) tracts.

1.6 Size distribution of polysomal poly(A)$^+$ and poly(A)$^-$u$^+$RNAs

To characterise further the polysomal poly(A)$^+$ and poly(A)$^-$u$^+$RNAs species, the sedimentation properties of poly(A)$^+$ and poly(A)$^-$u$^+$RNAs were examined under completely denaturing conditions, in order to exclude any possible aggregation artifacts. Fig 4 shows the sedimentation properties of [^{3}H] uridine labelled polysomal poly(A)$^+$ and poly(A)$^-$u$^+$RNAs in 98% (v/v) formamide-sucrose gradients (Ross 1976). [^{3}H] uridine labelled Friend cell polysomal RNA was centrifuged on a parallel gradient to provide sedimentation markers. The poly(A)$^+$ and poly(A)$^-$u$^+$RNAs appear to sediment as two discrete, but heterogeneous, components, with a mean value of about 18s and 20s respectively.

These sedimentation characteristics are consistent with those reported for Friend cell polysomal poly(A)$^+$ RNA (Getz et al 1975), and BHK/21 cell poly(A) binding RNA

Figure 4

Sedimentation behaviour of polysomal poly(A)$^+$ and poly(A)$^-$u$^+$RNAs in denaturing sucrose-formamide gradients

Polysomal poly(A)$^+$ and poly(A)$^-$u$^+$RNAs, prepared from Friend cells labelled with 20µci/ml of [^{3}H] uridine for 2 hrs, were ethanol precipitated and treated as described in Methods Section 5.4. The denatured RNAs were layered on 8% to 20% (w/v) sucrose gradients containing 98% (v/v) formamide, 2mM EDTA, 10mM tris-HCl, pH7.5, and centrifuged at 32,000 rpm/min in the Spinco SW56 rotor for 22 hrs at 30°C.

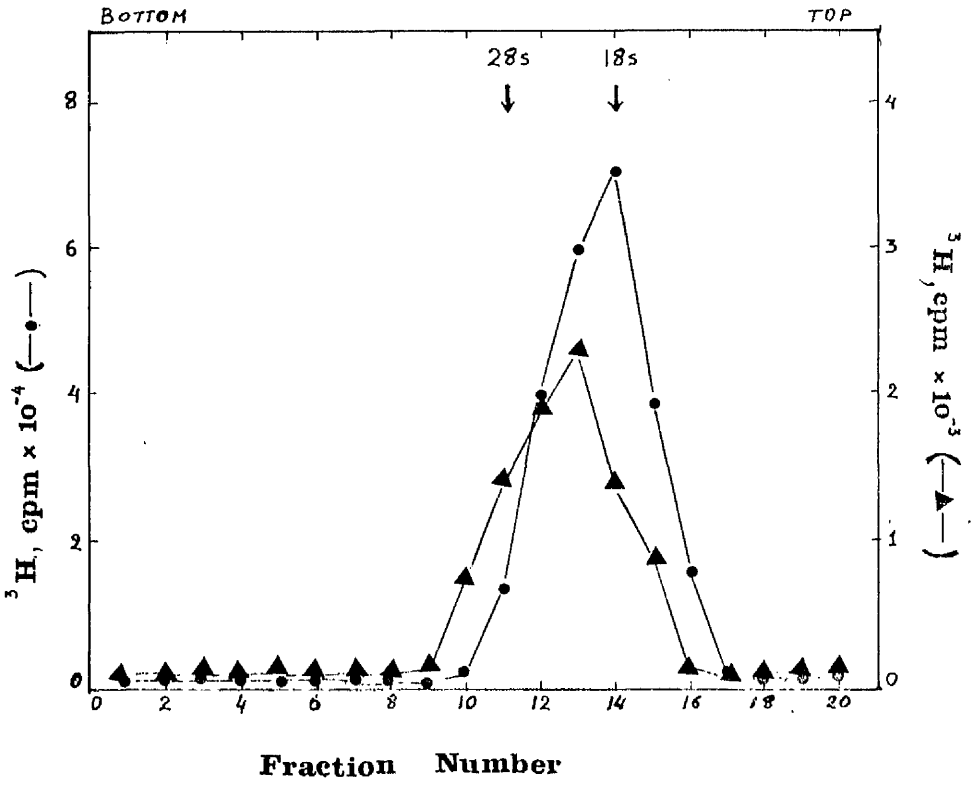
Friend cell polysomal RNA was centrifuged in parallel gradients to provide markers for comparison of sedimentation (see arrows).

Fractions were collected and their content of 5% (w/v) trichloroacetic acid-precipitable radioactivity was determined (see Methods Section 18.1).

•——• [^{3}H] poly(A)$^+$RNA

▲——▲ [^{3}H] poly(A)$^-$u$^+$RNA

Figure 4



(Burdon et al 1976). Similar results have also been obtained for poly(A)$^+$ and poly(A)$^-$ mRNAs from other cell types (Milcarek et al 1974, Nemer et al 1974, Spradling et al 1975, Greenberg 1976). Thus, polysomal poly(A)$^-$u$^+$RNAs are not likely to be derived from poly(A)$^+$RNA by degradation. Also, the sedimentation behaviour observed indicates that Friend cell poly(A)$^-$u$^+$RNA does not belong to a class of very small, "U-rich" RNA molecules, such as those found in polysomes of chick muscles (Bester et al (1975).

1.7 Sequence homology between polysomal poly(A)$^+$ and poly(A)$^-$u$^+$RNAs

The co-existence of distinct poly(A)$^+$ and poly(A)$^-$u$^+$RNAs species in Friend cells prompted the question of whether or not these RNAs constitute classes of distinctly different genetic origin.

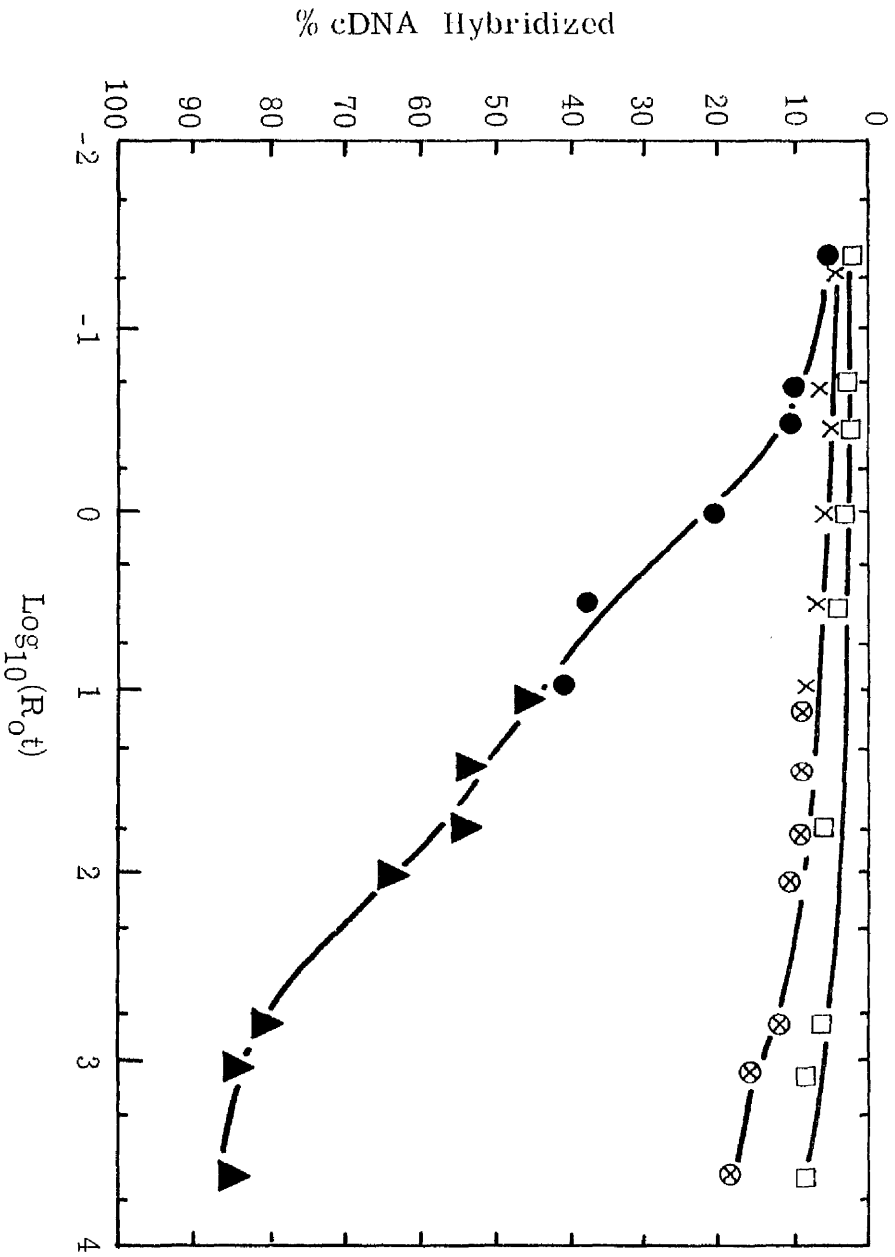
This was examined by using molecular hybridisation; [^{3}H]-labelled complementary DNA (cDNA) was prepared using purified polysomal poly(A)$^+$ RNA as a template (see Methods 10) for AMV reverse transcriptase. This [^{3}H] cDNA was incubated for various times, and with various amounts of its own template RNA, and the hybridisation of the [^{3}H] cDNA to this RNA was measured using a single-strand specific nuclease (S1) (Birnie et al 1974). The results are shown in Fig 5, where it can be seen that at least 90% of the cDNA can form nuclease-resistant hybrids with the template RNA.

Figure 5

Kinetics of hybridisation of complementary DNA (cDNA) prepared from Friend cell polysomal poly(A)$^+$RNA with polysomal poly(A)$^+$ and poly(A)$^-$u$^+$RNAs

Polysomal RNA was prepared and fractionated into poly(A)$^-$u$^+$ and poly(A)$^+$RNAs as described in Table 4. A portion of the poly(A)$^+$RNA was used to prepare [^{3}H] cDNA. RNA excess reactions were carried out using poly(A)$^+$RNA at 10μg/ml (●) and at 2,500μg/ml (▲), poly(A)$^-$u$^+$RNA at 10μg/ml (×) and at 2,500μg/ml (⊗) poly(U) at 1,000μg/ml (□). The ratios of RNA:cDNA used were 20 and 2,000:1. Hybridisation mixtures were made up to 1mg/ml in RNA by the addition, where necessary, of appropriate amounts of *E. coli* rRNA.

Figure 5


% cDNA Hybridized
100
90
80
70
60
50
40
30
20
10
0
-2
-1
0
1
2
3
4
$Log_{10}(R_o t)$

In parallel experiments, the annealing of the same [^{3}H] cDNA to poly(A)$^-$u$^+$RNA was measured. The data shown in Fig 5 indicate that only about 10% of the [^{3}H] cDNA complementary to poly(A)$^+$RNA is found in hybrids (when corrected for a poly(U) background hybridisation). The $\frac{Ro}{t}$ (Ro = initial RNA concentration in moles of nucleotide per litre, t = time in seconds) values used were sufficient to ensure that hybridisation was essentially complete. Since the preparation of cDNA employed only yields reverse-transcripts of the 3' terminal sequences of the template poly(A)$^+$RNA, these results suggest that the sequences of the poly(A)$^+$RNA adjacent to its 3' end display little sequence homology with the poly(A)$^-$u$^+$RNA sequences. These data support the view the poly(A)$^-$u$^+$RNAs from Friend cell polysomes appears to be a discrete class of poly(A)$^-$mRNA, with little direct sequence relationship to the poly(A)$^+$ RNA.

1.8 Template activity of polysomal poly(A)$^+$ and poly(A)$^-$u$^+$RNAs

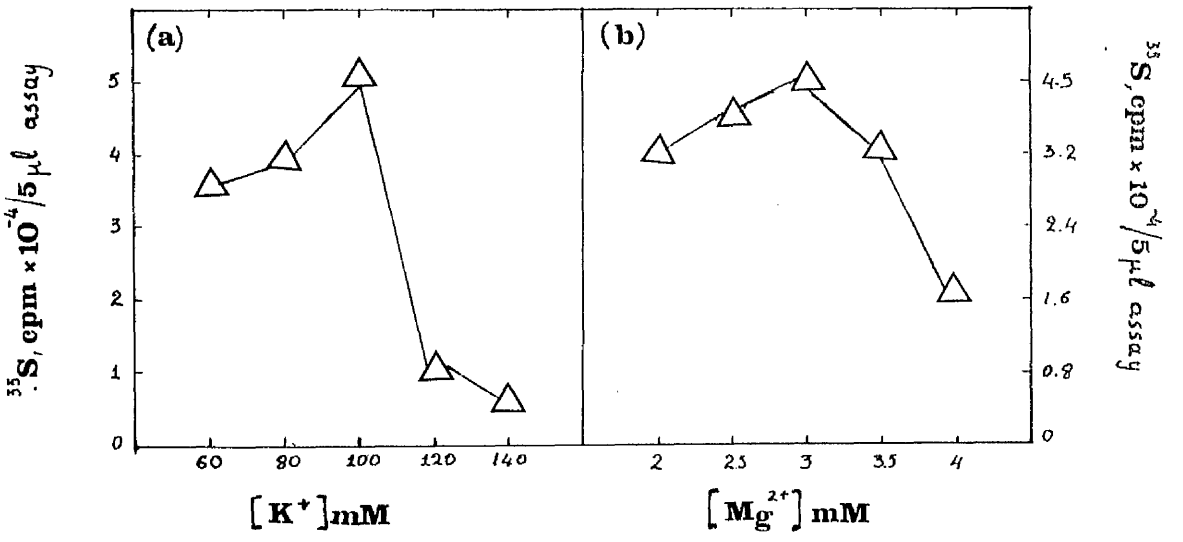
The characterisation so far of poly(A)$^-$u$^+$RNAs suggested that this may have a messenger function. This was investigated by attempting to translate the polysomal poly(A)$^-$u$^+$ RNA in a cell-free system. This approach has been utilised for a number of non-polyadenylated polysome-associated RNAs, in a variety of cell types (Fromson and Verma 1976, Sonenshein *et al* 1976, Ruderman and Pardue 1977, Kaufmann *et al* 1977, Ragg *et al* 1977, Geoghegan *et al* 1978, Whalen and Gross 1978).

Both Friend polysomal poly(A)$^+$ and poly(A)$^-$u$^+$RNAs were examined for template activity in an optimised, wheat germ, cell-free, polypeptide synthesising system. Initially the wheat germ preparation used was examined for optimal concentration of monovalent (K^+) and divalent (Mg^{++}) anions (Fig 6). In addition, spermine and spermidine were assayed for increased efficiency of polypeptide synthesis. The optimum spermine concentration gave an improved stimulation, compared to that of spermidine (Fig 7). In all these studies only the poly(A)$^+$RNA was used since the preparation of microgram quantities of poly(A)$^-$u$^+$RNA involved considerable work. Subsequently, conditions of 3mM Mg^{++}, 100mM K^+ and 150μM spermine were used as optimal conditions.

Under these conditions both poly(A)$^+$ and poly(A)$^-$u$^+$RNAs stimulated the incorporation of labelled [^{3}H]-leucine and [^{35}S]-methionine into hot trichloroacetic acid-precitable material (Table 9). Incorporation was linearly dependent on the amounts of the added poly(A)$^+$ or poly(A)$^-$u$^+$RNAs at low inputs (Fig 8), but at higher RNA inputs less incorporation than expected was observed. This decrease may be caused by residual traces of SDS or EDTA in the RNA preparation. As a control, an equivalent amount of Friend cell 28s RNA was found to give very little stimulation (Table 9). Interestingly, the addition of spermine appears to have a relatively greater effect on the stimulation of [^{3}H]-leucine incorporation directed by poly(A)$^-$u$^+$RNA, than that of poly(A)$^+$ RNA

Figure 6

Ionic requirements for polypeptide synthesis directed by Friend cell polysomal poly(A)$^+$RNA in a wheat germ cell-free protein synthesising system



Assay mixtures utilising [^{35}S] methionine and containing different concentrations of magnesium and potassium, were incubated for 60 min at 25°C with 7.6µg of poly(A)$^+$RNA, and hot trichloroacetic acid-precipitable radioactivity (—△—) was determined (see Methods Section 18.2).

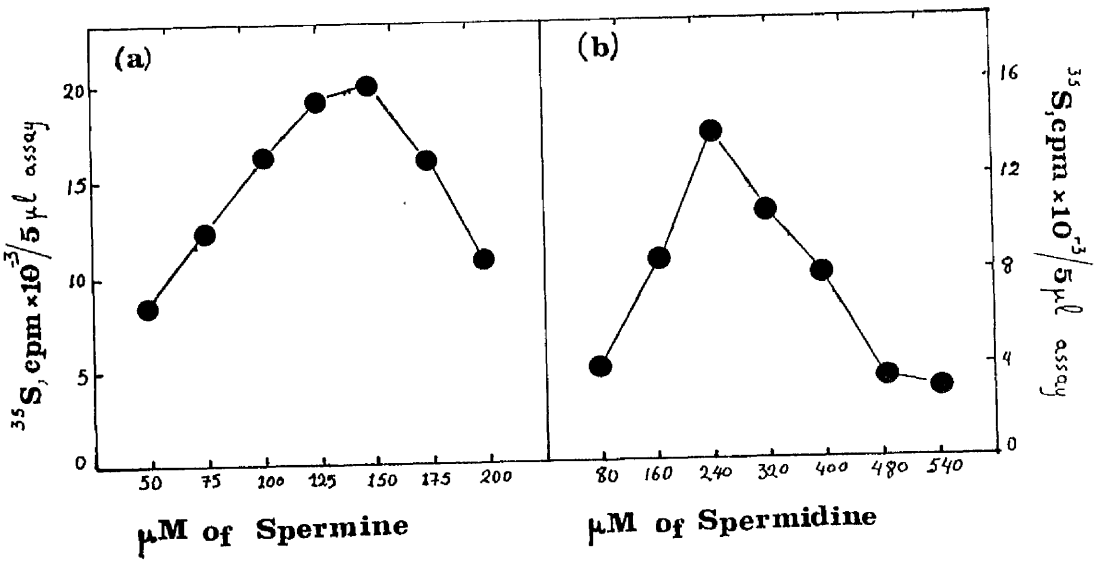
(a) Effect of KCl concentration on polypeptide synthesis at 3mM magnesium acetate

(b) Effect of magnesium acetate concentration on polypeptide synthesis at 100mM KCl

Incorporation due to endogeneous polypeptide synthesis (3,000-3,200 cpm per 5µl assay) was subtracted in each case.

Figure 7

Spermine or spermidine requirements for optimum polypeptide synthesis directed by Friend cell polysomal poly(A)$^+$RNA in a wheat germ cell-free protein synthesising system



Assay mixtures containing 3mM magnesium acetate, 100mM KCl and different concentrations of spermine (a) or spermidine (b) were incubated for 60 min at 25°C with 3.5µg of poly(A)$^+$RNA. Incorporation of [^{35}S] methionine into hot trichloroacetic acid-precipitable radioactivity was used as a measure of polypeptide synthesis and was determined as described in Methods Section 18.2.

Incorporation due to endogeneous protein synthesis (3,200-3,500 cpm per 5 assay) was subtracted in each case.

Figure 8

Stimulation of incorporation of [^{35}S] methionine into polypeptides by different amounts of added Friend cell polysomal poly(A)$^+$ or poly(A)$^-$u$^+$RNAs in a wheat germ cell-free protein synthesising system

Assay mixtures containing various amounts of either polysomal poly(A)$^+$ (—●—) or poly(A)$^-$u$^+$RNA (—•—) and [^{35}S] methionine were incubated for 60 min at 25°C under standard conditions. Trichloroacetic acid-precipitable was determined as described in Methods Section 18.2. The values quoted were obtained from two separate experiments. Bars (⊢——⊣) indicate the extreme of the measurements. Incorporation due to endogeneous protein synthesis (2,800-3,000 cpm per 5µlassay) was subtracted in each case.

Figure 8

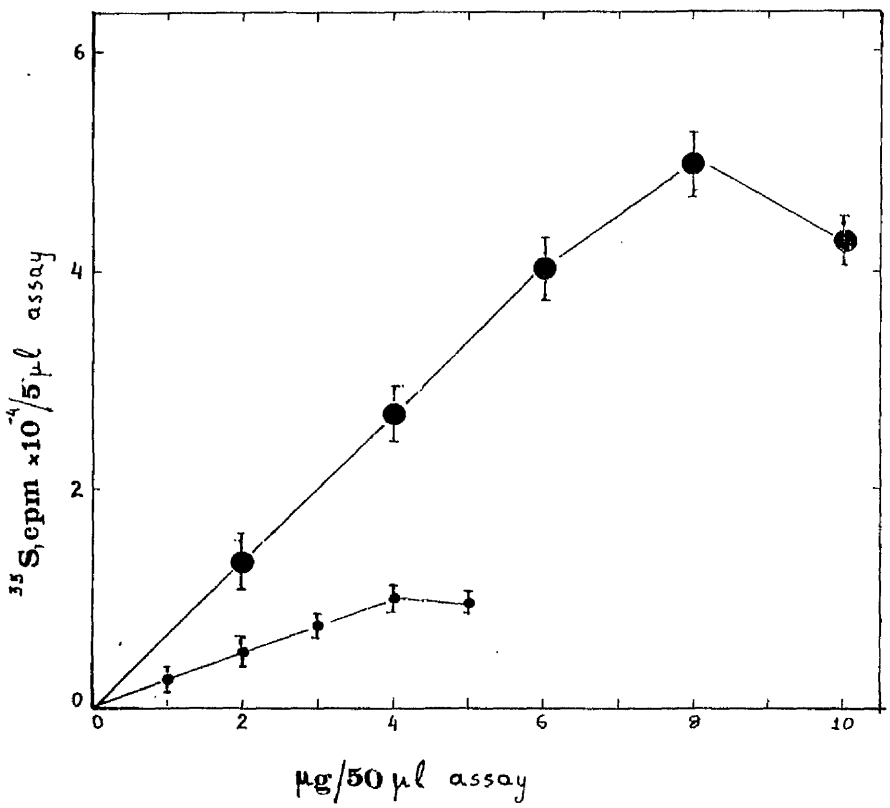


Table 9

In vitro incorporation of [^{35}S] methionine and [^{3}H] leucine into polypeptides directed by Friend cell polysomal poly(A)$^{+}$ and poly(A)$^{-}$u^{+}RNAs

RNA assayed	Amounts µg/50µl assay	[^{35}S] methionine incorporation cpm/5µl assay	[^{3}H] leucine incorporation cpm/50µl assay
Poly(A)$^{+}$RNA	4.0	24000	31000
Poly(A)$^{-}$u^{+}RNA	2.4	5800	9070
Poly(A)$^{-}$u^{+}RNA*	1.4	3895	4760
Poly(A)$^{+}$RNA + Poly(A)$^{-}$u^{+}RNA	2.3 + 1.9	21850	24400
28s rRNA**	4.0	2150	1730
no-RNA	-	2050	1850

Assay mixtures (50µl) containing various amounts of poly(A)$^{+}$ or poly(A)$^{-}$u^{+}RNAs or both, and either [^{35}S] methionine or [^{3}H] leucine were incubated for 60 min at 25°C under standard conditions. Incorporated radioactivity was estimated as described in Methods (Section 18.2). Incorporation due to endogeneous messenger activity was subtracted.

* Poly(A)$^{-}$u^{+}RNA retained upon repassage through poly(A)-sepharose

** Friend cell, 28s ribosomal RNA

(Table 10). Furthermore, the results shown in Fig 8 suggest that poly(A)$^+$ and poly(A)$^-$u$^+$RNAs differ in their translational efficiency in that poly(A)$^+$ is translated about 3-7 times more efficiently. This contrasts with the observations of Fromson and Verma (1976), and Kaufmann et al (1977), who concluded that both poly(A)$^-$ and poly(A)$^+$ mRNAs from sea urchin embryos and HeLa cells have the same translational efficiency. However, it should be made clear that poly(A)$^-$u$^+$RNA is only a particular fraction of non-polyadenylated mRNA and may have unique properties.

1.9 Analysis of the polypeptide products directed by polysomal poly(A)$^+$ and poly(A)$^-$u$^+$RNAs in a wheat germ system

The translation products obtained from the wheat germ system were initially analysed by one-dimensional SDS-polyacrylamide gel electrophoresis.

Poly(A)$^+$ and poly(A)$^-$u$^+$RNAs were translated in a wheat germ system using [^{3}H]-leucine or [^{35}S]-methionine as radioactive amino acid, and the size distribution of the synthesised labelled polypeptides analysed on 17.5% SDS-polyacrylamide gels. As can be seen in Fig 9, a heterogeneous pattern of polypeptides with molecular weights ranging from 5 x 10^3 - 70 x 10^3 daltons is obtained when either poly(A)$^+$ or poly(A)$^-$u$^+$RNAs is used to direct protein synthesis, with [^{35}S]-methionine or [^{3}H]-leucine as a label. The detection of two very low

Table 10

Effect of spermine on [^{3}H] leucine incorporation into polypeptides directed by Friend cell polysomal poly(A)$^+$ or poly(A)$^-$u$^+$RNAs in a wheat germ cell-free protein synthesising system

RNA assayed	Amounts (µg/50µl)	Spermine (150µM)	[^{3}H] leucine incorporation cpm x 10^{-3}/50µl assay
Poly(A)$^+$RNA	5.8	-	28.30
Poly(A)$^+$RNA	5.8	+	42.20
Poly(A)$^-$u$^+$RNA	3.2	-	5.15
Poly(A)$^-$u$^+$RNA	3.2	+	8.95
No-RNA	-	-	2.16
No-RNA	-	+	2.31

Under standard conditions (100mM KCl and 3mM Magnesium acetate) and in the presence or absence of 150µM spermine, 50µl reaction mixtures containing either poly(A)$^+$ or poly(A)$^-$u$^+$RNAs were incubated at 25°C for 60 min. Hot trichloroacetic acid-precipitable material was determined as described in Methods (Section 18.2). Results presented have been corrected for endogeneous activity.

Figure 9

One dimensional SDS-polyacrylamide gel electrophoresis of radioactive polypeptides synthesised in a wheat germ cell-free system in the presence of Friend cell polysomal poly(A)$^+$ or poly(A)$^-$u$^+$RNAs

Labelled polypeptides synthesised in a wheat germ cell-free system in the presence of polysomal poly(A)$^+$ or poly(A)$^-$u$^+$ RNAs were analysed for their size distribution on 17.5% SDS-polyacrylamide gels (see Methods Section 15.1).

The arrows indicate the distances migrated by standard protein of known molecular weight, which are bovine serum ablubin (BSA 68,000), glutamate dehydrogenase (GDH 53,000), lactate dehydrogenase (LDH 36,000), chymotrypsinogen (EHM 25,700).

Initially, the products synthesised by poly(A)$^+$ or poly(A)$^-$u$^+$ RNAs were analysed on separate gels, although the results have been co-plotted for comparative purposes (a and b). Later, products synthesised by poly(A)$^+$ or poly(A)$^-$u$^+$RNAs were made with different radioactive labels, mixed and analysed on the same gel using double radioactive label counting method (c and d) (see Methods Section 18.3).

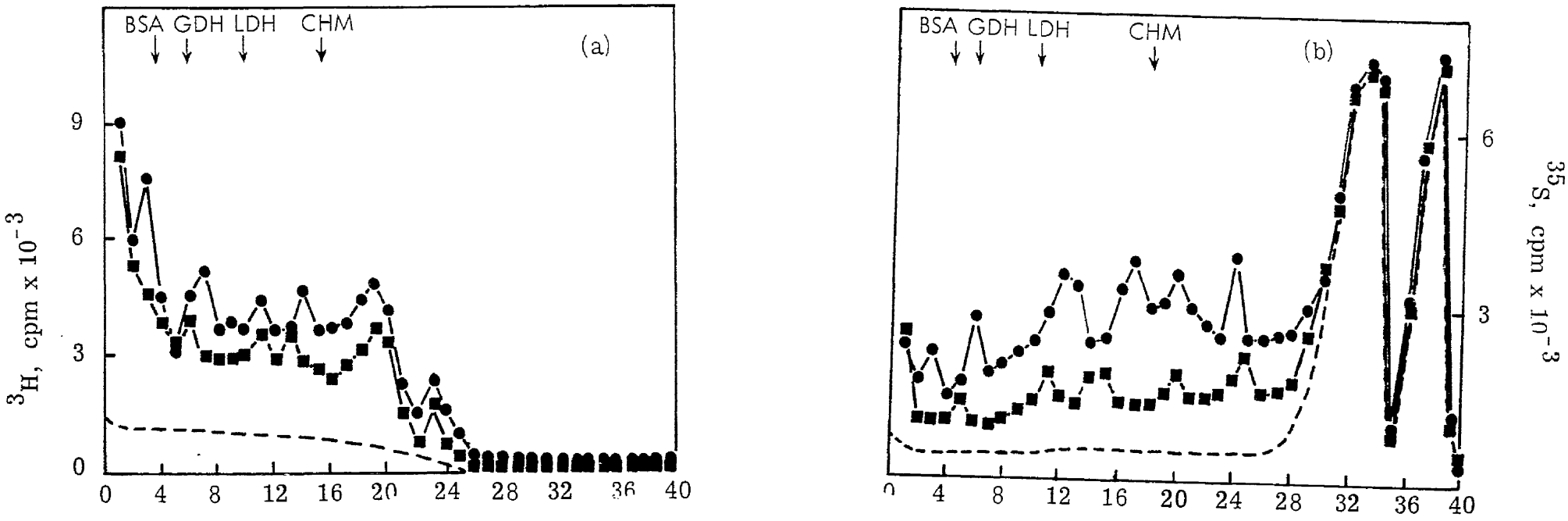
(a) [^{3}H] leucine labelled polypeptide products directed by poly(A)$^+$RNA (—●—) or poly(A)$^-$u$^+$RNA (—■—) or produced endogeneously (- - -)

(b) [^{35}S] methionine labelled polypeptide products directed by poly(A)$^+$RNA (—●—) or poly(A)$^-$u$^+$RNA (—■—) or produced endogeneously (- - -)

(c) [^{3}H] leucine labelled polypeptide products directed by poly(A)$^+$RNA (—●—) were mixed with [^{14}C] leucine labelled polypeptide products directed by poly(A)$^-$u$^+$RNA (—■—). [^{3}H] leucine labelled endogeneously produced polypeptide products (—○—) were also mixed with [^{14}C] leucine endogeneously produced polypeptide products (—□—)

(d) [^{35}S] methionine labelled polypeptide products directed by poly(A)$^+$RNA (—●—) were mixed with [^{3}H] methionine polypeptide products directed by poly(A)$^-$u$^+$RNA (—■—). [^{35}S] methionine labelled endogeneously produced polypeptide products (—○—) were also mixed [^{3}H] methionine endogeneously produced polypeptides (—□—).

Figure 9


BSA GDH LDH CHM
(a)
^{3}H, cpm x 10^{-3}
0
3
6
9
0 4 8 12 16 20 24 28 32 36 40
BSA GDH LDH CHM
(b)
^{35}S, cpm x 10^{-3}
3
6
0 4 8 12 16 20 24 28 32 36 40
Number of slices (2 mm)

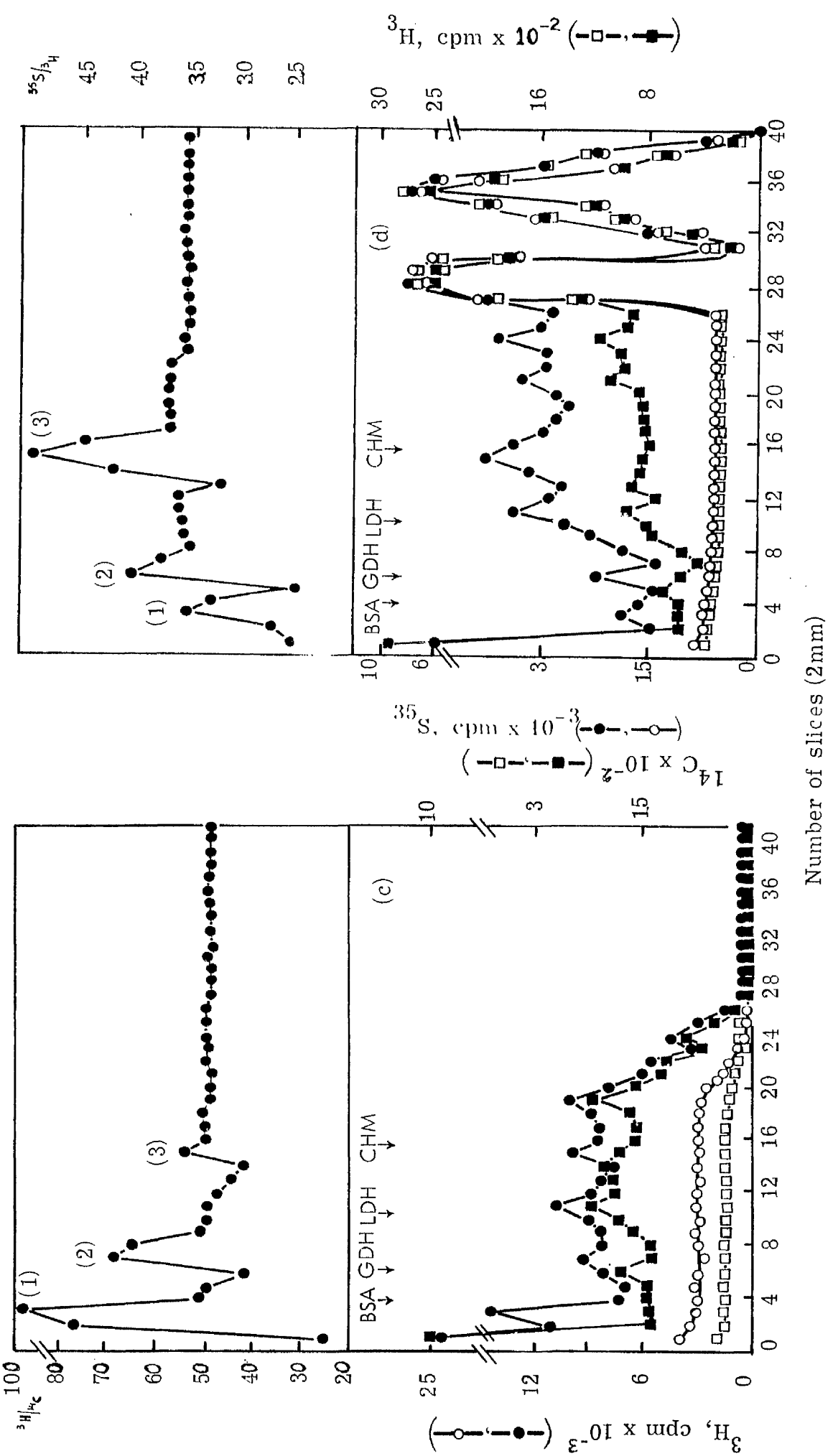

^{3}H, cpm x 10^{-2}
^{35}S, cpm x 10^{-3}
^{14}C x 10^{-2}
^{3}H, cpm x 10^{-3}
(1)
(2)
(3)
BSA GDH LDH CHM
(c)
(d)
Number of slices (2mm)

Figure 9

molecular weight polypeptides when [^{35}S]-methionine is employed as a label, could be a result of premature termination and release of a small peptide(s) (Davies et al 1975), or possibly the result of proteolytic cleavage near the N-terminus of a polypeptide, or both. Interestingly the profiles obtained (Fig 9) show apparent differences in the range of proteins encoded by poly(A)$^+$ or poly(A)$^-$u$^+$mRNAs. Many of the products encoded by Friend cell polysomal poly(A)$^+$ or poly(A)$^-$u$^+$mRNAS, however, exhibit broadly similar electrophoretic mobilities. To investigate this further, [^{3}H]-leucine was used to label the protein synthesis directed by poly(A)$^+$RNA and [^{14}C]-leucine used to label the protein synthesis directed by poly(A)$^-$u$^+$RNAs. An analogous experiment was carried out using [^{35}S]-methionine to label poly(A)$^+$ RNA directed polypeptides and [^{3}H]-methionine to label poly(A)$^-$u$^+$RNA directed polypeptides. After mixing the differently labelled polypeptide products, and running the mixtures on SDS-polyacrylamide gels, the profiles shown in Fig 9 c, d were obtained. These results support the previous observations regarding the size distribution of polypeptides directed by the two types of RNA (see also Discussion Section 1). The similarity is somewhat surprising since only 10% sequence homology was detected between poly(A)$^+$ and poly(A)$^-$u$^+$RNAs (Fig 5). It may be that some of the apparently similar products are simply the result of a fortuitous comigration of non-identical proteins on one dimensional gels. To clarify this further, a more detailed analysis on the translation

products is being attempted, utilising the greater resolution of a two dimensional gel electrophoresis techniques (O'Farrel 1975). The results of a pilot experiment using poly(A)$^+$mRNA is presented in Fig 10.

1.10 DNA sequence representation in polysomal poly(A)$^+$ and poly(A)$^-$u$^+$RNAs

Since a number of studies suggest that some of the poly(A)$^+$ RNA is transcribed from both middle repetitive and non-repetitive regions of DNA (see Introduction 6.2), an investigation was carried out to examine the DNA sequence classes represented in both polysomal poly(A)$^+$ and poly(A)$^-$u$^+$RNAs.

Trace amounts of [^{3}H] uridine labelled poly(A)$^+$ and poly(A)$^-$u$^+$RNAs were hybridised with a vast excess of fragmented total Friend cell DNA, and the hybrids assayed as ribonuclease-resistant, acid-precipitable material (see Methods Section 13). From the kinetics of hybridisation shown in Fig 11, at a Cot (Co = initial concentration of DNA in moles of nucleotide per litre, t = time in seconds) value of 40 about 6% of [^{3}H] poly(A)$^+$ RNA was hybridised, whilst this value is only 1-2% for the [^{3}H] poly(A)$^-$u$^+$RNA. The remainder of both RNA classes hybridised to unique DNA sequences, displaying a Cot $\frac{1}{2}$ (Co = initial concentration of DNA in moles of nucleotide per litre, $t_{\frac{1}{2}}$ = time of half reaction in seconds) of about 550. This suggests that the vast majority of both RNA classes are transcribed from unique DNA sequences. At the DNA:RNA ratio of 2000:1 used, about 40% of both [^{3}H]

Figure 10

Fluorograph of two-dimensional gel electrophoresis of radioactive polypeptides synthesised in a wheat germ cell-free system in the presence of Friend cell polysomal poly(A)$^+$RNA

[^{35}S] methionine labelled polypeptides in a wheat germ cell-free system in the presence of polysomal poly(A)$^+$RNA were analysed on two-dimensional electrophoresis as described in Methods Section 15.2. The gel was then fluorographed, dried and exposed to X-ray film for 6 weeks, as described in Methods Section 17.

Figure 10

Figure 11

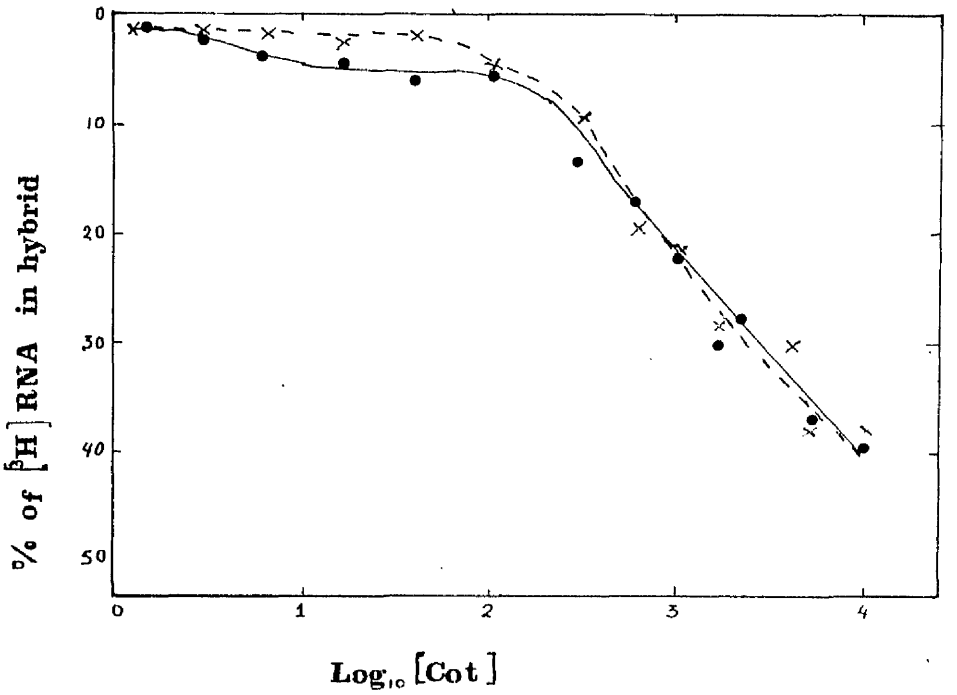
Hybridisation of [^{3}H] uridine labelled polysomal poly(A)$^+$ and poly(A)$^-$u$^+$RNAs from Friend cells with excess Friend cell DNA

Reaction mixtures containing either [^{3}H] poly(A)$^+$RNA (55,000 cpm/μg) or [^{3}H] poly(A)$^-$u$^+$RNA (14,000 cpm/μg) were hybridised with Friend cell DNA sheared to a mean size of 0.3 Kb (see Methods Section 12) at a DNA to RNA ratio of 2,000:1. The reaction mixtures were incubated in 0.12M phosphate buffer (see Methods Section 13) at 65°C for appropriate times, and hybrids analysed using pancreatic ribonuclease as described in Methods Section 13. The results were corrected using a control hybridisation of E. coli DNA and the appropriate RNA; 3-4% of the [^{3}H] RNA became ribonuclease-resistant when incubated with E. coli DNA.

×——× [^{3}H] poly(A)$^+$RNA

•——• [^{3}H] poly(A)$^-$u$^+$RNA

Figure 11



poly(A)$^+$ and poly(A)$^-$u$^+$RNAs are hybridised at a Cot = 10,000. Further hybridisation was undetectable with the conditions used, indeed a similar cessation of hybridisation has been detected by various other workers (Klein et al 1974, Spradling et al 1974, Nemer et al 1975). This has been interpreted as being due to the difficulty in obtaining true DNA-excess conditions for cell RNA sequences (Klein et al 1974), as well as a kinetic effect related to differences in rates of DNA/DNA duplex formation, and DNA/RNA hybridisation under these conditions (Davidson et al 1975).

1.11 Nature and location of "U-rich" regions in polysomal poly(A)$^-$u$^+$RNA species

Since it is known, that polysomal poly(A)$^+$RNA species contain a poly(A) segment (100-200 nucleotide long) attached at the 3' end of the RNA chain (see Introduction Section 6.1), it was of interest to examine the nature and the location of the "U-rich" region(s) in the polysomal poly(A)$^-$u$^+$RNA species.

To examine whether regions involved in binding to poly(A)-sepharose resemble the discrete size oligo(U) sequences (20-30 nucleotides long) found in HeLa polysomal poly(A)$^-$ RNA (Korwek et al 1976), or simply regions rich in uridylate residues as was suggested by preliminary data from BHK/21 poly(A)-binding RNA (Burdon et al 1976), the following experiment was devised. [^{32}P] polysomal poly(A)$^-$u$^+$RNA was digested with T1 ribonuclease (see

Methods Section 6.2), and the resultant oligonucleotides passed through a poly(A)-sepharose column. When the eluates were analysed for trichloroacetic acid-precipitable radioactivity, very little binding of the oligonucleotides was detected (0.9%-1.1% of total [^{32}P]radioactivity in poly(A)$^-$u$^+$RNAs). Attempts to determine the size of these fragments were unsuccessful since upon precipitation with ethanol only a small proportion of radioactivity was recovered. The inability of ethanol to precipitate these oligonucleotides is probably due to their small size (Cleaver and Boyer 1972). Since T1 ribonuclease cleaves specifically after guanosine residues, a small size of digestion fragments would imply that no long sequences of pure oligo(U) occur. Very possibly they are interupted by guanylate residues. Thus, the sequences responsible for binding to poly(A)-sepharose would appear to be merely "U-rich" regions, and not "pure" oligo(U) tracts. In addition the RNA material which failed to bind to either poly(U)- or poly(A)-sepharose was digested with T1 ribonuclease, and the digests chromatographed on a poly(A)-sepharose. As expected very little (0.01%) radioactivity appeared to bind.

The localisation of the "U-rich" region(s) in polysomal poly(A)$^-$u$^+$RNAs was investigated as follows: [^{3}H] uridine labelled poly(A)$^-$u$^+$RNAs was partially digested using M. lysodiekticus polynucleotide phosphorylase. This enzyme, in the presence of inorganic orthophosphate, is known to degrade polynucleotides from the 3'-OH terminus,

releasing nucleotide 5'-diphosphates (Singer et al 1960, Grunberg-Manago 1963). Fig 12 shows the results of an experiment where poly(A)$^+$ or poly(A)$^-$u$^+$RNAs were incubated with polynucleotide phosphorylase, in a phosphorolysis buffer (see Methods Section 7), for various times. The digested RNAs were phenol-chloroform extracted and examined for rebinding on poly(A)- or poly(U)-sepharose. The results shown in Fig 12 suggest that after 45 min of incubation in the presence of enzyme, about 80-90% of the poly(A)$^+$RNA failed to bind to poly(U) sepharose, while 65-75% of the poly(A)$^-$u$^+$RNA failed to bind to poly(A) sepharose. On the contrary, in the absence of enzyme, nearly 100% was found to be retained on the columns.

To monitor the extent of phosphorolysis, the digestion products obtained on incubating [^{3}H] uridine labelled poly(A)$^-$u$^+$RNA for 45 min with polynucleotide phosphorylase were chromatographed on DEAE-paper (see Methods Section 7). The results obtained are summarised in Fig 13. As can be seen about 9-12% of [^{3}H] uridine derived radioactivity was found to migrate to a position expected of nucleoside 5'- diphosphates. There was no indication of any small oligonucleotide products which would chromatograph between the origin and the position occupied by the nucleotide diphosphate products. The non-migration from the origin of most of the digestion products reflects the limitations of the chromatography system used, since oligonucleotides ranging in size from several nucleotides to several hundred nucleotides co-migrate. Thus in 45 min the extent of phosphorolysis is only 9-12%.

Figure 12

Effect of phosphorolysis of Friend cell polysomal poly(A)$^+$ and poly(A)$^-$u$^+$RNAs, by polynucleotide phosphorylase, on their binding to poly(A)- and poly(U)-sepharose

[^{3}H] uridine labelled poly(A)$^+$ or poly(A)$^-$u$^+$RNAs were incubated in the presence or absence of polynucleotide phosphorylase (see Methods Section 7). Aliquots were removed at various times, RNA was phenol-chloroform extracted and then chromatographed on an appropriate poly(U) or poly(A)-sepharose column. Bound RNAs were eluted with 90% (v/v) formamide in 10mM EDTA, 10mM tris-HCl, pH7.4, 0.2% (v/v) SDS. Bound and unbound RNA fractions were assayed for 5% (w/v) trichloroacetic acid-precipitable radioactivity. The RNA retained by each column is expressed as a percentage of the applied sample (corrected for the observed binding efficiency (see Table 5) of the poly(U)- or poly(A)-sepharose for poly(A)$^+$ or poly(A)$^-$u$^+$RNAs respectively). The values quoted were obtained from 2 separate experiments.

Binding of [^{3}H] poly(A)$^+$RNA to poly(U)-sepharose after incubation in the presence (⊢—●—⊣) or absence (⊢—•—⊣) of polynucleotide phosphorylase

Binding of [^{3}H] poly(A)$^-$u$^+$RNA to poly(A)-sepharose after incubation in the presence (⊢—■—⊣) or absence (⊢—◆—⊣) of polynucleotide phosphorylase

Bars indicate the extremes of the experiments.

Figure 12

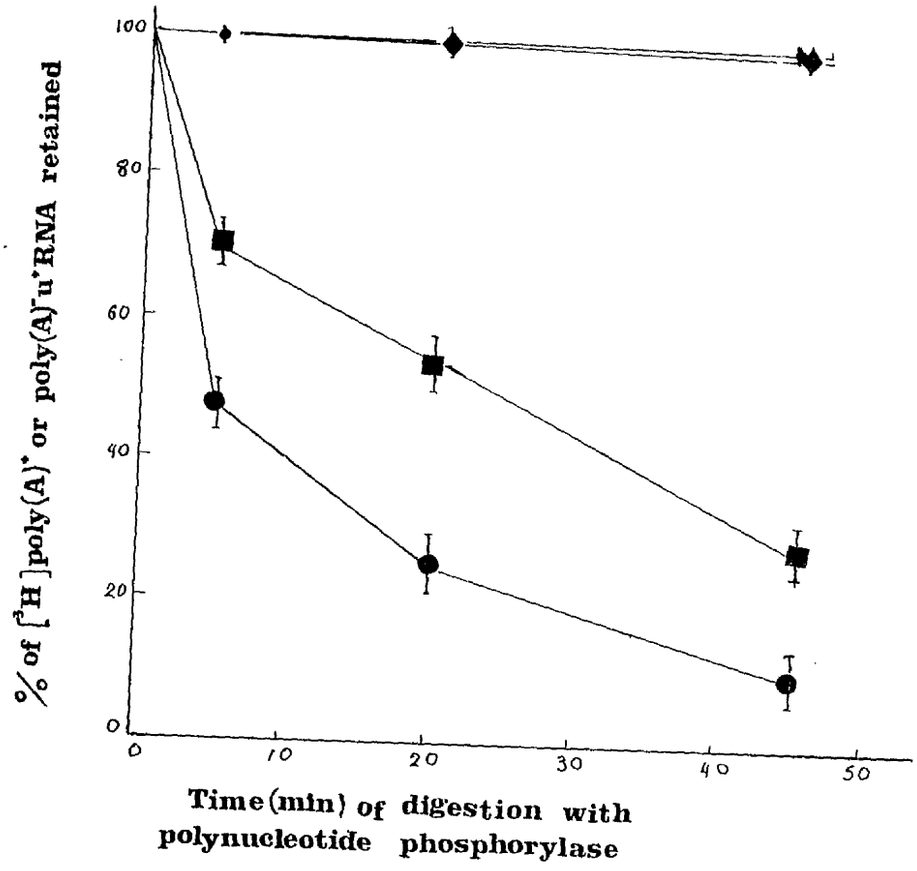
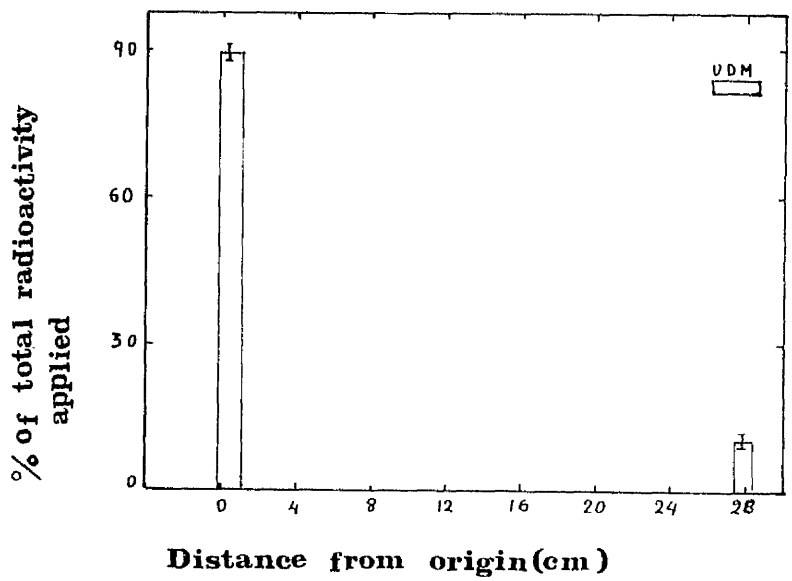

% of [³H]poly(A)* or poly(A)*u*RNA retained
100
80
60
40
20
0
0
10
20
30
40
50
Time (min) of digestion with polynucleotide phosphorylase

Figure 13

The phosphorolysis of [^{3}H] uridine labelled Friend cell polysomal poly(A)$^-$u$^+$RNA species by polynucleotide phosphorylase



[^{3}H] uridine labelled poly(A)$^-$u$^+$RNA was digested with polynucleotide phosphorylase for 45 min, and the resulting mixture was analysed by DEAE-paper chromatography (see Methods Section 7). Following chromatography, radioactivity was determined as described in Methods Section 18.4.

Results are expressed as a percentage of radioactivity applied to the DEAE-paper. The quoted values were obtained from two separate experiments. Bars (⟷) indicate the extremes of the measurements.

This resulted in the loss of 65-75% of the ability of poly(A)$^-$u$^+$RNA to bind to poly(A)-sepharose. Since polynucleotide phosphorylase is a 3' specific exonuclease, these results suggest that the majority of the poly(A)$^-$u$^+$ RNAs contain a "U-rich" region towards to the 3'-end of RNA chain, while about 25-35% of the poly(A)$^+$u$^+$RNAs may contain internal "U-rich" region(s) or both.

1.12 Elution characteristics of polysomal poly(A)$^+$ and poly(A)$^-$u$^+$RNAs

Having shown that polysomal poly(A)$^-$u$^+$RNA appears to lack long pure uridylate tracts, the binding to poly(A)-sepharose seems likely to be effected through "U-rich" region(s) occurring in the poly(A)$^-$u$^+$RNAs. These could form partially base paired structures in which the unmatched bases are looped out from the hybrid double helix (Fresco et al 1960).

To examine further the properties of these "U-rich" region(s), the respective binding properties of both poly(A)$^+$ and poly(A)$^-$u$^+$RNAs to poly(U)- and poly(A)-sepharose columns were investigated with regard to stability in different formamide concentrations in low salt. The rationale behind such a technique lies in the well documented denaturing ability of formamide with regard to double-stranded nucleic acids (Tso et al 1962, 1963, Pinter et al 1974, Sippel et al 1977). Indeed, under high salt conditions, it has been shown that a 1% increase in formamide concentration is equivalent to a 0.72^{o}C

decrease in the melting temperature of a DNA.RNA hybrid (McConaughy et al 1969). The melting temperature of a double-stranded nucleic acid is related directly to the hybrid length (Stenier and Beer 1961, Walker 1969), although base composition (Rilley et al 1966) and degree of mismatching (Walker 1969), also affect this parameter. Consequently, an examination of the elution profiles of the bound RNA classes with increasing formamide concentrations, should yield valuable information concerning both length and "purity" of "A-rich" or "U-rich" regions. Such an approach has been used to distinguish total cytoplasmic poly(A)$^+$RNAs, with respect to the lengths of their poly(A) sequences (Salditt-Georgieff et al 1976). However, this method suffers a limitation, in the sense that short tracts of high "purity" may be indistinguishable from longer tracts of lesser "purity".

Total polysomal RNA was chromatographed on poly(U)- and poly(A)-sepharose as described in Table 4. After the non-bound RNA has been washed through the columns, the bound RNAs were eluted with stepwise increasing formamide concentrations in low salt. As can be seen (Fig 14), the poly(A)$^+$RNA elutes as a single component at high formamide concentration ($\sim$ 40%), whilst the poly(A)$^-$u$^+$RNA elutes as a single component at much lower concentration ($\sim$ 7.5%). The elution profiles of poly(A)$^-$u$^+$RNA would suggest that most of the "U-rich" region(s) are approximately the same size although, clearly, the degree of mismatching in the bound hybrids requires characterisation

Figure 14

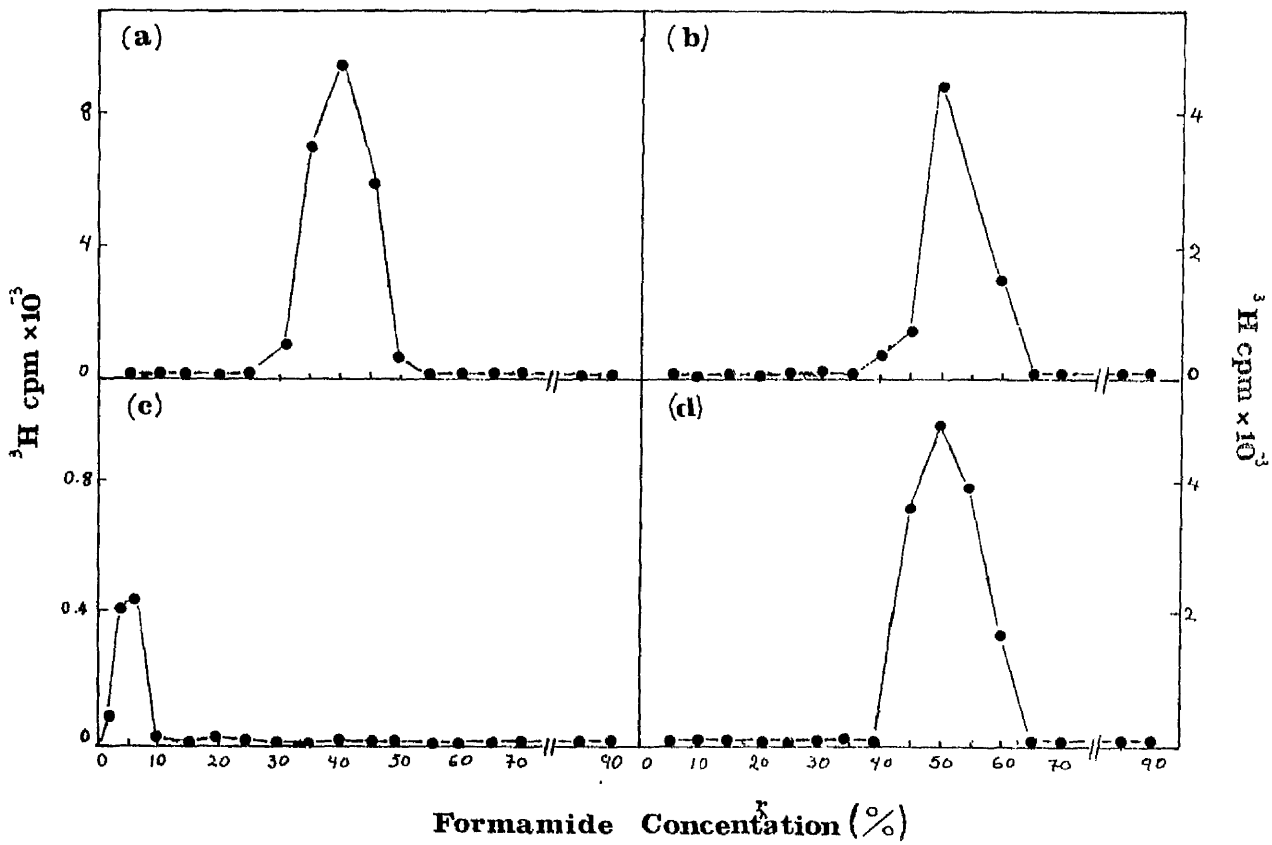
Effect of formamide concentration on elution of ribohomo polymers and Friend cell polysomal RNAs bound to poly(U)- or poly(A)-sepharose columns

[^{3}H] uridine labelled polysomal RNA was chromatographed on poly(U)- or poly(A)-sepharose columns (see Table 4). In addition, commercial [^{3}H] poly(U) or [^{3}H] poly(A) were chromatographed on poly(A)- or poly(U)-sepharose columns respectively, as above. The bound RNAs were then eluted stepwise with increasing concentration of formamide in 1mM EDTA, 10mM tris-HCl,(pH7.4), 0.2% (w/v) SDS at a temperature of 22°-23°C. Radioactivity was determined as described in Methods Section 18.1.

Elution profiles shown are:

(a) [^{3}H] poly(A)$^+$ polysomal RNA
(b) [^{3}H] poly(U)
(c) [^{3}H] poly(A)$^-$u$^+$ polysomal RNA
(d) [^{3}H] poly(A)

Figure 14


(a)
(b)
(c)
(d)
^{3}H cpm ×10^{-3}
^{3}H cpm ×10^{-3}
0
4
8
0.4
0.8
2
4
0 10 20 30 40 50 60 70 90
Formamide Concentration (%)

before any definite conclusion can be reached.

In order to show that the lengths of hybrids were actually limited by the lengths of the "U-rich" region(s), and not by the lengths of the poly(A) attached to the sepharose matrix, an experiment was performed in which commercial [^{3}H] poly(U) was bound to poly(A)-sepharose. This was found to require 50% formamide (Fig 14) for elution, and so it can be concluded the length of poly(A) attached to sepharose was not a limiting factor in hybridisation of poly(A)$^-$u$^+$RNA the column. A corresponding control was performed using [^{3}H] poly(A) and poly(U)-sepharose. Again 50% formamide required (Fig 14) establishing that, in this case, the poly(U) size was not limiting the hybrid length.

1.13 Nucleotide composition of polysomal poly(A)$^+$ and poly(A)$^-$u$^+$RNAs

The nucleotide analysis of the poly(A) binding RNA from BHK/21 cells revealed an unusual base composition with a high uridylate residues content ($\sim$ 30%) (Burdon *et al* 1976). In addition, non-polyadenylated mRNAs from sea urchin exhibit a high level of uridylate residues (Nemer *et al* 1974).

To ascertain if this is also the case for Friend cell polysomal poly(A)$^-$u$^+$RNA, [^{32}P] polysomal poly(A)$^+$ and poly(A)$^-$u$^+$RNAs were hydrolysed by alkali, and their nucleotide composition was determined. The results are shown in Table 11, where it can be seen that the value obtained for the percentage of the uridylate residues in

Table 11

Nucleotide composition of Friend cell polysomal poly(A)$^+$ or poly(A)$^-$u$^+$RNAs

RNA examined	Nucleotide composition (moles per 100 moles) CMP	AMP	GMP	UMP
Poly(A)$^+$ RNA	22.3	32.3	22.4	23.0
Poly(A)$^-$u$^+$RNA	23.3	18.4	27.2	31.1

Polysomal poly(A)$^+$ and poly(A)$^-$u$^+$RNAs prepared from Friend cells labelled with ortho [^{32}P] phosphate (see Methods Section 1.2 (c)) for 2 hrs, were isolated as described in Table 4. These RNAs were ethanol precipitated then alkali digested, and the resultant mononucleotides resolved by high voltage paper electrophoresis (see Methods Section 8). Radioactivity in each mononucleotide was determined as described in Methods (see Section 18.4 (i)). The values quoted represent the means of three determinations.

poly(A)$^-$u$^+$RNA is 31.1%. The observation can also be made that the guanosine content of the poly(A)$^-$u$^+$RNA seems quite high, whilst the cytidine content is fairly low.

1.14 Methylation of polysomal poly(A)$^+$ and poly(A)$^-$u$^+$RNA species

Since methylated nucleotides have been found in both poly(A)$^+$ and poly(A)$^-$ mRNAs from sea urchin embryos (Surrey and Nemer 1976), it was of interest to examine whether the Friend cell polysomal poly(A)$^-$u$^+$RNA contains methyl groups or not.

To this end Friend cells were labelled with [^{3}H] methyl methionine (see Methods Section 1.2), and polysomal poly(A)$^+$ and poly(A)$^-$u$^+$RNAs were prepared as described in Table 4. The level of [^{3}H] methyl label incorporation into each of these RNAs is given in Table 12. These results suggest that the poly(A)$^-$u$^+$RNA does contain methyl groups, although the amount of radioactivity with poly(A)$^-$u$^+$RNA was insufficient for further characterisation of the methylated nucleotides.

2. Friend cell nuclear RNA with high affinity for poly(A) or poly(U) sepharose

Having demonstrated the existence of distinct poly(A)$^+$ and poly(A)$^-$u$^+$ mRNAs in Friend cell cytoplasm, the investigation was extended to examine the nuclear RNA for any similar molecules, or molecules which might serve as their precursors.

Previously, Burdon *et al* (1976) had provided preliminary

Table 12

Incorporation of [^{3}H]-methyl methionine into Friend cell polysomal poly(A)$^+$ or poly(A)$^-$u$^+$RNAs

Duration of labelling (hrs)	cpm in Poly(A)$^+$RNA	Poly(A)$^-$u$^+$RNA
1	1950	105
4	6160	208

Friend cells were labelled with 20μci/ml of [^{3}H]-methyl methionine (see Methods Section 1.2 (b)). Polysomal poly(A)$^+$ and poly(A)$^-$u$^+$RNAs were prepared as described in Table 4, and further purified by repeating the fractionation using fresh columns. Radioactivity was determined as described in Methods Section 18.1.

data for the existence of poly(A)$^-$ hnRNA species from BHK/21 cells, having a high affinity for poly(A). Dubroff et al (1975), using poly(U) filters reported the existence of two classes of hnRNA from sea urchin, one class containing poly(A), the other lacking poly(A), but containing oligo(A) sequences. Subsequently, Dubroff (1977) reaffirmed these observations and, using poly(A)-sepharose column, also characterised a "U-rich", poly(A)$^-$ hnRNA.

2.1 Fractionation of Friend cell nuclear RNA by virtue of their relative affinities for poly(A) or poly(U) sepharose

The protocol used again involved binding to poly(U)- or poly(A)-sepharose columns, as for the cytoplasmic RNA studies (see Section 1.1). For these studies Friend cells were labelled with [^{3}H] uridine for 1 hr, and nuclear RNA prepared by the phenol-chloroform method (Penman 1969). Isolated nuclear RNA was then characterised with regard to the ability to bind to a poly(U)-sepharose and poly(A)-sepharose as already described in Fig 14. The stepwise elution with increasing formamide concentration was also advantageous in investigating the possible existence of oligo(A) containing nuclear RNA species, as has been described recently in sea urchin hnRNA. Fig 15 shows that two distinct fractions were eluted from a poly(U)-sepharose column, one was characterised by a mid-point of elution at 10% formamide (such an RNA class does not contain long poly(A) tracts, but will have a short "A-rich" region(s), possibly oligo(A), henceforth referred to as

Figure 15

Effect of formamide concentration on elution of Friend cell nuclear RNA bound to poly(U)- or poly(A)-sepharose columns

Nuclear RNA was extracted from Friend cells which had been labelled with 20μci/ml of [^{3}H] uridine for 1 hr following a 30 min preincubation in the presence or absence of actinomycin D (0.04μg/ml). The isolated RNA was chromatographed on poly(U)- and poly(A)-sepharose columns as described in Table 4 and the bound RNAs were eluted stepwise with solutions of increasing formamide concentration in 1mM EDTA, 10mM tris-HCl, pH7.4. Radioactivity was determined as described in Methods Section 18.1.

(a) Elution profiles of [^{3}H] nuclear RNA bound to poly(U)-sepharose, prepared from actinomycin D treated (—▲—) or untreated (—•—) cells.

(b) Elution profiles of [^{3}H] nuclear RNA bound to poly(A)-sepharose, prepared from actinomycin D treated (—▲—) or untreated (—•—) cells

Figure 16

Elution profiles of nuclear poly(A)$^+$, poly(A)$^-$a$^+$ and poly(A)$^-$u$^+$RNA species

[^{3}H] uridine nuclear poly(A)$^+$, poly(A)$^-$a$^+$ and poly(A)$^-$u$^+$ RNAs were prepared by chromatography on appropriate sepharose columns as described in Fig 15. These RNAs were then rechromatographed on appropriate sepharose columns and eluted using stepwise increasing concentrations of formamide. Radioactivity was determined as described in Methods Section 18.1.

— x — Elution of poly(A)$^-$u$^+$RNA bound to poly(A)-sepharose

—•— Elution of poly(A)$^-$a$^+$RNA bound to poly(U)-sepharose

—■— Elution of poly(A)$^+$RNA bound to poly(U)-sepharose

Figure 15

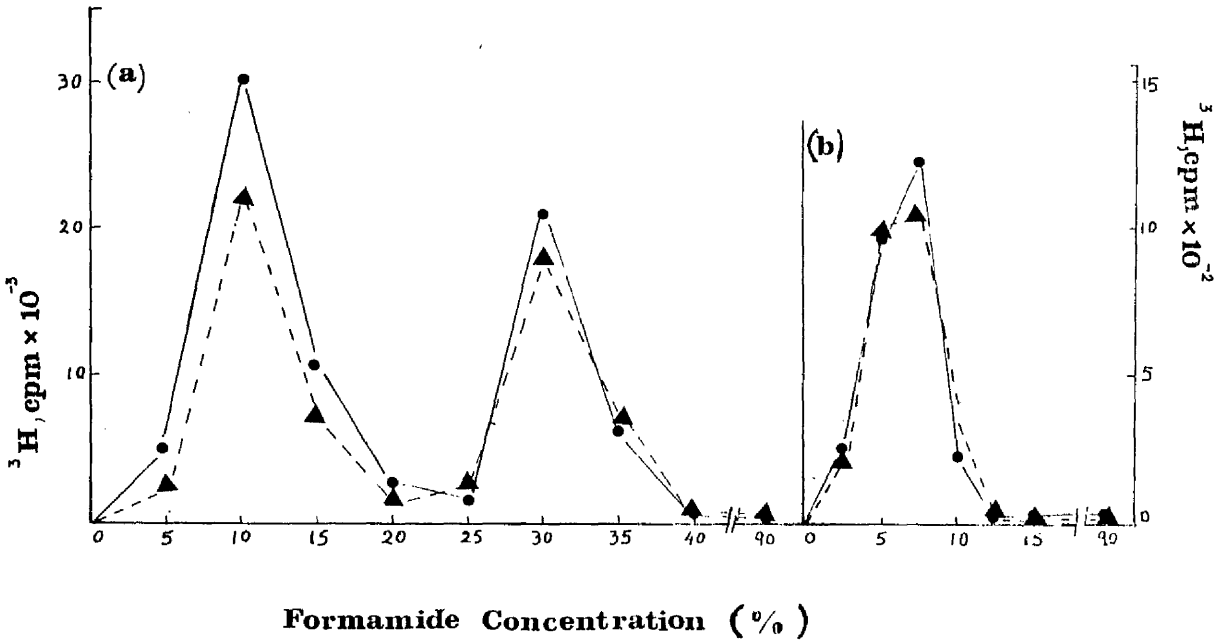
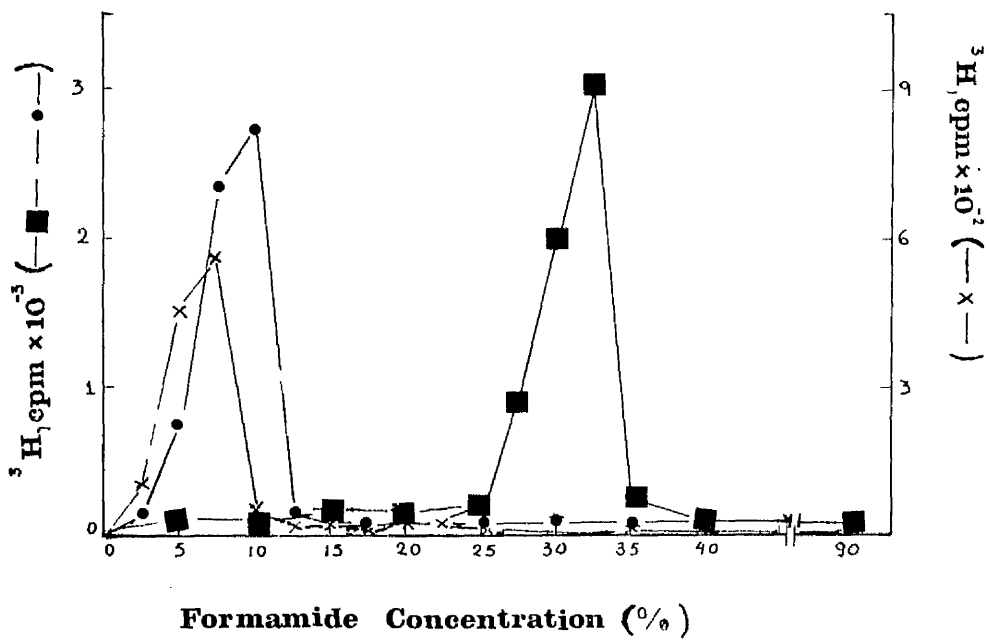


Figure 16



nuclear poly(A)$^-$a$^+$RNA), the other had an elution mid-point at 30% formamide (presumed to be due to long poly(A) tracts, henceforth referred to as nuclear poly(A)$^+$RNA). The RNA which failed to bind a poly(U)-sepharose column, but did bind to a poly(A)-sepharose column, was eluted as a single component at a mid-point at 7.5% formamide (this compares with the behaviour of cytoplasmic poly(A)$^-$u$^+$RNA, and therefore is referred to as nuclear poly(A)$^-$u$^+$RNA). A parallel experiment was performed using Friend cells which had been incubated in medium containing 0.04µg/ml of actinomycin D for 30 min prior to labelling. At this concentration actinomycin D effectively inhibits the synthesis of ribosomal RNA precursors, whilst having only a small effect on the synthesis of hnRNA (Penman et al 1968). The labelled nuclear RNA from these cells bound to poly(U)- or poly(A)-sepharose showed no differences in elution characteristics, compared to cells untreated with actinomycin D, nor was the ratio of radioactivity in poly(A)$^+$:poly(A)$^-$a$^+$:poly(A)$^-$u$^+$ nuclear RNA affected (Fig 15). This suggests that ribosomal RNA, or its precursors, do not belong to either poly(A)$^+$, poly(A)$^-$a$^+$ or poly(A)$^-$u$^+$ nuclear RNAs.

A large proportion of the material eluted from the poly(U)- or poly(A)-sepharose columns was shown to rebind on re-application to a column of poly(U)- or poly(A)-sepharose respectively (Table 13). Furthermore, this rebound RNA showed a similar elution characteristic towards formamide (Fig 16). Also RNA which failed to bind in the first instance, either to poly(A)-sepharose or poly(U)-sepharose

Table 13

Binding of nuclear poly(A)$^+$, poly(A)$^-$a$^+$, poly(A)$^-$u$^+$ and non-bound RNAs to poly(U)- or poly(A)-Sepharose

RNA examined	% of applied [^{3}H] RNA sample bound to Poly(U)-sepharose	Poly(A)-sepharose
Poly(A)$^+$RNA	88 ± 3	≤ 0.01
Poly(A)$^-$a$^+$RNA	83 ± 3	≤ 0.05
Poly(A)$^-$u$^+$RNA	≤ 0.01	78 ± 3
Non-bound RNA	≤ 0.01	≤ 0.01

[^{3}H] uridine labelled nuclear poly(A)$^+$, poly(A)$^-$a$^+$, poly(A)$^-$u$^+$ and non-bound RNAs were prepared as described in Fig 15. Ethanol precipitates of these RNAs were dissolved in binding buffer (see Methods Section 5.1.3) and chromatographed on both poly(U)- or poly(A)-sepharose columns. Bound RNAs were eluted with 90% (v/v) formamide in 10mM EDTA, 10mM tris-HCl ph7.4. Bound and unbound RNA fractions were assayed for 5% (w/v) trichloroacetic acid-insoluble radioactivity. Results are expressed as a percentage of total radioactivity of the applied RNA sample, ±1standard deviation. The quoted values were obtained from 7 separate experiments.

columns (henceforth referred to as non-bound nuclear RNA) again failed to bind on re-application to fresh columns (Table 13). Negligible binding was also observed on re-application of bound RNA to a "complementary column", e.g. material eluting from a poly(A)-sepharose column re-applied to a poly(U)-sepharose column (Table 13). This suggests that the nuclear RNA species form distinct classes as was found for the polysomal poly(A)$^+$ and poly(A)$^-$u$^+$RNA species. An interesting observation on these results is that the relative proportions of the three classes of RNA which become labelled is a function of the time of labelling (Table 14). This phenomenon is examined more closely in Section 3.

2.2 Integrity and size of Friend cell nuclear RNA and its classes

The suggested existence of distinct classes of poly(A)$^+$, poly(A)$^-$a$^+$ and poly(A)$^-$u$^+$ nuclear RNAs could merely be artifactual arising from degradation of molecules having two or more of these features.

To investigate this possibility, three different methods were employed to isolate Friend cell nuclei, and the elution profiles of RNA prepared from the three "types" of nuclei compared. Essentially, using Friend cells labelled with [^{3}H] uridine for 1 hr, nuclei were prepared using either a "citric acid" lysis of the cells (Getz et al 1975), or an NP40 lysis (see Methods Section 3.1). In the latter case, two types of nuclei were

Table 14

Incorporation of [^{3}H] uridine into nuclear poly(A)$^+$, poly(A)$^-$a$^+$ and poly(A)$^-$u$^+$RNAs as a function of time

Duration of labelling (min)	% of applied [^{3}H] nuclear RNA in Poly(A)$^+$RNA	Poly(A)$^-$a$^+$RNA	Poly(A)$^-$u$^+$RNA
15	19 ± 2.5	25 ± 3.0	1.80 ± 0.20
60	13 ± 1.5	16 ± 2.0	0.90 ± 0.10
120	7 ± 1.0	8.5 ± 1.5	0.25 ± 0.07

Friend cells were incubated with [^{3}H] uridine (20μci/ml) and samples withdrawn at various times. Nuclear poly(A)$^+$ poly(A)$^-$a$^+$ and poly(A)$^-$u$^+$RNAs were prepared from the withdrawn samples as described in Fig 15, the amount of [^{3}H] radioactivity in each was determined (Methods Section 18.1), and expressed as a percentage of the applied total [^{3}H] nuclear RNA sample, ± 1 standard deviation. The quoted values were obtained from 7 separate experiments.

prepared, those which had received a "sucrose-citric acid wash", and those which had not (see Methods Section 3.1). The prepared RNAs were then fractionated on poly(U)- and poly(A)-sepharose columns (results shown in Fig 17). If significant degradation of the nuclear RNAs occurs during the employed manipulations, then the elution profiles of the nuclear RNAs prepared from the three differently treated nuclei would be expected to be different. The results shown in Fig 17 indicate no such difference. In addition, a further control experiment was performed in order to assess whether the nuclear RNA is degraded during the isolation of nuclei from the cells. Total RNA from intact cells was prepared directly, in the presence of ribonuclease inhibitors such as heparin and dextran sulphate. Using these inhibitors Kwan et al (1977) were able to prepare high molecular weight cellular RNA from mouse spleen cells. The cellular RNA prepared in this way was chromatographed on poly(U)- and poly(A)-sepharose columns, and again the elution profiles remain the same (Fig 17), suggesting that no degradation is occurring. Finally, the elution profiles, from poly(U)- and poly(A)-sepharose columns, of high molecular weight HeLa and BHK/21 cell hnRNA (mean size of about 35s and 30s respectively), prepared using a 15 min label with [^{3}H] uridine following a 30 min pre-treatment with 0.04μg/ml actinomycin D (gift of Mr. Tom Strachan of this Department), were determined (Fig 18). These RNAs were then mixed with unlabelled Friend cell nuclei, and total RNA extracted. If degradation is occurring during extraction, it would be expected

Figure 17

Comparison of elution profiles of total cellular RNA with nuclear RNA isolated from nuclei which have been prepared by three different methods

Friend cells were labelled with 15μci/ml of [^{3}H] uridine for 1 hr and nuclear RNA isolated from nuclei which have been prepared by three different methods (see Text). Total cellular RNA was isolated from cells which had been labelled with 15μci/ml of [^{3}H] uridine for 10 min.

Cellular and nuclear RNAs were separately chromatographed on poly(U)- and poly(A)-sepharose (see Table 4) and bound RNAs were eluted as described in Fig 15. Radioactivity was determined as described in Methods Section 18.1. The values quoted were obtained from two separate experiments. Bars (⊢——⊣) indicate the extremes of the measurements.

The nuclear RNA prepared from nuclei by different methods was fractionated on poly(U)-sepharose(A) and poly(A)-sepharose(B). The elution profiles shown represent

(i) [^{3}H] nuclear RNA from cells lysed with citric acid and sucrose-citric acid washed nuclei

(ii) [^{3}H] nuclear RNA from cells lysed with NP-40 and sucrose-citric acid washed nuclei

(iii) [^{3}H] nuclear RNA from cells lysed with NP-40 and unwashed nuclei

Elution profiles of cellular RNAs bound on poly(U)-sepharose(A) and poly(A)-sepharose(B).

Figure 17

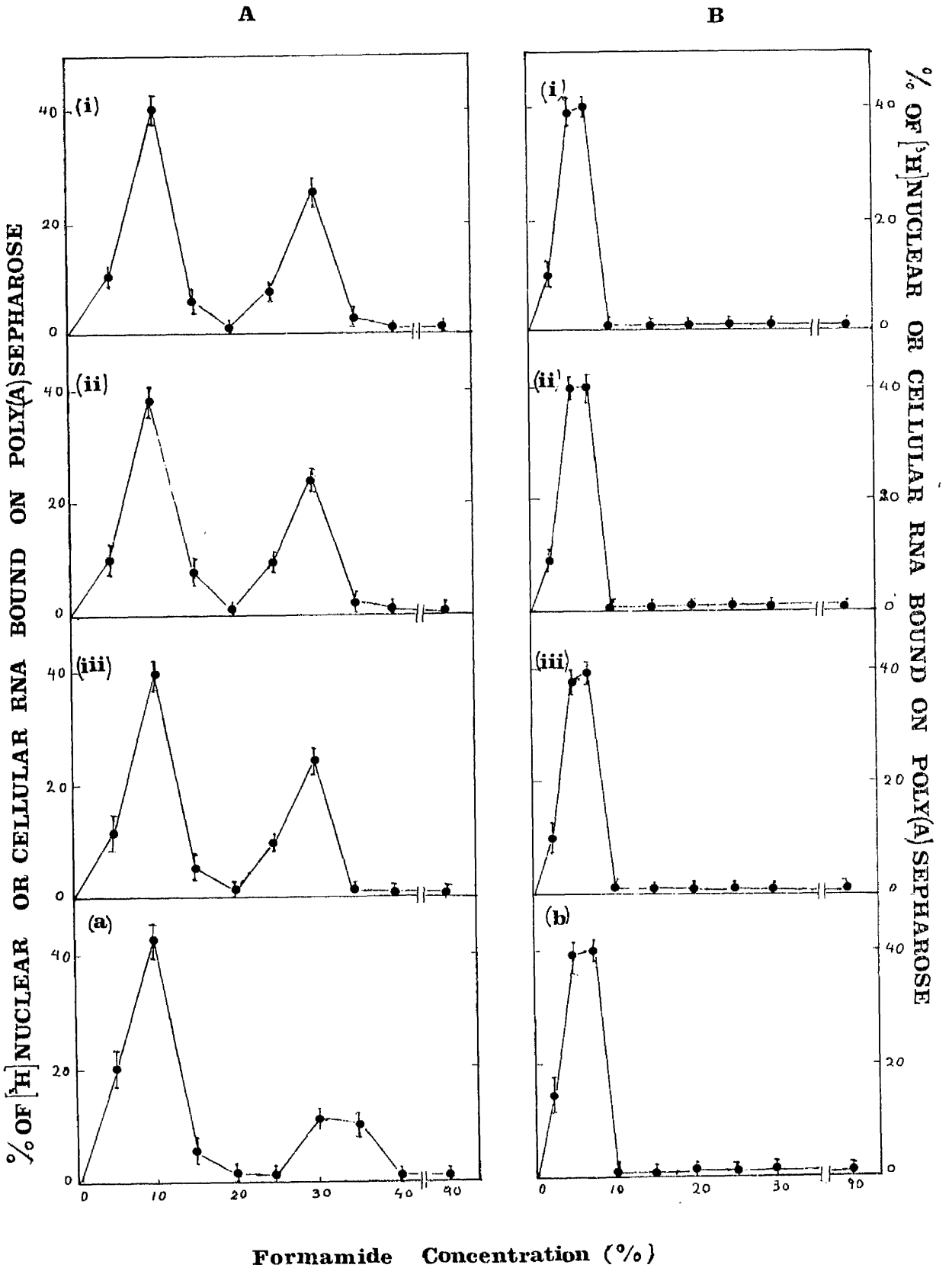

A
B
(i)
(ii)
(iii)
(a)
(b)
% OF [³H]NUCLEAR OR CELLULAR RNA BOUND ON POLY(A)SEPHAROSE
% OF [³H]NUCLEAR OR CELLULAR RNA BOUND ON POLY(A) SEPHAROSE
Formamide Concentration (%)

Figure 18

The effect of mixing [^{3}H] uridine labelled HeLa cell or BHK/21 cell hnRNAs with Friend cell nuclei on their subsequent affinity for poly(A)- and poly(A)-sepharose columns

Samples (0.1-0.3μg) of [^{3}H] uridine labelled hnRNA from HeLa and BHK/21 cells were mixed with about 10^7 unlabelled Friend cell nuclei and total nuclear RNAs extracted. Ethanol precipitates of this RNA mixture were dissolved in binding buffer (0.4M NaCl, 10mM EDTA, 10mM tris-HCl, pH7.4, 0.2% (w/v) SDS) and chromatographed on poly(U)- and poly(A)-sepharose columns as described in Fig 15. For comparison, samples of the original hnRNAs were also chromatographed in the same way. The bound RNAs were eluted with formamide of stepwise increasing concentration (see Fig 15). Radioactivity was determined as described in Methods Section 18.1. The values quoted were obtained from two separate experiments. Bars (⊢——⊣) indicate the extremes of the measurements.

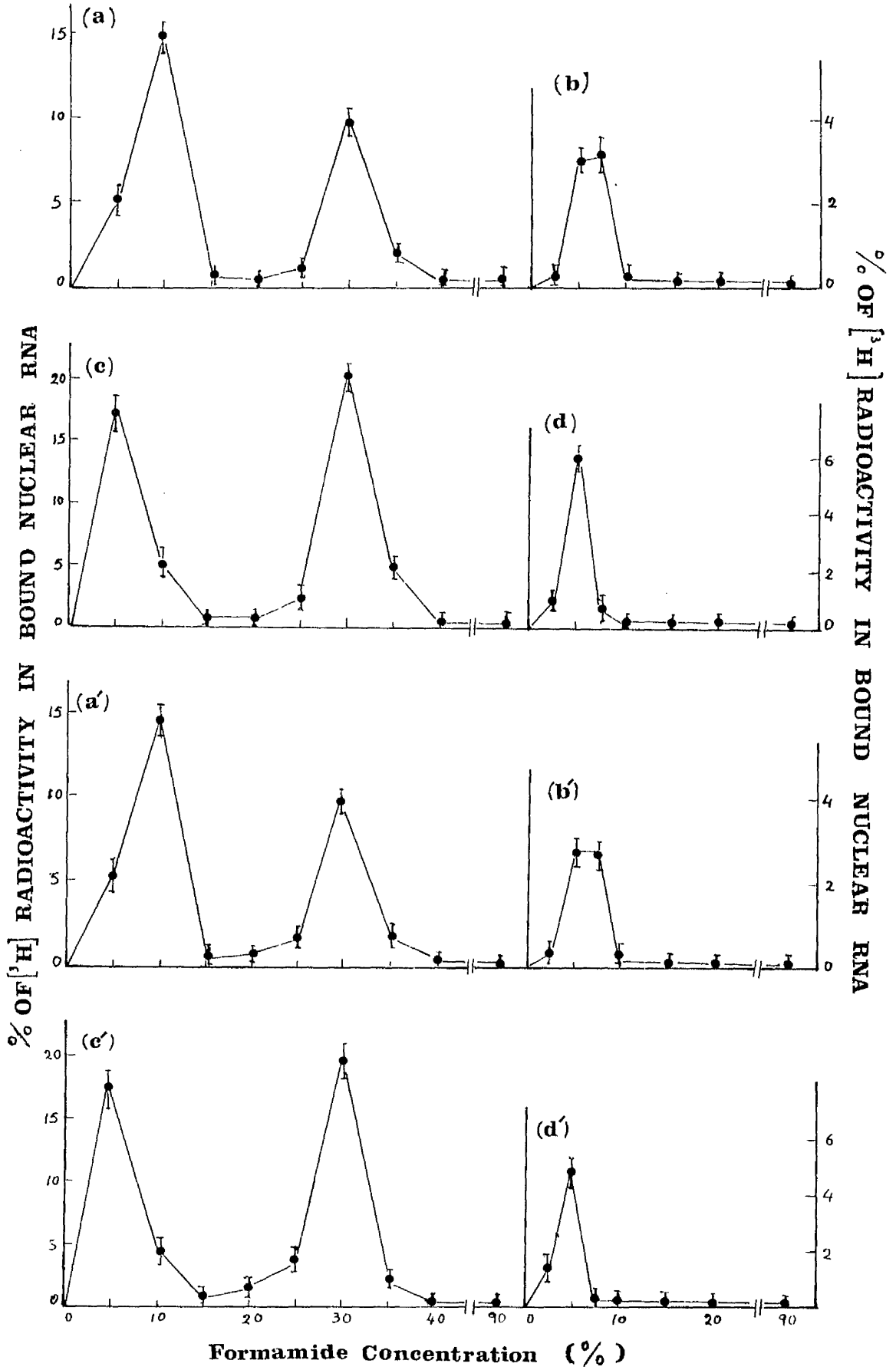
Elution profiles of original BHK cell hnRNA bound to
(a) Poly(U)-sepharose
(b) Poly(A)-sepharose

Elution profiles of original HeLa cell hnRNA bound to
(c) Poly(U)-sepharose
(d) Poly(A)-sepharose

Elution profiles of re-extracted BHK cell hnRNA bound to
(a') Poly(U)-sepharose
(b') Poly(A)-sepharose

Elution profiles of re-extracted HeLa cell hnRNA bound to
(c') Poly(U)-sepharose
(d') Poly(A)-sepharose

Figure 18


(a)
(b)
(c)
(d)
(a')
(b')
(c')
(d')
% OF [3H] RADIOACTIVITY IN BOUND NUCLEAR RNA
% OF [3H] RADIOACTIVITY IN BOUND NUCLEAR RNA
Formamide Concentration (%)

that some change in the elution characteristics of these added RNAs would occur. As Fig 18 shows no change seems to occur, again suggesting that degradation during extraction is minimal. It is interesting to note that all three classes of RNA, i.e. poly(A)$^+$, poly(A)$^-$a$^+$ and poly(A)$^-$u$^+$ nuclear RNA, also occur in all three of the RNAs used above, although relative proportions differ (Fig 17, Fig 18).

The next step in the characterisation of these RNA classes was an investigation of their sedimentation properties. In sedimentation studies, a major source of uncertainty in analysing hnRNA species has been the tendency of these molecules to form aggregates, and to remain aggregated even after being nicked, if rigorous denaturing conditions are not continuously employed (Macnaughton et al 1974, McKnight and Schimke 1974, Fedoroff et al 1977). Careful studies, however, have shown that formamide, used under the proper conditions, is an effective denaturant of RNA (Tso et al 1963, Pinter et al 1974, Sippel et al 1977, Stair et al 1977).

Using appropriate formamide gradients, as described in Methods (see Section 5.4), the sedimentation profile of total hnRNA shown in Fig 19 was obtained. As can be seen, total hnRNA displays a peak of sedimentation of about 28s under these conditions. When poly(A)$^+$ or poly(A)$^-$a$^+$ nuclear RNA species were analysed under the same conditions, it was found that although both of them display a similar peak of about 18s, the majority of the

Figure 19

Sedimentation behaviour of nuclear RNA and its classes in sucrose-formamide gradients

Friend cells were labelled with 15µci/ml of [^{3}H] uridine for 15 min and extracted nuclear RNA was chromatographed on poly(U)- and poly(A)-sepharose columns as described in Fig 15. Ethanol precipitates of total nuclear RNA as well as poly(A)$^+$, poly(A)$^-$a$^+$ and poly(A)$^-$u$^+$ nuclear RNAs were analysed on sucrose-formamide gradients, ran in parallel, as described in the legend to Fig 4.

Friend cell polysomal RNA was centrifuged in parallel gradients to provide sedimentation markers (see arrows).

Fractions were collected and their content of 5% (w/v) trichloroacetic acid-precipitable radioactivity was determined (see Methods Section 18.1).

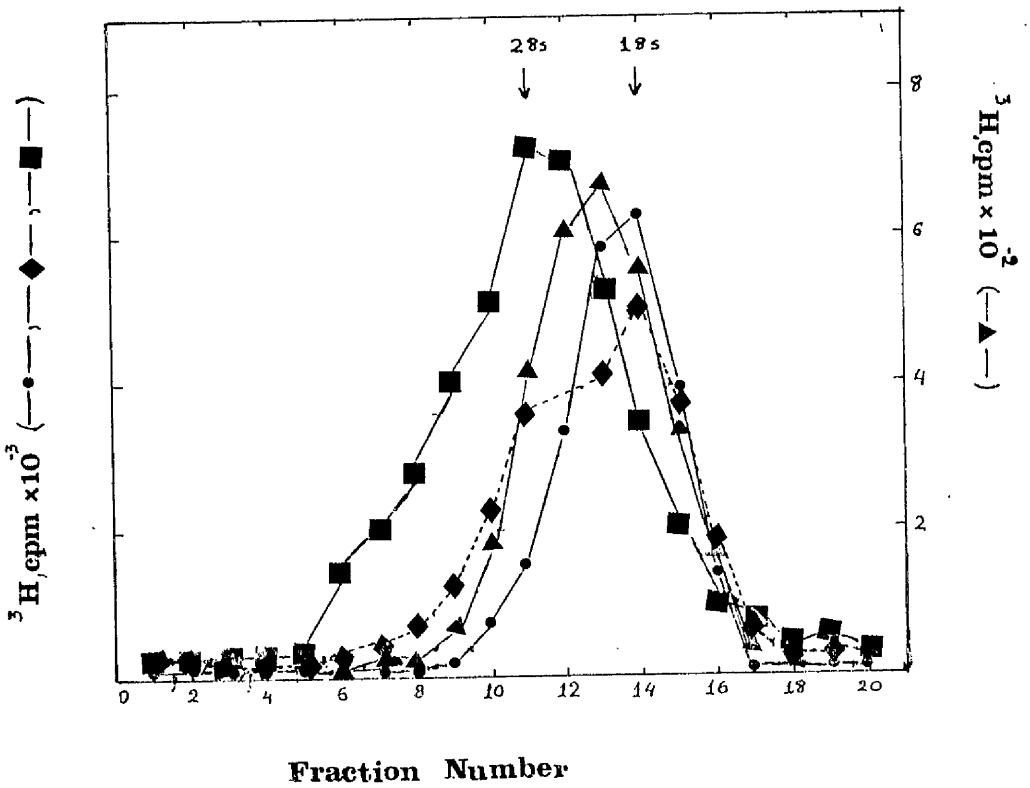
■——■ [^{3}H] total nuclear RNA

▲——▲ [^{3}H] poly(A)$^-$u$^+$RNA

•——• [^{3}H] poly(A)$^+$RNA

◆——◆ [^{3}H] poly(A)$^-$a$^+$RNA

Figure 19



poly(A)$^-$a$^+$ nuclear RNA molecules appear to be shifted into the higher molecular weight regions of the gradient. On the other hand the poly(A)$^-$u$^+$RNA displays a peak of about 20s (Fig 19). Similar values for the size of nuclear poly(A)$^+$ RNA from Friend cells is reported by Getz et al (1975), who found that when Friend cell nuclear poly(A)$^+$ RNA was analysed in a sucrose gradient, the majority of RNA molecules sedimented faster than 60s, while in a formamide-sucrose gradient this value was reduced to a mean value of 18s.

2.3 Characterisation of adenylic and uridylic regions from Friend cell nuclear RNA fractions

In view of the results obtained by Molloy et al (1974), who showed that a substantial proportion of poly(A)$^+$ and poly(A)$^-$a$^+$RNAs from HeLa cells failed to bind to poly(U)-sepharose, it was decided to examine the non-binding nuclear RNA fraction for any poly(A) or oligo(A) tracts. At the same time, the other RNA fractions (i.e. poly(A)$^+$, poly(A)$^-$a$^+$ and poly(A)$^-$u$^+$ nuclear RNAs) were examined for these characteristics. Nuclear RNA was prepared from Friend cells, which has been labelled for 2 hrs with ortho [^{32}P] phosphate. This nuclear RNA was fractionated into poly(A)$^+$, poly(A)$^-$a$^+$, poly(A)$^-$u$^+$RNA and non-bound nuclear RNA fractions, as described in Fig 15. Each of the RNA fractions was digested with a mixture of pancreatic and T1 ribonuclease, in a high salt buffer (see Methods Section 6.1), under these conditions adenylate homopolymeric sequences are resistant to digestion

(Beers 1960). The digests were then deproteinised, and RNA fragments precipitated with ethanol in the presence of 50μg/ml of yeast t-RNA. The ethanol precipitates were dissolved in binding buffer (0.4M NaCl, 10mM EDTA, 10mM tris-HCl, pH7.4, 0.2% (w/v) SDS), and chromatographed on a poly(U)-sepharose column. Thus any adenylate homopolymeric region(s) in each nuclear RNA fraction could be specifically detected. The results in Table 15 show the percentage of [^{32}P]radioactivity found as adenylate homopolymers in each of the examined RNA fractions. These results indicate that relatively little adenylate homopolymers occur in the non-bound nuclear RNA fraction. The investigation was carried further, however, and the size of adenylate homopolymers was examined on SDS-polyacrylamide gels. Fig 20 shows the results obtained from this procedure applied to the adenylate homopolymeric tracts, produced from poly(A)$^+$, poly(A)$^-$a$^+$ and non-bound nuclear RNAs. As can be seen, the sizes of adenylate homopolymeric tracts fall into two discrete classes - a poly(A) class of higher molecular weight than the t-RNA marker, and an oligo(A) class of much smaller molecular weight than the t-RNA marker, which co-migrate with the bromophenol blue tracker dye (see Fig 20). The oligo(A) tracts are distributed as follows: About 8-10% of the total [^{32}P]radioactivity in oligo(A) is found in the non-bound nuclear RNA fraction, about 2-4% was found in the poly(A)$^+$ nuclear RNA fraction, the rest occurring in the poly(A)$^-$a$^+$ nuclear RNA fraction (Fig 20 D). These results, taken together, suggest that

Table 15

Content of adenylate homopolymeric regions in nuclear RNA fractions from Friend cells

RNA examined	Percentage of digested sample bound recovered from poly(U)-sepharose
Poly(A)$^+$ RNA	5.2 ± 0.8
Poly(A)$^-$a$^+$RNA	1.3 ± 0.2
Poly(A)$^-$u$^+$RNA	Not detected
Non-bound RNA	0.08 ± 0.01

[^{32}P] labelled nuclear RNA was fractionated into poly(A)$^+$, poly(A)$^-$a$^+$, poly(A)$^-$u$^+$ and non-bound RNAs as described in Fig 15. Each RNA fraction was digested with a combination of pancreatic and T1 ribonuclease under the appropriate conditions (see Methods Section 6.2). RNA fragments were re-extracted from this mixture as described in Methods (Section 6.1), and chromatographed on a poly(U)-sepharose column. Bound RNA was eluted with 90% (v/v) formamide in 10mM EDTA, 10mM tris-HCl, pH7.4, 0.2% (w/v) SDS. Radioactivity was determined as described in Methods (Section 18.1). Results are expressed as a percentage of digested sample bound and recovered from poly(U)-sepharose ± 1 standard deviation. The values quoted were obtained from 3 separate experiments.

Figure 20

Electrophoretic behaviour of adenylate homopolymeric regions isolated from Friend cell nuclear RNA fractions

Adenylate homopolymeric regions prepared as described in Table 15, were electrophoresed on 12% SDS-polyacrylamide gels (see Methods Section 5.3).

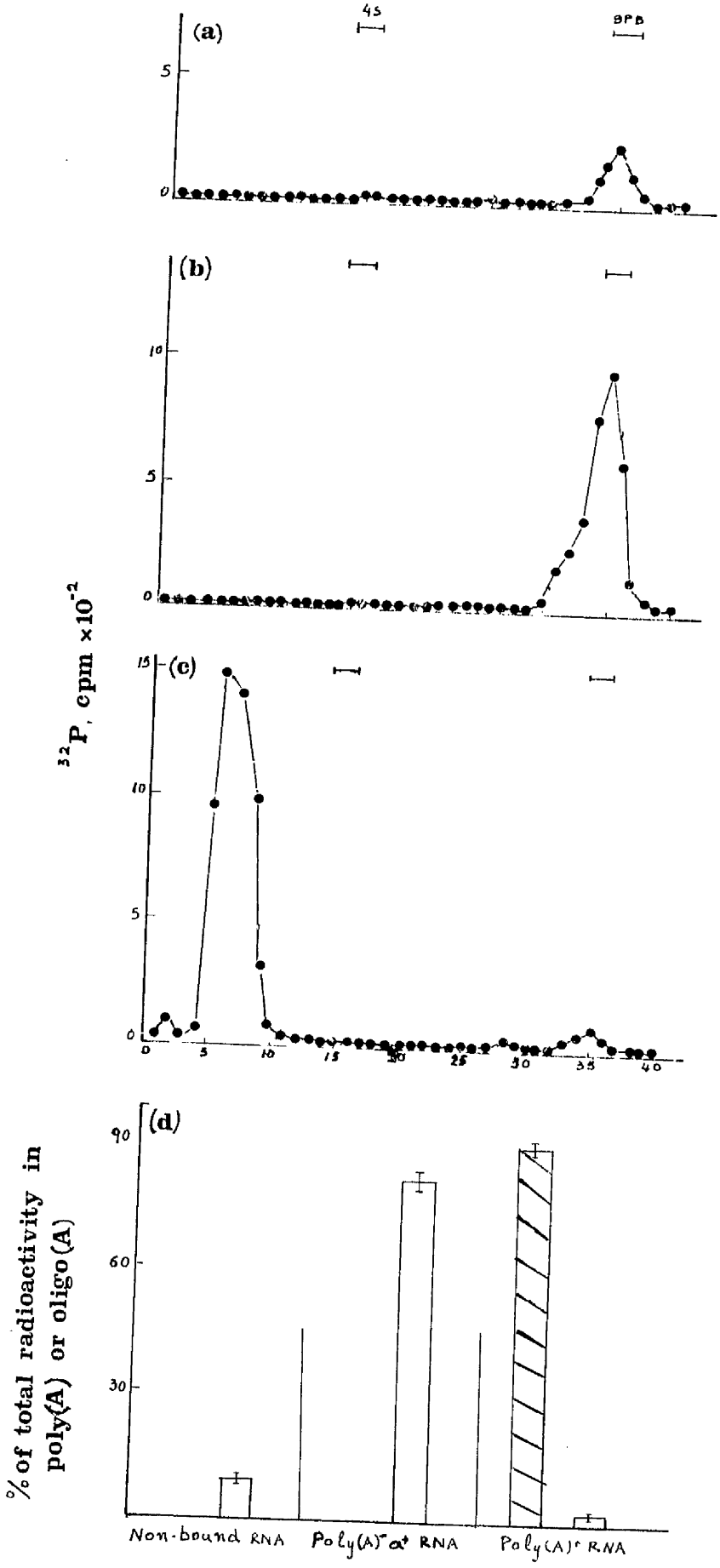
Adenylic homopolymer from:

(a) Non-bound RNA fraction
(b) Poly(A)$^-$a$^+$RNA fraction
(c) Poly(A)$^+$RNA fraction

4S, yeast t-RNA and BPB (bromophenol blue tracking dye) were co-electrophoresed to provide markers for comparison of electrophoretic mobility

(d) Poly(A) (shaded histogram) and oligo(A) (open histogram) distributions were obtained by summing the ^{32}P cpm migrating as poly(A) or oligo(A) on gels and expressing the amount present in each nuclear fraction as a percentage of the total poly(A) or oligo(A) recovered. Radioactivity was determined as described in Methods Section 18.3.

Figure 20


(a)
(b)
(c)
(d)
4S
BPB
^{32}P, cpm ×10^{-2}
% of total radioactivity in poly(A) or oligo(A)
Non-bound RNA
Poly(A)$^-$ RNA
Poly(A)$^+$ RNA

poly(U)-sepharose adequately retains Friend cell nuclear RNA molecules having large or small adenylic homopolymeric regions. A similar efficiency of poly(U)-sepharose for binding Friend cell nuclear poly(A)$^+$RNA species has also been reported by Humphries et al (1976). In addition, it is evident from these data, that nuclear RNA, containing large poly(A), can be efficiently separated from those containing only oligo(A). The low levels of oligo(A) found in the nuclear poly(A)$^+$RNA fractions suggests that possibly a small proportion of the nuclear poly(A)$^+$RNA molecules may also contain oligo(A) regions. Similar results have been reported by Nakazato et al (1974), who found that oligo(A) sequences are present in very few, if any, HeLa cell nuclear poly(A)$^+$RNA molecules.

Complementary studies were also undertaken to ascertain whether nuclear poly(A)$^-$u$^+$RNA contains either small stretches of oligo(U), or large stretches of "U-rich" sequences. For this purpose [^{32}P] labelled nuclear poly(A)$^-$u$^+$ RNA was digested with T1 ribonuclease, and the resultant oligonucleotides passed over a poly(A)-sepharose column. Following digestion, between 0.8-1.3% of the total [^{32}P] radioactivity in poly(A)$^-$u$^+$RNA is bound to poly(A)-sepharose. Recovery of this bound RNA, using ethanol precipitation, was unfortunately very low, as in the case of cytoplasmic "U-rich" regions of poly(A)$^-$u$^+$RNAs. These results suggest that the "U-rich" region(s) of Friend cell nuclear poly(A)$^-$u$^+$RNA might comprise small oligo(U) stretches, interrupted by guanine residues. Similar results were reported in a preliminary study in BHK/21 cell "U-rich" region(s) of nuclear poly(A) binding RNA (Burdon

et al 1976). This is in contrast to HeLa nuclear poly(A)$^-$ RNA, where a discrete size class of oligo(U) regions (20-30 nucleotides long) has been reported (Korwek et al 1976). Discrete oligo(U) sequences were undetectable in the non-bound Friend cell nuclear RNA fraction.

2.4 Double-stranded regions in Friend cell nuclear RNA and its classes

To examine further the structural features of these nuclear RNA classes, [^{3}H] labelled RNA from each fraction was digested with a mixture of pancreatic and T1 ribonuclease, at high ionic strength (see Methods Section 6.1) for 60 min, and acid insoluble radioactivity was determined. Table 16 shows the percentage of RNAase-resistant material in each nuclear RNA fractions e.g. poly(A)$^+$, poly(A)$^-$a$^+$, poly(A)$^-$u$^+$, and non-bound nuclear RNAs. These RNA structures were found to be double-stranded RNA regions, as judged by their ability to bind to hydroxyapatite columns at low phosphate concentrations (see Methods Section 5.2). The results obtained are in general agreement with previous observations, where it was found that 3-5% of [^{3}H] uridine labelled hnRNA in Ehrlich ascites and HeLa cells is in the form of double-stranded RNA (d-s RNA) (Jelinek et al 1972, Ryscov et al 1972). The nature of the d-s RNA regions was further examined by following their elution from hydroxyapatite column, using a stepwise increase in temperature. As shown in Fig 21 the d-s RNA regions derived from [^{3}H] nuclear poly(A)$^+$, poly(A)$^-$a$^+$ and non-bound RNA fractions have distinctive melting profiles with high

Table 16

Double-stranded RNA regions in nuclear RNA

[^{3}H] nuclear RNA tested	% of digested sample bound and recovered from hydroxyapatite
Total nuclear RNA	3.5 ± 0.3
Poly(A)$^+$ RNA	4.7 ± 0.4
Poly(A)$^-$a$^+$RNA	2.3 ± 0.2
Poly(A)$^-$u$^+$RNA	1.2 ± 0.2
Non-bound RNA	5.6 ± 0.5

Total nuclear RNA was prepared from Friend cells which had been labelled for 1 hr with 15μci/ml of [^{3}H] uridine, and fractionated into poly(A)$^+$, poly(A)$^-$a$^+$, poly(A)$^-$u$^+$ and non-bound RNAs as described in Fig 15. Aliquots of each RNA fraction were digested with a mixture of pancreatic and T1 ribonuclease (see Methods Section 6.1) and the double-stranded RNA content was assayed by hydroxyapatite chromatography. Radioactivity was determined as described in Methods Section 18.1). Results are expressed as a percentage of digested sample bound and recovered from the hydroxyapatite column, ± 1 standard deviation. The quoted values were obtained from 5 separate experiments.

Figure 21

Effect of temperature on the binding of double-stranded RNA derived from Friend cell [^{3}H] nuclear RNA fractions to columns of hydroxapatite

Friend cells were labelled with 20μci/ml of [^{3}H] uridine for 60 min and nuclear poly(A)$^+$, poly(A)$^-$a$^+$, poly(A)$^-$u$^+$ and non-bound RNA fractions were isolated (see Fig 15). Double-stranded RNAs were prepared from each of these RNA fractions (see Methods Section 6.1), and loaded onto hydroxyapatite columns in 50mM phosphate buffer (see Methods Section 5.2).

Stepwise elution was carried out by raising the column temperature in increments of 10°C. At each temperature step the columns were washed with 5 column volumes of 50mM phosphate buffer.

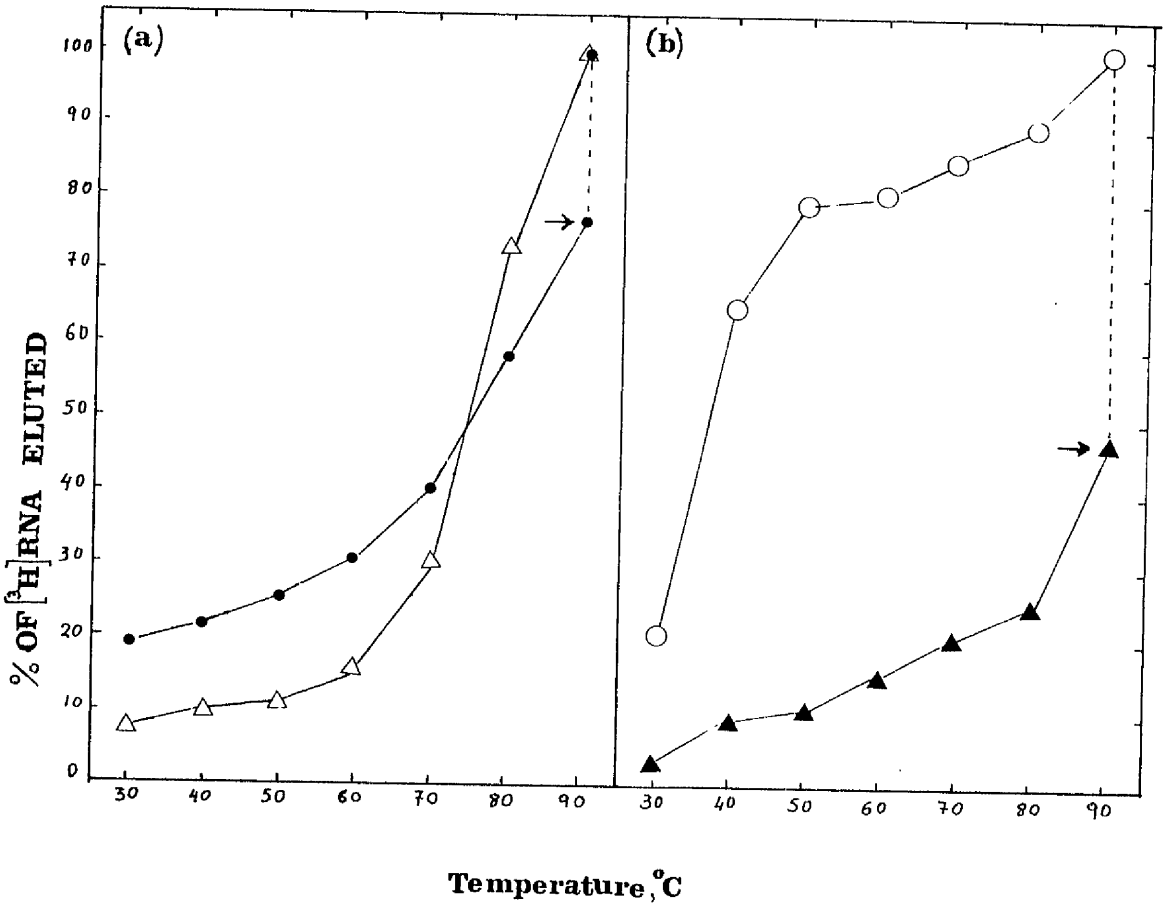
The results have been drawn to indicate the total material eluted up to and including the given temperature, this being expressed as a percentage of the original applied sample.

(a) Temperature elution of double-stranded derived from [^{3}H] nuclear poly(A)$^-$a$^+$ (△——△) or non-bound RNA (•——•) fractions bound to columns of hydroxyapatite

(b) Temperature elution of double-stranded derived from [^{3}H] nuclear poly(A)$^-$u$^+$RNA (O —— O) or poly(A)$^+$RNA (▲——▲) fractions bound to columns of hydroxyapatite

Elution with 500mM phosphate buffer (- - -) is indicated by the arrow.

Figure 21



melting temperatures, while the d-s RNA from the [^{3}H] nuclear poly(A)$^-$u$^+$RNA had a very much lower melting temperature (35^o-40^{o}C). Again these results argue for the existence of three distinct RNA classes with different types of d-s RNA regions.

3. Stability of nuclear and cytoplasmic poly(A)$^+$ and poly(A)$^-$u$^+$RNAs

The previous demonstration that polysomal poly(A)$^-$u$^+$RNA could function as messenger, coupled with the detection of a discrete nuclear poly(A)$^-$u$^+$RNA, at once raised questions concerning the metabolism and processing of this RNA class. Further, since at least some of these messengers are transcribed from genes distinct from those coding for poly(A)$^+$ mRNA, it was decided to compare the transcription and processing of poly(A)$^+$ and poly(A)$^-$u$^+$RNA species. To this end a pulse-chase experimental approach was employed. Pulse-chase measurements appears to be one of the most effective techniques for the direct determination of the stability of RNA molecules following synthesis, as well as allowing the determination of any products that may result from its intracellular processing. Such an approach does not suffer from the uncertainties that are introduced when drugs like actinomycin D are used to block transcription (Penman et al 1968, Levis and Penman 1977, Chernovskaya and Lerman 1977), and is also more sensitive than direct labelling experiments, using an equilibrium labelling approach (Brandhorst and McConkey 1974, Perry et al 1974). In this respect, two

basic "pulse-chase" approaches were examined, those of Warner et al (1966) and Scholtissek (1971). The former relies upon a pulse label of [^{3}H] uridine being "chased" by a vast excess of unlabelled uridine and cytidine. The latter procedure involves a chase by uridine, cytidine and glucosamine, following treatment with glucosamine.

As can be seen in Fig 22, the "glucosamine-uridine" treatment quickly prevents a further incorporation of [^{3}H] uridine into nuclear RNA, and radioactive RNA continues to appear in the cytoplasm for up to 60 min, (i.e. presumably the time taken for the various pulse labelled nuclear RNA species (pre-rRNA, pre-tRNA, hnRNA, pre-5sRNA) to be processed and transported to the cytoplasm). The technique that did not involve glucosamine treatment appeared less effective; labelling of nuclear RNA continues for 30 min after addition of uridine and cytidine.

To ensure that the "chase" situation established using glucosamine, is not simply due to direct inhibition of RNA synthesis, but is due to dilution of label from the cellular UTP pools, RNA synthesis was monitored using ortho [^{32}P] phosphate label. As shown in Fig 23, the incorporation of ortho [^{32}P] phosphate into nuclear RNA in the "glucosamine-uridine" treated cells, is almost identical with that in "glucosamine-uridine" untreated cells, for at least 4 hrs. These results suggest that the shrinkage of the UTP pools is apparently not so great as to interfere drastically with the rate of nuclear RNA synthesis. Additionally, when the accumulation of [^{32}P]

Figure 22

Comparison of "pulse-chase" techniques using Friend cells

A culture of Friend cells was concentrated (see Methods Section 1.3) and halved. One half was incubated for 60 min with glucosamine at a final concentration of 20mM the other receiving no such treatment. Both cultures were then labelled with 30µci/ml of [^{3}H] uridine for 15 min. In the glucosamine-treated culture the chase was initiated by the addition of glucosamine, uridine and cytidine (see Methods Section 1.3). In the untreated cells the chase was initiated by the addition of four volumes of fresh medium containing unlabelled cytidine and uridine each 17.5mM. Aliquots of cells were removed at the indicated time and radioactivity in total nuclear (a) and cytoplasmic (b) RNAs was determined (see Methods Section 18.1).

Results are expressed as percentages relative to the value at the start of the chase (indicated by the arrow), which is taken as zero time. For convenience of representation, the results are plotted with the zero time taken at 100%. The quoted values were obtained from 6 separate experiments. Bars indicate the extremes of the measurements.

—●— , [^{3}H] nuclear RNA (a) or cytoplasmic RNA (b) under "chase" conditions of 15mM unlabelled uridine and cytidine, of each

—◇— , [^{3}H] nuclear RNA (a) or cytoplasmic RNA (b) under "glucosamine-uridine" conditions

Figure 22

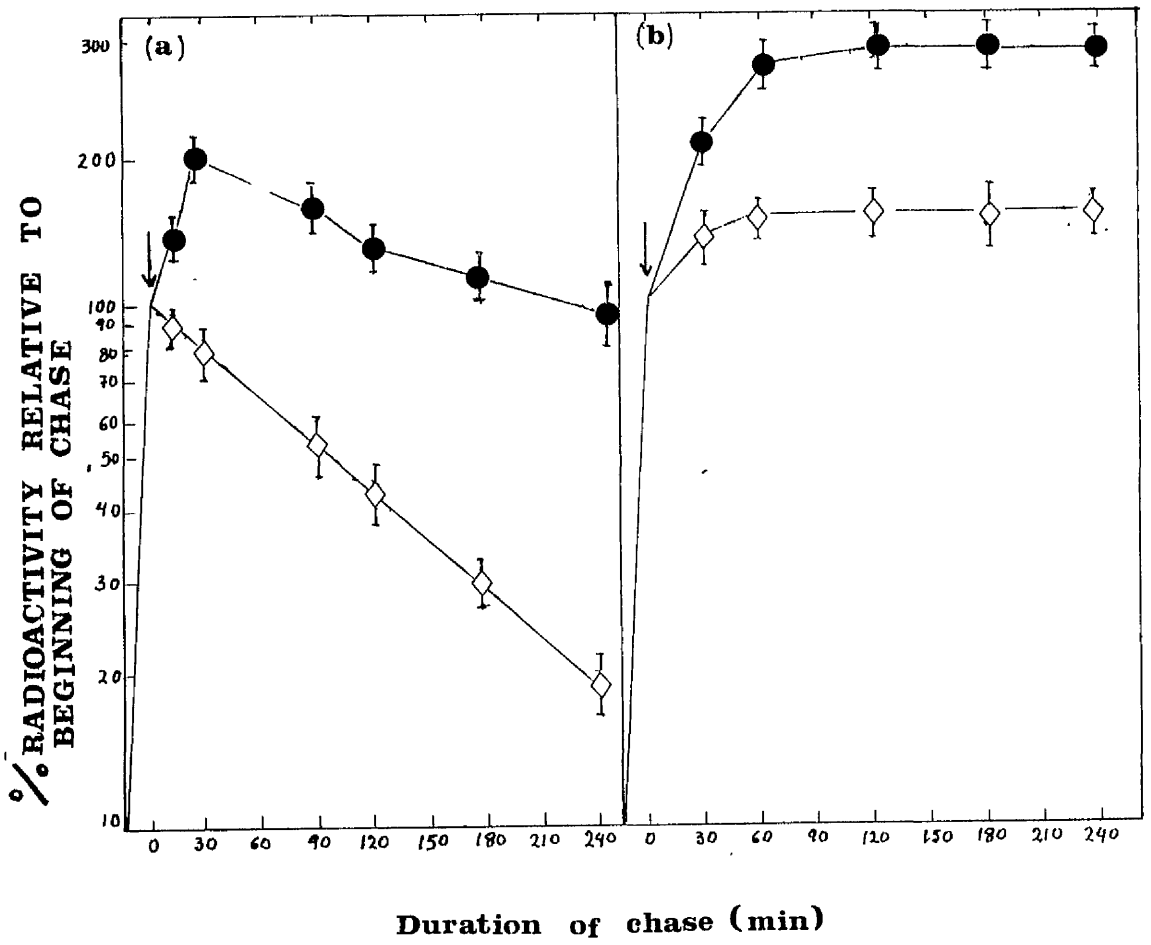

(a)
(b)
% RADIOACTIVITY RELATIVE TO BEGINNING OF CHASE
300
200
100
90
80
70
60
50
40
30
20
10
0
30
60
90
120
150
180
210
240
Duration of chase (min)

Figure 23

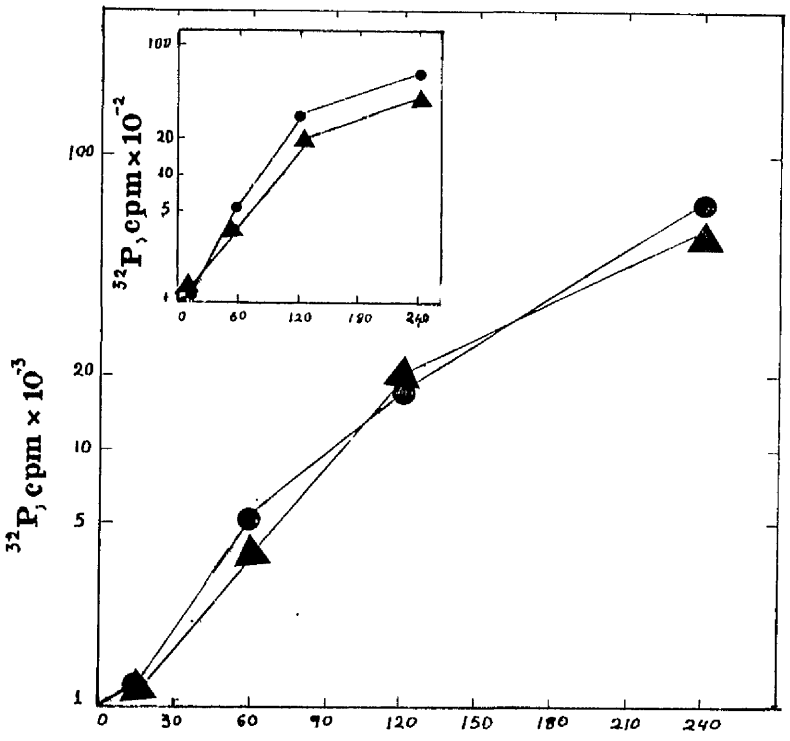
Effect of a "glucosamine-uridine" chase on Friend cell nuclear RNA synthesis as judged by ortho[^{32}P] phosphate incorporation

A culture of Friend cells was concentrated (see Methods Section 1.3) and halved. One half was incubated for 60 min with glucosamine at a final concentration of 20mM the other receiving no such treatment. In the glucosamine-treated culture labelling was initiated by the addition of four volumes of fresh medium containing 6.25mM uridine, 6.25mM cytidine, 26.5mM glucosamine and 5µci/ml of ortho[^{32}P] phosphate. The untreated cells were simply incubated with four volumes fresh medium containing 5µci/ml of ortho[^{32}P] phosphate. Aliquots of cells were removed at the indicated times and nuclei and cytoplasm were prepared as described in Methods Section 3.1. Isolated nuclei were incubated for 20 hrs in 0.3M NaOH at 37°C and acid-soluble radioactivity was determined (see Methods Section 11).

Accumulation of [^{32}P] nuclear RNA in the presence (▲) or absence (●) of the "glucosamine-uridine" treatment

Inset: Cytoplasmic RNA was prepared and the accumulation of [^{32}P] poly(A)$^{+}$RNA in the cytoplasm was followed in the presence (▲) or absence (•) of the "glucosamine-uridine" treatment

Figure 23


^{32}P, cpm × 10^{-3}
100
20
10
5
1
0
30
60
90
120
150
180
210
240
^{32}P, cpm × 10^{-2}
100
20
40
5
1
0
60
120
180
240
Time of ^{32}P label (min)

label into cytoplasmic RNA is followed, little difference is observed between "glucosamine-uridine" treated and untreated cells. This suggests that glucosamine has little effect on the nuclear processing, and transport to the cytoplasm of at least poly(A)$^+$ RNA species. Thus, the "glucosamine-uridine" chase procedure appears to be effective in Friend cells, clone M_2, and can therefore be reasonably used to examine the turnover of unstable RNA species in these cells.

3.1 Stability of nuclear poly(A)$^+$ and poly(A)$^-$u$^+$RNAs

It has been demonstrated (see Table 14) that the relative proportions of nuclear poly(A)$^+$ and poly(A)$^-$u$^+$RNAs is dependent on the time of labelling, suggesting that nuclear poly(A)$^-$u$^+$RNA is metabolised at a different rate, compared to nuclear poly(A)$^+$ RNA. The availability of an effective "pulse-chase" protocol, allows a much more meaningful and precise study of the metabolism of these RNA classes to be undertaken. To this purpose, Friend cells were pre-incubated with glucosamine for 60 min, then labelled for 15, 60 or 120 min with [^{3}H] uridine. The "chase" was initiated by the addition of glucosamine uridine and cytidine as described in Methods (see Section 1.3). Aliquots of cells were taken at successive time intervals, nuclei and cytoplasm were prepared (see Methods Section 1.3). Nuclear RNA was prepared and chromatographed on poly(U)- and poly(A)-sepharose columns as described in Fig 15. The [^{3}H] poly(A)$^-$a$^+$ and poly(A)$^+$ RNAs were eluted from the poly(U)-sepharose with 15% (v/v) and 90% (v/v) formamide

Figure 24

Stability of Friend cell [^{3}H] nuclear poly(A)$^+$ and poly(A)$^-$u$^+$RNAs following a 15, 60 or 120 min label with [^{3}H] uridine

Friend cells were pretreated with glucosamine as described in Methods Section 1.3, and then labelled with 30μci/ml of [^{3}H] uridine for 15, 60 or 120 min. In all cases the chase was initiated by the addition by glucosamine, uridine and cytidine as described in Methods Section 1.3. Aliquots of cells were removed at the indicated times and labelled cytoplasmic and nuclear RNAs isolated as described in Methods Section 4.1. [^{3}H] nuclear RNA was chromatographed on poly(U)- and poly(A)-sepharose columns (see Fig 15) and bound [^{3}H] RNA was eluted as described in the text. Radioactivity was determined as described in Methods Section 18.1.

Results are expressed as percentages relative to the value at the start of the "chase", which is taken as zero time. For convenience of representation results are plotted with the zero time value taken as 100%. The quoted values were obtained from two different experiments. Bars indicate the extreme of the measurements.

Stability of [^{3}H] nuclear poly(A)$^+$RNA (—●—) or [^{3}H] nuclear poly(A)$^-$u$^+$RNA (—□—) following a labelling period of (a) 15 min (b) 60 min (c) 120 min was monitored.

Stability of [^{3}H] nuclear poly(A)$^-$a$^+$RNA (—○—) or [^{3}H] nuclear non-bound RNA (—△—) following a labelling of (d) 15 min (e) 60 min (f) 120 min was monitored.

In each case the labelling period was followed by a "glucosamine-uridine" chase.

Figure 24

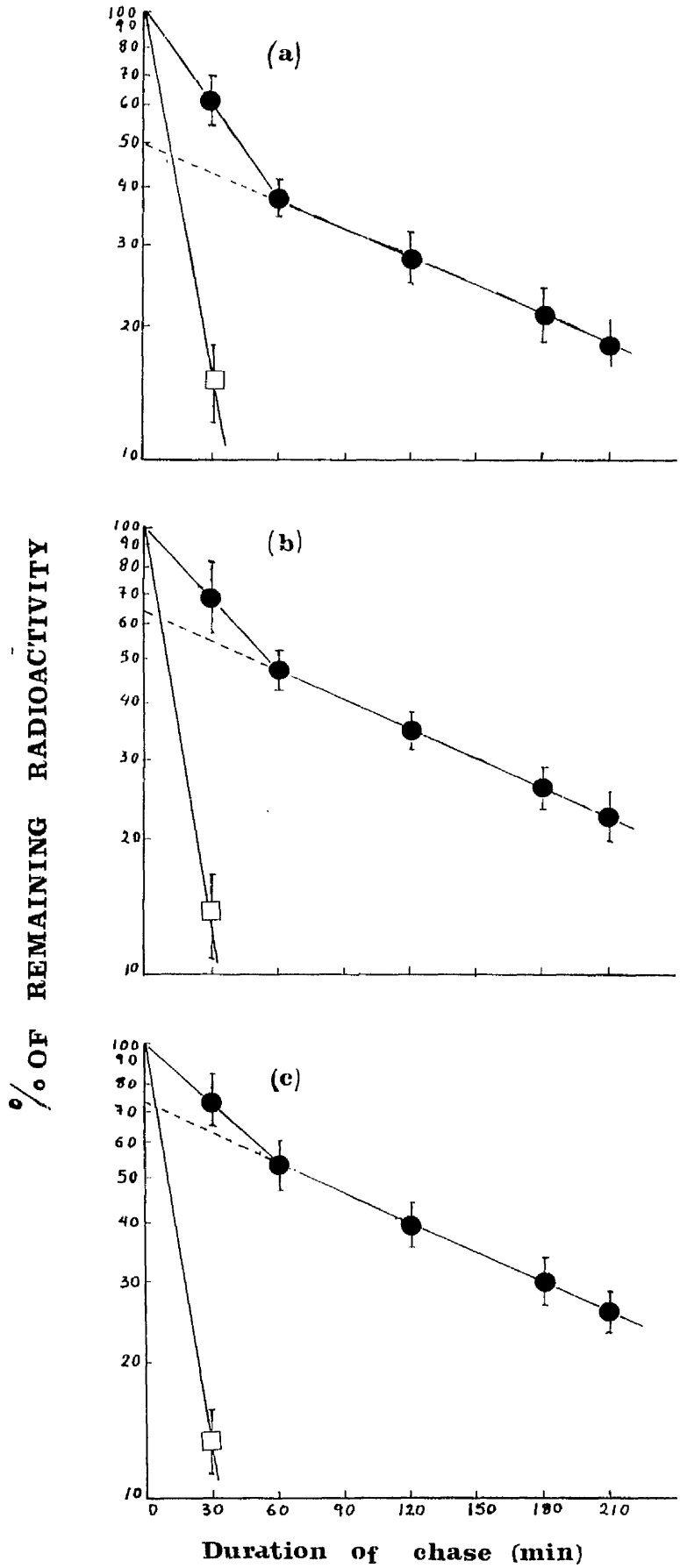
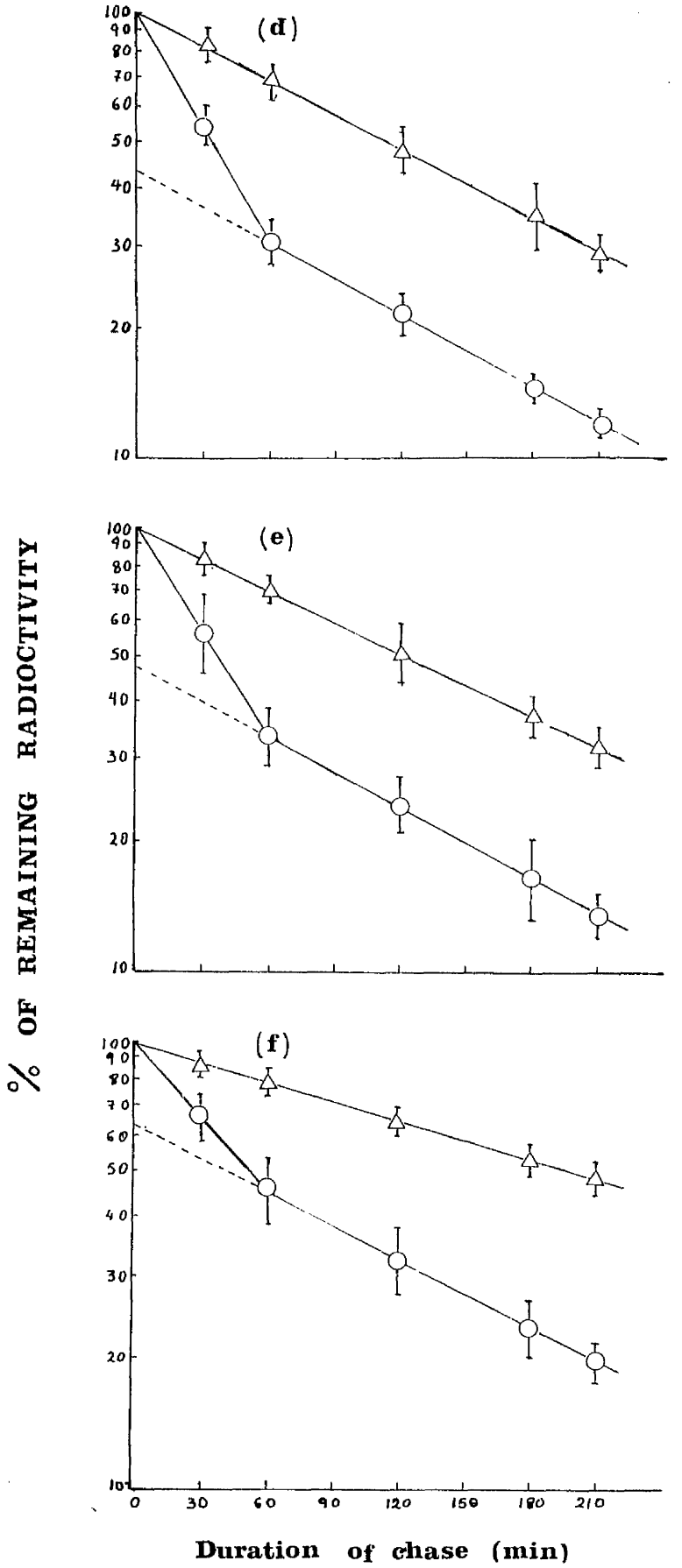

(a)
(b)
(c)
% OF REMAINING RADIOACTIVITY
100
90
80
70
60
50
40
30
20
10
0
30
60
90
120
150
180
210
Duration of chase (min)

Figure 24 (contd)



in10mM EDTA, 10mM tris-HCl, pH7.4, 0.2% (w/v) SDS respectively. Poly(A)$^-$u$^+$RNA was eluted from the poly(A)-sepharose with 90% (v/v) formamide in 10mM EDTA, 10mM tris-HCl, pH7.4, 0.2% (w/v) SDS. The amount of [^{3}H] uridine radioactivity found in poly(A)$^+$ and poly(A)$^-$u$^+$RNAs at each time is expressed as a proportion of the radioactivity incorporated at the beginning of the chase period, and the results are plotted as a function of time, on a semi-log graph (Fig 24).

Considering, firstly, the data relating to nuclear poly(A)$^+$ RNA, it can be seen that the decay rate deviates from simple first order kinetics for all labelling times used (Fig 24). In all cases, the data are interpreted to indicate simultaneous turnover of two subpopulations, each exhibiting first order decay kinetics, but having different kinetic constants. By applying graphical correction procedure (Lengyel and Penman 1977, Herman and Penman 1977) to resolve the two decay rates, half-lives of 22 min ("short-lived" poly(A)$^+$ RNA) and 130 min ("long-lived" poly(A)$^+$ RNA), were obtained. These components comprise 51% and 49% respectively of the 15 min labelled poly(A)$^+$ RNA. Furthermore, it can be seen in Fig 24 that regardless of the labelling time employed, the same half-lives of 22 and 130 min are obtained for the two subpopulations. The time of labelling, however, does affect the relative proportions within the total label of poly(A)$^+$ RNA. After 1 hr of labelling the proportions of "long-lived" and "short-lived" components are 63% and 37% respectively, whilst after 2 hrs of labelling, the proportions are 73%

and 37% respectively. In fact, assuming the proportions and half-lives obtained from a 15 min label, it is possible to calculate theoretical proportions of "short-lived" and "long-lived" components for 1 hr and 2 hrs labelling periods, which are in close agreement with those actually obtained (see Discussion). Such calculations predict "steady-state" proportions of 83% and 17% for "long-lived" and "short-lived" poly(A)$^+$ RNA respectively (see Discussion Section 6).

These results, taken together, suggest that there are at least two populations of nuclear poly(A)$^+$ RNA with different metabolic behaviour. Similar results have also been reported for Drosophila nuclear poly(A)$^+$ RNA using a "glucosamine-uridine" chase approach (Levis and Penman 1977), and for rat liver poly(A)$^+$ hnRNA, using an equilibrium labelling approach (Chernovskaya and Lerman 1977). In addition, poly(A)$^+$- adjacent hnRNA sequences in HeLa cells appear also to contain two kinetically different components (Herman and Penman 1977).

Considering the data for poly(A)$^-$u$^+$RNA, it can be seen that these RNA species can be characterised as one class of molecules, with an apparently uniform half-life of 10-12 min (Fig 24). These results are in agreement with earlier preliminary observations, where nuclear poly(A) binding RNA from BHK/21 cells was found to be a very unstable class of RNA molecules (Burdon et al 1976).

The detection of two sub-populations, of different half-

lives, in nuclear poly(A)$^+$ RNA could conceivably be an artifact, related to polyadenylation of labelled nuclear poly(A)$^-$ RNA during the chase. To eliminate such a possibility the metabolism of nuclear poly(A)$^+$ RNA was also examined in the presence of the drug cordycepin, an effective inhibitor of polyadenylation (Darnell et al 1971, Jelinek et al 1973, LaTorre et al 1973). With cordycepin added at the beginning of the chase, the results shown in Fig 25 were obtained. Under such conditions, the "short-lived" component of nuclear poly(A)$^+$ RNA turnover with only a slightly different half-life, whilst the half-life of the "long-lived" poly(A)$^+$ RNA component is reduced by 10-20%. The half-life of poly(A)$^-$ u$^+$RNA, however, is unaltered under these conditions. Thus, the possibility that polyadenylation of nuclear poly(A)$^-$ RNA component, cannot be ruled out. Even so, however, it still appears as if nuclear poly(A)$^+$ RNA has at least a "short-lived" and a "long-lived" component. It should be stressed that the results obtained from these studies should be viewed with caution since cordycepin is a drug, and it may cause severe side effects.

3.2 Stability of Friend cell nuclear poly(A)$^-$a$^+$ and non-bound RNAs

Along with the studies on the kinetic behaviour of nuclear poly(A)$^+$ RNA, the kinetic behaviour of the poly(A)$^-$a$^+$RNA fraction and non-bound RNA fraction were examined. This work seemed especially relevant in view of the evidence that some poly(A)$^-$ hnRNA might contain cytoplasmic

Figure 25

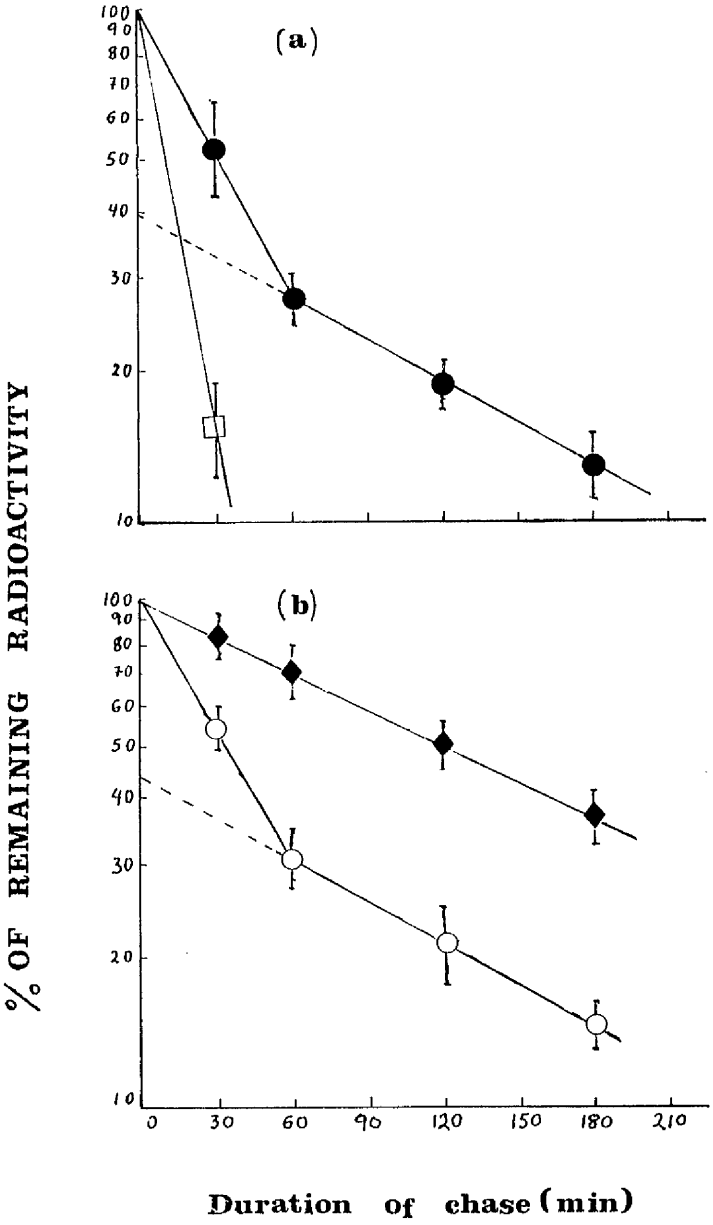
Effect of cordycepin on the stability of [^{3}H] uridine labelled poly(A)$^+$, poly(A)$^-$a$^+$, poly(A)$^-$u$^+$RNA and non-bound RNA fractions

Friend cells were treated with glucosamine as described in Methods Section 1.3, and then labelled with 30μci/ml of [^{3}H] uridine for 15 min. Chase was initiated using glucosamine, uridine and cytidine as described in Methods Section 1.3 in the presence of cordycepin (30μg/ml). Aliquots of cells were removed at the indicated times and [^{3}H] labelled cytoplasmic and nuclear RNAs were prepared. Nuclear RNA was chromatographed on poly(U)- and poly(A)-sepharose columns as described in Fig 15 and bound [^{3}H] RNAs were eluted as described to the legend of Fig 24. Radioactivity was determined as described in Methods Section 18.1.

Results are expressed as in Fig 24. The quoted values were obtained from two separate experiments. Bars indicate the extremes of the measurements.

Stability of (a) [^{3}H] nuclear poly(A)$^+$ RNA (—●—) and
[^{3}H] nuclear poly(A)$^-$u$^+$RNA (—□—)
(b) [^{3}H] nuclear poly(A)$^-$a$^+$RNA (—○—)
and [^{3}H] nuclear non-bound RNA (—◆—)

Figure 25


(a)
(b)
100
90
80
70
60
50
40
30
20
10
0
30
60
90
120
150
180
210
% OF REMAINING RADIOACTIVITY
Duration of chase (min)

poly(A)$^+$ mRNA sequences (Hames and Perry 1977), and that there may be messenger sequences adjacent to oligo(A) tracts (Kinniburg and Martin 1976).

The results shown in Fig 24 suggest that the decay kinetics of poly(A)$^-$a$^+$RNA, like poly(A)$^+$RNA, can also be described in terms of two sub-populations with half-lives of 20 and 120 min. The relative proportions of these sub-populations again being dependent on the period of labelling (Fig 24). Consistent with these results are the observations of Herman and Penman (1977), who reported that fragments of HeLa cell nuclear RNA, containing oligo(A) tracts, appears to decay biphasically. Conversely, the decay kinetics of the non-bound RNA fraction seem to indicate only a single kinetic component, having the relatively long half-life of about 120 min (Fig 24). Furthermore the metabolic behaviour of poly(A)$^-$a$^+$ and non-bound RNA fractions appears not to be affected in the presence of cordycepin (Fig 24). This suggests that both the poly(A)$^-$a$^+$ and non-bound RNAs may not serve as precursors to nuclear poly(A)$^+$ RNA.

Studies concerning the metabolism of poly(A)$^-$ hnRNA have been reported in Drosophila (Levis and Penman 1977) and in rat liver (Chernovskaya and Lerman 1977), where it was found that poly(A)$^-$ hnRNA exhibits a half-life of 15 and 30 min respectively.

3.3 Formation of Friend cell poly(A)$^+$ and poly(A)$^-$u$^+$ mRNA during a "pulse-chase"

The efficiency of the "glucosamine-uridine" procedure,

has also made possible the determination of the kinetics of appearance of poly(A)$^+$ and poly(A)$^-$u$^+$ mRNA in the cytoplasm.

Cytoplasmic RNA from the cells used in Fig 24 were isolated and examined with regard to their ability to bind on poly(U)- and poly(A)-sepharose columns. The results obtained are shown in Fig 26. Considering firstly the data relating to cytoplasmic poly(A)$^+$ mRNA, it can be seen that the emergence of poly(A)$^+$ mRNA continues for at least 2 hrs, regardless of labelling period. Subsequently a decrease in the amount of [^{3}H] uridine labelled poly(A)$^+$ mRNA is observed. This appears to be due to the decay of "short-lived" poly(A)$^+$ mRNA, rather than a cessation of emergence of poly(A)$^+$ mRNA, since from Fig 23 (inset), it has been shown that the amounts of [^{32}P] radioactivity in poly(A)$^+$ mRNA continues to increase throughout a 4 hrs "chase" period. In fact, very "short-lived" poly(A)$^+$ mRNA (half-life 15-60 min) has been reported in a variety of cell lypes (see Introduction Section 6.3). Furthermore, it should be noted that the percentage of poly(A)$^+$ mRNA accumulated, following a 15, 60 or 120 min labelling period, differs significantly (Fig 26). This can be explained by the fact that after 15 min of labelling only a small portion of newly synthesised [^{3}H] uridine labelled poly(A)$^+$ mRNA has been transported, compared with the amounts following a labelling time of 1 hr or 2 hrs. A similar continuation in appearance of poly(A)$^+$ mRNA after the "chase" has also been reported in HeLa cells (Herman and Penman 1977),

Figure 26

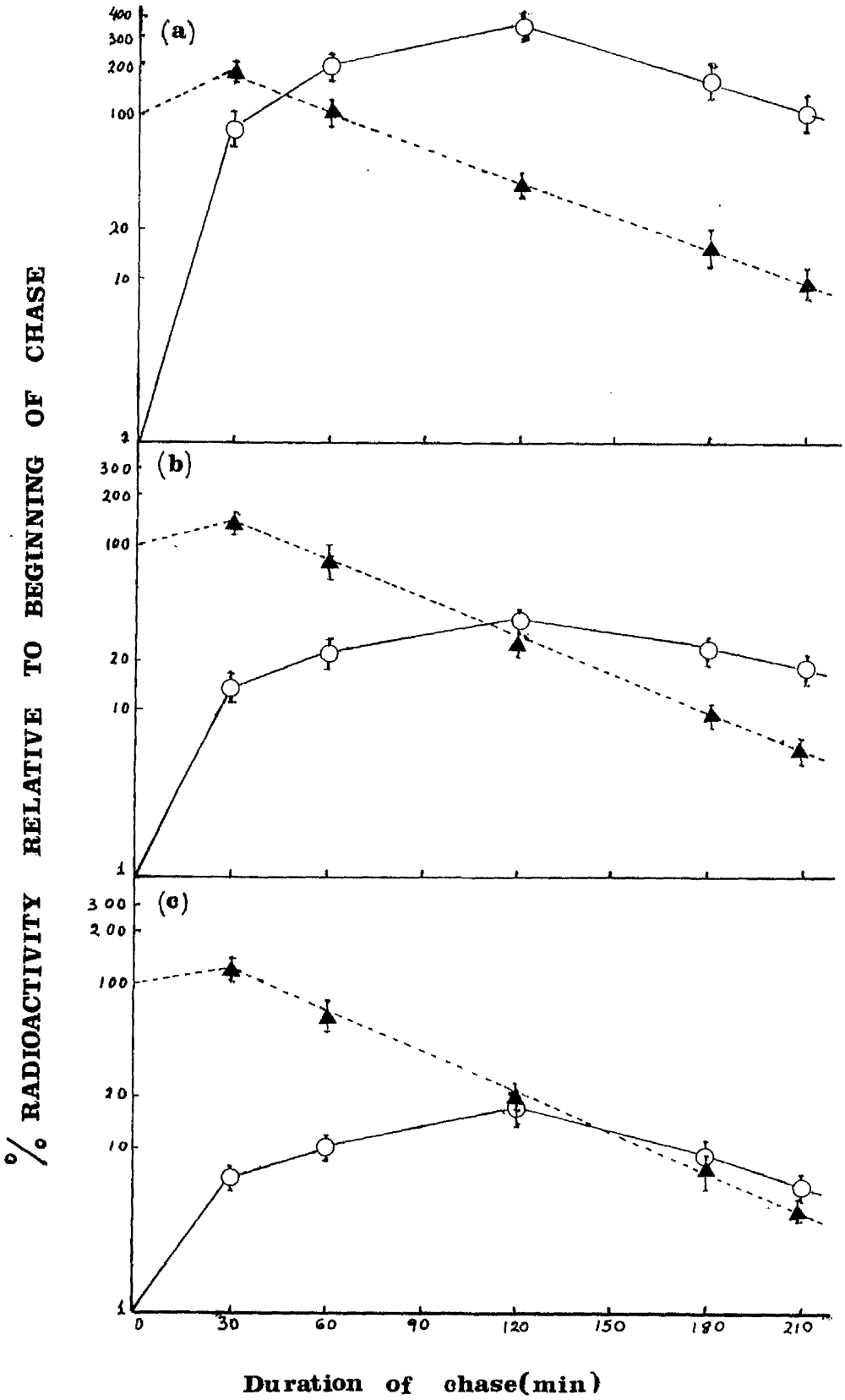
Appearance of poly(A)$^+$ and poly(A)$^-$u$^+$RNAs in the cytoplasm during a "pulse-chase"

Cytoplasm was prepared from Friend cells as outlined in the legend to Fig 24. [^{3}H] cytoplasmic poly(A)$^+$ and poly(A)$^-$u$^+$RNAs were then prepared as described in Table 4.

Results are expressed as percentages relative to the value at the start of the chase, which is taken as a zero time. For convenience of representation the results for [^{3}H] poly(A)$^+$RNA are plotted relative to the zero time value which is taken as 1%, whilst those of the [^{3}H] poly(A)$^-$u$^+$RNA are plotted relative to the zero time which is taken as 100%. The quoted values were obtained from two separate experiments. Bars indicate the extremes of the measurements.

The appearance of [^{3}H] poly(A)$^+$RNA (——o——) and [^{3}H] poly(A)$^-$u$^+$RNA (——▲——) in the cytoplasm after various labelling times (a) 15 min (b) 60 min (c) 120 min was monitored. In each case the labelling period was followed by a "glucosamine-uridine" chase.

Figure 26


(a)
(b)
(c)
400
300
200
100
20
10
1
300
200
100
20
10
1
300
200
100
20
10
1
0
30
60
90
120
150
180
210
% RADIOACTIVITY RELATIVE TO BEGINNING OF CHASE
Duration of chase(min)

and Chinese Hamster Ovary (CHO) (Bachellerie et al 1978). The observed decay kinetics of the nuclear poly(A)$^+$ RNA, coupled with the relatively long period during which cytoplasmic poly(A)$^+$ mRNA accumulates, suggests that cytoplasmic poly(A)$^+$ mRNA is derived from both "long-lived" and "short-lived" nuclear poly(A)$^+$ RNA components (see also Discussion Section 7).

Considering the data for the cytoplasmic poly(A)$^-$u$^+$ mRNA, it can be seen (Fig 26) that the amounts of [^{3}H] uridine in poly(A)$^-$u$^+$ mRNA emerging into the cytoplasm, increases only for the first 30 min of the chase and then decreases rapidly, suggesting that poly(A)$^-$u$^+$ mRNA is a labile RNA species. These results are consistent with the preliminary data obtained for BHK/21 cell cytoplasmic poly(A) binding RNA (Burdon et al 1976). An interesting observation from Fig 26 is that 30 min after the "chase" the relative percentage increase for both poly(A)$^+$ and poly(A)$^-$u$^+$ mRNAs are similar. However, since these two RNAs appear to exhibit considerably different metabolic behaviour (Fig 26), with poly(A)$^-$u$^+$ mRNA being more labile, it may be that poly(A)$^-$u$^+$RNAs undergoes a more rapid transport from the nucleus. To investigate this possibility, Friend cells were labelled with [^{3}H] uridine, and aliquots of cells were taken after short time intervals. The levels of cytoplasmic poly(A)$^+$ and poly(A)$^-$u$^+$ mRNAs were determined, and as Fig 27 shows the poly(A)$^-$u$^+$ mRNA does indeed appear to leave the nuclei earlier, compared to poly(A)$^+$ mRNA.

Figure 27

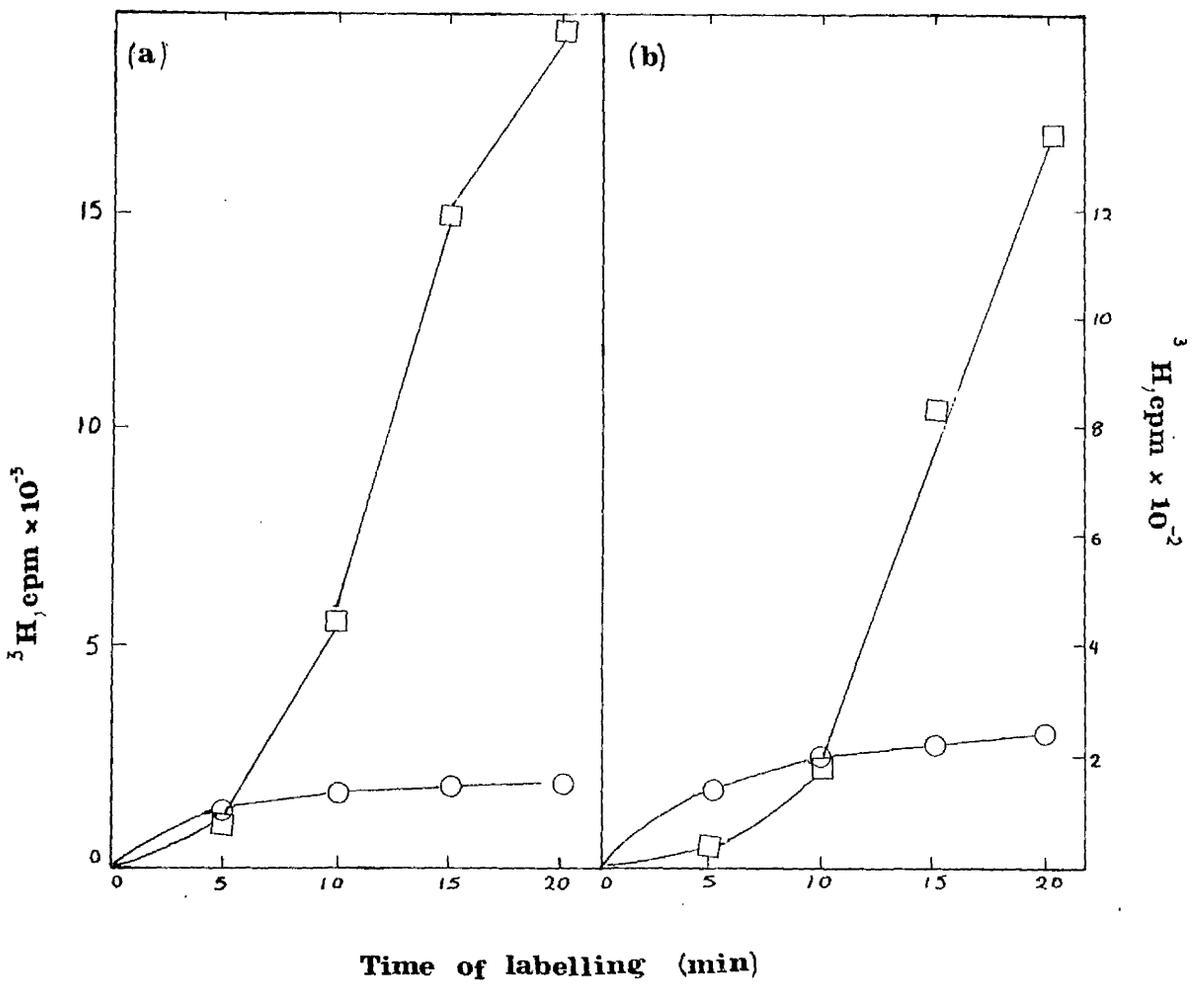
Time course of appearance of poly(A)$^+$ and poly(A)$^-$u$^+$RNAs in the nuclei (a) and cytoplasm (b) of Friend cells

Friend cells were labelled with 20μci/ml of [^{3}H] uridine and aliquots were removed at the indicated time intervals. Nuclear and cytoplasmic RNAs were prepared as described in Methods Sections 4.1 and 4.2, and then examined for the levels of [^{3}H] radioactivity in both poly(A)$^+$ and poly(A)$^-$u$^+$RNAs (see Table 4 and Fig 15). Radioactivity was determined as described in Methods Section 18.1.

□———□ poly(A)$^+$RNAs

○———○ poly(A)$^-$u$^+$RNAs

Figure 27



3.4 Conversion of Friend cell nuclear poly(A)$^+$ and poly(A)$^-$u$^+$RNAs to cytoplasmic poly(A)$^+$ and poly(A)$^-$u$^+$RNAs

The observed rates of decay of nuclear poly(A)$^+$ and poly(A)$^-$u$^+$RNAs actually represent the sum of at least two processes, intranuclear degradation and conversion to cytoplasmic poly(A)$^+$ and poly(A)$^-$u$^+$ mRNAs (see also Introduction 5.4.6).

To examine further the conversion of nuclear poly(A)$^+$ and poly(A)$^-$u$^+$RNAs to cytoplasmic messenger species, the data in Fig 25 can be expressed in its original form, rather than the more convenient percentage. Following a labelling period of 15, 60 and 120 min, and a "chase-time" of 30 or 120 min for nuclear poly(A)$^+$ and poly(A)$^-$u$^+$ RNAs respectively, the results presented in Table 17 were obtained. These time points were chosen in order to avoid the apparent decay of labile poly(A)$^+$ or poly(A)$^-$u$^+$ mRNA molecules in the cytoplasm, that is obvious after 2 hrs and 30 min, respectively. In addition, when the sizes of nuclear and cytoplasmic poly(A)$^+$ and poly(A)$^-$u$^+$ RNAs were examined at the beginning of the chase, and 60 min later, no significant change was found, compared to those in Fig 4 and Fig 19 (data not shown). These results suggest that after a short labelling period (15 min) and "chase", 20-30% of the [^{3}H] uridine nuclear poly(A)$^+$ RNA is converted to cytoplasmic poly(A)$^+$ mRNA, while 20-24% of the [^{3}H] uridine nuclear poly(A)$^-$u$^+$RNA is transported to the cytoplasm. In contrast to these results for a short label, longer labelling periods

Table 17 Intracellular re-distribution of [^{3}H] uridine labelled poly(A)$^+$ and poly(A)$^-$u$^+$RNAs under the conditions of a "glucosamine-uridine" chase

Experiments	Cellular compartment	Time of labelling (min)	Poly(A)$^+$RNA present at the beginning of the chase (cpm x 10^{-3})	Poly(A)$^+$RNA present after 2 hrs chase (cpm x 10^{-3})	Net change * (cpm x 10^{-3})	Poly(A)$^-$u$^+$RNA present at the beginning of the chase (cpm x 10^{-2})	Poly(A)$^-$u$^+$RNA present after 30 min chase (cpm x 10^{-2})	Net change * (cpm x 10^{-2})
1	Nuclei	15	45.2	12.5	- 32.7	41.1	8.9	- 32.2
	Cytoplasm		3.2	12.9	+ 9.7	9.3	17.1	+ 7.8
2	Nuclei	15	113.4	34.1	- 77.2	108.7	15.2	- 93.5
	Cytoplasm		8.3	25.8	+ 17.5	19.1	37.4	+ 18.3
1	Nuclei	60	125.3	43.1	- 82.2	92.1	15.4	- 76.7
	Cytoplasm		28.2	40.9	+ 12.7	24.7	37.9	+ 13.2
2	Nuclei	60	185.6	61.3	-124.3	125.5	16.3	-109.2
	Cytoplasm		40.9	54.5	+ 13.6	39.8	55.1	+ 15.3
1	Nuclei	120	220.7	44.3	-125.9	78.6	15.6	- 63.0
	Cytoplasm		61.5	73.7	+ 12.2	40.7	49.2	+ 8.5
2	Nuclei	120	178.5	54.1	-124.4	64.2	8.9	- 55.3
	Cytoplasm		49.8	57.1	+ 7.3	31.1	36.7	+ 5.6

* Difference between amount at the end of chase and amount initially present

For experimental details see the legends of Fig 24

(60 or 120 min) result in a much smaller proportion of nuclear poly(A)$^+$ RNA being transported. After 1 hr labelling, only 10-14% of the nuclear poly(A)$^+$ RNA is transported, and after 2 hrs labelling, only 6-9% of the nuclear poly(A)$^+$ RNA is transported. The results for the poly(A)$^-$u$^+$RNA also indicate that longer labelling periods result in transport of a smaller proportion of nuclear poly(A)$^-$u$^+$RNAs (Table 17), although the proportional decrease in transport for this fraction is considerably less than for the poly(A)$^+$ RNA. This apparent decrease in transport when longer labelling periods are considered, is a result of the larger amounts of radioactive poly(A)$^+$ and poly(A)$^-$u$^+$ mRNAs present in the cytoplasm at the start of the chase, compared to the transported RNAs. These results are consistent with those obtained by LaTorre et al (1973), who reported that 25% of L-cells [^{3}H] uridine nuclear poly(A)$^+$ RNA is transported to the cytoplasm, in cells "chased" with high doses of actinomycin D, following a pulse label of 15 min. A similar value of 10%-30% has been obtained for HeLa cells [^{3}H] uridine labelled poly(A)$^+$ hnRNA, using a "glucosamine-uridine" chase (Herman and Penman 1977). A somewhat larger value of about 40% is obtained for Drosophila [^{3}H] uridine poly(A)$^+$ hnRNA (Levis and Penman 1977).

3.5 Stability of nuclear poly(A)$^+$, poly(A)$^-$a$^+$, poly(A)$^-$u$^+$ and non-bound RNA species from induced Friend cells

Burdon et al (1976) reported that the growth pattern of

BHK/21 cells influenced the metabolic behaviour of cytoplasmic and nuclear poly(A) containing and poly(A) binding RNA species.

This raised the possibility that induced and non-induced Friend cells, clone M_2, might show differences in this parameter, since Friend cells, on induction by, for example Dimethylsulphoxide, proceed to display many of the characteristics of normal erythroid differentiation (Harrison 1976).

The methods used to examine this problem were simply those employed for non-induced Friend cells, but applied to induced cells. Essentially, the same experimental procedures used to investigate the metabolic behaviour of RNA in non-bound cells, were applied to study induced cells. Cells induced using DMSO (see Methods Section 1.1), were given a 15 min label with [^{3}H] uridine following glucosamine treatment (see Methods Section 1.3), and "chase" conditions initiated by adding glucosamine, uridine and cytidine, as described in Methods (Section 1.3). The results of this work are shown in Fig 28. No significant differences in the metabolic behaviour of the nuclear RNA classes can be distinguished, when these results are compared to the analogous results for non-induced cells, shown in Fig 24 a, e. The two cytoplasmic mRNA classes also show no detectable differences in metabolic behaviour, between induced and non-induced cells.

These results suggest that the induction process, although

Figure 28

Metabolic behaviour of nuclear and cytoplasmic poly(A)$^+$ and poly(A)$^-$u$^+$RNAs from induced Friend cells

Induced Friend cells (see Methods Section 1.1) were pre-treated with glucosamine as described in Methods Section 1.3, and then labelled with 30µci/ml of [^{3}H] uridine for 15 min. Chase was initiated using glucosamine, uridine and cytidine as described in Methods Section 1.3. Aliquots of cells were removed at the indicated times and cytoplasmic and nuclear RNAs were prepared. These RNAs were examined for the levels of [^{3}H] radioactivity in both poly(A)$^+$ and poly(A)$^-$u$^+$RNAs (see Fig 24 and Fig 25). Radioactivity was determined as described in Methods Section 18.1.

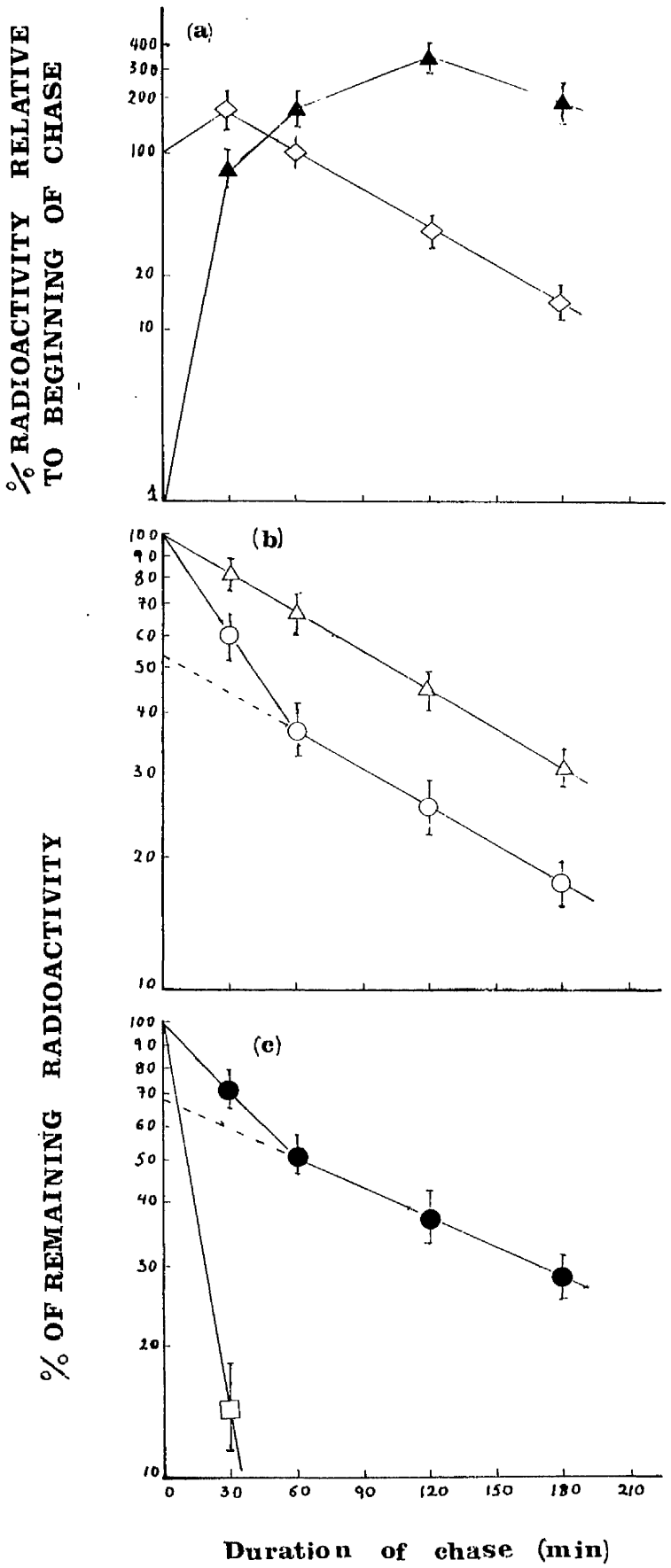
Results are expressed as in Fig 24 for the nuclear poly(A)$^+$ and poly(A)$^-$u$^+$RNAs and as in Fig 26 for the cytoplasmic poly(A)$^+$ and poly(A)$^-$u$^+$RNAs.

(a) Appearance of [^{3}H] poly(A)$^+$ (——▲——) and poly(A)$^-$u$^+$ (——◇——) RNAs in the cytoplasm during a "glucosamine-uridine" pulse chase

(b) Stability of [^{3}H] nuclear poly(A)$^-$a$^+$ (——o——) and non-bound (——△——) RNAs

(c) Stability of [^{3}H] nuclear poly(A)$^+$ (——●——) and poly(A)$^-$u$^+$ (——□——) RNAs

Figure 28


% RADIOACTIVITY RELATIVE TO BEGINNING OF CHASE
% OF REMAINING RADIOACTIVITY
(a)
(b)
(c)
400
300
200
100
20
10
1
90
80
70
60
50
40
30
0
30
60
90
120
150
180
210
Duration of chase (min)

leading to increased levels of globin messenger sequences (Gilmour et al 1974, Minty et al 1978) has small effect on the overall metabolism of both nuclear and cytoplasmic RNA.

DISCUSSION

DISCUSSION

1. The occurrence of non-polyadenylated mRNA

The existence of a class of eukaryotic mRNA having a 3'-terminal sequence of poly(A) is now well established (Brawerman 1974, Greenberg 1975). It is now clear, however, that not all mRNAs have this structural feature. In particular, histone messengers were shown to lack poly(A) (Adesnik et al 1972a), and more recently other poly(A)$^-$ mRNAs have been identified in a number of eukaryotes, including HeLa cells (Milcarek et al 1974), Slime molds (Lodish et al 1974), sea urchin embryos (Nemer et al 1974), rat mammary gland (Rosen et al 1975), L-cells (Greenberg 1976), trout testis (Geramu and Dixon 1976), mouse liver (Chernovskaya et al 1976, Grady et al 1978), mouse sarcoma 180 ascites cells (Sonenshein et al 1976, Geoghegan et al 1978), BHK/21 cells (Burdon et al 1976), muscle cells (Whalen et al 1976, Whalen and Gross 1978), plant cells (Gray and Gashmore 1976, Ragg et al 1977), xenopus embryo and ovaries (Ruderman and Pardue 1977) and mouse fibroblasts transferred by polyomavirus (Grady et al 1978). The poly(A)$^-$ mRNA from sea urchin embryos (Nemer et al 1974) and BHK/21 cells (Burdon et al 1976) have been demonstrated to exhibit a high content of uridylate residues. In the case of BHK/21 cells at least some of the poly(A)$^-$ mRNA did in fact have a high affinity for poly(A) (Burdon et al 1976). Behaviour towards poly(A) was, unfortunately, not examined in the case of sea urchin poly(A)$^-$ mRNA. Using poly(A)-sepharose affinity chromatography the poly(A)$^-$u$^+$mRNA can be isolated from polysomes

of Friend cells (clone M_2) in the same way as poly(A)$^+$ mRNA can be isolated using poly(U)-sepharose. The control experiments described in this study (see Results Section 1.3) suggest that the Friend cell polysomal poly(A)$^-$u$^+$mRNA does not appear to be of rRNA origin, nor to contain material arising from some form of non-specific binding to poly(A)-sepharose. In addition, polysomal poly(A)$^-$u$^+$mRNA is released from polysomes by EDTA in the manner expected of mRNA, and after deproteinisation sedimented in sucrose-formamide gradients with a size distribution resembling that already found for mRNA. From the structural standpoint the polysomal poly(A)$^-$u$^+$mRNA appears to lack poly(A) sequences greater than 6-8 nucleotides long. The binding of polysomal poly(A)$^-$u$^+$mRNA to poly(A)-sepharose appears to be due to an uridylate-rich region(s) which, however, seems to contain other nucleotides, notably guanylate residues. The location of most of this "U-rich" region(s) of poly(A)$^-$u$^+$mRNA molecules appears to be the 3'-terminus. Interestingly, an estimation of uridine composition of the "U-rich" region(s) can be made from the data using polynucleotide phosphorylase (see Results Section 1.11). Essentially, both poly(A)$^+$ and poly(A)$^-$u$^+$mRNAs were incubated with polynucleotide phosphorylase until the majority (about 90%) of the poly(A)$^+$ RNA failed to bind to poly(U)-sepharose. Assuming a length of poly(A) between 150-200 nucleotides and an equal reaction rate of polynucleotide-phosphorylase with poly(A)$^-$u$^+$mRNA, the uridine released from poly(A)$^-$u$^+$mRNA should originate from 3' terminal 150-200 nucleotides. Since the average

size of poly(A)$^-$u$^+$mRNA is about 3,000 nucleotides and knowing its base composition, it can be calculated that approximately 900 nucleotides are uridylate residues. Assuming that [^{3}H]-uridine is incorporated uniformly, release of 9-12% of total uridine during polynucleotide phosphorylase treatments suggests that 80-110 nucleotides of uridine have been released. On this basis, the uridine composition of the 3' terminal region is between 40-80%. This suggests that the "U-rich" region, in contrast to the poly(A) region, is not a "pure" homopolymer. This difference in purity would explain the significantly different elution profiles for poly(A)$^+$ and poly(A)$^-$u$^+$mRNAs, since the presence of other bases within the "U-rich" region will generate mis-matches on binding to poly(A)-sepharose. Such mis-matched hybrids will be eluted by a lower formamide concentration compared to that concentration required to elute pure poly(A) from poly(U)-sepharose. Furthermore, the cross-hybridisation results shown in Fig 5 at first sight suggests that polysomal poly(A)$^-$u$^+$ mRNA does not arise either as a result of deadenylation of poly(A)$^+$mRNA, or by random cleavage of poly(A)$^+$ RNA during preparation. In fact the average size of poly(A)$^-$ u$^+$mRNA is somewhat greater than that of poly(A)$^+$mRNA. However, the data does not demonstrate a complete lack of sequence homology between the two mRNA populations. A low level of sequence homology would be expected if the polysomal poly(A)$^-$u$^+$mRNA contained a few messenger sequences in relatively high concentration (abundant class) which also occur in high concentration in poly(A)$^+$mRNA class.

This could be further examined by preparing cDNA complementary to the "abundant" class of Friend cell polysomal poly(A)$^+$mRNA, and examining the kinetics of hybridisation of this cDNA with an excess of polysomal poly(A)$^-$u$^+$mRNA. In fact, Kaufmann et al (1977) presents data where it can be seen that cDNA prepared from the "abundant" class of HeLa cells poly(A)$^+$mRNA hybridises with HeLa poly(A)$^-$mRNA. From their data, they suggest that 10% of the "abundant" poly(A)$^+$mRNA sequences are present in poly(A)$^-$mRNA at relatively high concentration. Indeed, hybridisation studies, using cDNA prepared from poly(A)$^+$mRNA, have generally shown a small level of sequence homology between poly(A)$^+$ and poly(A)$^-$ mRNA populations obtained from such diverse sources as HeLa cells (Milcarek et al 1974), sea urchin embryos (Nemer et al 1974) and Ehrlich ascites mitochondria (Lewis et al 1976).

Interestingly, when Friend cell polysomal poly(A)$^+$ and poly(A)$^-$u$^+$mRNA were translated in a wheat germ cell-free protein synthesising system and the products analysed on one dimensional SDS-polyacrylamide gels, a measure of the similarity between these translation products can be obtained by comparing the ratio of either [^{3}H]/[^{14}C] or [^{35}S]/[^{3}H] in each gel slice. Identical translation products would contain the same number of radioactive leucine residues, and hence should exhibit the same ratio of ^{3}H to ^{14}C when this ratio is plotted against its position in the gel. In contrast different polypeptides would contain differing amounts of [^{3}H] and [^{14}C] and would exhibit an irregular

pattern on the same plot. Thus, the ratio of radioactivities, plotted in Fig 9, suggests dissimilar proteins as "peaks" or "troughs", and similar products, which give more constant ratios, a horizontal plot. It can be seen from Fig 9 that certain products (ranging from 36 x 10^3 to 70 x 10^3 daltons) seem to be encoded primarily by poly(A)$^+$ mRNA (see Fig 9, "peaks" 1, 2 and 3), while others (ranging from 30 x 10^3 to 50 x 10^3 daltons, see Fig 9 "troughs" 4 and 5) are mainly the translation products of poly(A)$^-$u$^+$ mRNA. Many translation products of both mRNAs, on the other hand, appear quite similar, as judged by the fact that the ratio of radioactivities is constant between molecular weights 63 x 10^3 - 68 x 10^3 and 30 x 10^3 - 47 x 10^3 daltons (Fig 9). That this similarity is not simply due to the presence of contaminating poly(A)$^+$mRNA sequences in the poly(A)$^-$u$^+$mRNA preparation may be concluded from a consideration of the results of a cross-hybridisation reaction between cDNA prepared by poly(A)$^+$mRNA and poly(A)$^-$u$^+$mRNA (Fig 3). For example, if only 1% of the poly(A)$^+$ mRNA sequences were present in the poly(A)$^-$u$^+$mRNA preparation, then one would expect a hybridisation curve similar to that for the homologous reaction, but "shifted" to the right by a factor of 100 or 2 log Rot units. This would produce a hybridisation figure of 50-60% at the largest Rots used instead of the 10% observed in this study. Using one dimensional gel electrophoresis, strong similarities have previously been shown between the translation products of poly(A)$^+$ and poly(A)$^-$mRNAs from sea urchin

embryos (Ruderman and Pardue 1977), HeLa cells (Kaufmann et al 1977), muscle cells (Whalen and Gross 1978) and mouse sarcoma 180 cells (Sonenshein et al 1976, Geoghegan et al 1978). At the present time, however, simple co-migration of non-identical polypeptides encoded by Friend cell polysomal poly(A)$^+$ and poly(A)$^-$u$^+$mRNAs cannot be ruled out. Also, when the protein products encoded by HeLa cell poly(A)$^+$ and poly(A)$^-$mRNAs were analysed by Kaufmann et al (1977) using two-dimensional gel electrophoresis three classes of polypeptides were revealed - one class of proteins (about 10) were detected among only the poly(A)$^-$mRNA products, a second class of proteins (about 40) were produced only by poly(A)$^+$mRNA, and a third class of proteins (about 40) were coded from both poly(A)$^+$ and poly(A)$^-$mRNAs (Kaufmann et al 1977). It would be interesting to speculate upon whether this situation holds in various other systems.

The existence of "abundant" poly(A)$^+$ and poly(A)$^-$mRNAs coding for the same protein may be a general feature of eukaryotes. The most striking example of this "polymorphism" is the histone mRNA which has been found in both poly(A)$^-$ and poly(A)$^+$ forms (Levenson and Marcu 1976, Ruderman and Pardue 1977, Ruderman and Pardue 1978). Poly(A)$^+$ and poly(A)$^-$mRNAs have also been found for a variety of proteins including protamine (Geramu and Dixon 1976), actin (Sonenshein et al 1976, Whalen and Gross 1978), and casein (Rosen et al 1975) these being nuclear, cytoplasmic and secreted proteins respectively. Furthermore

the occurrence of poly(A)$^+$ and poly(A)$^-$mRNAs coding for the same protein raises some questions of whether these poly(A)$^-$mRNAs could represent the transcription product of separate genes, lacking a signal for poly(A) addition, or the transcript of such separate genes might be polyadenylated in a normal fashion, but be more susceptible to the cytoplasmic processes that lead to size reduction and loss of the poly(A) segment. Evidence supporting the former possibility came from studies with histone genes where it was found that histone mRNAs from two different developmental stages of sea urchin embryos were dissimilar in sequences (Kunkel and Weinberg 1978) and were possibly translated to give protein products of slightly different primary structure (Newrock et al 1978). Also the presence of poly(A)$^-$mRNA (i.e. protamine, actin) on polysomes (Iatrou and Dixon 1977, Geoghegan et al 1978) suggests that these mRNAs are functional and not simply a "stage" of poly(A)$^+$mRNA degradation.

Although a proportion of the "abundant" class of poly(A)$^+$ and poly(A)$^-$mRNAs from HeLa cells appears to contain similar sequences, the low "abundance", high sequence complexity, mRNA fraction of both poly(A)$^+$ and poly(A)$^-$mRNA populations appears to contain distinct sequences. Indeed, Grady et al (1978) prepared a DNA probe complementary to the cellular RNA of mouse liver and cultured mouse cells. This was achieved by hybridising highly labelled single-copy mouse liver, or cultured mouse cell DNA, to total cellular RNA from both cell types, then isolating the

hybrid. Using the DNA (expressed DNA) from this hybrid they examined the complexity of polysomal poly(A)$^+$ and poly(A)$^-$mRNAs. This method is particularly useful because it can detect the low "abundancy" high complexity mRNA class. They found that poly(A)$^+$ and poly(A)$^-$mRNAs shares very little sequence homology, with 30-40% of the total mRNA complexity belonging to the poly(A)$^-$mRNA. An even higher value for the complexity of the poly(A)$^-$mRNA has been reported in sea urchin embryos, where it was found that about 90% of the mRNA complexity is derived from poly(A)$^-$mRNA (Nemer et al 1974, Galau et al 1974, McColl and Aronson 1978). The fraction of the mRNA mass occupied by these low "abundancy" mRNAs was calculated to be less than 10% (Galau et al 1974) of the total mRNA. This appears to be the situation for the low "abundancy" poly(A)$^+$mRNA in a variety of cell types, including Friend cell (Birnie et al 1974, Kleiman et al 1977), HeLa cell (Bishop et al 1974), L-cells (Ryffel and McCarthy 1975), Drosophila (Levy and McCarthy 1975), mouse liver (Young et al 1977) mouse brain (Young et al 1977) mouse kidney (Hastie and Bishop 1977) and chick embryo muscle culture (Paterson and Bishop 1977). Thus it appears that messengers belonging to the low "abundance" class are present in a small number of copies in the range 1-30. This makes this class virtually undetected in cell-free protein synthesising systems where "abundant" mRNA is present in the range of thousand copies.

2. Possible function of poly(A)$^-$mRNA

Although several well known proteins (e.g. protamine, actin) as well as others (Ruderman and Pardue 1977, Kaufmann et al 1977, Geoghegan et al 1977) appear to be coded by both poly(A)$^+$ and poly(A)$^-$mRNAs, the physiological significance of the poly(A)$^-$mRNAs is still obscure. Nevertheless, a close examination of data presented by Ruderman and Pardue (1977) and Whalen and Gross (1977) for sea urchin and muscle cells, respectively, suggest that the role of poly(A)$^-$mRNA may be most significant during early developmental or pre-terminal differentiation stages. Ruderman and Pardue (1977) have analysed the pattern of labelled polypeptides (in one-dimensional SDS-polyacrylamide electrophoresis) synthesised when poly(A)$^+$ and poly(A)$^-$ mRNAs, prepared from the various developmental stages of sea urchin embryos, were translated in a wheat germ cell-free protein synthesising system. Their data suggest, that although sea urchin egg poly(A)$^-$mRNA encodes a wide variety of non-histone proteins in vitro, fewer such non-histone products are detected in the translation products of morula or gestrula poly(A)$^-$mRNAs. Similarly, it was found that poly(A)$^-$mRNA from fused muscle cells encodes considerably fewer non-histone proteins compared to the poly(A)$^-$mRNA from dividing muscle cells. Particularly 30% of the translatable actin mRNA is found in the poly(A)$^-$mRNA from dividing muscle cells, whilst only 10% of the translatable activity is found in poly(A)$^+$mRNA from fused muscle cells. This apparent greater prevalence

of "abundant" poly(A)$^-$mRNAs in cell types which are not "highly-differentiated" may possibly be due to the requirement of these cells to respond rapidly to both internal or external changes. A very rapid response of these cells may well involve poly(A)$^-$mRNAs; in fact, since the very act of polyadenylation takes about 5-120 min (see Introduction Section 5.4.6) the probability of a messenger being adenylated may depend on a number of interacting factors, and may vary depending on the functional status of the cell or even within the cell cycle.

That this might be the case is suggested by the data of Burdon et al (1976) who found that the ratio of [^{3}H] uridine labelled polysomal poly(A) containing and poly(A) binding mRNAs is altered when BHK/21 cells are in the growing or resting stage. A change in the ratio of poly(A)$^+$ to poly(A)$^-$mRNAs has also been reported in regenerating mouse liver after partial hepatectomy (Chernovskaya et al 1976). In addition, in initial experiments the ratio of [^{3}H] uridine labelled polysomal poly(A)$^+$ and poly(A)$^-$u$^+$ mRNAs is decreased when Friend cells, clone M_2, are induced with DMSO.

3. Possible function of 3' terminal sequences

The presence of an untranslated poly(A) tract at the 3' end of some messenger has led to considerable debate regarding the function of these structures. It was initially thought that poly(A) was related in some way to the processing and transport of the nuclear precursors of mRNA (Darnell et al 1973). This hypothesis was based on

kinetic (Jelinek et al 1973) and cordycepin chase (Adesnik et al 1972b, Mendecki et al 1972) experiments. A number of experimental findings, however, cast some doubt on the validity of these suggestions. Firstly, histone mRNA, for example, does not contain poly(A), yet this molecule undergoes transport to the cytoplasm which was perceptibly very rapid, in comparison to poly(A)$^+$mRNA species (Schochetman and Perry 1972). This delay in appearance of poly(A)$^+$ RNA (see Introduction Section 5.4.6) compared to the histone poly(A)$^-$mRNA may be due to the time taken for polyadenylation. A similar, faster, transport was also observed for Friend cell poly(A)$^-$u$^+$mRNA, compared to poly(A)$^+$mRNA (see Results Fig 27). Furthermore, recent evidence based on hybridisation (Herman et al 1976, Ryffel 1976, Levy et al 1977, Minty et al 1977) or pulse-chase experiments (Herman and Penman 1977, Levis and Penman 1977) suggest that a considerable amount of nuclear poly(A)$^+$ RNA is confined to the nucleus, and never reaches the cytoplasm. In addition, the occurrence of cytoplasmic polyadenylation of mRNA (Slater and Slater 1974), as well as the presence of 3'-OH poly(A) of certain viruses transcribed and replicated in the cytoplasm (Kates 1970, Yogo and Wimmer, Armstrong et al 1972, Johnston and Base 1972) suggest that polyadenylation is not a compulsory event for processing or transport of mRNA sequences.

It has been argued that if polyadenylation is of uncertain function, in terms of nuclear events, then possibly its function is in the cytoplasm. Recently a number of studies

have explored the relationship between capacity of a messenger to direct protein synthesis in an *in vitro* system(s), and the presence (Soreq *et al* 1974, Williamson *et al* 1974) or accessibility (Munoz and Darnell 1974) of poly(A). For this purpose several groups of investigators carried out experiments where the poly(A) segment was removed from poly(A)$^+$ globin mRNA by specific enzymatic treatment, and the translational efficiency of poly(A)$^-$ globin mRNA to that of "native" compared in various *in vitro* protein synthesis system. Both poly(A)$^+$ and poly(A)$^-$ globin mRNA were found to be translated with the same efficiency under these conditions. Additionally, when poly(A) of poly(A)$^+$ mRNA was blocked as a poly(A).poly(U) hybrid, the translational efficiency of poly(A)$^+$mRNA and "blocked" poly(A)$^+$mRNA in a wheat germ system was again found to be the same (Munoz and Darnell 1974). Furthermore, the "naturally" occurring poly(A)$^+$ and poly(A)$^-$mRNAs in HeLa cell and sea urchin appear to have the same translational efficiency in a wheat germ system (Fromson and Verma 1976, Kaufmann *et al* 1977). The lower translational efficiency of Friend cell polysomal poly(A)$^-$u$^+$mRNA compared to poly(A)$^+$mRNA (see Results Fig 8), may be explained if this class of RNA comprises only a part of the total poly(A)$^-$mRNA population in Friend cells. On the other hand, considerable intrinsic differences in translational efficiency have been observed, even for poly(A)$^+$mRNAs, for example those for α and β globin (Lodish 1971). Thus the Friend cell poly(A)$^-$u$^+$RNA may simply exhibit a "natural" low translational efficiency. It is interesting to note that the

relatively greater translational efficiency of polysomal poly(A)$^-$u$^+$mRNA, compared to poly(A)$^+$mRNA, observed in the presence of spermine (see Table 10), is consistent with the observation of Igarashi et al (1975) who, using synthetic polynucleotides and E. coli cell free systems, found that the stimulation of polypeptide synthesis by spermidine depends on the uracil content of the "synthetic messenger" used. Another argument for the function of the cytoplasmic poly(A) is whether it is required for the stability of mRNA. To this end the stabilities of both poly(A)$^+$ globin mRNA and enzymatically deadenylated globin mRNA were followed, subsequent to injection into Xenopus laevis oocytes (Huez et al 1974). These experiments showed that "native" globin mRNA is very stable and efficiently translated over several days, whilst poly(A)$^-$ globin mRNA is rapidly degraded (Marbaix et al 1975). The minimal length of poly(A) segment required for stability was found to be 30 nucleotides (Nudel et al 1976). Re-addition of a poly(A) segment to deadenylated globin mRNA restored its functional stability on injection of frog oocytes (Huez et al 1975). Furthermore, it was found that histone poly(A)$^-$mRNA was as unstable as deadenylated globin mRNA in oocytes (Huez et al 1977), whilst enzymatic adenylation of HeLa histone poly(A)$^-$mRNA increased its half-life in oocytes significantly (Huez et al 1978). The role of poly(A) in mRNA stability is, however, not firmly established since deadenylated mengo virus mRNA appears to have the same stability as mengo virus poly(A)$^+$mRNA following injection into frog oocytes

(Revel and Groner 1978). It is possible that specific proteins may bind to the poly(A) segment and protect it from exonuclease degradation, thereby protecting the mRNA (Soreq et al 1974, Bergman and Brawerman 1977). In addition, proteins associated with poly(A), or the poly(A) itself, can be viewed as factors promoting the interaction of poly(A)$^+$mRNA with sub-cellular structures (i.e. membranes). However, the stability effect of poly(A) on mRNAs in oocytes seems incompatible with the observation that poly(A)$^-$mRNAs and poly(A)$^+$mRNAs have similar stabilities in HeLa and sea urchin embryos (Milcarek et al 1974, Nemer et al 1974).

In terms of the functional role of the "U-rich" region(s), the observations of Schweiger and Mazur (1975), who detected both cytoplasmic and nuclear protein(s) from rat liver having a high affinity for poly(U), seem quite suggestive. Possibly such protein(s) are analogous to the protein(s) found associated with poly(A) segments of poly(A)$^+$mRNA (see Introduction Section 6.1). It would certainly be of considerable interest to characterise the proteins associated with both Friend cell poly(A)$^+$ and poly(A)$^-$mRNAs.

4. Poly(A)$^-$u$^+$mRNA a special class of poly(A)$^-$mRNA

A special feature reported in this study is the presence of a "U-rich" region in at least some of the poly(A)$^-$mRNA molecules from Friend cells. This raised questions concerning the origin and functional role of the poly(A)$^-$u$^+$ mRNAs, and of the "U-rich" region in particular.

Results presented in this study (Fig 11) indicate that

Friend cell poly(A)$^-$u$^+$mRNAs are transcripts of unique DNA sequences. This is consistent with the work of Nemer *et al* (1975) with sea urchin embryos, although in this latter work only total poly(A)$^-$mRNA was examined. This is in marked contrast to poly(A)$^+$mRNAs, which appear to have a proportion of sequences (20-30%) transcribed from the middle repetitive class of DNA sequences (see Introduction Section 6.2).

Contrasting behaviour between poly(A)$^-$u$^+$mRNAs and poly(A)$^+$ mRNAs in Friend cells was also observed with respect to turnover in the nucleus and transport from the nucleus. The results in Fig 24 and 27 indicate that the possible precursors of poly(A)$^-$u$^+$mRNA appear to be subjected to more rapid turnover, and the exported poly(A)$^-$u$^+$mRNA species tend to appear more rapidly in the cytoplasm than is the case with the possible precursors of poly(A)$^+$mRNAs.

The differences in stability of poly(A)$^-$u$^+$ and poly(A)$^+$ RNAs within the Friend cell nucleus also seems to be reflected in their cytoplasmic stabilities. Generally, analysis of poly(A)$^+$mRNA stability in most eukaryotic systems has revealed the presence of classes of poly(A)$^+$ mRNA with distinct metabolic behaviour (see Introduction Section 6.3). Similar results have been also reported for poly(A)$^+$mRNA, in DMSO induced and non-induced Friend cells, line 745 (Aviv *et al* 1976, Lowenhaupt and Lingrel 1978). The behaviour of sea urchin embryo poly(A)$^-$mRNA during a chase appears to parallel that of poly(A)$^+$mRNA, suggesting that the poly(A)$^-$mRNA may have similar classes

of distinct metabolic behaviour. However, the results shown in Fig 26 indicate that Friend cell poly(A)$^-$u$^+$mRNAs is relatively unstable compared to the poly(A)$^+$mRNA. This discrepancy can be explained on the basis that the poly(A)$^-$u$^+$mRNA may be a specific sub-class of the total Friend cell poly(A)$^-$mRNA. Interestingly, the metabolic behaviour of poly(A)$^+$ and poly(A)$^-$u$^+$mRNAs from both induced and non-induced Friend cells appears to be similar.

Another point in relation to the stability of poly(A)$^-$u$^+$ mRNAs is that, despite their relatively rapid turnover in the cytoplasm, they nonetheless achieve sufficient cytoplasmic concentration to give major products on translation in a cell-free system. Presumably this is a result of their relatively rapid transport into the cytoplasm permitting a measurable steady state cytoplasmic level. A similar situation has also been noted for some "short-lived" HeLa poly(A)$^+$mRNAs (Lenk et al 1978).

The translatability of poly(A)$^-$u$^+$mRNA serves to confirm the view that this RNA class does function as a messenger and, further, the results shown in Fig 9 suggest that at least some of the translation products are perhaps distinct from those coded for by poly(A)$^+$mRNA. There is also the possibility that some of the products, however, may be the same as those coded for by poly(A)$^+$mRNA (This was discussed in Section 1). One aspect of the translation of the poly(A)$^-$u$^+$mRNAs which has not been investigated in this study relates to the question of translation of the "U-rich" region(s). If this region is translated, it will be in contrast to the results obtained for

the messengers studied so far, in which the 3' terminus is not translated (Greenberg 1975, Zezerov 1977). Should translation occur, however, the high proportion of uridine residues coupled with the observed guanylate content could be expected to give, on the basis of the genetic code, a strongly hydrophobic C-terminus to any polypeptide(s) produced in this way.

A final point which must be noted in connection with poly(A)$^-$u$^+$mRNA is its relationship to the total A$^-$mRNA population. Approaches to the question of whether poly(A)$^-$u$^+$mRNA is merely a sub-class of poly(A)$^-$mRNA, or whether all poly(A)$^-$mRNAs also contain a "U-rich" region(s), could involve both a detailed study of purified messenger ribonucleoprotein particles (mRNPs) and use of inhibitors of rRNA transcription or transport, although the former method would be preferable.

5. Friend cell nuclear poly(A)$^+$ and poly(A)$^-$u$^+$RNAs

The detection of polysomal poly(A)$^+$ and poly(A)$^-$u$^+$mRNAs prompted the examination of Friend cell nuclear RNA for possible precursors of these messengers. Initially, the total nuclear RNA was examined for RNA species having high affinity for poly(A)- or poly(U)-sepharose and, when such species were found, the scope of the investigation was extended to examine the possible metabolic relationships between these nuclear RNA species and their cytoplasmic counterparts. Initially, Friend cell nuclear RNA was fractionated into two general classes, one containing and one lacking poly(A) (i.e. poly(A)$^+$ and poly(A)$^-$ RNAs). Separation of eukaryotic nuclear RNA

into these classes was once thought to be a problem. Molloy et al (1974), using poly(U)-sepharose affinity chromatography reported that 50-90% of HeLa cell nuclear poly(A)$^+$RNA failed to bind on the columns, however Nakazato et al (1974) using oligo(dT)-cellulose and prior heat denaturation of HeLa nuclear RNA, reported a complete separation of nuclear poly(A)$^+$ and poly(A)$^-$RNAs. The method employed in this study utilises the greater binding efficiency of poly(U)-sepharose columns (Nemer et al 1975, Salditt-Georgiev et al 1976), together with a prior heat denaturation of the nuclear RNA, and so combines the advantages of both these methods. Furthermore, the results shown in Fig 15 indicate that Friend cell nuclear poly(A)$^-$RNA can be further resolved into poly(A)$^-a^+$, poly(A)$^-u^+$ and non-bound RNA fractions by using a system of poly(A)- and poly(U)-sepharose columns, as well as stepwise elution with increasing formamide concentrations. Interestingly, these distinct classes of nuclear RNA, namely poly(A)$^-a^+$ and poly(A)$^-u^+$RNAs, can also be detected in HeLa and BHK/21 cells (see Results Section 2.2). This confirms the earlier observations of Burdon et al (1976) and Fraser (1975) for the existence of poly(A)$^-$ nuclear RNAs with high affinity for poly(A) in HeLa and BHK/21 cells. Evidence for the existence of nuclear poly(A)$^-$ RNA with high affinity for poly(A) has also been reported in sea urchin (Dubroff 1977). In addition, the detection of a distinct class of nuclear poly(A)$^-a^+$RNA has been shown (see Results Section 2.1 and 2.3), although this has not been investigated in detail. This RNA class merits closer

attention, however, since it appears to exist in many eukaryotic systems (Edmonds et al 1976, Kinniburg and Martin 1976), and possibly may contain messenger RNA sequences (Kinniburg and Martin 1976) or may play some other significant role. The three nuclear RNA classes detected (i.e. poly(A)$^+$, poly(A)$^-$a$^+$ and poly(A)$^-$u$^+$RNAs) can be viewed as a population of naturally occurring molecules, rather than degradation products of much larger "precursor" molecules. Such "precursor" molecules would be expected to contain poly(A)$^+$, oligo(A) and "U-rich" regions and, perhaps as a result of random degradation, to give rise to products forming a single mode of sedimentation. Friend cell nuclear RNA, however, appears to contain these three RNA classes regardless of the method used to prepare nuclei or RNA (see Results Section 2.2), and the elution profile pattern of HeLa and BHK/21 cells hnRNA from poly(U)- or poly(A)-sepharose columns remain unchanged after mixing with unlabelled Friend cell nuclei followed by re-extraction of the RNA.

The size of Friend cell nuclear poly(A)$^+$RNA appears to be relatively small (mean size of 18s) compared to nuclear poly(A)$^+$RNA from other eukaryotes (Derman and Darnell 1974, Dubroff and Nemer 1975, Ryffel 1976, Schmincke et al 1976, Levis and Penman 1977), although similar values have been reported previously for total nuclear poly(A)$^+$ RNA from Friend cells (Getz et al 1975), rat liver (Sippel et al 1977) and Embryonal carcinoma cells (Jacquet et al 1977) who all used a denaturing sucrose-formamide gradient.

Interestingly, when nuclear poly(A)$^+$RNA from Friend and Embryonal carcinoma cells were analysed on non-denaturing SDS-sucrose gradients, the majority of the RNA molecules were found to sediment faster than 45s, even though, in the case of Embryonal carcinoma cells, the nuclear poly(A)$^+$ RNA was denatured using DMSO (see McKnight and Schimke 1974) prior to application on the non-denaturing gradients. Presumably, such discrepancies are due to the well known formation of high molecular weight aggregated RNA molecules, under conditions which are not strictly denaturing (Tso et al 1963, Macnaughton et al 1974, Pinter et al 1974, Sippel et al 1977).

The finding of two general classes of RNA, poly(A)$^+$ and poly(A)$^-$, in the nucleus, corresponding to the two general cytoplasmic classes was not unexpected. Again, as for the cytoplasmic RNA, at least some of the nuclear poly(A)$^-$ RNA molecules have been shown to possess "U-rich" region(s), as judged by their ability to bind to poly(A)-sepharose columns. This raises the possibility that the nuclear poly(A)$^-$u$^+$RNA class is a precursor to the cytoplasmic poly(A)$^-$u$^+$mRNA class. This possibility is also supported by both the kinetic evidence presented in this study, and by the similarity in elution profiles of nuclear and cytoplasmic RNAs. Further insight into this problem could be obtained by hybridisation studies.

A further point for consideration concerns the origin of the "U-rich" region(s) - do these arise as a result of transcription, possibly from (dA-dT) tracts, or as

some post-transcriptional event (or both). Evidence for the transcriptional origin of the "U-rich" region was obtained by Burdon et al (1976) using BHK/21 cells, who could detect no incorporation of [^{3}H] uridine into nuclear poly(A)-binding RNA under conditions of high actinomycin D concentration. This behaviour is in contrast to that obtained for incorporation of [^{3}H] adenosine into performed hnRNA molecules (Darnell et al 1971). The existence of (dA-dT) tracts in BHK/21 cell nuclear DNA was also reported by Shenkin and Burdon (1974), and this again, supports the notion of transcriptional origin of the "U-rich" region. A possible flaw in this evidence could be the existence of an oligo(U) - (and oligo(G)) - polymerase, which are inhibited by high concentration of actinomycin D. As further speculation one may ask at which "level(s)" the "U-rich" region is functional, if indeed, it has a function. That is, is(are) its function(s) expressed in the nucleus or cytoplasm (or both). That its function may be related to development is suggested by the results of Dubroff (1977), who found different relative levels of nuclear RNA with high affinity for poly(A)-sepharose at different developmental stages in sea urchin embryos. Furthermore, that Friend cell nuclear poly(A)$^+$ and poly(A)$^-$u$^+$ classes of RNA do indeed form functionally discrete populations of molecules and are not interelated by any precursor-product relationship is suggested by a number of observations presented in this study. Firstly, the exclusive binding of poly(A)$^+$RNA to poly(U)-sepharose columns and of poly(A)$^-$u$^+$RNA to poly(A)-

sepharose columns suggests that molecules possess either a poly(A) tract or "U-rich" tract but not both, although the possibility of molecules having intra-molecular poly(A). "U-rich" hybrids cannot be entirely excluded. Furthermore, the kinetics of appearance in the cytoplasm (see Fig 27) argue strongly against poly(A)$^-$u$^+$RNA being a product of some poly(A)$^+$RNA precursor since the production of poly(A)$^+$RNA itself seems to require much more time (5-120min, see Introduction 5.4.6) than the production of poly(A)$^-$u$^+$RNA. This argument is also supported by the results presented in Fig 25 showing that blockage of polyadenylation by the drug cordycepin has no effect on the metabolic properties of nuclear poly(A)$^-$u$^+$RNA. Although the above evidence does suggest a lack of a precursor-product relationship between poly(A)$^+$ and poly(A)$^-$u$^+$ RNAs, further studies involving hybridisation techniques are really required before a definitive statement may be made.

6. Metabolic behaviour of Friend cell nuclear RNA and its classes

As it is shown in Fig 21, total Friend cell nuclear RNA appears to decay as a uniform class of RNA molecules with a half-life of 90 min. Similar values have recently been reported for a variety of eucaryotes including HeLa cells (Herman and Penman 1977), avian immature red blood cells (Spohr et al 1974) and duck proerythroblasts (Stair et al 1977). The experiments performed were insufficient to identify any possible stable nuclear RNAs, although

extremely stable nuclear RNAs (half lives in the range 4-20 hrs) have been reported in Xenopus (Anderson and Smith 1976) and pigeon bone marrow cells (Gasaryan et al 1977). As mentioned previously Friend cell nuclear RNA can be separated into at least four classes (i.e. poly(A)$^+$, poly(A)$^-$a$^+$, poly(A)$^-$u$^+$ and non-bound RNAs). Interestingly, each of these nuclear RNA classes exhibits a distinct metabolic character. The results shown in Fig 24 indicate the existence of highly labile poly(A)$^-$u$^+$RNAs. The RNA which binds neither to poly(U)- nor poly(A)-sepharose will be largely composed of rRNA precursors, tRNA precursors, 5s RNA precursors and poly(A)$^-$u$^-$hnRNA, displaying a much longer half-life of 120 min. In contrast to the above classes which behave as single components, the poly(A)$^+$ and poly(A)$^-$a$^+$RNAs appears to be comprised of at least two metabolically distinct sub-populations, one "short"-lived and other "long"-lived (see Results Section 3.1). Such "long"-lived nuclear poly(A)$^+$RNA has also been reported in HeLa cells (Herman and Penman 1977), Drosophila cells (Levis and Penman 1977), CHO cells (Bachelleric et al 1978), and mouse 3T6 (Hendrickson and Johnson 1978). Interestingly, the biphasic turnover of nuclear poly(A)$^+$ RNA does not appear to be due to some disorder of processing arising out of the continuous culture of cells, since similar data have been reported in mouse liver (Chermovskaya and Lerman 1977). Furthermore, the kinetics of decay of newly synthesised nuclear poly(A)$^+$RNA gave an opportunity to estimate the theoretical values for the ratio of "short" and "long" lived nuclear poly(A)$^+$RNA

after various times of labelling, and further, to calculate the ratio of these two metabolically distinct sub-populations at steady-state. These calculations and their mathematical basis are presented below:

If the rate of synthesis of nuclear poly(A)$^+$RNA is represented by S and the decay constant by K, then the change in the amount (M) of nuclear poly(A)$^+$ in cell at any given moment in time is given by the equation:

$$\frac{dM}{dt} = S - KM \qquad (1)$$

(It being assumed that RNA decays with first-order kinetics and is synthesised with zero order kinetics).

Solving the equation (1), the change in the amount (M) of nuclear poly(A)$^+$RNA is given by:

$$M(t) = \frac{S}{K}(1 - e^{-Kt}) + Mo\ e^{-Kt}$$

where Mo is the concentration at t = 0

Considering the labelled species and setting Mo = 0 for t = 0 at the beginning of the labelling period, then:

$$M(t) = \frac{S}{K}(1 - e^{-Kt}) \qquad (2)$$

Thus S and K can be determined from the experimental data. The rate constant K is directly related to the half-life ($t_{\frac{1}{2}}$) of the nuclear poly(A)$^+$RNA by:

$$K = \frac{\ln 2}{t_{\frac{1}{2}}} \qquad (3)$$

Using this relationship, estimates of K can be made from the experimentally determined half-lives. Then, utilising the values K_1 and K_2 obtained for the "short" and

"long" lived nuclear poly(A)$^+$RNA respectively the rates of synthesis can be estimated from the following expressions:

$$M_1(T) = \frac{S_1}{K_1}(1 - e^{-K_1T})$$

$$M_2(T) = \frac{S_2}{K_2}(1 - e^{-K_2T})$$

Where M_1 and M_2 are amounts of "short" and "long" lived poly(A)$^+$RNA respectively present following a labelling period T(mins). Hence, knowing M_1 and M_2 from the experimental data, S_1 and S_2 can be computed. Using these values of S_1, S_2, K_1 and K_2 the relationship in equation (2) may be used to calculate the relative amounts of "long" and "short" lived poly(A)$^+$RNA for any period of labelling. In this way, the different relative amounts of "short" and "long" lived poly(A)$^+$RNA present following the different labelling periods (see Results Section 3.1) used were shown to be consistent.

After a long time of labelling, the relative proportions of M_1, M_2 will be at steady-state level.

A steady-state, $\frac{dM}{dt} = 0$ and so $S = KM$ i.e. $M = \frac{S}{K}$

Hence, $M_1{}^S = \frac{S_1}{K_1}$ and $M_2{}^S = \frac{S_2}{K_2}$ where the superscript S denotes steady-state amounts.

Using this the ratio of "long" lived poly(A)$^+$RNA to "short" lived poly(A)$^+$RNA is given by $\frac{M_2{}^S}{M_1{}^S} = \frac{S_2}{K_2} \times \frac{K_1}{S_1} = \frac{83}{17}$

The relative amounts of nuclear poly(A)$^+$RNA at the steady-state may be regarded as giving the actual ratio of these RNAs within the cell and so the ratio of "long" to "short"

lived Friend cell nuclear poly(A)$^+$RNA may be estimated as about 5.A value of 1.6 has been found for Drosophila cells (Levis and Penman 1977). In addition, by applying the values obtained for HeLa cells poly(A) adjacent nuclear RNA sequences in the described equation a value of about 6 is obtained. Whether or not the observed differences in the ratio has something to do with the evolutionary differences between such diverse species is an open question.

7. Transport of Friend cell nuclear poly(A)$^+$ and poly(A)$^-$u$^+$RNAs to the cytoplasm

The problem of the extent of nuclear poly(A)$^+$RNA transport to the cytoplasm is rather controversial. Perry et al (1974), using L-cells, found that the rate of labelling of cytoplasmic poly(A) reached a maximum at a time when the specific activity of nuclear poly(A) continued to rise for a period of several hours. They suggested that not all nuclear poly(A)$^+$RNAs are transported to the cytoplasm as poly(A)$^+$RNA but a certain fraction is degraded in the nucleus. In a similar study, however, Puckett et al (1975) reported that in HeLa cells all nuclear poly(A) is transported to the cytoplasm. A further insight on the extent of transport nuclear poly(A)$^+$RNA has recently been obtained from pulse-chase experiments. Herman and Penman (1977), using "chase" conditions which do not inhibit the continuous synthesis of hnRNA, reported that 3-6% of the rapidly labelled [^{3}H] uridine hnRNA of HeLa cells is transported to the cytoplasm as poly(A)$^+$RNA. Levis and

Penman (1977), using the same methodology estimated that at least 40% of the [^{3}H] uridine rapidly labelled nuclear poly(A)$^+$RNA from Drosophila cells is transported to the cytoplasm as poly(A)$^+$RNA. In a similar study, but using Friend cells I found that about 20-30% of the rapidly labelled [^{3}H] uridine nuclear poly(A)$^+$RNA is transported to the cytoplasm as poly(A)$^+$RNA. A value of about 25% has been reported for L-cells (Lattore et al 1973) and 70% is for Slime molds (Lodish et al 1973) respectively. The extent of conversion of nuclear poly(A)$^+$RNA to cytoplasmic poly(A)$^+$RNA appears to be influenced by whether the cells in question are in growing or resting stage (Johnson et al 1975, Burdon et al 1976), although species differences may also play a role (Lengyel and Penman 1975). Interestingly, the extent of conversion may have been a selective factor during evolution, based on the gradation observed when a number of organisms are examined over the phylogenic scale e.g. Slime molds (70%), Drosophila cells (40%), Friend cells (20-30%), L-cells (25%), HeLa cells (10-24%).

Furthermore, the data presented in Fig 24 and 26 suggest that both "short" and "long" lived Friend cell nuclear poly(A)$^+$RNA may give rise to cytoplasmic poly(A)$^+$RNA, since the half-life of the "short" lived nuclear poly(A)$^+$ RNA is only about 22 min, whilst poly(A)$^+$RNA continues to emerge in the cytoplasm for at least 2 hrs. Similar results have been reported for HeLa cells (Herman and Penman 1977) and CHO cells (Bachelleric et al 1978).

The conversion of Friend cell nuclear poly(A)$^-$u$^+$RNA to cytoplasmic poly(A)$^-$u$^+$RNA appears to be about 20-30%. The extent of transport may again be dependent on the functional state of the cells, evidence for this being the work of Burdon et al (1976). That only a proportion of sea urchin poly(A)$^-$hnRNA is transported to the cytoplasm is also supported by the data of Galau et al (1974) Nemer et al (1974), Hough et al (1975), McColl and Aronson (1978). Here the complexity of gastrula and bastula total hnRNA is about 10 times that of total polysomal mRNA. Interestingly, it was found in both hnRNA and mRNA populations examined, that the poly(A)$^+$RNA population represented only a small proportion of the total complexity. It should be stressed, however, that in all these "pulse-chase" studies only the actual decay kinetics of possible mRNA precursors is followed. It would be of interest to complement these studies with studies following the decay kinetics of the actual messenger sequences themselves using, possibly, an m-DNA preparation (see Galau et al 1974, Hames and Perry 1977) especially in relation to the distribution of messenger sequences between the "long" and "short" lived classes.

The observed transport of only a 10-40% of the total possible mRNA precursors sequences to the cytoplasm may be explained in two general ways. Firstly, the existence of larger hnRNA precursors which are processed to smaller molecules prior to transport could account for this e.g. β-globin and ovalbumin gene precursors (see Introduction Section 5.4.6). Alternatively, there may be a class of

RNA whose sequences are never transported. A gene which is transcribed but never transported would fall into this latter class, and would contribute to the nuclear RNA label without appearing in the cytoplasm. Evidence for such behaviour was obtained by a variety of workers. Kleene and Humphries (1977) reported that in the blastula and pluteus stage of sea urchin development hnRNA sequences appear to display very similar complexities, whilst the respective mRNA sequences differ remarkably (Galau et al 1976). Recently Wold et al (1978) reported that virtually all the blastula complex mRNA sequences were present in hnRNA from adult sea urchin tissues, although most of these mRNA sequences were absent from the polysomal mRNA of these tissues. Furthermore, Humphries et al (1976) found mouse β-globin messenger sequences in nuclear RNA from mouse liver and brain. Similarly, Roop et al (1978) found ovalbumin messenger sequences in nuclear RNA from chicken liver and spleen.

ADDENDUM

An initial inspection of the size distribution of the prepared cDNA (Fig 2) suggests a mean size of 300-400 nucleotides. A more accurate interpretation of the data, however, would be provided by a plot of actual numbers of fragments versus size. Such a plot would give a lower mean size than that suggested by Fig 2, since many more small fragments are required to give the equivalent radioactivity of larger fragments. In view of the relatively small size of the cDNA fragments compared to the template poly(A)$^+$mRNA, it would be of interest to extend the experiments performed using a cDNA of longer length, possibly by following the methodology employed by Mackedonski and McConkey (1978). Alternatively, it may be possible to optimise conditions for preparing cDNA to total Friend cell poly(A)$^+$mRNA in a similar manner to that employed by a number of workers using specific poly(A)$^+$mRNA (Efstratiadis et al 1976, Friedman and Rosbash 1977, Buell et al 1978). In this way a more accurate estimation of the sequence complementarity between poly(A)$^+$mRNA and poly(A)$^-$u$^+$mRNA would be possible. In addition, it would be desirable to achieve higher Rot values for the reaction to clarify the nature of the apparent "plateau" obtained (Fig 5).

Although the cell-free translation data presented in Section 1.8 (Results) suggest that poly(A)$^-$u$^+$mRNA may serve as a messenger, the possibility exists that

the translation products are simply due to a stimulation of certain endogeneous wheat-germ messengers, in some way, although 28S ribosomal RNA is without effect (Table 9). To test for such a possibility, a number of controls are possible. Firstly, another cell-free system could be employed, for example reticulocyte lysate system (Pelham and Jackson 1976), and the translation products examined. The characterization of translation products directed by poly(A)$^{-}$u^{+}mRNA should also be performed using two-dimensional gel electrophoresis (O'Farell 1975) for both wheat germ and reticulocyte lysate cell-free system. This would permit a more accurate definition of the translation products. Additional studies to complement the above might employ immunological techniques using, for example, antibodies to total wheat germ protein and antibodies to total Friend cell protein. The former could be used as a probe for endogeneous translation, whilst the latter would provide a means of detecting translation products directed by Friend cell mRNAs.

The melting characteristics of duplexes formed between poly(U) and a variety of sizes of adenylate segments have been studied (Steiner and Beers 1961, Walker 1969). Significant differences in melting temperature have been observed for duplex sizes up to 100 nucleotide pairs long (Steiner and Beers 1961, Walker 1969). No differences can be observed in duplexes larger than 100 nucleotides pairs long. In addition, Bautz and Bautz (1964) have

reported that the melting temperature of poly(AN).poly(U) [where N is any nucleotide and is varied between 0-30%] is affected by the degree of mismatching. A 30% mismatch lowers the melting temperature by 20°C in 0.5M salt. Equivalent results would seem to be obtainable with denaturing solvents; 1% increase in formamide the concentration is equivalent to a 0.72°C decrease in the melting temperature of a DNA.RNA hybrid under 0.1M salt conditions (McConaughy et al 1969). The dramatic difference in the elution profile of [^{3}H] poly(A)$^-$u$^+$mRNA compared to pure [^{3}H] poly(U) (Fig 14) presumably reflects a fairly high degree of mismatching in the poly(A)$^-$u$^+$mRNA.poly(A) hybrids. Probably the size of the "U-rich" region plays a minor role in this respect, since it appears to be relatively long (100-200 nucleotides long, see Discussion, Section 1). Another possible factor is provided by the observation of Kallenbach et al (1973) who found a diminution of melting temperature due to non-duplexed regions, this being under low salt conditions which are similar to these used here. This latter factor may explain the slightly different elution profiles of [^{3}H] nuclear, polysomal poly(A)$^+$mRNAs and [^{3}H] poly(A).

There would appear to be a discrepancy in the results presented for the stability of nuclear poly(A)$^+$RNA, since it is known that a number of precursors for specific messengers e.g. globin, have half-lives of about 5-10

minutes (Bastos and Aviv 1977). This may be compared to the lowest half-life suggested from the presented data of 22 minutes (Fig 24, Fig 28). Such a discrepancy probably highlights the problems of comparing an average result obtained from a large number of different molecules to the corresponding result from a specific member of the class. Thus, the members of the class having relatively short half-lives, such as globin, become "obscured" by those members having relatively longer half-lives possibly as long as 50 minutes. Possibly, a greater resolution of this class could be obtained by examining the metabolic behaviour using smaller time intervals. It is striking that the half-life of 5-10 minutes quoted for specific messenger precursors is similar to that obtained for the nuclear poly(A)$^-$u$^+$RNA. This suggests that the nuclear poly(A)$^-$u$^+$RNA should be examined for specific messenger sequences, possibly by using a specific DNA "probe" complementary to the messenger in question in a DNA-excess hybridization reaction. With regard to the more stable nuclear poly(A)$^+$RNA component (Fig 24, Fig 28), the possibility of cytoplasmic contamination is one which must be carefully considered. Other workers (Humphries et al 1976, Minty et al 1978) using similar techniques for preparation of nuclei, found that steady-state nuclear RNA could be contaminated with up to 1-2% of the globin mRNA sequences present in the cytoplasm. However, after exposure to [^{3}H] uridine for 15 minutes, the total nuclear poly(A)$^+$RNA has at least 10 times more radioactivity than the cytoplasmic poly(A)$^+$RNA (Table 17).

Thus, a cytoplasmic contamination of 1-2% will only produce a contamination of 0.1-0.2% in terms of radioactivity. This suggests that the observed proportion of long-lived nuclear poly(A)$^+$RNA (50% of the total nuclear [^{3}H] poly(A)$^+$RNA, Fig 24) is not significantly due to a cytoplasmic contamination. Indeed, a cytoplasmic contamination of the order of 50% would still only provide contribution of about 5% to the measured radioactivity.

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