HEAT-SHOCK GENE EXPRESSION 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 Instructions to Authors (Biochem. J. (1978) 169, 5-27), with the following additions.

BHK/21	Baby Hamster kidney
BSS	Balanced salt solution
cDNA	complementary DNA
CHO	Chinese Hamster ovary
cpm	counts per minute
DEP	Diethylpyrocarbonate
ds	double stranded
DMSO	dimethylsulphoxide
DNase I	Deoxyribonuclease I
DTT	Dithiothreitol
Hepes	4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid
hnRNA	heterogeneous nuclear RNA
hnRNP	heterogeneous nuclear ribonucleoprotein
hsp	heat-shock protein
kb	kilobases
NP-40	Nonidet P-40 (Non-ionic detergent)
poly(A)$^+$ mRNA	polyadenylated mRNA species
poly(A)$^-$ mRNA	non-polyadenylated mRNA species
poly(A)$^-$u$^+$ RNA	non-polyadenylated RNA species having high affinity for poly(A)
PPLO	Pleuropneumonia-like organism
PPO	2, 5 diphenyloxazole
RSB	Reticulocyte standard buffer
SDS	Sodium dodecyl sulphate
TEMED	N, N', N', Ntetramethylethylenediamine

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SUMMARY

Increase of the incubation temperature of HeLa cells to 40°C for 5 mins followed by recovery of the cells at the normal growth temperature, results in the induction of three particular protein bands (heat-shock protein bands) of molecular weights 100,000, 72,000 - 74,000 and 37,000 daltons. These protein bands are resolved into more than one polypeptides each. Maximum synthesis of these heat-shock polypeptides takes place 2 hrs after the recovery from the heat-shock treatment and declines thereafter. The induction of the heat-shock polypeptides is blocked by actinomycin D, suggesting that their production may be regulated at the transcriptional level.

Heat-shock treatment of HeLa cells results in degradation of pre-existing polysomes. Normal polysomal profile is obtained 1-2 hrs after the heat-shock treatment. mRNA synthesis is slightly affected during the 2 hrs after the heat shock as judged by the incorporation of [^{3}H]-uridine into poly(A)$^+$ mRNA molecules.

In vitro translation, using a rabbit reticulocyte lysate cell-free protein synthesising system, of polysomal mRNA from HeLa and Friend cells has shown that a number of polypeptides are encoded by both poly(A)$^+$ and poly(A)$^-$ mRNAs. Using the same method, it was shown that the prominent 72,000 - 74,000 heat-shock protein band is encoded by seven mRNA species, all of which exist in both poly(A)$^+$ and poly(A)$^-$ forms. The heat-shock polypeptides encoded by these seven mRNA species are designated as α, α', β, γ, δ, ε and ζ.

The poly(A)$^-$ mRNAs coding for the 72,000 - 74,000 polypeptides have been shown to lack the poly(A) tails detected in poly(A)$^+$ mRNAs by the following criteria:

1) Inability of these specific poly(A)$^-$ mRNAs to be retained by poly-(U)-Sepharose after successive chromatography cycles carried out at 20°C or 4°C.

2) Limited retention of partially purified poly(A)$^-$ mRNAs coding for the 72,000 - 74,000 dalton polypeptides by poly-(U)-Sepharose and distinct elution profile of the retained material from that of poly(A)$^+$ mRNA molecules.

Even though increased synthesis of heat-shock proteins declines 4 hrs after the heat-shock treatment, both poly(A)$^+$ and poly(A)$^-$ mRNAs coding for most of the 72,000 - 74,000 heat-shock polypeptides can be detected by the method of *in vitro* translation in the cytoplasm of heat-shocked HeLa cells 6 hrs after the heat-shock treatment. *In vitro* translation of polysomal and post-polysomal RNA fractions detected some of the heat-shock mRNAs in both polysomal and post-polysomal fractions 6 hrs after the heat-shock with a slight enrichment in the post-polysomal fraction.

Double stranded cDNA was synthesised from cytoplasmic poly(A)$^+$ RNA isolated from heat-shocked HeLa cells and cloned into the pst site of the plasmid pBR322. The method of differential *in situ* colony hybridisation and the technique of two-dimensional gel electrophoresis of polypeptides synthesised *in vitro* by mRNAs that hybridise specifically to recombinant plasmids bound to filter discs, have been used to characterise 3 cDNA heat-shock clones. pHS2 contains a cDNA segment that hybridises to mRNA coding for the hspγ, pHS3 contains a cDNA segment that hybridises to mRNAs coding for hspβ, while pHS6 has an inserted cDNA segment that hybridises to mRNA coding for hsp's β, δ and ε.

Immobilisation of the recombinant plasmid DNA from these clones

on filter discs and hybridisation with [^{32}P]- labelled "partially" purified poly(A)$^-$ mRNA which was shown to code for the seven 72,000 - 74,000 hsp's, showed that the poly(A)$^-$ mRNAs coding for hsp γ share common sequences with their polyadenylated counterparts. On the other hand, poly(A)$^-$ mRNAs coding for hsp's β, δ and ε did not hybridise to cDNA sequences derived from the corresponding poly(A)$^+$ mRNAs.

INTRODUCTION

1. Eukaryotic messenger RNA

In living cells the genetic information encoded in the sequence of bases in the double helix of DNA is transcribed into a complementary sequence of RNA bases to form the molecule known as messenger RNA (mRNA), which is subsequently translated into protein. The initial discovery of mRNA was made in bacterial cells. Hersh ey, Dixon and Chase (1953) observed that when E. coli cells were infected with T-even bacteriophages, bacterial DNA transcription stopped and new DNA molecules were formed. Hall and Spiegelman (1961) concluded from hybridisation reactions that the RNA synthesised after infection was a T-even specific RNA. The concept that this newly synthesised RNA constituted a messenger between the DNA and the sites of protein synthesis was postulated by Jacob and Monod (1961). These investigators showed that the viral-induced RNA became attached to pre-existing ribosomes but was metabolically much less stable than 16S and 23S rRNA. Early attempts to identify mRNA in eukaryotes centred mainly on the nucleus. Progress came when investigators shifted from looking for mRNAs in the nucleus to the polysomes (Warner et al., 1963) and employed the following criteria: rapidity of labelling and relative instability compared to other types of RNA (Penman et al., 1963), heterogeneous sedimentation pattern (Henshaw et al., 1965), possession of a "DNA-like" base composition (Davidson, 1969) and their release from polysomes by EDTA or puromycin in the form of slowly sedimenting ribonucleoproteins (Darnell, 1968; Penman et al., 1968; Perry and Kelley, 1968; Mathews, 1973). The existence of mRNAs which are not associated with polysomes, however, has also been reported (Preobrazensky and Spirin, 1978; Vincent et al., 1981) and there is evidence suggesting that certain mRNAs extractable from total cytoplasm are absent or undetectable in polysomal mRNA (Levy and Rizzino, 1977).

1.1. Eukaryotic mRNA 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) A poly(A) segment at the 3' end

Evidence for the occurence of poly(A) sequences in cytoplasmic mRNA was first obtained from the work of Edmonds and Caramela (1969). Later on, Edmonds and colleagues (1971) showed that poly(A) is absent for nucleolar RNA (rRNA), but is present in the RNAs of both nucleoplasm and cytoplasm. The location of the poly(A) segment in mRNA is known to be the 3' end. 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). It is now known that polyadenylation occurs in the nucleus of the nuclear mRNA-precursor molecules (see Introduction, Section 9.1.). Labelled adenosine first incorporated into the poly(A) segment of hnRNA molecules can be "chased" into polysomal mRNA (Darnell et al., 1971; Mendecki et al., 1972). The poly(A) segment of mammalian mRNAs has been shown to undergo a process of gradual size decrease after its appearance in the cytoplasm (Sheiness and Darnell, 1973; Gorski et al., 1974; Merkel et al., 1975; Nokin et al., 1976; Palatnik et al., 1979). A small amount of poly(A), about 8 bases, has been shown to be added in mammalian cytoplasmic mRNAs. These newly added terminal bases are subsequently removed in the shortening reaction (Diez and Brawerman, 1974). Upon fertilisation of sea urchin eggs, in particular, almost complete turnover of the poly(A) tail has been reported which is then followed by extensive polyadenylation in the cytoplasm (Wilt, 1977).

The detection of poly(A) segment in mRNAs has allowed the isolation of mRNAs by affinity chromatography on poly(U)-Sepharose or oligo(dT)-cellulose. Even though a large proportion of cellular mRNAs contain poly(A) tails ($poly(A)^+$ mRNAs), there is a class of mRNAs which seem to lack poly(A) tail ($poly(A)^-$ mRNAs). Poly(A) segments have also been found in mRNAs from

mitochondria (Perlman et al., 1973) and viruses (Kates, 1970) and yeast (Fahrner et al., 1980).

(b) A 5' "cap"

"Capping" occurs on the nuclear mRNA precursors early after transcription (see Section 9.1.). Pulse chase experiments have shown that label in the "caps" can be followed from hnRNA to mRNA (Perry and Kelley, 1976). Two types of caps are detected in mRNAs: type I (m^7G(5')ppXmY) and type II (m^7G(5')ppp(5')XmYmZ). Type I "caps" are also found in hnRNA molecules, while type II "caps" are only found in cellular mRNAs (Friederici et al., 1976). It has recently been reported that cap II methyltransferase activity is found exclusively in the cytoplasm of HeLa cells, while cap I methyltransferase activity is also detected in the nucleus (Langberg and Moss, 1981).

The function of the 5' "cap" is not completely clear. Two types of experiments have suggested that "caps" are important for efficient translation. The first has shown that "decapped" or uncapped mRNAs are translated in vitro in a variety of systems less efficiently than "capped" mRNAs (Shatkin, 1976). The second has shown that 7 methylguanosine 5' monophosphate ($m^7G5'p$) inhibits the translation of "capped" mRNAs. However, mRNAs which are "uncapped" in vivo can be translated in vitro without suffering inhibition by $m^7G5'p$ (Shafritz et al., 1976; Weber et al., 1976; Hickey et al., 1976). So, even though the 5' terminal "cap" has been shown to promote mRNA translation in vitro (Shatkin, 1976) the existence of uncapped eukaryotic mRNAs which are efficiently translated both in vivo and in vitro argues against the absolute necessity of the 5' "cap" for ribosome binding and efficient translation (Kozak, 1978).

(c) Internal methylation

Internal methylation produces N^6 methyladenosine (m^6A) and smaller amounts of N^5 methylcytosine (m^5C) in the mRNA molecules (Shatkin, 1976). The location of m^6A in the poly(A)$^+$ mRNA was suggested to be in

both 3' and 5' portions of mouse cells (Perry et al., 1975) but only in the 5' portion of HeLa cells poly(A)$^+$ mRNA (Salditt-Georgieff et al., 1976). Both poly(A)$^+$ and poly(A)$^-$ non-histone mRNAs of sea urchin embryos have been shown to contain internal m^6A (Surrey and Nemer, 1976). However, internal m^6A has not been detected in mouse and rabbit α- and β-globin mRNA (Cheng and Kazazian, 1977; Heckle et al., 1977; Lockard and Rajbhandary, 1976), histone mRNAs from sea urchin embryos (Surrey and Nemer, 1976), and HeLa cells (Stein et al., 1977) and Bombyx mori silk fibroin mRNA (Yang et al., 1976).

(d) Careful size measurements have indicated that eukaryotic 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, 1975). Sequence analysis has revealed the existence of non-translated regions at the 5' and 3' ends of various eukaryotic messages (Lewin, 1980). For example, rabbit β-globin mRNA contains 53 untranslated nucleotides at the 5' end and 95 untranslated nucleotides at the 3' end (Baralle, 1977; Proudfoot, 1977). Similar results have been obtained for α-globin mRNA (Lockhard and Rajbhandary, 1976), chick ovalbumin mRNA (McReynolds et al., 1978) and mouse dihydrofolate reductase mRNA (Nunberg, 1980).

The length of the 3' noncoding region varies (Proudfoot and Brownlee, 1976) and it's role is still unclear. Recent data of Kronenberg, Robert and Efstratiadis (1979) have shown that the 3' noncoding region of rabbit β-globin mRNA is not required for in vitro translation. This comes in agreement with the results of Nunberg (1980) who detected a dihydrofolate reductase mRNA lacking the 850 nucleotides long 3' noncoding sequence and which codes for dihydrofolate reductase in translation assays. In globin, ovalbumin and in an immunoglobulin mRNA, the hexanucleotide AAUAAA occurs about 20 bases before the 3' terminus (Proudfoot and Brownlee, 1976). This hexanucleotide within the 3'-noncoding region has been suggested to serve as a signal for polyadenylation (Proudfoot and Brownlee, 1976) or

termination (Nunberg, 1980).

Sequence analysis of the non-coding sequences of rabbit and human β-globin mRNAs has revealed that the 3' noncoding sequences are quite well conserved throughout evolution (Kafatos et al., 1977; Proudfoot, 1977). The 3' noncoding regions of mouse dihydrofolate mRNA, hen ovalbumin mRNA, mouse and human β-globin mRNA are represented as a continuous region in the genome uninterrupted by intervening sequences (Nunberg, 1980).

The 5'-noncoding sequences are also variable in size (Baralle and Brownlee, 1978). Part of the 5'-noncoding region may be involved in ribosome binding (Lewin, 1980).

1.2. Isolation and characterisation of individual messengers

The isolation of individual mRNA species until recently was possible only for proteins synthesised in large quantity by some particular cell type. Relying upon the presence of large amounts of a single messenger in the polysomes, it was possible to obtain somewhat purified preparations from which the mRNA could be characterised. The paradigm for this approach is globin mRNA, which comprises a 9-11S fraction representing some 2% of the total RNA of the avian or mammalian reticulocyte (Chantrenne et al., 1967). Another approach, which can be applied to messenger RNAs present in reasonably large but not such overwhelming amounts, is to precipitate polysomes engaged in synthesis of some protein by reaction with antibody against the protein. This approach was first used successfully with immunoglobulin mRNA in myeloma cells and albumin in rat liver (Schechter, 1974; Taylor and Schimke, 1974). The more recent development of cloning technology since has made it possible to isolate any mRNA by virtue of its reaction with a cloned sequence. It is also possible to use immunoprecipitation of polysomes to obtain a preparation of mRNA sufficiently purified to allow cloning techniques then to be applied to obtain complete purity (Strair et al., 1977).

Isolated mRNA may be characterised by translation in two types of system. Reconstituted cell-free systems, comprising ribosomes, protein synthetic factors, and tRNAs, can be obtained from various sources. The most common are wheat germ, rabbit reticulocyte, and mouse ascites tumour cells but many others have been used (Roberts and Paterson, 1973; Pelham and Jackson, 1976; Mathews et al., 1972; McDowell et al., 1972). Usually the cell-free systems work relatively inefficiently. Each messenger is translated only a few times before activity ceases, generally within 90-20 minutes. The Xenopus oocyte provides an alternative in which the in vivo condition of the protein synthetic apparatus is assured. The system is more efficient and translation continues for 24-48 hrs. First characterised for globin mRNA, the oocyte system has since been used with many other mRNAs and it has been possible to follow coupled transcription and translation of injected DNA (Lane et al., 1971; Berns et al., 1972; Marbaix and Lane, 1972; De Robertis and Mertz, 1977).

1.3. Polysomal mRNA-protein complexes

Most of the messenger RNA in living cells is found associated with ribosomes in the polyribosomes or polysomes. Treatment of polysomal fractions with EDTA or puromycin results in dissociation of ribosomes and release of the mRNA as an mRNA-protein complex (mRNPs). The released mRNP bands on CsCl at 1.40-1.45 $g\text{-}cm^{-3}$ (Perry and Kelley, 1968). Isolation of polysomal mRNP was initially performed by sucrose gradient sedimentation. Later on, electrophoresis of mRNP and affinity chromatography on oligo(dT) cellulose was adopted.

The polypeptide composition of the polysome released mRNPs has been studied in various cells: KB cells (Venrooij et al., 1981), HeLa cells (Kumar and Pederson, 1975), Ehrlich ascites (Barrieux et al., 1975), L cells (Greenberg, 1977; Setyono and Greenberg, 1981), mouse kidneys (Irwin et al., 1975). Usually two major proteins of 49,000-52,000 and 73,000-78,000 daltons were found associated with most of the mRNA species

investigated. However, the presence and number of additional proteins was variable and these variations may arise from the different methods employed for the isolation of mRNP (Preobrazhensky and Spirin, 1978). Better information has been obtained with individual mRNPs. For example, the 15S globin mRNP released from polysomes with EDTA has been shown to contain a major 73,000 dalton polypeptide, 7 minor basic polypeptides with molecular weights ranging from 45,000 to 68,000 dalton and 5 acidic components in the 80,000 to 130,000 molecular weight (Vincent et al., 1981).

The 73,000-78,000 dalton protein has been shown to be associated with the poly(A) tail of the mRNA molecules of reticulocyte and L-cell polysomes (Blobel, 1973) and in HeLa cells (Kish and Pederson, 1976; Schwartz and Darnell, 1976). The poly(A) tail of polysome dissociated globin mRNP has been shown to be associated with two proteins of molecular weights of 73,000 and 47,000 (Vincent et al., 1981). These poly(A) associated proteins of globin mRNP have been shown to be associated with sequences adjacent and nonadjacent to the poly(A) tail (Goldenberg et al., 1980). It has also been suggested that these interactions may result in the folding back of the mRNA on itself (Goldenberg et al., 1980). Such secondary structures at the 3' end of the mRNA molecule could play an important role in the termination of mRNA translation (Pelham, 1978), diminishing the possibility of read-through as described for globin mRNA (Geller and Rich, 1980). It has also been proposed that more than one of the 73,000 M: proteins may be aligned along the poly(A) tail (Venrooij et al., 1981). Such a possibility is supported by the recent finding that the 3'-poly(A)-ribonucleoprotein of mRNA has a characteristic repeating structure with a periodicity of about 27 residues (Baer and Kornberg, 1980). In the case of L-cell and duck mRNPs, it has been proposed that the poly(A)-associated proteins are in dynamic equilibrium with free proteins (Setyono and Greenberg, 1981; Vincent et al., 1981).

Little is still known about the role of the other proteins associated with mRNA molecules. It had been initially proposed that the 52,000 dalton protein associated with mRNA in Ehrlich ascites cells could be identical to the 52,000 dalton subunit of eIF-2 (Barrieux and Rosenfeld, 1977), but this conclusion is not supported by further investigation (Barrieux and Rosenfeld, 1978).

2. Relation of mRNA to the genome

The extraordinarily large amount of potential genetic information in animal DNA 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 molecules. The first approach is based on saturation hybridisation in which trace amounts of highly radioactive single-copy DNA are annealed to saturation with an excess unlabelled RNA. In this case the complexity of mRNA is calculated from the percentage of unique DNA driven into DNA-RNA hybrids (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), prepared using reverse transcriptase, and the poly(A)$^+$ mRNA itself (Birnie et al., 1974; Bishop et al., 1974).

Even though in some cases both approaches have been reported to yield qualitatively similar results (Bishop et al., 1974; Axel et al., 1976; Hahn et al., 1980), in others widely different estimations of complexity have been obtained (Ryffel and McCarthy, 1975; Bantle and Hahn, 1976; Kleinman et al., 1977).

In general, cDNA measurements tend to underestimate complexity because of the difficulty in estimating the high complexity low abundance class of mRNAs. An additional problem is the validity of the assumption that cDNA accurately reflects the number and distribution of mRNA sequences. Another initial drawback of this method was its restriction to poly(A)$^+$

mRNAs. However, this drawback has been overcome since cDNA can be transcribed from poly(A)$^-$ mRNA using oligonucleotides as primers (Van Ness et al., 1979).

On the other hand, the method of saturation hybridisation has two major disadvantages. 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 (< 4%) making any small variation quite significant.

Using the experimental approaches mentioned above, it has been shown that about 1-4% of single copy DNA is transcribed into poly(A)$^+$ mRNA (Bishop et al., 1974: Galau et al., 1974; Birnie et al., 1974; Bantle and Hahn, 1976; Kleinman et al., 1977; Ryffel and McCarthy, 1975). A much larger value, 5-8%, was obtained for polysomal mouse fibroblast mRNA (Grady and Campbell, 1975). Also, in mouse brain cells the percentage of single copy DNA hybridising with polysomal mRNA was about 8% (Van Ness et al., 1979), almost half of which was hybridising only with polysomal poly(A)$^-$ mRNA.

In general, these data suggest that about 10^4-10^5 genes are expressed during the life span of a higher eukaryote (Galau et al., 1974; Grady et al., 1978; Bishop et al., 1974; Bantle and Hahn, 1976). Some of the gene transcripts are present in only a few copies per cell, while others occur in relatively large numbers per cell (Galau et al., 1974; Bishop et al., 1974; Birnie et al., 1974). The transcript numbers of the expressed genes are dependent upon the developmental state of the cell (Birnie et al., 1974; Galau et al., 1974; Hereford and Rosbash, 1977; Paterson and Bishop, 1977).

The same techniques of saturation and kinetic hybridisation had been employed to find out whether abundant mRNAs are transcribed from genes present as multiple copies in the genome. 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; Wilson and Melli, 1976). At the same time ovalbumin gene in chick oviduct (Harris et al., 1973) and the δ-crystallin gene in chick embryonic lens (Zelenka and Piatigorsky, 1976) have been shown to exist in a single copy while fibroin gene is found in less than 2-3 copies in the genome of Bombyxmori (Suzuki et al., 1972; Gage and Manning, 1976). Similarly, there are two copies for α-globin in the human genome and a single copy for human β-globin gene (Toltoshev et al., 1977; Old et al., 1976; Ottolenghi et al., 1975). Most of these results have been lately confirmed by the use of restriction enzyme analysis of the genome.

3. Nature of eukaryotic DNA

Before going into the description of the organisation of some protein coding genes and the biosynthesis of the precursor mRNA molecules in the nucleus, it is important to describe the nature of eukaryotic DNA from which these molecules originate by the process of transcription.

It was in 1968 that Britten and Kohne demonstrated that eukaryotic DNA sequences fall into three frequency classes: a highly repetitive, an intermediate repetitive and a unique fraction. Molecular hybridisation studies have allowed the examination and characterisation of the various frequency DNA sequences. Thus it was shown that unique DNA consists of the sequences which code for most enzymes, while rRNA, tRNA and histone mRNA are thought to be transcribed from middle-repetitive DNA.

A large proportion of the DNA from eukaryotic organisms is organised with alternating regions of unique and repeated sequences. In Drosophila, middle-repetitive sequences of average length 5,600 bases long are interspersed with non-repetitive sequences longer than 13,000 base pairs long (Manning et al., 1975). A different interspersion ratio has been detected in other genomes. Unique sequences of about 1,000 base pairs are interspersed with repetitive sequences of about 300 base pairs for about 50% of the genome and unique sequences of several thousand

base pairs are interspersed with repetitive sequences for a further 30-40% in Xenopus, slime mould, protostome and human genome (Davidson et al., 1973; Firtel and Kindle, 1975; Angerer et al., 1975; Schmid and Deininger, 1975). The 300 nucleotide interspersed sequences are closely related to one another and in particular contain a highly conserved 30 nucleotide segment present in both human and hamster DNA (Jelinek et al., 1980). These sequences have been shown to be transcribed as part of the hnRNA (Robertson et al., 1977) and to serve in vitro as templates for RNA polymerase III (Duncan et al., 1979). Small RNAs associated with cytoplasmic poly(A)$^+$ RNA have been shown to be complementary to these sequences in Chinese hamster cells (Jelinek and Leinwand, 1978). It has also recently been reported that poly(A)$^+$ RNA from HeLa cells contains sequences complementary to this repeated family (Calabretta et al., 1981). Similar repeated sequences have been shown to exist amongst human globin genes (Fritsch et al., 1980). Longer repeated sequences (about 5,000 base pairs long) have also been reported in mammalian genome (Singer, 1982). One of these sequences has been shown to exist downstream from the end of the human β-globin gene (Adams et al., 1980).

4. Eukaryote gene structure

A unique characteristic of eukaryotic structural genes is that they have intervening sequences known as "introns" within the gene itself. The length of these intervening sequences varies and so does the number of intervening sequences within genes. For example: Xenopus laevis vitellogenin genes contain at least 33 introns, while chicken and human preproinsulin gene contains only two introns (Breathnach and Chambon, 1981) and chicken ovalbumin gene seven introns (Royal et al., 1979). Current evidence suggests that these intervening regions are transcribed into RNA (see Section 12) and then removed by internal processing events in the cell nucleus until the RNA transcript is the size of the final mRNA product (see Section 13). Intervening sequences appear to have a wider

evolutionary freedom for mutation than the coding sequences of the DNA (Lomedico et al., 1979; Abelson, 1979).

Intervening sequences have also been discovered in DNA sequences giving rise to 28S rRNA in Drosophila and 21S rRNA in yeast (Abelson, 1979). Some, but not all, yeast tRNA genes have been shown to contain intervening sequences (Abelson, 1979). Intervening sequences have not been detected in genes coding for histones (Kedes, 1979) or human interferon (Nagata et al., 1980; Houghton et al., 1981).

5.0. Organisation of specific protein-coding nuclear genes

5.1. Globin genes

Detailed analysis of the physical map both in rabbit and mouse has shown that the β-globin gene does not exist as a contiguous stretch of DNA, but is present in three coding blocks (exons) separated by both a large and a small intervening sequence (or intron) (Van den Berg et al., 1978). Hybridisation of clon ed β-globin fragments with globin mRNA allowed the formation of "R loops" and thus the visualisation of the larger intervening sequence (Tilgham et al., 1978)[a,b] while the detection of the small intervening sequence was more difficult. A detailed sequence examination of the large and small intervening sequences from mouse and rabbit β-globin genes showed them to be about equal length (large, 646 and about 580 base pairs, small 115 and 126 base pairs for mouse and rabbit respectively (Abelson, 1979), and moreover they occur in precisely the same positions relative to the coding sequence. On the other hand the homologous intervening sequences show very little sequence similarity which is mostly evident at the junctions with coding sequences (Abelson, 1979). Intervening sequences have also been detected in chicken β-globin genes (Richards et al., 1979) and human β, δ and γ genes (Little et al., 1979).

Similarly, the mouse α-globin genes have two inserts at positions analogous to the β genes, although of smaller size (Nishioka and Leder, 1979).

5.2. Ovalbumin genes

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

5.3. Vitellogenin genes

Studies of Xenopus laevis cDNAs have shown that vitellogenin is encoded by a small family of at least four genes (Wahli et al., 1979). These genes fall into two pairs. Members of a pair are about 95% homologous in sequence and 80% homologous to members of the other pair (Wahli et al., 1980). The genes coding for both members of one of these pairs have been cloned and have shown marked similarities. In both genes the mRNA-coding sequence of 6,000 base pairs is interrupted 33 times (Wahli et al., 1980). The introns interrupt the structural sequences at homologous positions in both genes (Wahli et al., 1980).

5.4. Insulin genes

Chicken and human have a single preproinsulin gene (Perler et al., 1980; Bell et al., 1980). Cloning and sequencing of these genes has revealed that each one contains two introns similarly located. One within the sequences coding for the mRNA's 5' untranslated region and the other interrupting the C-peptide coding region (Perler et al., 1980; Bell et al., 1980). Rat, however, has two genes one of which contains two introns similar in structure to human and chicken genes while the second one lacks the intron interrupting the C-peptide coding region (Lomedico et al., 1979; Cordell et al., 1979)

6. Eukaryotic RNA polymerases.

The apparatus responsible for the production of RNA is the enzyme RNA polymerase. Unlike the situation in prokaryotic cells, eukaryotes have several DNA dependent RNA polymerases. It was Roeder and Rutter in 1969 who separated the different RNA polymerases and classified them as RNA polymerase I, II and III according to their order of elution from DEAE-Sephadex column by increasing ionic strength of ammonium sulphate. RNA polymerase I has been shown to be of nucleolar origin while RNA polymerae II and III exist in the nucleoplasm. It is known that the eukaryotic RNA polymerases are macromolecular multi-subunit enzymes with a molecular weight around 500,000 (Paule, 1981). In all the examined cases they have been shown to consist of two high molecular weight subunits larger than 100,000 daltons and a number of smaller subunits (Jendrisak and Burgess, 1977; Teissère et al., 1977; Guilfoyle et al., 1976). It seems that the large subunits of the three enzyme classes are of different sizes (Chambon, 1975; Jendrisak and Burgess, 1977). The different classes of RNA polymerase have been shown to operate optimally under different conditions. For example, RNA polymerase I requires low ionic strength and Mg^{2+} for maximum activity, while RNA polymerases II and III operate optimally at high ionic strength and Mn^{2+} (Roeder, 1976). The different classes are also differentially affected by α-amanitin. In animal, plant and insect cells RNA polymerase II is inhibited by low levels of α-amanitin whereas RNA polymerase I is not affected even at very high levels (Jacob, 1973). RNA polymerase III is affected by α-amanitin but at higher concentrations.

Data from experiments with α-amanitin had initially suggested that RNA polymerase II is responsible for the transcription of heterogeneous nuclear RNA (hnRNA), while RNA polymerase I for rRNA synthesis (Bitter and Roeder, 1978). It has recently been proposed that RNA polymerase II is also responsible for the transcription of some small

nuclear RNAs (snRNAs) (Zieve, 1981) while RNA polymerase III is responsible for the transcription of tRNA genes and genes coding for small RNA species (Weil et al., 1979; Wu, 1980). Experiments with soluble cell-free systems from cultured human cells has revealed that accurate initiation of genes encoding mRNA species requires not only RNA polymerase II but additional soluble factors (possibly proteins) (Weil et al., 1979b; Luse and Roeder, 1980; Wu, 1978; Wasyslyk et al., 1980).

Since differential alterations in the rate of synthesis of the major classes of RNA (rRNA, mRNA, tRNA) has been observed during a number of physiological changes and given that they are transcribed by different enzymes a question arising is whether changes in gene activity are modulated by changes in the amounts of the RNA polymerases. Supporting evidence for this possibility has been reported for regenerating liver (Roeder, 1977). Also, it has recently been suggested that the increased concentration of RNA polymerase I is responsible for the high rate of nucleolar transcription in rat liver (Yu, 1980). On the other hand, data showing that during the development of Xenopus laevis when qualitative and quantitative changes in gene expression occur, the absolute and relative amounts of RNA polymerases remain the same (Roeder et al., 1974), argue against it.

7. Structure of chromatin

In eukaryotic cells DNA is not found naked but in chromosomes or chromatin during interphase. Chromatin consists of DNA complexed with histone and non-histone proteins and RNA. Biochemical and biophysical studies have shown that the structure of chromatin is considerably ordered. It consists of an array of repeating units (nucleosomes). The core of each nucleosome consists of 145 base pairs DNA fragment arranged around the outside of an octamer of histones. The octamer contains two copies of H2A, H2B, H3 and H4 histones. Each nucleosome is separated from the next one by a segment of DNA, which is termed "linker" DNA. The length of the linker varies from about 15 base-pairs to about 100 base-pairs

depending upon the organism and tissue from which the nucleosomes are isolated (Felsenfeld, 1978; Mathis et al., 1980). It has been proposed that shorter repeat lengths are correlated with higher levels of gene activity (Morris,1976; Thomas and Thomson, 1977), although this proposal does not seem to hold for lower eukaryotes (Lohr . . and Ide 1979). Early experiments had suggested that histone H_1 is associated with the linker DNA between core particles (Varshavsky et al., 1976; Whitlock and Simpson, 1976), but recent data have suggested that histone H_1 is localised at the point where DNA enters and exits from the core particle (Thoma et al., 1979).

Two levels of higher organisation of chromatin structure have been reported. The first appears as a thin filament 100 Å in diameter and the second as a thicker fibre with a diameter of 200-300 Å. The thin fibre is almost certainly a linear array of nucleosome cores in contact with one another, while the thick fibre seems to be generated by coiling of the thin filament (Felsenfeld, 1978; McGhee and Felsenfeld, 1980). At low ionic strength, the 100 Å fibril appears upon electron microscopy in a zigzag configuration with a two-nucleosome repeat (Thoma et al., 1979). When the ionic strength is raised a new form of structures begin to form. The most compact structures are consistent with a superhelical model of 6-7 nucleosomes per turn organised in a 300 Å filament (McGhee and Felsenfeld, 1980). A number of hydrodynamic and electron microscopic studies have suggested that histone H_1 may play a role in the formation of the highly ordered form of chromatin. For example, readdition of histone H_1 to H_1-depleted chromatin leads to chromatin contraction (Thoma and Køller, 1977).

Two approaches have been used to find out whether transcriptionally active chromatin is organised in nucleosomes: that of electron microscopy and digestion with nucleases. The use of electron microscopy has revealed nucleosome-like beads interspersed between the transcribing RNA polymerase molecules in the Balbiani rings of Chironomus tentans, which

is an example of nonmaximally transcribed gene (Lamb and Daneholt, 1979). On the other hand, studies on the morphology of very actively transcribed genes in Drosophila melanogaster embryo chromatin (McKnight and Miller, 1976) and in the putative transcriptional unit of silk fibroin gene (McKnight et al., 1976) have revealed no nucleosome-like particles between the molecules of RNA polymerase. Similarly, nucleosomes appear to be absent from very actively transcribed ribosomal genes in Xenopus oocytes (Scheer, 1978) and in Oncopeltus fasciatus (Foe et al., 1976). Electron microscopy data on the structure of spacers amongst the rRNA genes are conflicting. In Drosophila melanogaster the spacers amongst the rRNA genes exhibit "beaded" structure characteristic of nucleosomes (Laird et al., 1976), while in Xenopus oocytes they do not (Scheer, 1978).

Nuclease digestion has revealed that "active" chromatin is more susceptible to DNase I digestion. This was initially shown in the case of globin genes in chick erythrocytes (Weintraub and Groudine, 1976). Hypersensitivity to DNase I has since been detected for several "active" genes: the ovalbumin gene in hen oviduct (Garel and Axel, 1976), the ribosomal genes in various organisms (Stalder et al., 1978; Mathis et al., 1980) and the induced heat-shock loci in Drosophila tissue culture cells (Wu et al., 1979). Even though DNase I hypersensitivity is localised to messenger-coding sequences along a DNA segment (Mathis et al., 1980), this does not seem to be due to the transcriptional machinery since non-transcribed genes have also been shown to be hypersensitive to DNase I digestion. For example, the sequences coding for the preshock mRNA population in Drosophila are as sensitive to DNase I before and after the heat shock treatment (Biessmann et al., 1977). Similarly, a complex subset of genes rarely represented in the mRNA population of hen oviduct cells is as sensitive to DNase I as the ovalbumin gene (Garel et al., 1977). This hypersensitivity to DNase I may reflect changes in the nucleosome particle (e.g. formation of half nucleosomes). There is evidence that

the "high mobility group" (HMG proteins) may promote DNase I sensitivity. For example, the sensitivity of globin genes to DNase I is reversed when the HMG proteins are eluted with 0.35M salt and restored when the eluate is added back to the washed chromatin (Weisbrod and Weintraub, 1979).

Several data had initially proposed that "active" chromatin is not preferentially degraded by micrococcal nuclease. For example, in avian reticulocytes the globin genes which are actively transcribed exhibit the same pattern of digestion by micrococcal nuclease as all other chromatin DNA (Weintraub and Groudine, 1976). However, it has been recently shown that micrococcal nuclease can recognise some features of "active" chromatin. For example, expressed ovalbumin gene sequences have been shown to be preferentially excised in hen oviduct (Bloom and Anderson, 1978). Similarly, the excision of "active" sequences by micrococcal nuclease has been demonstrated for the Drosophila heat-shock loci (Wu et al., 1979) and for "active" rRNA genes in Xenopus (Reeves, 1978). There is also suggestive evidence that "active" chromatin is specifically recognised by DNase II (Mathis et al., 1980).

8. Sequences involved in the control of transcription

Prokaryotic promoters are those sequences of the DNA indispensable for initiation of transcription to which RNA polymerase binds. They contain a sequence of homology related to the 5' TATAATG 3' (Pribnow box) located about 10 base pairs upstream from the mRNA start site (Rosenberg and Court, 1979; Siebenlist et al., 1980). A second region of homology referred to as "recognition region" has been found in a region 35 base pairs upstream from the mRNA start site (Rosenberg and Court, 1979; Siebenlist et al., 1980).

From the information obtained so far from eukaryotic genes it seems that more than one elements could be important for transcription of eukaryotic genes. One element, which corresponds to the "ATAA" box region has been detected 25-30 base pairs upstream from the mRNA start site of all as yet sequences eukaryotic mRNA coding genes transcribed by RNA polymerase II with the exception of adenovirus 2 early gene (Baker et al., 1979) and papovavirus late genes (Breathnach and Chambon, 1981). A region that includes the "ATAA"

box has been shown to be required for faithful in vitro transcription of rabbit globin genes (Grosveld et al., 1981) and for the major adenovirus late genes (Hu and Manley, 1981). Also, a single base change (ATAA to AGAA) reduces the level of in vitro transcription from the conalbumin gene by a factor of 10 (Wasylyk et al., 1980). In contrast, deletion of sequences including the TATAA box from the 5' end of the SV40 early genes has little or no effect on the amount of early transcripts made in vitro (Mathis and Chambon, 1981) or in vivo (Benoist and Chambon, 1981). The TATAA box is believed to be necessary for accurate initiation (Paul, 1982), because both in those instances in which it is naturally missing (Benoist and Chambon, 1981) and in those in which it has been deleted multiple initiation points are observed (Benoist and Chambon, 1981; Grosveld et al., 1982).

A second element, the CAAT or Chambon box located 70-80 bases upstream the 5' end of mammalian globin genes (Efstratiadis et al., 1980) and many other sequenced genes (Benoist et al., 1980). This sequence seems to be required for in vivo transcription of α-globin genes (Mellon et al., 1981) and β-globin genes (Grosveld et al., 1981). Its deletion from α-globin (Mellon et al., 1981) and β- globin genes (Grosveld et al., 1981) has been shown to result in decrease of transcription. On the other hand, deletion of the same region in sea urchin H2A histone genes stimulates RNA synthesis (Grosschedl and Birnstiel, 1980a).

Sequences farther upstream from the 5' end of sea urchin histone gene are required for transcription of sea urchin histone H2A genes in Xenopus oocytes (Grosschedl and Birnstiel, 1980) and the SV40 early genes (Benoist and Chambon, 1981) in cells in culture. Although these sequences have no homology to each other, they have similar effects on transcription. The SV40 sequences can enhance the transcription of a cloned rabbit β-globin gene transfected into HeLa cells (Banerji et al., 1981).

Little is as yet known about the sequences specifying termination

of transcription. Some sequence homologies have been reported around the polyadenylation site (Benoist et al., 1980) apart from the AATAAA region found about 20 nucleotides before the 3' terminus of various mRNAs and suggested to serve as site for termination of transcription of polyadenylation (Proudfoot and Brownlee, 1976; Nunberg, 1980). However, no site-directed mutagenesis experiments have as yet confirmed the role of these sequences in termination of transcription.

9. Heterogeneous nuclear RNA

The question of the origin of mRNA has been under investigation for some time. An initial puzzle was the relationship of mRNA to a family of nuclear RNAs designated as heterogeneous nuclear RNA. Heterogeneous nuclear RNA (hnRNA) is the "DNA-like" fraction of nucleoplasmatic RNA which is being rapidly labelled in the nucleus and exhibits heterogeneous size distribution. This class of nuclear RNA is distinct from the precursors of rRNA which are of nucleolar origin, have discreet homogeneous sizes and are being labelled later on (Weinberg, 1973).

9.1. Structural features of hnRNA

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:

(a) A long (180-230 nucleotides) poly(A) segment at the 3' end of some hnRNA molecules. The absence of long (dT) regions in eukaryotic genomes (Shenkin and Burdon, 1974) and data showing that poly(A) addition and hnRNA biosynthesis are differentially affected by actinomycin D and cordycepin (3' deoxyadenosine) (Darnell et al., 1971; Darnell et al., 1973), led to the conclusion that the poly(A) tail is added post-transcriptionally. Polyadenylation takes place by adding one base at a time to nascent large hnRNA molecules or to smaller older hnRNA molecules (Derman and Darnell, 1974). A poly(A) polymerase activity has been identified in both vaccinia virions and infected cells (Moss et al., 1973; Brakel and Kates, 1974) and enzyme

activities able to synthesise poly(A) have been already reported (Edmonds and Abrams, 1960; Winters and Edmonds, 1973).

(b) A short (20-40 nucleotides) internally located oligo(A) segment which is transcribed from the DNA template (Nakazato et al., 1974). These oligo(A) regions are not found in poly(A)$^+$ hnRNA and appear to be absent from mammalian mRNA (Venkatesan et al., 1979). It should be noted however, that Dyctiostelium mRNA does contain internally located oligo(A) segments 20-30 nucleotides long (Lodish et al., 1974).

(c) Short oligo uridylate-rich (oligo(U)) stretches have been found in the hnRNA (Molloy et al., 1974) and mRNA (Korwek et al., 1976) from HeLa cells. In hnRNA these regions occur at the 5' terminus of at least some hnRNA molecules (Molloy et al., 1974) and appear to be transcribed from repetitive regions of the genome (Molloy et al., 1972). Additionally, oligo(U) stretches have also been reported in non-polyadenylated (poly(A)$^-$ hnRNA molecules in HeLa cells (Korwek et al., 1976), BHK/21 cells (Burdon et al., 1976). The possibility of formation of double or treble-stranded regions between oligo(U) and oligo(A) or poly(A) regions has been discussed either as a means for compaction or of "looping-out" of processing sites (Dubroff, 1977; Kish and Pederson, 1977).

(d) A "cap" structure of type I:$m^7G^{5'}ppp^{5'}N'mpN''p$ at the 5' terminus of the hnRNA molecule. Recent data of Salditt-Georgieff and colleagues (1980) have shown that capping occurs very rapidly on the primary transcripts. Even though it was initially believed that caps can be added either to the 5' terminus of the primary transcript or to internal sites after processing of the primary transcript (Schibler and Perry, 1976), recent data from analysis of the composition of the cap I structures of both large and small hnRNA favour the model by which hnRNAs can initiate with any of the four nucleotides and that capping occurs very close to or at the start of hnRNA (Salditt-Georgieff et al., 1980). In support of this hypothesis are data from analysis of precursor molecules for

chicken ovalbumin (Roop et al., 1980), silk fibroin (Tsuda et al., 1979) and mouse β-globin genes (Weaver et al., 1979). Internal N^6-methyl adenines (m^6A) are also found in hnRNA molecules (Salditt-Georgieff et al., 1976).

(e) Mammalian hnRNA also contains double-stranded regions which are resistant to the action of ribonucleases (Jelinek and Darnell, 1972). 4% of the mass of HeLa cell hnRNA is involved in these double-stranded structures (Robertson et al., 1977) and it has been shown that the sequences involved in them are derived from repetitive elements of the genome (Jelinek et al., 1974; Jelinek, 1977). Such double-stranded regions of hnRNA have also been shown to be less tightly bound to proteins than the single-stranded regions (Calvet and Pederson, 1978). The function of these double-stranded regions is not yet clear. Since globin mRNAs have been shown to contain sequences complementary to the double-stranded regions of hnRNA (Ryscov et al., 1976), it is possible that double-stranded regions might serve as cleavage sites for endonucleolytic processing involved in the biogenesis of mRNA. It has also been postulated that the attachment of hnRNA to the so-called "nuclear skeleton" is mediated via the double-stranded regions of hnRNA (Herman et al., 1976).

10. Relationship of hnRNA to mRNA

That some of the hnRNA molecules may serve as precursors of mRNA molecules has been accepted for many years now, even though direct evidence has been obtained only recently. A number of experiments had given indirect evidence for such a relationship.

Labelling kinetic experiments have shown that some of the nucleotides present in the hnRNA enters the cytoplasm (Herman and Penman, 1977). It has also been reported that the poly(A) tail which exists at the 3' end of some hnRNA molecules is conserved and transported to the cytoplasm (Nevins and Darnell, 1978; Puckett et al., 1975, 1976). Kinetic analysis has shown that, in mouse L-cells, the cap I structures

of mRNA are derived from the cap I structures of hnRNA (Perry and Kelley, 1976). Also, comparison of cap-containing sequences in mRNA and hnRNA of the same cells showed a similar sequence composition of hnRNA and mRNA caps (Schibler and Perry, 1976). At the same time, a number of experiments demonstrated homology between mRNA and hnRNA sequences. For example, hybridisation of genomic DNA complementary to mRNA ("mDNA") to various pulse-labelled hnRNA fractions in L-cells suggested that a substantial proportion of the large rapidly labelled hnRNA molecules are potential precursors of mRNA, whilst the small poly(A)$^+$ hnRNA may consist of partly processed derivatives of these molecules, enriched in mRNA sequences (Hames and Perry, 1977).

That mRNA is generally derived from a larger primary transcript constituting a part of the hnRNA population was also suggested from results obtained by the use of the U.V. transcription mapping technique. For example, in HeLa cells it was shown that the length of long nascent hnRNA molecules coincides with the length of the genomic sequence transcribed, while the transcription unit of HeLa cell mRNA was estimated to be at least 3 times larger than the actual size of the mRNA itself and coincided with the transcription unit length of hnRNA (Goldberg et al., 1977).

11. The primary transcript

With the development of the molecular cloning technique it has become possible to identify the primary transcripts of some protein coding genes and to investigate whether the primary transcript contains any sequences transcribed from flanking regions of the corresponding genes.

It seems that initiation of transcription may occur at the nucleotide corresponding to the first capped nucleotide of the mature mRNA as it is suggested from the analysis of precursor molecules for chicken ovalbumin (Roop et al., 1980), silk fibroin (Tsuda et al., 1979), mouse β-globin genes (Weaver et al., 1979) and adenovirus 2 major late transcription unit (Ziff and Evans, 1978). However, initiation of transcription upstream from the cap site followed by rapid processing of the primary

transcript can not be ruled out (Breathnach and Chambon, 1981).

Termination of transcription may occur at (or very close to) the poly(A) addition site as in the case of ovalbumin gene (Roop et al., 1980) or beyond the poly(A) addition site, in which case the 3' ends of the mature mRNAs are generated by endonucleolytic cleavage site. The latter has been shown to occur in the case of adenovirus-2 major late transcription unit (Ziff, 1980), the units for early regions 2 and 4 (Nevins et al., 1980) and the SV40 late region (Ford and Hsu, 1978). Read-through across polyadenylation site can occur as evidenced by the two size mRNAs for gene X(Heilig et al., 1980) and probably for the dihydrofolate reductase gene (Nunberg et al., 1980). An interesting example of read-through is that of the production of the membrane bound form of immunoglobulin M when transcription stops downstream the first polyadenylation site, so that the primary transcript contains two more exons coding for the hydrophobic C-terminus of the protein (Rogers et al., 1980). For the production of the secreted form of the protein transcription stops at the first polyadenylation site and since the two immunoglobulins are produced at different stages of development, it has been suggested that there may be developmental stage-specific recognition of different polyadenylation site (Early et al., 1980).

12. Precursors of specific mRNAs

12.1. Globin mRNA precursor

A 15S globin mRNA precursor has been detected in mouse foetal liver cells (Ross, 1976), Friend cells (Curtis and Weissmann, 1976) and mouse spleen (Kwan et al., 1977). In erythroid cells 15S hnRNA is processed to mature mRNA within 20 minutes (Ross and Knecht, 1978). Examination of this 15S transcript showed it to be capped (Curtis et al., 1977) and to have a 150 nucleotides long poly(A) terminus added post-transcriptionally (Curtis and Weissmann, 1976; Ross and Knecht, 1978; Curtis et al., 1977). Electron microscopy has revealed that no R loops

are formed between 15S RNA and cloned β-globin genes (Tilgham et al., 1978), while more recent data have demonstrated the existence of the β-globin gene intervening sequences in 15S hnRNA (Smith and Lingrel, 1978; Kinniburgh et al., 1978). It has also been shown that both the 5' and 3' terminal sequences of 15S transcript and the mature globin mRNA map at the same site on the genomic DNA (Weaver et al., 1979), suggesting that no sequences are excised from the 5' end during maturation. However, one can not exclude the existence of a larger primary transcript which is rapidly converted to 15S RNA especially since the existence of a 27S globin-precursor has been reported by others (Bastos and Aviv, 1977; Strair et al., 1978).

12.2. Ovalbumin mRNA precursors

Ovalbumin mRNA precursors of variable length have been detected in oviduct nuclei as judged by their ability to hybridise to both structural and intervening sequences of ovalbumin gene (Roop et al., 1978). The largest of them is 7,800 nucleotides long and is believed to be the primary transcript of the ovalbumin gene (Tsai et al., 1980a). This 7,800 nucleotides long nuclear RNA is 200 nucleotides longer than the ovalbumin gene. Since no transcripts of the flanking regions from the 5' or 3' end of the gene have been detected in any of the nuclear precursors (Tsai et al., 1980b) and given that most of the 7,800 nucleoties long nuclear RNAs has been shown to contain a poly(A) tail (Tsai et al., 1980a) the difference in the length between the ovalbumin gene and the 7,800 nucleotides long RNA may be accounted for by the length of the poly(A) tail. The smaller nuclear RNA precursors of ovalbumin RNA are believed to be processing products of the 7,800 nucleotides long nuclear RNA (Roop et al., 1978).

12.3. Immunoglobulin precursor mRNAs

Nuclear precursors of immunoglobulin light chain mRNA have been detected in myeloma cells by hybridising cloned cDNA to nuclear RNA sedimenting through sucrose gradients (Gilmore-Herbert and Wall, 1978). Three size classes of nuclear RNA were shown to be complementary to

immunoglobulin light chain mRNA : 40S, 24S and 13S nuclear and a sequential precursor-product relationship was demonstrated between these RNAs. The size of the largest, 40S, nuclear RNA is in good agreement with the U.V. mapped transcription unit size (10,000 nucleotides) of myeloma light chain immunoglobulin mRNA (Gilmore-Herbert et al., 1978). However, according to more recent data of Schibler and colleagues (1980), the largest detected nuclear RNA precursor of light chain immunoglobulin mRNA is about 5,300 nucleotides long. The discrepancy between the results of this group and those of Gilmore-Herbert and Wall (1978) might be due to the fact that the size estimate of the largest nuclear precursor of the latter group was based on sedimentation velocity in acqeous sucrose gradients. It is also possible that this size discrepancy is related to transcription unit size of different κ light chain genes.

The largest detected precursor of heavy chain immunoglobulin mRNA was shown to be about 11,000 nucleotides long (Schibler et al., 1980) and most of these precursor nuclear RNAs have been shown to be polyadenylated.

13. Processing of pre-mRNA molecules

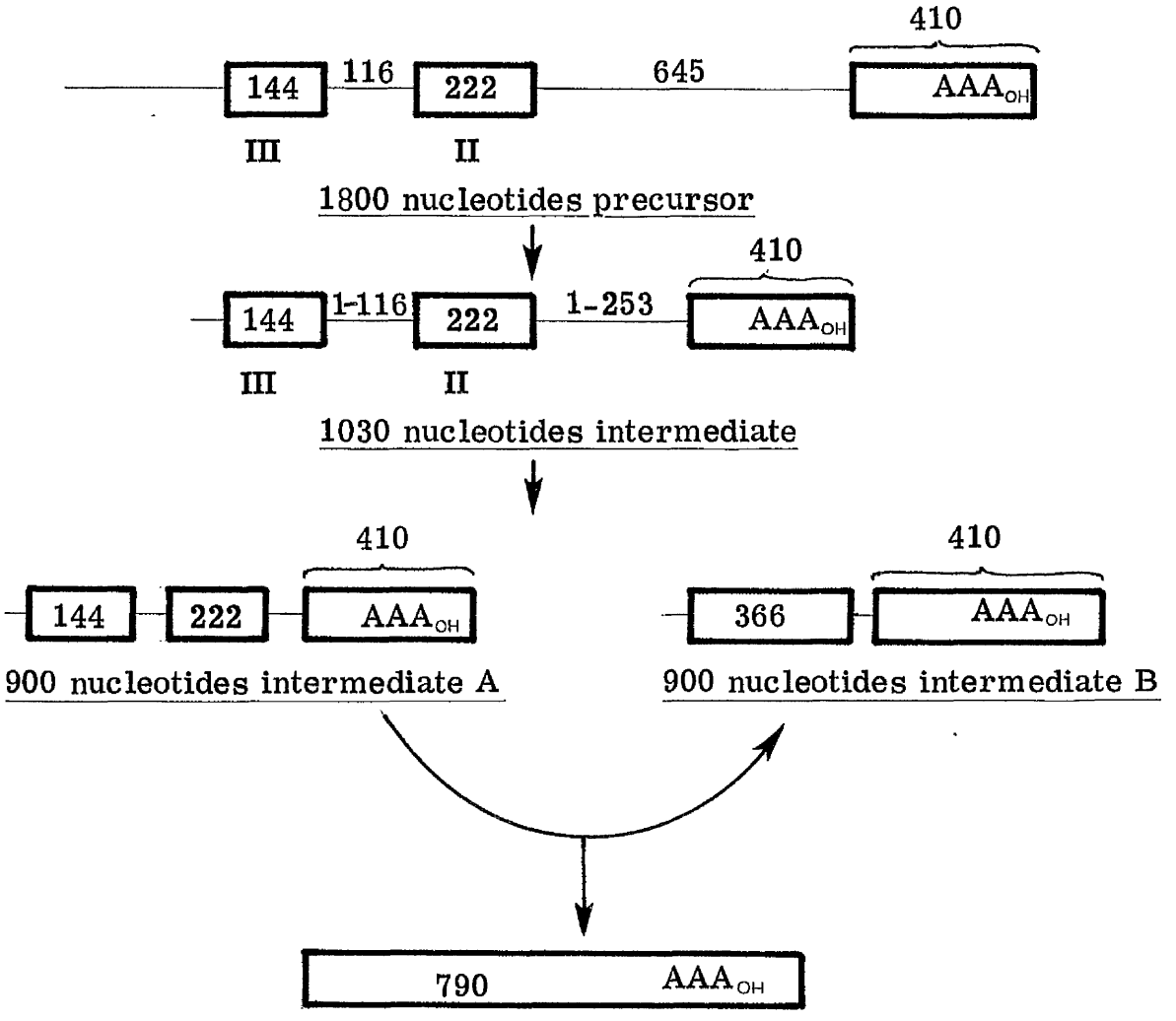
13.1. Pre-mRNA processing intermediates

Excision of the "introns" from the nuclear precursors of mRNA may take place in several steps. For example, many intermediates have been identified in the splicing pathway of adenovirus-2 late mRNAs (Ziff, 1980). Similarly, the mouse β-globin mRNA precursor has been reported to require at least 2 splicing events to excise the large intron transcript (see Figure 1), while three distinct stepwise pathways have been identified for the removal of an intron transcript in the chick a-2 collagen mRNA precursor (Avvedimento et al., 1980).

Studies on vitellogenin (Ryffel et al., 1980) and ovomucoid (Tsai et al., 1980; Nordstrom et al., 1979) putative mRNA precursors suggest that there is not a rigid order of removal of intron transcripts

FIGURE 1

Processing of mouse β-globin mRNA precursor



Mature β-globin mRNA

The globin RNA-specific fragments are noted by boxes and the intervening and 5' terminal RNA sequences by the lines. The lengths of the various segments are indicated. The Roman numerals refer to the RNA-specific fragments.

(Adopted from Kinniburg and Ross, 1979).

from these molecules, even though preferred orders of excision may exist as reported for the ovomucoid precursor (Tsai et al., 1980). The 5' terminal splice has been reported to occur before others for the maturation of adenovirus-2 major late leader (Berget and Sharp, 1979) and some adenovirus early messengers (Weber et al, 1980).

13.2. Splicing signals

Sequence studies of the junctions between coding and intervening sequences have led to the proposal that the intron begins with the dinucleotide GT at its 5' end and ends with the dinucleotide AG at its 3' end (Abelson, 1979; Breathnach and Chambon, 1981). Even though this rule seems to apply for most of the sequenced protein-coding genes (Breathnach and Chambon, 1981), it does not seem to apply for tRNA genes (Abelson, 1979) and ribosomal genes (Wild and Sommer, 1980). The GT-AG rule may also not apply strictly to the intermediate splicing events that may be involved in the stepwise removal of sequences within a given intron transcript (Avvedimento et al., 1980).

Since the consensus sequences are very simple, it had been suggested that other factors such as secondary structure, sequences within other introns or even within distant exons might be important for correct splicing. However, extensive secondary structures involving intron transcripts do not seem to be necessary for correct splicing, since correct splicing occurs in SV40 mutants which lack a large segment of the large T intron (Thimmapaya and Schenk, 1979; Volckaert et al., 1979). Extensive sequences of the exons may also be removed in vitro without stopping the splicing process in vivo (Hamer and Leder, 1979).

Unlike the eukaryotic tRNA situation (Peebles et al., 1979) no enzymatic splicing activity has yet been isolated for mRNA, although the reactions have been detected in isolated nuclei to which various soluble cytoplasmic and nuclear fractions have been added (Blanchard et al., 1978; Hamoda et al., 1980). It remains still unknown whether the enzyme(s)

involved in splicing require molecules other than the substrate. A class of small nuclear RNAs (snRNAs) has been suggested to play a role in splicing (Lerner et al., 1980; Rogers and Wall, 1980) by hybridising near the splice points thus "guiding" the splicing enzymes (Murray and Holliday, 1979). Indeed, the nucleotide sequence at the 5' end of one of them, U_1 RNA, exhibits complementarity to splice junctions (Lerner et al., 1980; Rogers and Wall, 1980). A similar role has been postulated for the small RNAs (VA RNAs) specifically coded by adenovirus DNA (Mathews, 1980). The observation that polyadenylation may precede splicing and that all spliced molecules are polyadenylated has led to the suggestion that poly(A) could align splicing sites by formation of triple-stranded structures with sequences around both donor and acceptor sites (Bina et al., 1980).

13.3. Splicing and RNA transcript stability

Several experiments have suggested that splicing is required for the accumulation of stable RNA. For example, precise deletion of SV40 late gene splice junction results in rapid degradation of the transcripts (Lai and Khoury, 1979), while introduction of a pair of splice sites from mouse β-globin gene into the intronless SV40 16S RNA gene results in the formation of stable, spliced RNA (Gruss and Khoury, 1980). Similarly, mutants of SV40 early genes where the acceptor splice junction has been removed also failed to produce stable RNAs or proteins (Volckaert et al., 1979; Gruss and Khoury, 1980). It has also been suggested that splicing may be linked to transport of the RNA from the nucleus to the cytoplasm (Volckaert et al., 1979; Murray and Holliday, 1979b; Hamer and Leder, 1979). However, the existence of unspliced mRNAs such as histone mRNAs (Kedes, 1979), poly(A)$^+$ mRNAs for adenovirus polypeptide 1X (Alestrom et al., 1980) and interferon a-1 (Nagata et al., 1980) has suggested that other transport mechanisms may also exist. Indeed, very recently the synthesis of an unspliced cytoplasmic message by an adenovirus 5 deletion mutant has been reported (Carlock and Jones, 1981).

13.4. Splicing and control of gene expression

There have been speculations that splicing with its apparent link to the production of stable mRNA, may be used as a control mechanism. These speculations have been based on indirect experimental data. For example, infection of undifferentiated F-9 murine teratocarcinoma cells by SV40 does not lead to the production of early mRNA possibly due to a block at the splicing level (Segal et al., 1979) while the block is lifted in in vitro differentiated cells (Segal and Khoury, 1979). Similarly, late in adenovirus infection of HeLa cells poly(A)$^+$ hnRNA is transcribed from the cellular genome but no mature mRNA reaches the cytoplasm, possibly because the splicing machinery is taken over by their viral counterparts (Beltz and Flint, 1979).

It was only very recently that splicing was shown to be directly involved in the control of gene expression during infection with adenovirus. Analysis of pulse-labelled cytoplasmic and nuclear RNA both early and late in infection has revealed that it is through differential splicing that L_1 precursor RNA early in infection is processed to give one mRNA molecule, while later in infection it gives three distinct mRNA molecules (Nevins and Wilson, 1981).

14. Association of hnRNA molecules with proteins

In the nucleus of eukaryotic cells, hnRNA is found associated with proteins to form hn-RNP particles. In animal cells, in particular, the buoyant density of hnRNPs is about 1.4g-cm^3 which corresponds to a protein:RNA ratio of about 4:1 by mass. The use of nucleases specific for single or double-stranded regions of hnRNP has shown that, in HeLa cells intramolecular double-stranded regions of hnRNA are essentially free of associated protein (Calvet and Pederson, 1978). Nuclease protection experiments have also revealed that mRNA-homologous sequences in hnRNP are extensively complexed with protein (Munroe and Pederson, 1981).

Several investigators have examined the number and size of proteins associated with hnRNA in various cell types, but their results differ depending on the method applied. In any case, polypeptide chains of about 40,000 dalton have always been reported to be the main, or one of the main protein components of hnRNP (Preobrazhensky and Spirin, 1978). Ultraviolet light-induced crosslinking of proteins to hnRNA has recently revealed that, in HeLa cells, a complex set of polypeptides is associated with hnRNA (Mayrand et al., 1981). It has also been shown that the polypeptides associated with poly(A)$^+$ hnRNA have the same mobilities with those associated with poly(A)$^-$ hnRNA (Mayrand et al., 1981). However, Setyono and Greenberg (1981) using the same method in L-cells have recently reported that one protein of molecular weight 60,000 is found associated with poly(A)$^+$ hnRNA at higher degree than with poly(A)$^-$ hnRNA. This polypeptide was shown to be associated with the poly(A) tail of hnRNA (Setyono and Greenberg, 1981). It also seems that in both HeLa and L-cells the proteins associated with hnRNA are different than those associated with cytoplasmic mRNA (Mayrand et al., 1981; Setyono and Greenberg, 1981).

15. Free-mRNA-protein complexes

Messenger RNA molecules are not only found associated with polysomes in the cytoplasm of eukaryotic cells, but also in a "free" form associated with proteins to form the free mRNPs. It has been proposed that there are two populations of free mRNPs: one containing mRNA species with a polyribosomal translated counterpart - possibly in equilibrium with each other - and another containing the mRNA fully repressed in a given cell at a given time (Vincent et al., 1981). In cells growing exponentially in culture such as Chinese Hamster cells, in which complete sequestering of mRNA sequences is unlikely, the same mRNA sequences have been found in both polysomal and free mRNPs (Walters et al., 1979). However, the balance between polysomal and free mRNA appears to be dependent upon the growth conditions. For example, increase of the temperature of incubation

of L-cells results in polysome disaggregation and increase in the free mRNP molecules (Schochetman and Perry, 1972). Also, changes in the gene expression during maturation of avian erythrocytes may also be explained by the selective gradual sequestration of some mRNA species into an inactive form (Stewart et al., 1976).

The fact that free mRNPs contain mRNAs which are in an inactive form, raised the question whether the protein content of the free mRNPs is responsible for this inactivation. Reports from various laboratories have offered rather conflicting data. Even though it has been reported that free mRNPs from avian erythroblasts (Gander et al., 1973), chick embryonic muscle cells (Jain and Sarkar, 1979) and HeLa cells (Liautard et al., 1976) are more protein rich than polysomal mRNPs, recent data from Setyono and Greenberg (1981) have shown that this is not the case in L-cells. Comparison of the proteins associated with globin mRNA in polysomal and free mRNPs has recently shown that, indeed, one of the two free globin mRNPs in duck erythroblasts is more protein rich (Vincent et al., 1981).

There are also conflicting results concerning the poly(A)-protein complex in polysomal or free mRNPs. Some investigators have concluded that the 73,000-78,000 protein is also found associated with poly(A) in free mRNPs (Liautard et al., 1976; Jain and Sarkar, 1979; Setyono and Greenberg, 1981) while others claim it is not (Gander et al., 1973; Vincent et al., 1981).

The possibility that the association of certain protein(s) with specific mRNAs may serve as a recognition sign for repression of these mRNAs has been raised by the finding that non-globin mRNAs from duck erythroblasts which are long-term repressed are associated with a 19,000 molecular protein which is not found in free globin mRNPs (Vincent et al., 1977). It is also of interest that two types of globin free mRNPs have been detected in duck erythroblasts: one 16S and another 13S. The 13S

globin mRNP contains 5 polypeptides which are not detected in the 16S globin mRNP.

16. Protein synthesis in eukaryotes

The final stage in gene expression is the translation of the genetic information from mRNA to proteins. In eukaryotic cells translation occurs on the ribosomes in the cytoplasm and involves a large number of ribosomal proteins, initiation factors and other components.

16.1. Mechanism of translation

The mechanism of protein synthesis involves three different processes: initiation, elongation and termination.

16.1.1. Initiation

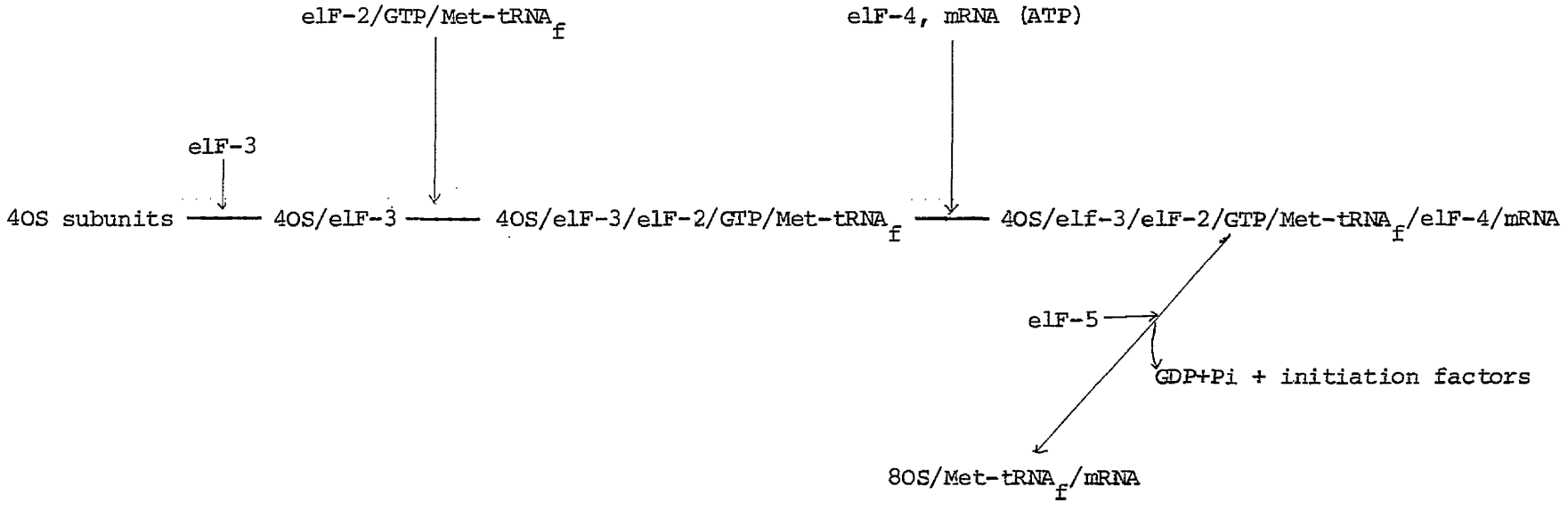
The initiation process in eukaryotic protein synthesis has been recently reviewed by Hunt (1980).

In eukaryotes, initiation of protein synthesis involves many individual steps which are presented in Figure 2. The first step is the binding of initiator tRNA (Met-$tRNA_f$) to 40S ribosomal subunit. 40S subunit is bound to an anti-association factor eIF-3 which prevents it from binding to 60S ribosomal subunit. The binding of Met-$tRNA_f$ to this 40S/eIF-3 complex requires initiation factor eIF-2 and GTP and results in the formation of a ternary complex of Met-RNA_f with GTP and EIF-3 and eIF-2 (see Figure 2).

The second step involves the binding of mRNA to this ternary complex which requires initiation factor eIF-4 and ATP. It is still not clear by which mechanism the 5' end of mRNA is recognised by the ribosome. In prokaryotes, binding has been proposed to be facilitated by observed complementarity of the 5' end of mRNA with sequences in 16S rRNA. A similar situation does not seem to hold for eukaryotes. A "scanning" model has been proposed by Kozak (1978) by which ribosomes bind somewhere at the 5' end of the mRNA and work their way to the first AUG codon. In support of this model are data showing that tobacco mosaic virus RNA can

Figure 2

The sequence of reactions in initiation of translation



Adopted from Hunt (1980).

bind a second ribosome on the left side of the first AUG codon (Fillipowicz and Haenni, 1979). Recognition of the "cap" may be achieved through a protein. A 24,000 dalton protein has been isolated from ribosomes by affinity chromatography on m^7GDP Sepharose and shown to be crosslinked to mRNA when it is bound in initiation complexes (Sonenberg et al., 1979).

The third stage of initiation involves binding of the large ribosomal subunit (60S). The joining reaction probably occurs when the Met-$tRNA_f$ anticodon has engaged the initiator AUG codon and requires an addition initiation factor called eIF-5. The set of reactions taking place in this stage are the following: (1) Met-$tRNA_f$ becomes bound in the P site of 60S subunit; (2) The GTP is hydrolysed and; (3) all the initiation factors leave the ribosome (Peterson et al., 1979).

16.1.2. Elongation

Elongation is defined as the addition of amino acids one at a time to a growing polypeptide in a sequence dictated by mRNA. The process of polypeptide chain elongation involves two ribosomal sites and occurs in three steps.

In step I, a ternary complex of aminoacyl-tRNA, GTP and elongation factor eFla (Molecular weight ≈ 53,000) binds to the A site of the ribosome. The charging or loading of tRNA with an amino acid precedes the formation of the ternary complex and is catalysed by aminoacyl-tRNA synthetases. Binding of ternary complex to the A site results in hydrolysis of GTP and release of the Efla-GDP complex. Another elongation factor, EF1B (molecular weight ≈ 30,000 is involved in the recycling of EFla (Clark, 1980).

Peptide bond formation constitutes Step II of elongation and is catalysed by the enzyme peptidyl transferase. This step is followed by the translocation step III which involves movement of peptidyl t-RNA from A site to P site and requires elongation factor EF-2 and GTP hydrolysis (Clark, 1980).

16.1.3. Termination

Termination involves the release of the completed polypeptide chain from the mRNA-ribosome complex. A single eukaryotic release factor, RF, has been isolated from rabbit reticulocytes, mammalian liver and insect cells (Goldstein et al., 1970; Ilan, 1973). This release factor has been shown to recognise all three termination codons UAA, UAG or UGA (Konecki et al., 1977). GTP dependent binding of RF to the A site seems to invoke esterase activity at the peptidyl transferase centre, resulting in the hydrolysis of peptidyl-tRNA. After GTP hydrolysis RF is released, the ribosome dissociates into subunits and initiation can occur again (Caskey, 1980).

17. Translational control

In prokaryotic cells, with short lived mRNAs, regulation of gene expression occurs mainly at the transcriptional level. In eukaryotic cells, on the other hand, in which mRNAs have a longer life span, gene expression is controlled not only at the transcriptional but also at the translational level. Regulation of translation in eukaryotic cells takes place, in part, during the initiation of polypeptide chains and this may explain why eukaryotic initiation factors are more numerous and structurally more complex than their prokaryotic counterparts (Weissbach and Ochoa, 1976).

Two major mechanisms of translational control are known to operate in eukaryotic cells. The first one is called quantitative and the second qualitative control.

17.1. Quantitative control of translation

Quantitative control of protein synthesis occurs in circumstances when the rate of protein synthesis in eukaryotic cells alters in a way that affects the translation of all mRNAs to a similar extent. The only cell where there is an understanding of the mechanism involved is the reticulocyte, the protein synthesis of which is inhibited by 1) the absence

of haem; 2) double-stranded RNA and 3) oxidised glutathione. It seems that in all three cases of inhibition the same component of translation is affected.

The absence of haem from rabbit reticulocytes affects drastically protein synthesis (Mathews et al., 1973). It seems that this is due to the activation of an inhibitor hemin-controlled inhibitor (HCI), which has been shown to be a cyclic-AMP independent phosphokinase that phosphorylates the 38,000 dalton subunit of eIF-2 (Farrell et al., 1977). Phosphorylation of the 38,000 dalton subunit of eIF-2 affects its binding to a stimulating protein (ESP) which enhances the ability of eIF-2 to form the ternary initiation complex (De Haro et al., 1978a; De Haro and Ochoa, 1978b). Similarly, low concentrations of double-stranded RNA activate an eIF-2 kinase and a translational inhibitor (Farrell et al., 1977) while addition of oxidised glutathione in reticulocytes also results in phosphorylation of the 38,000 subunit of eIF-2 and subsequently inhibition of protein synthesis (Clemens et al., 1975). Translation inhibitors with eIF-2 protein kinase activity, have also been isolated from rat liver (Delaunay et al., 1977), undifferentiated Friend leukemia cells (Pinphanichakarn et al., 1977), HeLa cells (Weber et al., 1975) and mature erythrocytes (Freedman et al., 1974).

17.2. Qualitative control

In addition to the co-ordinate regulation of the translation of the mRNAs for all proteins in a cell, discrimination between different cellular mRNA has been reported in eukaryotic cells. Stimulation or inhibition of the translation of mRNAs for specific proteins can also be found where the metabolism of a cell is dramatically altered by virus infection or by interferon action.

17.2.1. Differential translation of cellular mRNAs

In several systems the amount of protein synthesised is not directly related to the amount of mRNA present. For example, in rabbit

reticulocytes the α and β globin chains are known to be present in the ratio of 1:1 while α-globin mRNA is present in excess of β-mRNA (Lodish, 1971). Since identical rates of elongation and termination have been reported for α and β globins, the α:β ratio is possibly regulated at the initiation step (Lodish and Jacobsen, 1972). Several investigators have tried to find out whether the two globin mRNAs differ in their requirement for some initiation factor. Addition of initiation factors eIF-4A and eIF-4B in reticulocytes has been reported to relieve the inbalance *in vitro* (Kabat and Chappel, 1977). It has also been proposed that an RNA molecule may be involved, since the ratio α:β globin synthesis is increased in nuclease-treated reticulocytes (Stewart *et al.*, 1977). Indeed, the presence of such an RNA (translational control RNA) has been reported in embryonic muscle mRNAs (Bester *et al.*, 1975; Heywood *et al.*, 1975). A similar RNA molecule has also been reported to stimulate α-globin synthesis (Bogdanovsky *et al.*, 1973).

17.2.2. *Viral inhibition of host protein synthesis*

Many, but not all, eukaryotic viruses specifically inhibit host protein synthesis. A number of different mechanisms may be involved. For example, in cells infected with vaccinia virus the takeover of protein synthesis may be related to the high concentration of viral mRNA relative to host mRNA (Cooper and Moss, 1979). During poliovirus infection, however, this is achieved by the loss of "cap" binding activity in infected cells (Trachsel *et al.*, 1980) which results in preferential translation of poliovirus mRNA. Similarly, reovirus induces a gradual modification in the cap dependence of the host translational apparatus (Skup *et al.*, 1981). In other cases, like that of encephalomyocarditis viral infection, higher affinities of viral mRNAs for initiation factors have been suggested to be responsible for translational control in infected cells (Golini *et al.*, 1976).

17.2.3. *Interferon*

The production of interferon by cells infected by viruses probably

results in translational control of viral mRNAs. Synthesis of interferon may also be induced with double-stranded RNA. Addition of double-stranded RNA to interferon-treated cells results in the activation of a protein kinase that phosphorylates eIF-2 and another protein of unknown function (Kimchi et al., 1979). A second consequence is the activation of an enzyme that catalyses the synthesis of the trinucleotide pppA2'p5" A2'p5'A (Kerr and Brown, 1978) which activates a ribonuclease that degrades mRNA (Slattery et al., 1979) and a phosphodiesterase that degrades the CCA end of tRNAs (Schmidt et al(1979)

Even though all these translational inhibitors are not specific for viral mRNAs, they may affect the translation of those mRNAs having higher affinities for ribosomes (i.e. viral mRNAs) (Metz, 1975).

18. mRNA stability

The level of mRNA in cytoplasm depends upon both the rate of production and transport from the nucleus and the rate of degradation.

Messenger RNA stability may be assessed by two types of criterion: physical and functional. Early experiments on the stability of mRNA made use of the transcriptional inhibitor actinomycin D, as well as continuous labelling and pulse-chase experiments. In this way it was shown 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 and L-cells decay as a single component with a half life of about 60-90 min and 10-15 hrs 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 (Quellette et al., 1976), resting lymphocytes (Berger and Cooper, 1975) and insect cells (Spradling et al., 1975). In addition,

Perry et al (1976), in a more accurate study, obtained data from poly(A)$^+$ mRNA turnover in L cells which can be detected in terms of two mRNA classes with half lives of 3.5 and 18 hrs.

Although analysis of poly(A)$^+$ mRNA stability in eukaryotic cells has revealed stable, or relatively stable classes of mRNA with half lives ranging from 3-24 hrs, short lived mRNAs have been detected in a variety of cells such as Aedes cells (Spradling et al., 1975), kidney cells (Quellette et al., 1976), HeLa cells (Puckett et al., 1975) and Drosophila cells (Lengyel and Penman, 1977). Extremely short half-life has also been reported for interferon mRNA (Cavalieri et al., 1977). These estimates of mRNA half-lives apply only to poly(A)$^+$ mRNAs. Two mRNA species with very short half-lives (13 min) have been detected early in the development of Dictyostelium discoideum (Margolskee and Lodish, 1980b).

Poly(A) tail has been suggested to play a role in stabilising the mRNAs from data which showed that deadenylated globin poly(A)$^+$ mRNAs which were microinjected into Xenopus oocytes were less stable than the native ones (Huez et al., 1975) and from the work of Sheiness and Darnell (1975) which correlated the size of poly(A) tail to the mRNA's half-life. However, later results of Desphande and colleagues (1978) showed that the decay of a2u-globulin mRNA was not related to the length of the poly(A) tail found in these molecules. On the other hand, recent data of Palatnik and colleagues (1980) have even suggested that in Dictyostelium discoideum mRNA molecules with shorter poly(A) tails are more stable than those with longer poly(A) tails. Also, both native and deadenylated human fibroblast interferon mRNAs when microinjected in Xenopus oocytes exhibit similar half-lives (Pravinkumar et al., 1978). In agreement with this come results which have shown that non-histone mRNAs in HeLa, Aedes and sea urchin embryos (Milcarek et al., 1974). Spradling et al., 1975; Nemer et al., 1975; have similar decay rates with those of poly(A)$^+$ mRNAs from the same cells.

The stability of mRNA may be dependent upon the differentiation state of a particular cell. For example, the stability of myosin mRNA was shown to increase throughout differentiation (Buckingham et al., 1974). However, despite initial reports which had suggested that late in differentiation of murine erythroleukemia cells the half-life of globin mRNA decreases from 50 hrs to 17 hrs (Lowenhaupt and Lingrel, 1978; Lowenhaupt and Lingrel, 1979), it has recently been shown that the half-life of globin mRNAs in terminally differentiated cells is also 50 hrs (Volloch and Hausman, 1981). Also in Dictyostelium discoideum the half-lives of mRNAs during growth and differentiation have been shown to remain the same (Margolskee and Lodish, 1980a). On the other hand, recent data have shown that adenovirus mRNAs from transcription units 1A and 1B are more stable late in infection of HeLa cells than they are early in infection (Wilson and Darnell, 1981). Stability of mRNA has also been shown to be subject to hormone control. For example, in chicken oviduct upon induction with estrogen ovalbumin mRNA has a half-life of about 24 hrs. Withdrawal of the hormone is followed by a rapid decline in the survival of ovalbumin mRNA, which does not follow first-order kinetics, but accelerates in rate with time, reaching a value corresponding to a half-life of about 3 hrs (Palmiter and Carey, 1974).

AIMS

From this introduction it is clear that our knowledge of factors regulating eukaryotic gene expression is still far from complete. A particular area of uncertainty is the role of mRNAs that are either polyadenylated or non-polyadenylated. The general aim of this work at the outset was to compare the structure, origin and the possible role of polyadenylated and non-polyadenylated mRNAs coding for a specific cellular protein. However, this led to more detailed consideration of the heat-shock response in HeLa cells as well as the mRNAs for specific heat-shock proteins.

MATERIALS AND METHODS

MATERIALS

1. Biological

Rabbit reticulocyte lysate was a product of New England Nuclear, Manheim.

2. Radiochemicals

[5,6-^{3}H] uridine	40 Ci/mmol	Radiochemical Centre,
[γ-^{32}P] ATP	5,000 Ci/mmol	Amersham
[5-^{3}H] deoxy cytidine	21 Ci/mmol	
L-[^{35}S]-methionine	$\sim$ 1,000 Ci/mmol	New England Nuclear, Manheim.

3. Chemicals

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

4-(2-hydroxyethyl)-1 piperazine-ethanesulphonic acid (Hepes)	
2-Amino-2-hydroxymethyl propane-1,3 diol (Tris)	
Dithiothreitol (DTT)	Sigma (U.K.), London, England.
Chloramphenicol	"
Ampicillin	"
Tetracycline	"
Triton X-100	Koch-Light Laboratories Ltd., Colnbrook, England.
Trichloroacetic acid	"
2-mercaptoethanol	"
Streptomycin	Glaxo Pharmaceuticals, London.
Penicillin	"
Calf serum	"
Minimal Essential Medium amino acids	Biocult Laboratories Ltd., Paisley, Scotland.

Minimal Essential Medium	Biocult Laboratories Ltd.,
vitamins.	Paisley, Scotland.
Horse serum	"
Actinomycin D	Calbiochem Ltd., Hereford, England.
poly(U)-Sepharose	Pharmacia, Uppsala, Sweden.
poly(A)-Sepharose	"
Sephadex G100	"
dATP	P-L Biochemicals Inc.
dGTP	"
dCTP	"
dTTP	"
oligo(dT)	"
Formamide (Fluka)	Fluoreschem Ltd., Derbyshire, England.
Selectron filters	Scheicher and Schiell GmbH, D-3354, W. Germany.
Nitrobenzyloxymethyl paper	"
Whatman 3MM paper discs	H. Reeve-Engel and Co., Ltd., London, England.
Ampholines	LKB
Endonuclease AluI	Uniscience Ltd., Cambridge, England.
T4 polynucleotide kinase	Boehringer Co., London, Ltd.
Fuji-X-ray film	Fuji Photo Film Co. Ltd., Tokyo.
Hyamine hydroxide	Fisons Scientific Apparatus, Loughborough, Leics., England.

METHODS

1. Cell culture

1.1. Routine maintenance of cells

1.1.1. Growth of HeLa cells

HeLa cells (Gey et al., 1962) were maintained as monolayers in either rotating 80 oz clear glass Winchester bottles (House and Wildy, 1965), or at the bottom of small glass scintillation vials. The growth medium was the Glasgow modification of Eagle's minimal essential medium (see Table 1) supplemented with 10% (v/v) calf serum, penicillin (100 units/ml) and streptomycin (100 µgs/ml). Bottles were seeded with 2×10^7 cells in 180ml growth medium and grown in an atmosphere containing 5% (v/v) CO_2 at 37°C. When small scintillation vials were used, 0.5×10^6 cells were grown for 48 hrs at the bottom of the glass scintillation vials in 5ml of the already described medium.

1.1.2. Growth of Friend murine leukaemia cells

Friend murine leukaemia cells, clone M_2, were used in some 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 1) supplemented with 2X glutamine (584 mg/l), non-essential amino acids (Table 2), 15% (v/v) horse serum, penicillin (100 units/ml) and streptomycin (100 µgs/ml). Growth was initiated by inoculating cells, as a suspension, into stirrer culture vessels containing 1-1.5l of medium. The inoculum was chosen to give an initial cell density of $0.5 - 0.6 \times 10^5$ cells per ml. Cultures were maintained at a temperature of 37°C and in an atmosphere of 5% (v/v) CO_2 for 3

TABLE 1

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	nicotinamide	2.0
l-leucine	52.5	pyridoxal Hcl	2.0
L-lysine	73.1	riboflavin	0.2
L-methionine	14.9	thiamin Hcl	2.0
L-phenylalanine	33.0		
L-threonine	47.6		
L-tyrosine	36.2		
L-valine	46.9		

Inorganic salts and other components	mg/litre
$Cacl_2\ 6H_2O$	393.0
Kcl	400.0
$MgSO_4 \cdot 7H_2O$	200.0
Nacl	6,800.0
$NaH_2PO_4\ 2H_2O$	140.0
D-glucose	4,500.0
$NaHCO_3$	2,240.0
Phenol red	15.3

TABLE 2

Non-Essential Amino Acid Mixture for Minimum Essential Medium Eagle

Amino acids	mg/litre
L-alanine	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

days before harvesting, at which time the cultures 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).

1.2. Subculture of HeLa cells

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

1.3. Contamination checks

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 in seven days.

(b) Fungal contamination.

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

(c) Mycoplasma (PPLO pleuropneumonia-like organism) 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 seven days, and examined microscopically for the characteristic "fried egg" appearance of PPLO colonies.

1.4. Heat-shock treatment of HeLa cells

Heat-shock treatment was carried out by immersion of the 80 oz bottles or small scintillation vials in a 45°C water bath for 5 mins.

1.5. Radioactive labelling procedures

1.5.1. Labelling of HeLa cell proteins with [^{35}S]- methionine

HeLa cells (grown at the bottom of small scintillation vials) were washed with 5 ml minimal essential medium minus methionine containing 10% (v/v) dialysed calf serum. Dialysed calf serum was prepared by putting calf serum into sterile dialysis tubing and dialysing at 4°C against sterile Balanced Salts Solution (BSS) [0.116M Nacl, 5.4mM Kcl, 1mM $MgSO_4$, 1mM NaH_2PO_4, 1.8mM $Cacl_2$, 0.002% phenol red] whose pH was adjusted to 7.0 with 5.6% (w/v) $NaHCO_3$. The medium was then removed and the cells were labelled with 10μCi L-[^{35}S]- methionine [∿1,000 Ci/mmol] in 1ml of the above medium.

1.5.2. Labelling of HeLa cell RNA with [^{3}H]- Uridine

The 180 ml of culture medium was removed from the 80 oz bottles and replaced by 50 ml of fresh, prewarmed medium containing 50μCi of [^{3}H]- Uridine (∿40Ci/mmol).

2. Harvesting of cells

2.1. Harvesting of HeLa cells

The culture or radioactive medium was decanted from the 80 oz bottles and the cell monolayer was washed twice with approximately.

50ml of ice cold BSS (see above). The cells were then scraped into 10ml of cold BSS with a rubber wiper and collected by centrifugation at 800g for 10 mins at $4^{o}C$ and washed twice by resuspending 10 pellet volumes of BSS followed by centrifugation as described above. In cases when the cells would be used for the preparation of polysomes BSS contained cycloheximide at a concentration of 10µg/ml.

2.2. Harvesting of Friend cells

Friend cells were centrifuged at 800g for 5 min at $4^{o}C$ directly from the medium and washed twice with BSS.

3. Cell fractionation

3.1.1. Nuclei and cytoplasm from HeLa cells

HeLa cells were disrupted as described by Penman (1969). The washed cell pellet was resuspended in hypotonic RSB buffer (10mM Nacl, 1.5mM $Mgcl_2$, 10mM Tris-Hcl pH 7.4) at a density of 5×10^7 cells/ml. After leaving for 10 mins at $4^{o}C$ cells were broken in a stainless steel Dounce homogeniser (clearance 0.992" diameter). 10 strokes were usually sufficient to obtain almost complete cell breakage, as monitored by phase contrast microscopy. The nuclei were removed as a pellet by centrifugation at 800g for 10 mins at $4^{o}C$. The supernatant remaining after the 800g spin will henceforth be referred to as cytoplasm in this study. All operations were carried out at $0.4^{o}C$.

3.1.2. Nuclei and cytoplasm from Friend cells

This step was carried out as described by Katinakis and Burdon (1981).

All operations were carried out at $0-4^{o}C$. The washed cell pellet was resuspended in lysing buffer (0.14M Nacl, 1.5mM $MgSO_4$, 10mM Tris-Hcl, pH 7.4) 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 3-6 mins (lysis being monitored by phase contrast microscopy), then the lysed cells were centrifuged at 800g for 5 mins in order to remove nuclei.

3.2.1. Polysomes, sub-polysomes and cytosol from HeLa cells

This step was carried out as described by Penman et al., (1969).

The cytoplasm (see Section 3.1.1.) was made up to 0.5% with respect to deoxycholate, 0.5% with respect to Brij-58 and layered onto 15-30% (w/v) sucrose gradients in RSB. Gradients were centrifuged at 27,000 r.p.m. for 110 mins at 4°C in the Beckman SW27 rotor and then harvested by pumping through a Gilford 2,000 recording spectrophotometer set at 260nm. Polysomal and post-polysomal fractions (as indicated in Figure 30A), were separated, made up to 0.2M Nacl and alcohol precipitated overnight at -20°C by the addition of 2 volumes of absolute alcohol.

3.2.2. Polysomes from Friend cells

Polysomes were prepared from Friend cells as described by Katinakis and Burdon (1981). Polysomes were pelleted from the cytoplasm (see Section 3.1.2.) by centrifugation through 2M sucrose in lysing buffer (see Section 3.1.2.) at 230,000 x g_{av} for 3 hrs at 4°C in the 8 x 25 Ti MSE fixed-angle rotor.

3.3. Preparation of [^{35}S]-labelled proteins from HeLa cells for subsequent SDS gel electrophoretic analysis

After the removal of the radioactive medium from the small glass scintillation vials and the washing of the cell monolayer with BSS, the cells were lysed by addition of 0.250 ml of SDS sample buffer (2% SDS, 10% glycerol, 3% 2-mercaptoethanol, 50mM Tris/Hcl pH 6.8, 0.1mM phenylmethylsulphonyl fluoride). The lysate was sonicated using a Dawes soniprobe at 7A for 10 seconds then heated to 100°C for 2-3 mins prior to the addition of bromophenol blue (0.01%) as a

marker for subsequent SDS/polyacrylamide gel electrophoresis. The cell lysates were stored at -20°C.

4 RNA isolation

4.1. Precautions against ribonuclease contamination

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

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

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

4.2. Preparation of cytoplasmic RNA

The cytoplasm (see Section 3.1.) was made up to 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-isoamyl alcohol, followed by chloroform-isoamyl alcohol alone (Penman (1966). RNA was precipitated from the acqeuous phase by the addition of 0.1 volumes 2M Nacl plus 2 volumes absolute alcohol. After 20 hrs at -20°C RNAs were collected by centrifugation at 12,000g for 20 mins at -10°C.

4.3. Preparation of polysomal, post-polysomal and cytosol RNA

(a) Polysomal RNA

The polysomal fraction (see Section 3.2.) was collected by centrifugation at 12,000g for 20 mins at -10°C and the pellet was dissolved in NETS buffer (0.1m Nacl, 10mM EDTA, 10mM Tris-Hcl, 0.5% (w/v) SDS, pH 7.4). The RNA was extracted as described for cytoplasmic RNA (see Section 4.2.).

(b) Post-polysomal and cytosol RNA

Ethanol precipitates of post-polysome and cytosol were resuspended in NETS buffer and RNAs were extracted as described for cytoplasmic RNA (see Section 4.2.).

5. RNA fractionation

5.1. Affinity chromatography

5.1.1. Preparation of poly-(U) Sepharose 4B

0.3g of poly-(U) Sepharose (Pharmacia) was swollen in 3ml of 1M Nacl (pH 7.5) at 4°C and then poured into a small column (2.0 x 0.6 cm) made by blocking a 5ml pipette with a small glass bead. All glassware was siliconised (Repelcote, Hopkins and Williams), to prevent binding of RNA to the glass. Before the fractionation, the columns were washed with 10 column volumes of [0.1M Nacl, 10mM EDTA, 0.2% SDS, 10mM Tris pH 7.4] followed by 10 volumes of 90% (v/v) formamide in [0.5% SDS, 10mM Tris, 10mM EDTA pH 7.4] and equilibrated by washing with 10 column volumes of binding buffer [0.2% N-lauroyl sarcosine, 0.4M Nacl, 10mM Tris, 10mM EDTA pH 7.4].

From this step and further on SDS is substituted by N-lauroyl sarcosine because the latter is soluble at high salt concentrations.

5.1.2. Preparation of poly-A()-Sepharose 4B

Poly-(A)-Sepharose was obtained from Pharmacia (Great Britain Ltd.) and 1ml columns were prepared according to the manufacturer's

instructions by resuspending 0.3 gr of dry gel in 3 ml of 1M Nacl (pH 7.5) and packaging into small columns made by blocking a 5 ml pipette with a small glass bead. Washing and equilibration of the columns was carried out as already described for poly-(U)-Sepharose columns.

5.1.3. Separation of poly(A)$^+$ and poly(A)$^-$ RNA

The method described by Katinakis and Burdon (1981) for the separation of poly(A)$^+$ and poly(A)$^-$ RNA was used.

A pellet of ethanol precipitated RNA (not exceeding 1,500 µgs) was resuspended in a small volume (0.2 or 0.5 ml) of binding buffer (see Section 5.1.1.) heated at 70^{o}C for 5 mins, cooled rapidly on ice and immediately applied to the top of a poly-(U) Sepharose column. The sample was eluted with binding buffer at a slow flow rate, at room temperature (unless otherwise stated), such that the sample took approximately 10 mins to pass through the column. The small eluted fraction was reapplied to the column and eluted at the same rate. Then, the unbound material (poly(A)$^-$ RNA) was eluted with 10 column volumes of binding buffer, whilst the bound material (poly(A)$^+$ RNA) was eluted with 10 column volumes of 90% (v/v) formamide in (0.5% N-lauroyl sarcosine, 10mM EDTA, 10mM Tris, pH 7.4). Poly(A)$^+$ and poly(A)$^-$ RNA fractions were pooled and precipitated overnight with 2 volumes of ethanol at -20^{o}C.

For better separation of poly(A)$^+$ RNA from poly(A)$^-$ RNA, poly(A)$^-$ RNA fractionated after one cycle of affinity chromatography was, in some cases, subjected to another two cycles of affinity chromatography as follows: The unbound material eluted from the first poly-(U) Sepharose column was subjected to a second cycle of affinity chromatography on a second poly-(U) Sepharose column. The unbound material eluted from the second poly-(U) Sepharose column was then subjected to another cycle of affinity chromatography on a third poly(U) Sepharose column.

5.1.4. Isolation of poly(A)$^-$RNA species with high affinity for poly-(A) Sepharose

Poly(A)$^-$ RNA with high affinity for poly-(A) Sepharose was isolated as described by Katinakis and Burdon (1981).

Poly(A)$^-$ RNA dissolved into binding buffer (see Section 5.1.1.) was applied to a poly-(A) Sepharose column. The small eluted fraction was re-applied to the column. Finally the unbound material was eluted with 10 volumes of binding buffer, while the bound material was eluted with 10 column volumes of 90% (v/v) formamide in (0.5% N-lauroyl sarcosine, 10mM EDTA, 10mM Tris pH 7.4). Bound (poly(A)$^-$u$^+$) and unbound poly(A)$^-$u$^-$) material was pooled and precipitated overnight with 2 volumes of ethanol at -20^{o}C.

5.2. Sucrose gradient analysis of RNA

Sucrose density gradient centrifugation of cytoplasmic RNA was carried out according to the procedure of Kelley et al., (1980). Approximately 0.7 mg of cytoplasmic poly(A)$^-$ RNA or 0.3 mg of poly(A)$^+$ RNA from heat-shocked HeLa cells were dissolved in 1mM EDTA, 10mM Hepes pH 7.5, heated at 65^{o}C for 10 mins, cooled rapidly on ice and immediately layered on a 5-20% (w/v) linear sucrose gradient in the same buffer. The gradients were centrifuged in a Beckman SW40 rotor at 35,000 rpm for 16 hrs at 4^{o}C. About 27 fractions were collected and the RNA from each fraction was alcohol precipitated, collected by centrifugation (see Section 4.2.) dissolved in sterile distilled water and its optical density was measured in U.V. at 260nm. Before any further analysis the RNA from each fraction was again ethanol precipitated.

6. In vitro translation of RNA

Poly(A)$^+$ and poly(A)$^-$ RNA was translated *in vitro* in an mRNA dependent rabbit reticulocyte cell-free protein synthesising system (obtained from New England Nuclear Mannheim). The rabbit reticulocyte lysate had been prepared by the manufacturers as described by Pelham and Jackson (1976). The assay mix, of final volume 25µl, was prepared by mixing in order: 5µl of L-[^{35}S]-methionine (1,000 Ci/mmol), 5µl of translational cocktail (which according to the manufacturers contained spermidine, creatine phosphate, dithiothreitol and guanosine triphosphate in Hepes buffer), 2µl of 1M potassium acetate, 0.5µl of 32.5mM magnesium acetate, 10µl rabbit reticulocyte lysate and 2µl RNA sample in distilled water.

According to the manufacturers all components (except of the RNA sample) had been optimised with respect to all of the reagents for maximum incorporation into protein. Assays were incubated at 37^{o}C for 60 mins.

7. Analysis of the cell-free products of translation

7.1. Preparation of translation mixtures for one-dimensional gel electrophoresis

Appropriate amounts of translation mixtures containing the labelled products of *in vitro* translation were mixed with equal volume of [2% (w/v) SDS, 3% (v/v) 2-mercaptoethanol, 50mM Tris-Hcl pH 6.8] and heated at 100^{o}C for 2 mins prior to the addition of bromophenol blue (0.01%).

7.2. Preparation of translation mixtures for two-dimensional gel electrophoresis

The assay mixtures containing the labelled products of translation were mixed with equal volume of lysis buffer, containing 9.5M Urea, 2% (w/v) NP-40, 1% ampholines (pH 5-7), 1% ampholines (pH 3.5-10), (LKB-Ltd.), 5% (v/v) 2-mercaptoethanol.

7.3. SDS-polyacrylamide gel electrophoresis

120 mm of 8.75% slab gels (29.2 : 0.8, acrylamide: bisacrylamide) overlaid with 10-20 mm of 3% stacking gel were prepared by the method of Le Stourgeon and Beyer (1977). After application of the sample a current of 20mA was applied until the bromophenol blue dye front passed into the resolving gel, then the current was increased to 40mA. The run was terminated when the bromophenol blue tracker dye had migrated to a position close to the end of the gel. Molecular weight (20,000-200,000) calibration proteins (Combithek, Boehringer, Mannheim) comprising Escherichia coli RNA polymerase (a 39,000, b 155,000, b' 165,000), bovine serum albumin (68,000, and soya bean trypsin inhibitor (21,500)) were also run. After the end of the electrophoresis the gels were fixed in 7.5% acetic acid, 5% methanol and prepared for fluorography. In some cases the gels were stained with 0.25% (w/v) Coomassie brilliant blue in 9% acetic acid, 45% methanol.

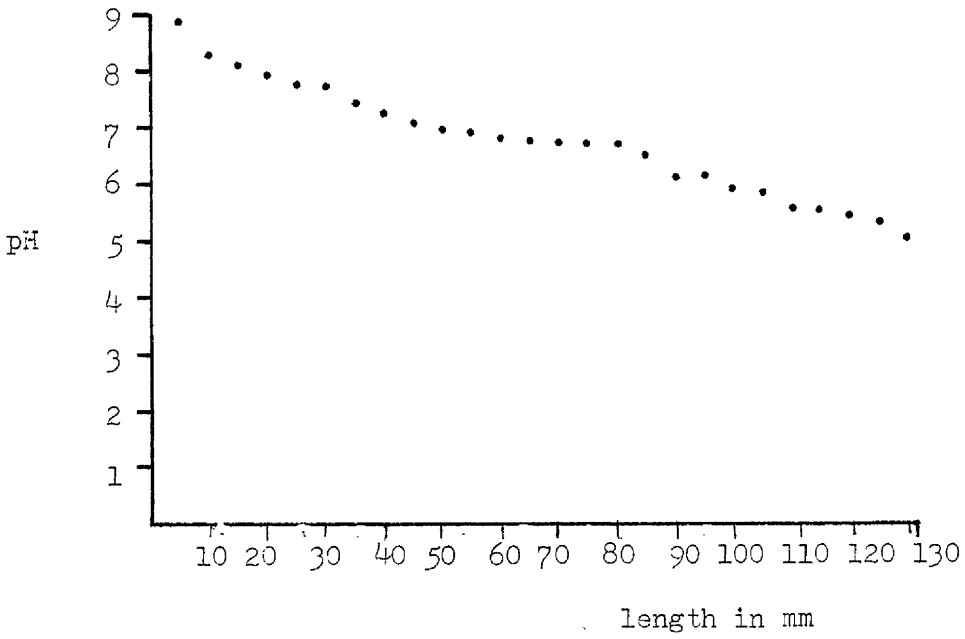
7.4. Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was carried out according to the procedure of O'Farrell (1975) with the modification that the ampholytes used for the first dimension isoelectric focusing consisted of 1% ampholytes (LKB) pH 5-7 and 1% ampholytes pH 3.5-10 respectively.

Briefly, the first dimension involved electrofocusing in a 160mm x 3mm tube gel at 7,200 volts x hours at room temperature. Then the isoelectric focusing gel was extruded and stored in 5ml of SDS sample buffer [10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2.3% (w/v) SDS, 0.0625M Tris-Hcl pH 6.8] at -70^{o}C. Before the second dimension polyacrylamide electrophoresis the isoelectric focusing gel was thawed and fused with 1% agarose in SDS sample buffer at the top of a polyacrylamide slab gel. The pH gradient of the

FIGURE 3

pH gradient in isoelectric focusing gels



After isoelectrophocusing at 7,200 volts x hours, the isoelectric focusing gel was extruded and cut in 5mm sections which were placed in vials containing .2ml of degassed H_2O. These vials were capped and shaken for 5 to 10 mins before the pH reading was taken.

isoelectric focusing gels was determined by cutting the gel in 5mm sections which were placed in vials containing 2ml of degassed H_2O. Each vial was capped and shaken before the pH reading was taken, using a microelectrode. Electrophoresis in the second dimension was performed as described in Section 7.3. The equilibration step preceeding the second dimension electrophoresis [O'Farrell (1975)] was omitted since a substantial number of proteins was found to diffuse off the gel during this step.

In cases when the second dimension was run immediately after the electrofocusing, the tube gel was allowed to stand for 1 min in 5ml of SDS sample buffer and then loaded on the slab gel as already described.

8. Fluorography

Gels were processed for fluorography according to the method of Bonner and Laskey (1974). The gels were immersed in two successive baths of dimethylsulphoxide (DMSO) (Sigma), for a total period of 1 hr and then impregnated with 2,5-diphenyloxazole (PPO) by immersion in 4 volumes of 20% (w/v) PPO in DMSO for 3 hrs. The gels were then soaked in water for 1 hr and dried under vacuum onto Whatman 3MM chromatography paper.

A fluorograph was obtained by placing a sheet of Fuji-RX X-ray 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.* The films were developed for 6 mins using DX-80 developer. 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.

* The two dimensional gels were loaded with ^{35}S-methionine labelled samples which contained a total of 100,000 cpm. The fluorographs of these gels were usually exposed for two weeks.

9. Molecular cloning of complementary DNA (cDNA) reverse transcribed from poly(A)$^+$ RNA isolated from heat-shocked HeLa cells

It was carried out in collaboration with Dr. Cato as described in a publication of Cato and colleagues (1981). The various steps (see Figure 4) are described briefly below:

1) Synthesis of complementary DNA (cDNA) and addition of poly(dC) tail

cDNA was synthesised as described by Ohno et al., (1980) in 1ml of reaction mixture containing 50µg of poly(A)$^+$ mRNA from heat-shocked HeLa cells, 1mM each of dATP, dGTP, dTTP and [^{3}H]-dCTP (21 Ci/mmol), 50mM Tris-Hcl (pH 7.9 at 42^{o}C), 10mM $Mgcl_2$, 10mM dithiothreitol, 5µg oligo (dT) and 225 units of AMV reverse transcriptase (supplied by Dr. J Beard), one unit was defined as the incorporation of one nmol of dNTP into acid insoluble product in 10 mins at 37^{o}C). After incubation for 1 hr at 42^{o}C the reaction was stopped by the addition of 1/20 volume of 0.5M EDTA (pH 7.4) at 0^{o}C. The reaction mixture was extracted with phenol and passed through a Sephadex G-100 column. The fractions containing cDNA were collected and treated with 0.1M NaOH for 20 mins at 60^{o}C. cDNA was then ethanol precipitated.

Double stranded cDNA was synthesised in 0.2ml reaction containing 0.6µg of cDNA, 67mM potassium phosphate buffer (pH 7.4), 6.7mM $Mgcl_2$, 5mM dithiothreitol, 1mM of each dATP, dCTP, dGTP, dTTP and 90 units/ml of E.coli DNA polymerase I (Klenow fragment). Before the addition of the enzyme, the reaction mixture containing cDNA was heated at 70^{o}C for 5 mins, then cooled slowly at 20^{o}C to allow the formation of hairpin loop structure at the 3' end of the cDNA. Incubation was at 20^{o}C for 2 hrs, then more enzyme was added and

FIGURE 4

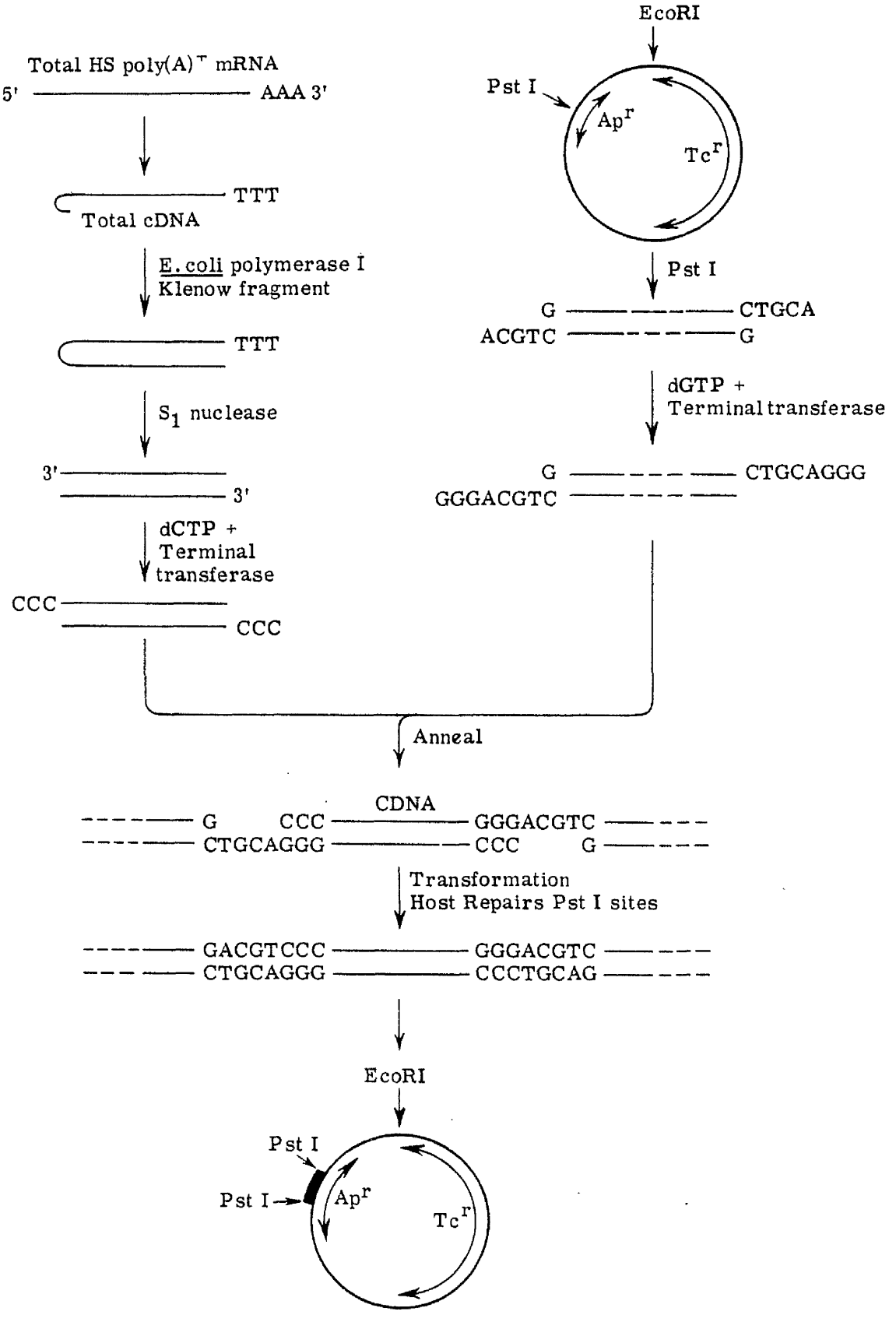
Procedure used for the insertion of double-stranded cDNA derived from heat-shocked HeLa cell poly(A)$^+$ RNA into the Pst 1 site of the plasmid pBR322

Ap^r, ampicillin resistance gene

Tc^r, tetracycline resistance gene

Eco Rl, Pst 1, restriction enzyme cleavage sites

ds cDNA, double-stranded cDNA


Total HS poly(A)⁺ mRNA
5' AAA 3'
TTT
Total cDNA
E. coli polymerase I
Klenow fragment
TTT
S1 nuclease
3'
3'
dCTP +
Terminal
transferase
CCC
CCC
EcoRI
Pst I
Ap^r
Tc^r
Pst I
G CTGCA
ACGTC G
dGTP +
Terminal transferase
G CTGCAGGG
GGGACGTC
Anneal
CDNA
G CCC GGGACGTC
CTGCAGGG CCC G
Transformation
Host Repairs Pst I sites
GACGTCCC GGGACGTC
CTGCAGGG CCCTGCAG
EcoRI
Pst I
Pst I
Ap^r
Tc^r

incubation was carried on for another 2 hrs. The reaction was stopped as described above and chromatographed on Sephadex G-100 column. The fractions containing double stranded cDNA were pooled. Double stranded cDNA was extended at the 3' end with the addition of homopolymer dC tails as described by Roychoudhury et al (1976) by incubating double stranded DNA with dCTP and the enzyme terminal transferase.

2) Purification of plasmid DNA and addition of poly(dG) tails

Plasmid DNA was prepared according to the method of Clarke and Carbon (1976).

500ml culture of bacteria were amplified with chloramphenicol (200µg/ml) for 16-20 hrs and the bacteria were collected by centrifugation. The pellet was resuspended in 200ml of cold TE buffer (10mM Tris, 1mM EDTA pH 8) and the bacteria were again collected by centrifugation. The pellet was then suspended in 12.5ml cold 12.5% sucrose in 50mM Tris (pH 8). At this point 2.5ml of fresh lysosome solution (10 mg/ml in 250mM Tris pH 8) was added and the whole was left on ice for 5 mins. Then 20ml of ice-cold detergent solution (1ml 10% Triton X-100, 12.5ml 0.5M EDTA pH 8.5, 5ml 1M Tris pH 8, 81.5ml H_2O) was added to promote lysis. The whole was swirled gently on ice for 10 mins until the solution was somewhat cleared and highly viscous and was then centrifuged at 25,000 rpm for 30 mins at 4°C in an SW27 rotor. The supernatant was removed and 0.9g CsCl plus 0.1ml of EtBr (10 mg/ml) were added per ml of cleared lysis supernatant, which was then centrifuged at 45,000 rpm for 72 hrs at 4°C in a 60 Ti rotor. The DNA band was visualised under L.W. U.V. illumination in a darkened room and the lower band was collected in a syringe by side puncture using a 16-18 gauge needle. Ethidium bromide was removed from DNA by three extractions with isoamyl

alcohol. CsCl was removed by dialysis and the DNA was ethanol precipitated. Plasmid DNA was digested with Pst 1 and tailed with poly(dG) as described by Cozens et al., (1980).

3) Annealing of poly(dC) extended cDNA with poly(dG) extended plasmid DNA

This step was carried out according to the method of Wensink et al., (1974) by annealing poly(dG) terminated plasmid DNA to poly(dC) terminated plasmid DNA in 0.1M NaCl in TE buffer (see above) for 10 mins at 65°C and then for 2 hrs at 45°C.

4) Transformatin of Escherichia coli HB101 cells

Transformation of E. coli HB101 cells was carried out according to the method of Dagert and Ehrlich (1979).

A single bacterial colony from an overnight L-agar plate was inoculated into 50ml of L-broth and incubated, with shaking, at 37°C until A650 reached 0.2 The culture was chilled for 10 mins one ice, the cells harvested and resuspended in 20ml of cold 0.1M $CaCl_2$ and incubated at 0°C for 20 mins. They were again harvested and resuspended in 0.1M $Cacl_2$.

Transformation was carried out by adding 0.01ml of recombinant plasmid DNA (4ng) to 0.1ml of cell suspension. The mixture was incubated on ice for 10 mins and then at 37°C for 5 mins. It was then diluted with 2ml of L-broth and incubated for 1 hr at 37°C with shaking. 10µl of the culture was spread on L-agar plate supplemented with tetracycline. Transformants were selected on ampicillin and tetracycline plates.

5) In situ hybridisation of transformants.

319 tetracycline resistant, ampicillin sensitive (tet^r amp^s) were prepared for hybridisation as described by Grunstein and Hogness (1975) with the modifications of Humphries et al., (1978).

Millipore nitrocellulose sheet was cut into circles (85mm diameter), autoclaved and placed on agar in 90mm petri-dishes. Bacterial colonies containing cDNA were transferred to the filter surface by streaking with sterile toothpicks. The plates were incubated overnight at 37°C. The filters bearing bacterial colonies produced by incubation overnight were placed upon Whatman 3MM paper and wetted with 0.5M NaOH for 10 mins. They were then treated with 1M Tris-Hcl pH 7.4 and finally with 1.5M Nacl, 0.5M Tris-Hcl (pH 7.4) followed by incubation with proteinase K in (0.015M sodium citrate, 0.15M sodium chloride pH7.0). The filters were then rinsed with 0.03M sodium citrate, 0.3M sodium chloride pH 7.0, dried and baked at 80°C for 2 hrs. The baked filters were pretreated at 68°C for 5-20 hrs in 0.045M sodium citrate, 0.45M sodium chloride (pH 7.0).

Cytoplasmic poly(A)$^+$ RNAs from control and heat-shocked HeLa cells labelled with $^{\gamma}$-[^{32}P] ATP (5,000 Ci/mmol) at a specific activity 10^7 cpm/µg was hybridised with replica filters in a sealed polythene sac containing 10ml of 0.045M sodium citrate, 0.45M sodium chloride (pH 7.0) plus 0.5% SDS for 16-24 hrs at 68°C. The filters were then removed, washed with 0.03M sodium citrate, 0.3M sodium chloride pH 7.0 plus 0.5% SDS, dried in the air and exposed at -70°C to X-ray film (Fuji Photo Film Co. Ltd., Tokyo). By comparing the amount of hybridisation of poly(A)$^+$ RNA from heat-shocked cells to that of poly(A)$^+$ RNA from normal HeLa cells, it was possible to identify clones containing cDNA sequences complementary to specific RNAs whose synthesis is increased after heat-shock.

10. Identification of the clones

10.1. Preparation of diazobenzyloxymethyl-paper

Synthesis of diazobenzyloxymethyl paper was carried out according to the method of Alwine et al., (1977) starting from nitrobenzyloxymethyl paper (Schleicher and Schuell, Anderman Co., Ltd., Surrey). The nitrobenzyloxymetyl paper (NBM-paper), was reduced to aminobenzyloxymethyl paper (ABM-paper) by treating it with 2.5ml of 20% (w/v) dithionite per cm^2 of NBM paper for 30 mins at 60°C with shaking. The ABM paper was washed for 20 mins with several washes of water, followed by a 20 mins wash with 30% (v/v) acetic acid. Then it was washed again with water for an extra 20 mins. Just before the reaction with single stranded nucleic acids, ABM paper was converted to diazobenzyloxymethyl form (DBM) by treatment with a solution containing 1.3ml of freshly prepared solution of $NaNo_2$ (10mg/ml) per 50ml of 1.2M Hcl for 30 mins at 4°C. The volume of solution used was about 1.7ml per cm^2 of ABM-paper. At the beginning and the end of this period the solution was checked for free HNO_2 with starch-iodide paper. Then the paper was washed five times for 5 mins each with ice-cold sterile water (10ml/cm^2), followed by two washes (10 mins each) with 2.5ml (per cm^2 of DBM paper) of ice-cold 25mM phosphate buffer (pH 6.5). Upon washing the paper turned bright yellow. It was kept cold until transfer began, no more than 15 mins later.

10.2. Plasmid DNA binding to diazobenzyloxymethyl-paper (DBM-paper)

The binding of recombinant plasmid DNA to the DBM-paper was carried out as described by Smith et al., (1979).

10µg of recombinant plasmid DNA were digested with endonuclease AluI (Uniscience Ltd., Cambridge), ethanol precipitated and redissolved in 25mM potassium phosphate buffer (pH 6.2). Then,

following the addition of 4 volumes of dimethylsulphoxide, the DNA was heated for 10 mins at 80°C and cooled on ice. Discs of DBM-paper (1cm diameter) were incubated with 10 µgs of denatured plasmid DNA in small glass vials at 4°C in the dark, overnight. Then the filters were washed twice with sterile distilled water and treated with NaOH (0.4M) for 30 mins at 37°C followed by three washes in sterile distilled water and storage in 10mM Tris-Hcl (pH 8.0), 50% (v/v) formamide at 4°C.

10.3. Hybridisation and elution of unlabelled RNA from DNA filters

The whole procedure was carried out as described by Smith et al., (1979) with the modifications of Cato et al., (1981).

The DBM filters to which recombinant DNA was covalently bound were hybridised in 0.5ml of buffer (0.75M Nacl, 0.1M Tris Hcl (pH 7.5), 1mM EDTA, 0.5% SDS, 50% (v/v) formamide) containing 125µg of total unfractionated cytoplasmic RNA from heat-shocked cells in 24-well microtitre plates (Linbro, Flow Laboratories, Connecticut U.S.A.). Hybridisation was carried out with gentle shaking at 40°C for 16 hrs. Thereafter the filters were washed twice in 5 ml of (0.015M sodium citrate, 0.15M sodium chloried (pH 7.0), 0.5% SDS, 2mM EDTA) at room temperature for a total of 10 mins. This was followed by three washes in 5 ml of the same buffer at 60°C for 15 mins. Three more washes in 5ml of 0.0015M sodium citrate, 0.015M sodium chloride (pH 7.0), 0.1% SDS, 2mM EDTA at 60°C for 15 mins were carried out. The filters were then rinsed twice for 2 mins in 10mM Tris-Hcl, pH 7.5, 2mM EDTA. The mRNAs that hybridised to the filters were finally eluted twice in 150µl of 10mM Tris Hcl (pH 7.5), 2mM EDTA, 90% (v/v) formamide at 43° for a total of 30 mins with agitation by placing the 24-well micro titre plates on a rotary incubator. The eluted RNA was made 0.3M sodium acetate (pH 5.3), and

2µg yeast tRNA was added and precipitated overnight in 5 volumes ethanol at -70°C. The RNA was collected by centrifugation and reprecipitated in 70% (v/v) ethanol, 0.3M sodium acetate overnight at -70°C. The RNA was again collected by centrifugation and dried under vacuum.

11. End labelling of RNA

End labelling of RNA was carried out according to the method of Maziels (1976).

About 1µg of RNA was subjected to mild alkaline hydrolysis by heating it at 90°C for 5 or 10 mins in 50mM Tris Hcl (pH 9.5). Hydrolysis was carried out in a sealed cappilary in a total volume of 4µl. Kinase labelling was in 11µl reactions containing 50mM Tris Hcl (pH 9.5), 10mM $Mgcl_2$, 5mM dithiothreitol, 5% glycerol, 50 Ci [γ-^{32}P]-ATP (5,000 Ci/mmol), 1µg of RNA and 3µl of T4 polynucleotide kinase (Boehringer Co., London Ltd.). The reaction continued for 45 mins at 37°C and generated RNA with a specific activity of about 10^7 cpm/µg. The 11µl assay buffer was mixed with 2µl of 2M ammonium acetate, 5µgs tRNA and 90µl ethanol, chilled in ethanol/dry ice for 5 mins and spun in Microfuge. The pellet was dissolved in 25µl of 0.3M sodium acetate (pH 5.0) and 75µl of ethanol was added. The whole was mixed, chilled and centrifuged. The pellet was washed with 1ml ethanol, spun again and dried under vacuum.

12. Spot hybridisation

Spot hybridisation was carried out as described by Cozens et al., (1980).

Recombinant plasmid DNA from different clones was covalently bound at different spots of an 150mm x 20mm DBM-paper strip following the procedure described in Section 10.1. The filter was hybridised overnight with [^{32}P]-labelled RNA at 42°C in 10ml of 50% (v/v)

formamide in 0.075M sodium citrate, 0.75M sodium chloride (pH 7.0). It was then washed with 200 ml of the above buffer at 42°C for 30 mins followed by two washes with 1 litre of 0.015M sodium citrate, 0.15M sodium chloride (pH 7.0), at 65° for a total of 2 hrs. Finally the filter was washed with 1 litre of 0.0015M sodium citrate, 0.015M sodium chloride (pH 7.0), at 65°C for 1 hr. The solution was then drained and the strip was placed in Saron wrap and exposed on Fuji-X-ray film.

13. Determination of radioactivity

1) Radioactivity in RNA molecules was determined by precipitation of RNA with ice-cold trichloroacetic acid (5% (w/v)), and collection of the precipitates on a millipore filter (0.45µm pore size). Following drying (60 mins at 60°C), radioactivity was determined by adding 5ml of toluene scintillator fluid composed of 0.5% PPO (w/v) in Toluene.

2) Detection of radioactivity incorporated into proteins

From each cell-free assay duplicate 1µl aliquots were spotted on Whatman 3MM 2.5cm paper discs which were then dropped into a beaker containing 10% (w/v) trichloroactic acid (TCA). The TCA was brought to a 100°C for 10 mins at the end of which ice was added and the TCA was poured off as radioactive waste. The filters were then washed with a mixture of 10% (w/v) TCA, hydrogen peroxide, formic acid (2:1:1), followed by two washes with water, alcohol and acetone. Finally the discs were dried and placed into scintillation vials containing 0.5ml of 1.0M hyamine hydroxide solution in methanol. The vials were placed in a 60°C oven for 30 mins and the radioactivity was determined by adding 5ml of toluene scintillator fluid composed of 0.5% (w/v) PPO in Toluene.

RESULTS

1. Non-polyadenylated mRNAs as compared with adenylated mRNAs

Non-polyadenylated (poly(A)$^-$) mRNAs have been shown to be very similar to adenylated (poly(A)$^+$) mRNAs in a number of respects, size and base composition (Nemer et al., 1974), capping (Surrey and Nemer, 1976), association with protein (Greenberg, 1977), transcription from unique sequences of DNA (Nemer et al., 1975), rate of synthesis, entry to the cytoplasm and turnover (Milcarek et al., 1974; Nemer et al., 1974; Spradling et al., 1975) and translational efficiency (Fromson and Verma, 1976). However, experiments employing the method of saturation hybridisation by which the low abundance high complexity sequences can be detected (Hereford et al., 1977), have shown that, in mouse liver and mouse brain cells, poly(A)$^-$ mRNAs share little or no sequence homology with poly(A)$^+$ RNAs (Grady et al., 1978; Van Ness et al., 1979; Chikaraishi, 1979). This raises the question whether the translation products of poly(A)$^+$ and poly(A)$^-$ mRNA populations are distinct or not. To examine this question we used the method of in vitro translation in a rabbit reticulocyte cell-free protein synthesising system (Pelham and Jackson, 1976).

1.1. Coding potential of polysome associated poly(A)$^+$ and poly(A)$^-$ abundant mRNAs from HeLa cells

1.1.1. One dimensional analysis of the translation products of polysomal poly(A)$^+$ and poly(A)$^-$ mRNA

In HeLa cells, about 30% of polysome associated mRNA has been shown to lack poly(A) (Milcarek et al., 1974). In order to compare the coding potential of the polysome associated poly(A)$^-$ mRNAs to that of poly(A)$^+$ mRNAs, the following was done:

Polysomes were isolated as described in Methods (Section 3.2.) and polysomal RNA was extracted using the phenol-chloroform method of Penman et al (1966). The RNA was then fractionated into poly(A)$^+$ and poly(A)$^-$ by passing it twice through a poly-(U) Sepharose column (see

Methods, Section 5.1.3.). Poly(A)$^+$ and poly(A)$^-$ RNAs were then translated *in vitro* using an mRNA dependent rabbit reticulocyte cell-free protein synthesising system (Pelham and Jackson, 1976). As it is shown in Figure 5 the optimum ionic requirements were 83.0 mM with respect to K^+ and 0.78 mM with respect to Mg^{++}. The optimum concentration of RNA per 25µl of translation assay was 1µg of poly(A)$^+$ or 5µg of poly(A)$^-$ RNA (see Figure 6). When the products of poly(A)$^+$ and poly(A)$^-$ RNA were analysed on one dimensional acrylamide/SDS gels (see Figure 7), the following was observed:

1) Most of the translation products of both poly(A)$^+$ and poly(A)$^-$ RNAs have electrophoretic mobilities on SDS/polyacrylamide gels that are similar to the proteins that become labelled when HeLa cells are incubated with [^{35}S]- methionine at 37^{o}C for 1 hr.

2) Proteins encoded by poly(A)$^+$ or poly(A)$^-$ RNAs are qualitatively very similar as judged by their mobilities on one dimensional polyacrylamide/SDS gels.

3) However, quantitative differences do exist. For example, protein bands 1 and 2 seem to be enriched among poly(A)$^+$ RNA products, while protein band 4 is enriched among poly(A)$^-$ RNA products.

1.1.2. Two dimensional analysis of the in vitro translation products of polysomal poly(A)$^+$ and poly(A)$^-$ mRNA

A more detailed comparison of the two sets of *in vitro* products was afforded by the higher resolution of the two-dimensional gel electrophoresis of O'Farrell (1975). Figure 8 shows a two-dimensional analysis of polypeptides encoded *in vitro* by poly(A)$^+$ RNA (Figure 8A) or poly(A)$^-$ RNA (Figure 8B).

Comparison of the two fluorograms shows the following:

1) Most of the polypeptides encoded by poly(A)$^-$RNA are also encoded by poly(A)$^+$ RNA. Only 4 polypeptides (indicated by arrows in Figure 8B) are unique products of poly(A)$^-$ mRNA.

FIGURE 5

Ionic requirements for polypeptide synthesis directed by HeLa cell polysomal poly(A)$^+$ RNA in a rabbit reticulocyte cell free protein synthesising system

Assay mixtures utilising [^{35}S] methionine and containing a) different concentrations of magnesium and 83.04 mM Kcl or b) different concentrations of Kcl and 0.784mM magnesium acetate were incubated for 60 min at 37^{o}C with 0.75 g of poly(A)$^+$ RNA and hot trichloroacetic acid-precipitable radioactivity (- -) was determined (see Methods, Section 13.2.).

a) Effect of magnesium acetate concentration on polypeptide synthesis at 83.04mM Kcl.

b) Effect of Kcl concentration on polypeptide synthesis at 0.784mM magnesium acetate.

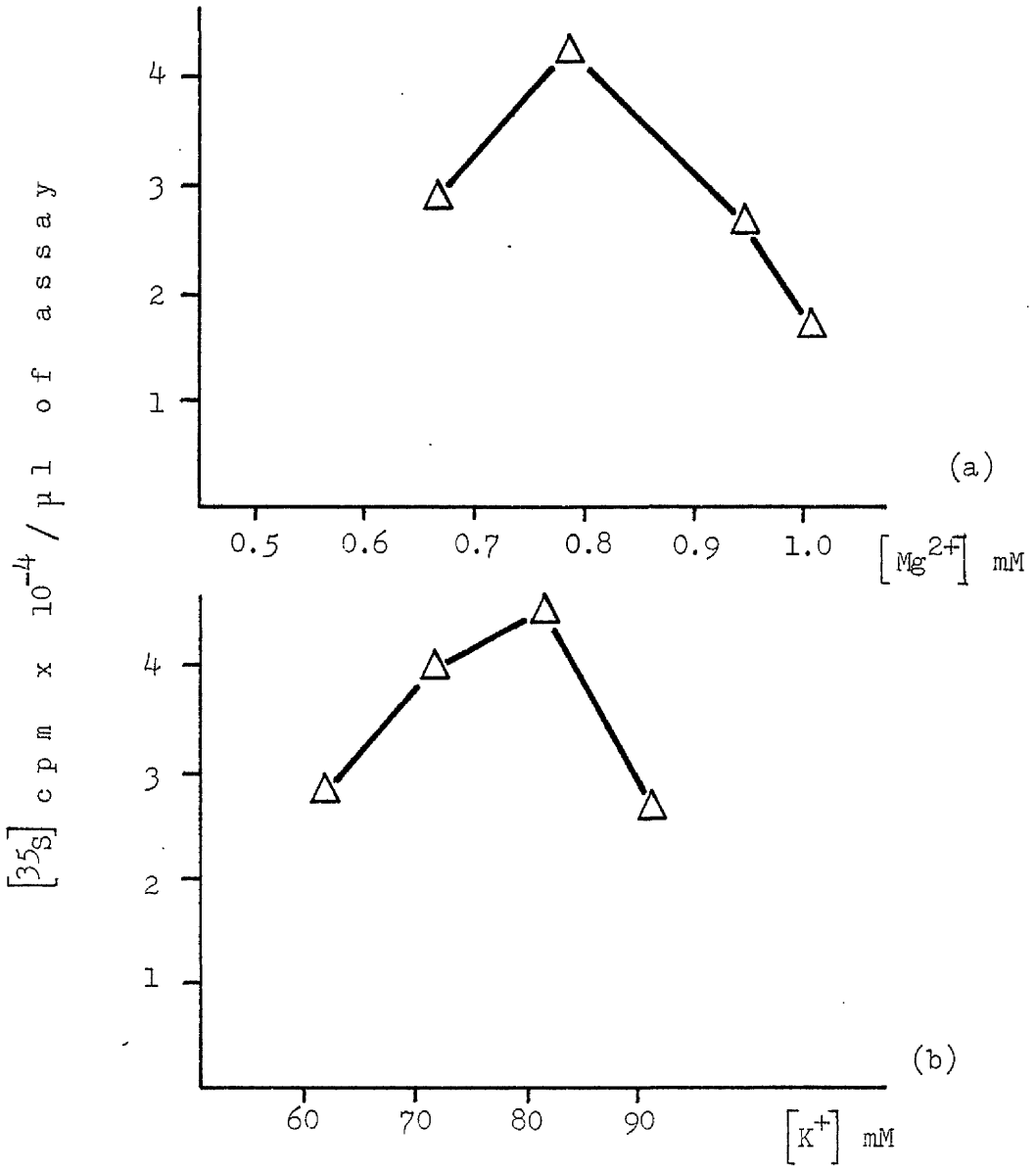

[35S] cpm x 10-4 / µl of assay
4
3
2
1
0.5
0.6
0.7
0.8
0.9
1.0
[Mg2+] mM
(a)
60
70
80
90
[K+] mM
(b)

FIGURE 6

Stimulation of incorporation of L-[^{35}S]-methionine into polypeptides by different amounts of added HeLa cell polysomal poly(A)$^+$ or poly(A)$^-$ RNA in rabbit reticulocyte cell-free protein synthesising system

Assay mixtures containing various amounts of a) polysomal poly(A)$^+$, or b) poly(A)$^-$ and [^{35}S] methionine were incubated for 60 min at 37^{o}C under standard conditions. Hot trichloroacetic acid-precipitable radioactivity was determined (see Methods, Section 13.2.). Incorporation due to endogenous protein synthesis (13,000-15,000 cpm) was subtracted in each case. Data are expressed as cpm of [^{35}S] methionine incorporated into proteins per μl of the translation assay.

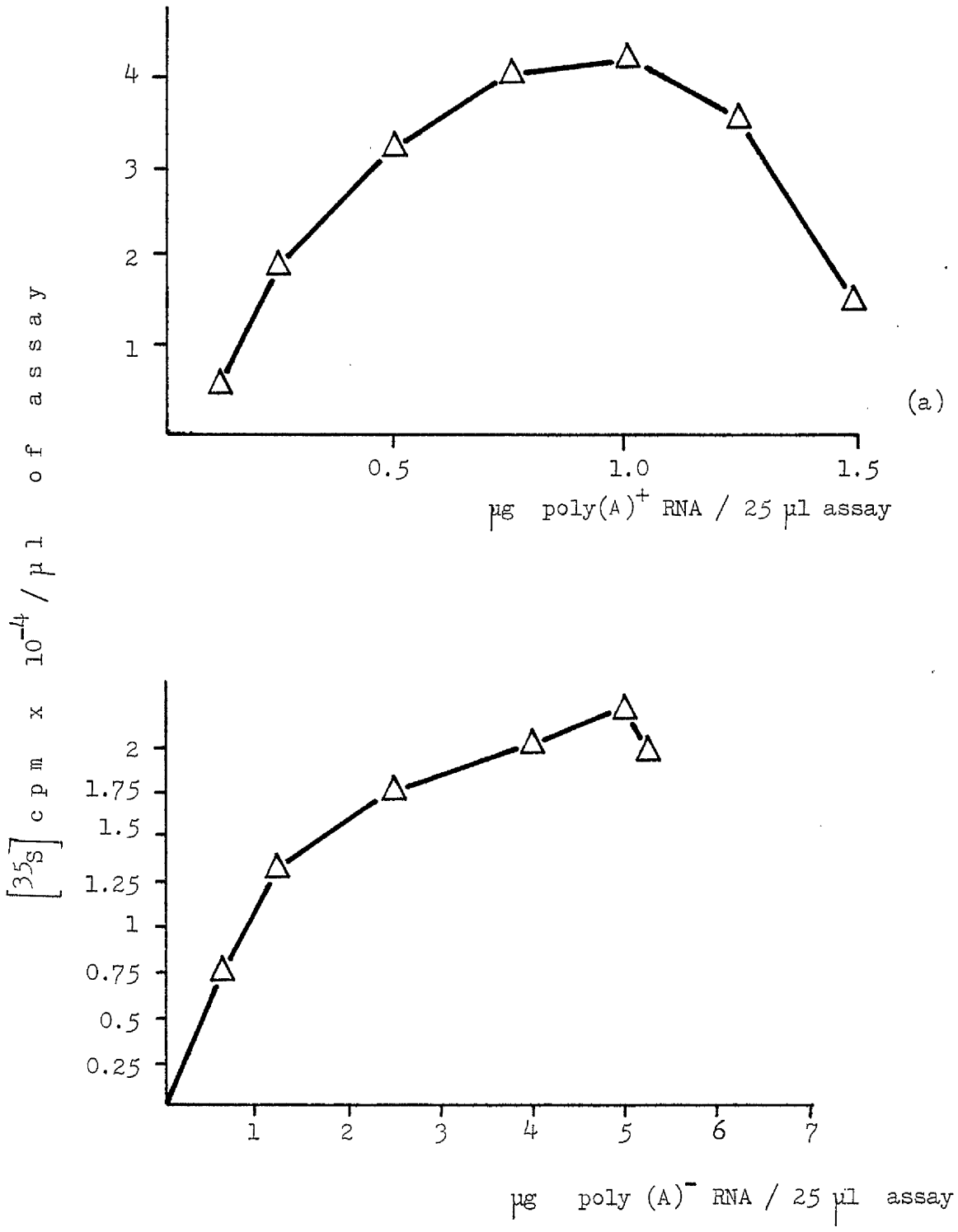

4
3
2
1
0.5
1.0
1.5
(a)
µg poly(A)+ RNA / 25 µl assay
[35S] cpm x 10-4 / µl of assay
2
1.75
1.5
1.25
1
0.75
0.5
0.25
1
2
3
4
5
6
7
µg poly (A)- RNA / 25 µl assay

FIGURE 7

Fluorogram of [^{35}S]-methionine labelled polypeptides resulting from the translation of polysomal poly(A)$^+$ and poly(A)$^-$ RNA from HeLa cells

Polysomal poly(A)$^+$ or poly(A)$^-$ RNA was *in vitro* translated in a rabbit reticulocyte cell;free protein synthesising system. Aliquots (not exceeting 10µl) of translation assays containing poly(A)$^+$ or poly(A)$^-$ RNA's translation products were loaded on a 12.5% polyacrylamide/SDS gel.

Lane 1 analysis of 10µl translation assays containing no exogenous RNA.

Lane 2 Translation products of poly(A)$^+$ RNA added at a concentration of 1µg/25µl assay.

Lane 3 Translation products of poly(A)$^-$ added at a concentration of 5µg/25µl assay.

Lane 4 Proteins labelled *in vivo* by incubating HeLa cells with [^{35}S] methionine for 1 hr at 37°C (see Methods, Section 3.3.).

Calibration proteins (Cornbithek, Boehringer, Mannheim) comprising of β subunit of *E. coli* RNA polymerase (155,000 daltons) bovine serum albumin (68,000 daltons), α subunit of *E. coli* polymerase (39,000 daltons), soya bean trypsin inhibitor (21,500 daltons) were also run. The position to which the markers migrated was determined by staining the gel with 0.25% (w/v) Coomassie brilliant blue in 9% acetic acid and 45% methanol before fluorography.

Bands 1 and 2 are those enriched among the products of poly(A)$^+$ RNA (Lane 2), while bands 3 and 4 are enriched among poly(A)$^-$ mRNA products (Lane 3).

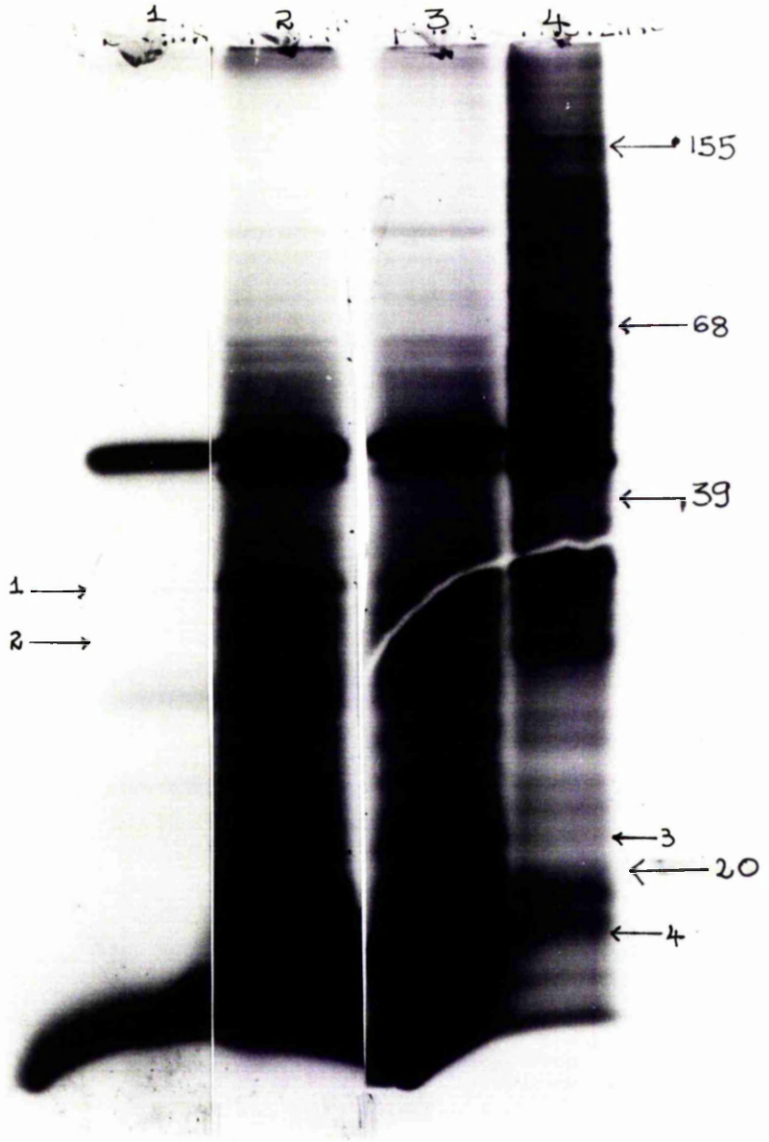

1
2
3
4
155
68
39
1
2
3
20
4

FIGURE 8

Fluorograms of two-dimensional polyacrylamide gels of [^{35}S]-methionine polypeptides resulting from the translation products of polysomal poly(A)$^+$ or poly(A)$^-$ RNA from HeLa cells

Polysomal poly(A)$^+$ or poly(A)$^-$ RNA was translated *in vitro* in a rabbit reticulocyte cell-free protein synthesising system and the products were analysed on two dimensional gels according to the method of O'Farrell (1975). The second dimension was 12.5% polyacrylamide/SDS gel.

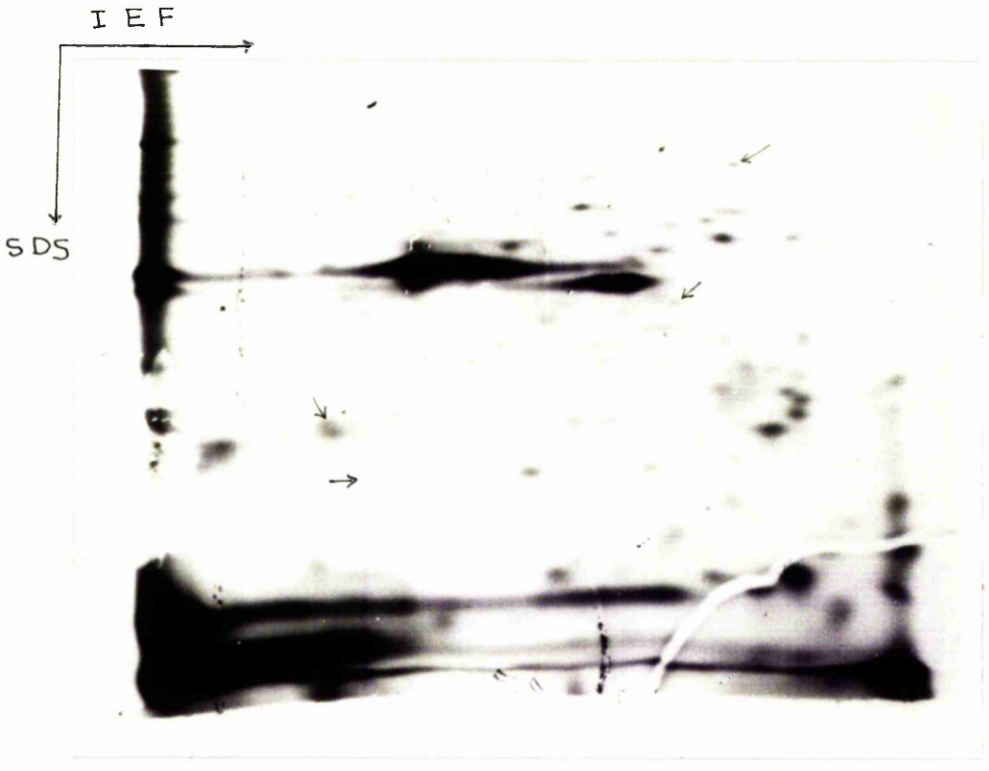
(a) Polypeptides encoded by polysomal poly(A)$^+$ RNA added at a concentration of 1µg/25µl assay.

(b) polypeptides encoded by polysomal poly(A)$^-$ RNA added at a concentration of 5µg/25µl assay.

The arrows in Figure 8A indicate polypeptide spots which are unique translation products of polysomal poly(A)$^+$ RNA.

The arrows in Figure 8B indicate polypeptide spots which are unique translation products of polysomal poly(A)$^-$ RNA.

(A)



(B)

2) 16 of the polypeptides encoded by poly(A)$^+$ RNA are only encoded by this RNA fraction, suggesting that a substantial portion of abundant mRNA sequences are only found in a polyadenylated form.

It should be mentioned that when total cytoplasmic rather than polysomal poly(A)$^+$ and poly(A)$^-$ RNA from HeLa cells was translated *in vitro* in wheat germ cell-free protein synthesising system and the products were analysed in two dimensions, a very similar result was obtained by Kaufmann and colleagues (1977). They had also found that 1) most of the poly(A)$^-$ mRNA products also appeared among the poly(A)$^+$ mRNA products. Only 6 minor proteins had been found to be unique products of poly(A)$^-$ mRNA and 2) many proteins, including 8 major ones, had exclusively appeared in the poly(A)$^+$ mRNA products.

1.2. Coding potential of polysomal poly(A)$^+$ and poly(A)$^-$ mRNA molecules from Friend cells

Since in HeLa cells only some of the abundant coding sequences exist in both poly(A)$^+$ and poly(A)$^-$ forms, the question arose whether the situation is the same in other mammalian cell lines. So, we tested the coding potential of polysomal poly(A)$^+$ and poly(A)$^-$ mRNA from Friend cells.

Polysomal poly(A)$^+$ and poly(A)$^-$ RNA from Friend cells (isolated as described in legend of Figure 9) was translated *in vitro* in a rabbit reticulocyte cell-free protein synthesising system and the products were analysed in two dimensions according to the method of O'Farrell (1975). Comparison of the products encoded by poly(A)$^+$ RNAs (Figure 9A) with those encoded by poly(A)$^-$ RNAs (Figure 9B) shows that, in Friend cells, most of the abundant mRNAs exist in both adenylated and non-adenylated form. Only one polypeptide spot (indicated by an arrow in Figure 9A) was found to be a unique product of poly(A)$^+$ RNA, while two others (indicated by arrows in Figure 9B) are only encoded by poly(A)$^-$ RNA.

FIGURE 9

Fluorograms of two-dimensional polyacrylamide gels of [^{35}S]-methionine polypeptides resulting from the translation products of polysomal poly(A)$^+$ or poly(A)$^-$ RNA from Friend cells

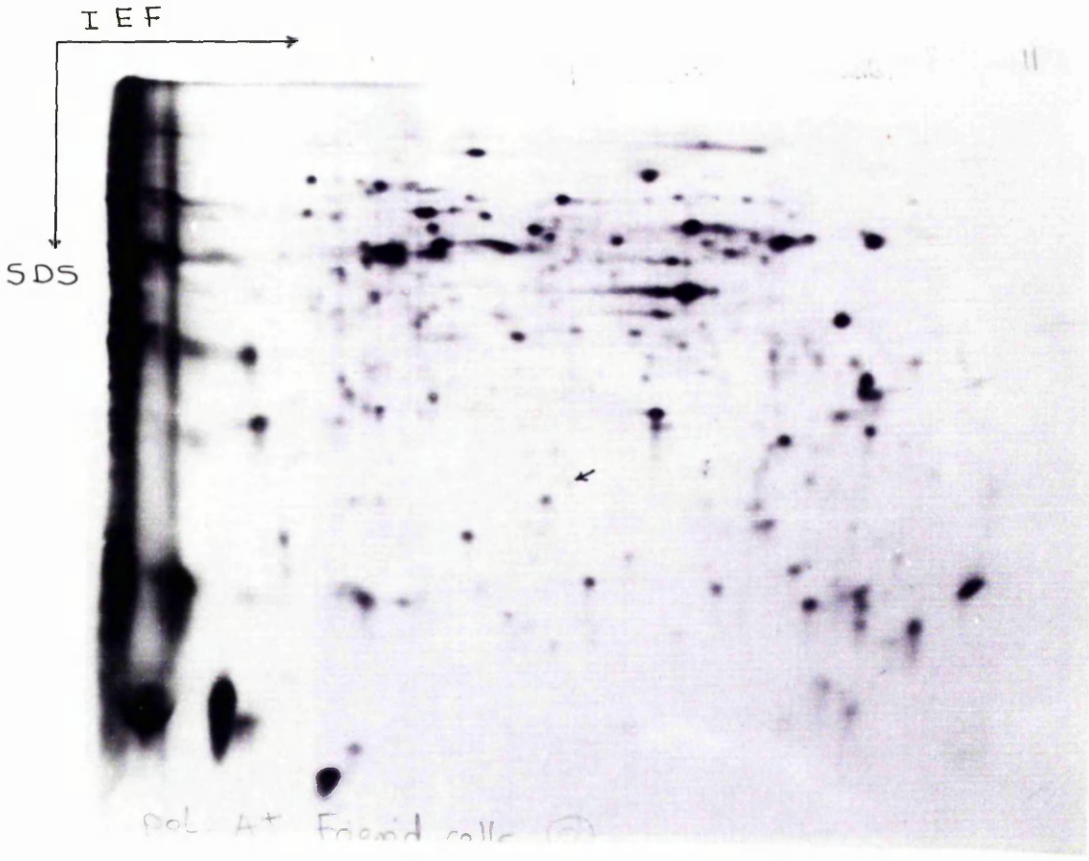
Polysomes were isolated from Friend cells as described in Methods (Section 3.2.2.) and polysomal RNA was extracted according to the method of Penman (1966).

Polysomal RNA was then fractionated in poly(A)$^+$ and poly(A)$^-$ according to the procedure described in Methods (Section 5.1.3.). Poly(A)$^+$ and poly(A)$^-$ RNA was translated *in vitro* under the conditions employed for *in vitro* translation of RNAs from HeLa cells. The products were analysed on two-dimensional gels according to the method of O'Farrell (1975). The second dimension was 12.5% polyacrylamide/SDS gel.

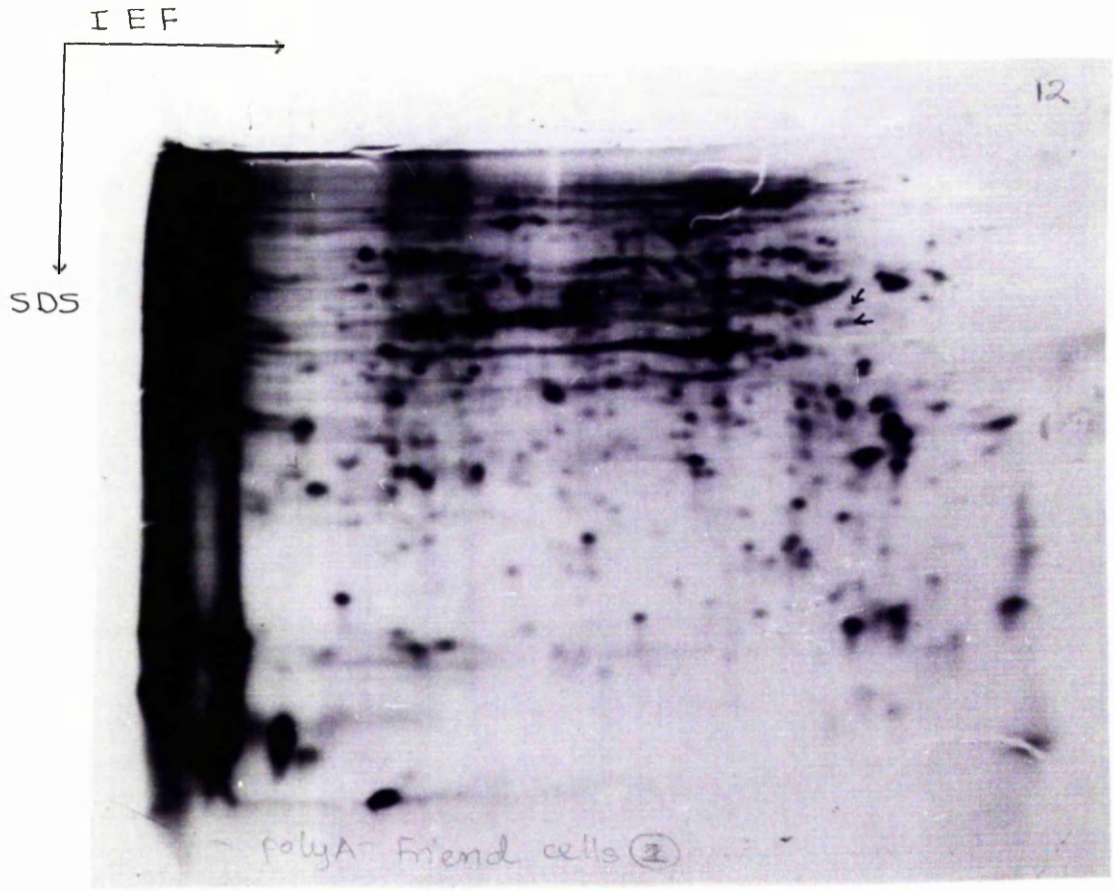
(a) Polypeptides encoded by polysomal poly(A)$^+$ RNA

(b) Polypeptides encoded by polysomal poly(A)$^-$ RNA.

The arrow in (a) indicates the protein which is only encoded by poly(A)$^+$ RNA, while those in (b) the proteins which are only encoded by poly(A)$^-$ RNA.



(A)



(B)

1.3. Conclusion

Despite the reported lack of sequence homology between the high complexity sequences of poly(A)$^+$ and poly(A)$^-$ mRNA molecules, the translation products of polysomal poly(A)$^-$ mRNA are not wholly distinct from those of poly(A)$^+$ RNA in two studied cases. In HeLa cells whilst several proteins do result from the translation of either poly(A)$^-$ mRNAs or poly(A)$^+$ mRNAs a considerable number of mRNAs appear to exist in poly(A)$^+$ and poly(A)$^-$ forms. In fact, in Friend cells it is the latter situation which is predominant.

2. Coding potential of a subset of poly(A)$^-$ mRNA molecules from HeLa cells with high affinity for poly(A) - Sepharose

Although a considerable number of specific mRNAs appear to exist in both adenylated and non-polyadenylated forms the reason for this bimorphism is obscure. Some of the proteins like histones, hen oviduct ovalbumin and the β form of actin in HeLa, Friend and other mammalian cells have been reported to be encoded by both poly(A)$^+$ and poly(A)$^-$ mRNA molecules (Ruderman and Pardue, 1977; Shapiro et al., 1975; Hodgson et al., 1979; Hunter and Garrels (1977); Kaufmann et al., 1977; Minty and Gros, 1980). Since three ovalbumin like genes have been reported in chick (Royal et al., 1979) and the existence of multiple actin and histone genes in several cell types is well documented (McKeown et al., 1978; Kindel and Firtel, 1978; Tobin et al., 1980; Fyrberg et al., 1980; Vandekerchove and Weber, 1978; Engel et al., 1981; Jacob et al., 1976; Kedes, 1979), a question that arises is whether poly(A)$^+$ and poly(A)$^-$ mRNAs coding for the above mentioned proteins are actually transcribed from different genomic sequences. This might explain the lack of sequence homology.

Alternatively poly(A)$^+$ and poly(A)$^-$ mRNAs coding for the same functional protein may have different stabilities. Whilst poly(A)$^-$ and poly(A)$^+$ mRNAs have similar rates of entry in the cytoplasm and decay in HeLa and sea urchin embryo cells(Milcarek et al., 1974; Nemer et al., 1974), globin

deadenylated mRNAs microinjected in Xenopus oocytes were found to be less stable than the corresponding polyadenylated mRNAs (Huez et al., 1974; Marbaix et al., 1975). To examine these questions an approach might be to separate poly(A)$^-$ sequences coding for a particular protein from the bulk of poly(A)$^-$ RNAs and use this in comparisons with poly(A)$^+$ mRNAs coding for the same protein.

A problem with poly(A)$^-$ RNA is that it mainly contains rRNA and tRNA molecules. To examine poly(A)$^-$ mRNA sequences in more detail an initial approach was to isolate a subclass of poly(A)$^-$ mRNAs which exhibit high affinity for poly(A) Sepharose (Katinakis and Burdon, 1981). These molecules were shown to be rich in uridylate sequences and were thus called poly(A)$^-$u$^+$ and they coded for a small number of proteins all of which were encoded by poly(A)$^+$ RNA (see below).

Since it has been reported that in HeLa cells half of the cytoplasmic poly-(U) sequences are detected in poly(A)$^-$ RNA molecules (Korwek et al., 1977), we employed the technique of Katinakis and Burdon (1981) to isolate poly(A)$^-$u$^+$ RNAs from HeLa cells and we tested their coding potential in a rabbit reticulocyte cell-free protein synthesising system.

2.1. Relative amounts of polysomal poly(A)$^+$, poly(A)$^-$u$^+$, poly(A)$^-$u$^-$ RNAs from HeLa cells

Polysomes were isolated from HeLa cells as described in Methods (Section 3.2.1.). RNA was extracted by using the phenol-chloroform extraction of Penman et al (1966). Total cytoplasmic RNA was isolated as described in Methods (Section 4.2.). Polysomal or cytoplasmic RNA was fractionated into poly(A)$^+$, poly(A)$^-$u$^+$ and poly(A)$^-$u$^-$ according to the method of Katinakis and Burdon (1981). RNA was dissolved into binding buffer (see Methods, Section 5.1.3.), denatured at 70^{o}C for 5 mins and then applied twice to a poly-(U) Sepharose column. The material retained by poly-(U) Sepharose is poly(A)$^+$ RNA. RNA which failed to get

bound is referred to as poly(A)$^-$ RNA. Poly(A)$^-$ RNA was then applied twice to a poly-(A) Sepharose column. RNAs bound to the column under these conditions are called poly(A)$^-$u$^+$, while those washed off the column are referred to as poly(A)$^-$u$^-$ RNAs and include rRNA, tRNA as well as non-adenylated mRNA molecules.

The data presented in Tables 3 and 4 show that in both polysomal and total cytoplasmic RNA population, poly(A)$^-$u$^+$ RNAs represent only a small proportion of the total mRNA population in HeLa cells. The fact that poly(A)$^-$u$^+$ RNAs relative concentration in polysomes is half of it's concentration in total cytoplasmic RNA (see Table 4) suggests that poly(A)$^-$u$^+$ RNA molecules are enriched in the post-polysomal fraction.

2.2. Coding potential of polysomal poly(A)$^-$u$^+$ RNA as compared with that of polysomal poly(A)$^+$ RNA

2.2.1. One-dimensional analysis of the translation products of polysomal poly(A)$^-$u$^+$ and poly(A)$^+$ RNA from HeLa cells

The coding potential of poly(A)$^-$u$^+$ and poly(A)$^+$ RNAs was tested by translating *in vitro* in a rabbit reticulocyte cell-free protein synthesising system. As judged from the incorporation of [^{35}S]-methionine into proteins, poly(A)$^-$u$^+$ RNA does not seem to have much stimulating effect on *in vitro* protein synthesis (see Table 5). When the products of poly(A)$^+$ and poly(A)$^-$u$^+$ RNA were analysed on one-dimensional acrylamide/SDS gel very few protein bands were shown to be encoded by poly(A)$^-$u$^+$ RNA (see Figure 10, Lane 2). Most of the bands seen are the products of the lysate's endogenous mRNA, while the rest are also encoded by poly(A)$^+$ RNA (see Figure 10, bands 1, 2, 3, 4, 5, 6).

2.2.2. Two-dimensional analysis of the translation products of polysomal poly(A)$^-$u$^+$ and poly(A)$^+$ RNA from HeLa cells

A more detailed comparison of the *in vitro* products was afforded by the higher resolution of the two-dimensional analysis of O'Farrell (1975).

TABLE 3

Quantitation of poly(A)$^+$, poly(A)$^-$ and poly(A)$^-$u$^-$ in polysomal and cytoplasmic RNAs from HeLa cells

FRACTION OF	POLYSOMAL in µgs		CYTOPLASMIC in µgs	
RNA	Expt.1	Expt. 2	Expt.1	Expt.2
poly(A)$^+$	43.2	30.0	100.0	80.0
poly(A)$^-$u$^+$	4.6	4.0	14.5	10.0
poly(A)$^-$u$^-$	1,600	1,020	2,700.0	2,280.0

TABLE 4

Expression of the poly(A)$^-$u$^+$ RNA amounts as % of A$^+$ or total RNA

	(A)$^-$u$^+$ % of (A)$^+$	(A)$^-$u$^+$ % of total RNA
POLYSOMAL	10.6 - 13.3	0.24 - 0.28
CYTOPLASMIC	12.5 - 14.5	0.51 - 0.61

Polysomes were isolated as described in Methods (Section 3.2.1.) RNA was isolated from polysomes (see Methods, Section 4.3.) or total cytoplasm (see Methods, Section 4.2.) and fractionated into poly(A)$^+$, poly(A)$^-$u$^+$ or poly(A)$^-$u$^-$ as described in Methods (Sections 5.1.3. and 5.1.4.). The optical density of all RNA fractions was measured at 260nm in U.V. light and the amounts of RNAs were calculated from the optical density values taking in account that 1 OD unit corresponds to 40µg RNA. The results in Table 4 express the amount of poly(A)$^-$u$^+$ RNA as % of the poly(A)$^+$ RNA or as % of the total RNA from the same fraction.

TABLE 5

In vitro incorporation of L-[^{35}S-methionine into polypeptides directed by polysomal poly(A)$^+$, poly(A)$^-$u$^+$ or poly(A)$^-$u$^-$RNA from HeLa cells

Polysomal RNA assayed	Amounts µg/25 µl	[^{35}S]-methionine incorporation cpm/ 1 assay
poly(A)$^-$u$^+$	1.0	200
poly(A)$^+$	1.0	43,258
poly(A)$^-$u$^-$	5.0	21,000

Assay mixtures (25µl) containing various amounts of polysomal poly(A)$^+$ or poly(A)$^-$u$^+$ or poly(A)$^-$u$^-$ RNA and [^{35}S]-methionine were incubated for 60 min at 37^{o}C under standard conditions. [^{35}S]-methionine radioactivity incorporated into proteins was estimated as described in Methods (Section 13.2.). Incorporation due to endogenous messenger activity was subtracted in each case.

FIGURE 10

Fluorogram of [^{35}S]-methionine labelled polypeptides resulting from the in vitro translation of polysomal poly(A)$^-$u$^+$ and poly(A)$^+$ RNA from HeLa cells

Polysomes were isolated from HeLa cells (see Methods, Section 3.2.1.) and the RNA was extracted by phenol-chloroform extraction (Penman et al., 1966). The RNA was then fractionated in poly(A)$^+$, poly(A)$^-$u$^+$ and poly(A)$^-$u$^-$ as described in Methods (Sections 5.1.3. and 5.1.4.). Poly(A)$^+$ or poly(A)$^-$u$^+$ RNA was in vitro translated in a rabbit reticulocyte cell-free system. Equal volumes of translation mixtures were loaded on Lanes 1 and 2. The gel was run as described in Methods (Section 7.3). The gel was then processed for fluorography as in Methods (Section 8.).

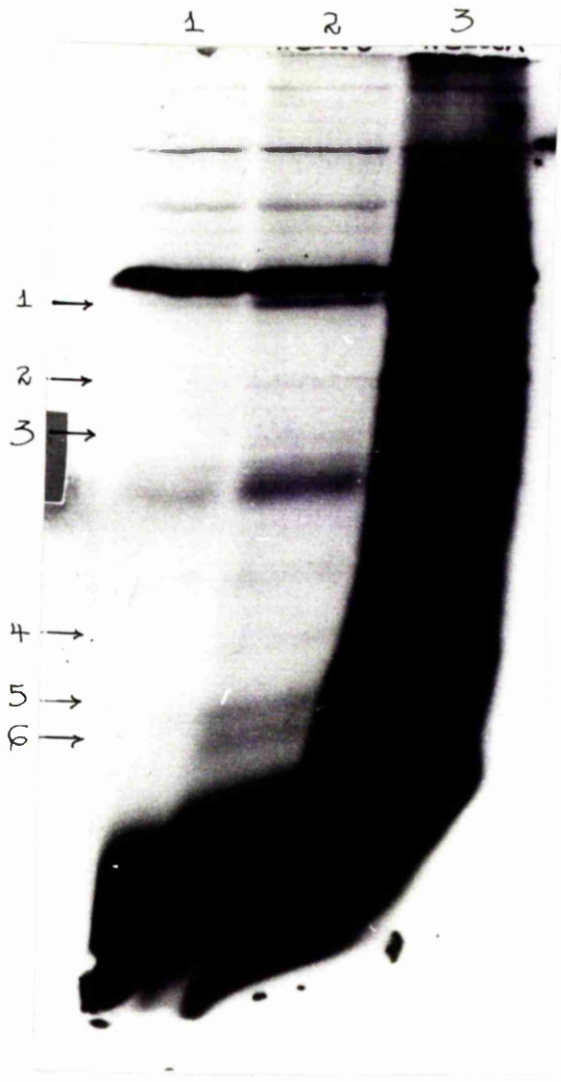
Lane 1 Translation products of lysate's endogenous mRNA.

Lane 2 Translation products of 1µg polysomal poly(A)$^-$u$^+$ RNA.

Lane 3 Translation products of 1µg polysomal poly(A)$^+$ RNA.

Protein bands 2, 3, 4, 5, 6 are detected among the products of polysomal poly(A)$^-$u$^+$ RNA, but not among the products of the lysate's endogenous mRNA.

Band 1 is detected among the products of the lysate's endogenous mRNA, but it is much enriched among the products of poly(A)$^-$u$^+$ RNA.


1
2
3
1
2
3
4
5
6

Comparison of the two-dimensional analysis of polypeptide spots encoded by lysate's endogenous mRNA (Figure 11A) with those encoded by polysomal poly(A)$^-$u$^+$ RNA (Figure 11B) shows that very few polypeptide spots are encoded by poly(A)$^-$u$^+$ RNA. This is in agreement with the conclusions drawn from one-dimensional analysis (see Figure 10). When the two-dimensional pattern of proteins encoded by poly(A)$^-$u$^+$ RNA was compared with that of proteins encoded by poly(A)$^+$ RNA, it was shown that all the polypeptide spots encoded by poly(A)$^-$u$^+$ RNA were found among the products of poly(A)$^+$ RNA (numbered polypeptide spots in Figure 12B).

2.3. Coding potential of cytoplasmic poly(A)$^-$u$^+$ and poly(A)$^-$u$^-$ RNA from HeLa cells

Polysomal preparations include only a portion of the total cytoplasmic poly(A)$^-$u$^+$ RNA sequences, the majority of which have been shown to exist in the post-polysomal fraction of the cytoplasm (see Results, Section 2.1.). Since it is possible that the polysomal poly(A)$^-$u$^+$ RNAs are not representative of the total cytoplasmic poly(A)$^-$u$^+$ RNA sequences, we isolated poly(A)$^-$u$^+$ RNA from total cytoplasm (see Legend, Table 3) and tested its coding potential in a rabbit reticulocyte cell-free protein synthesising system.

The incorporation of [^{35}S]-methionine into polypeptides as a result of cytoplasmic poly(A)$^-$u$^+$ RNA's translation was again at the level of the incorporation due to the lysate's endogenous mRNA *in vitro* translation (Table 6). One-dimensional

FIGURE 11

Fluorogram of two-dimensional polyacrylamide gels of [^{35}S]-methionine polypeptides resulting from the translation of polysomal poly(A)$^-$u$^+$ RNA from HeLa cells

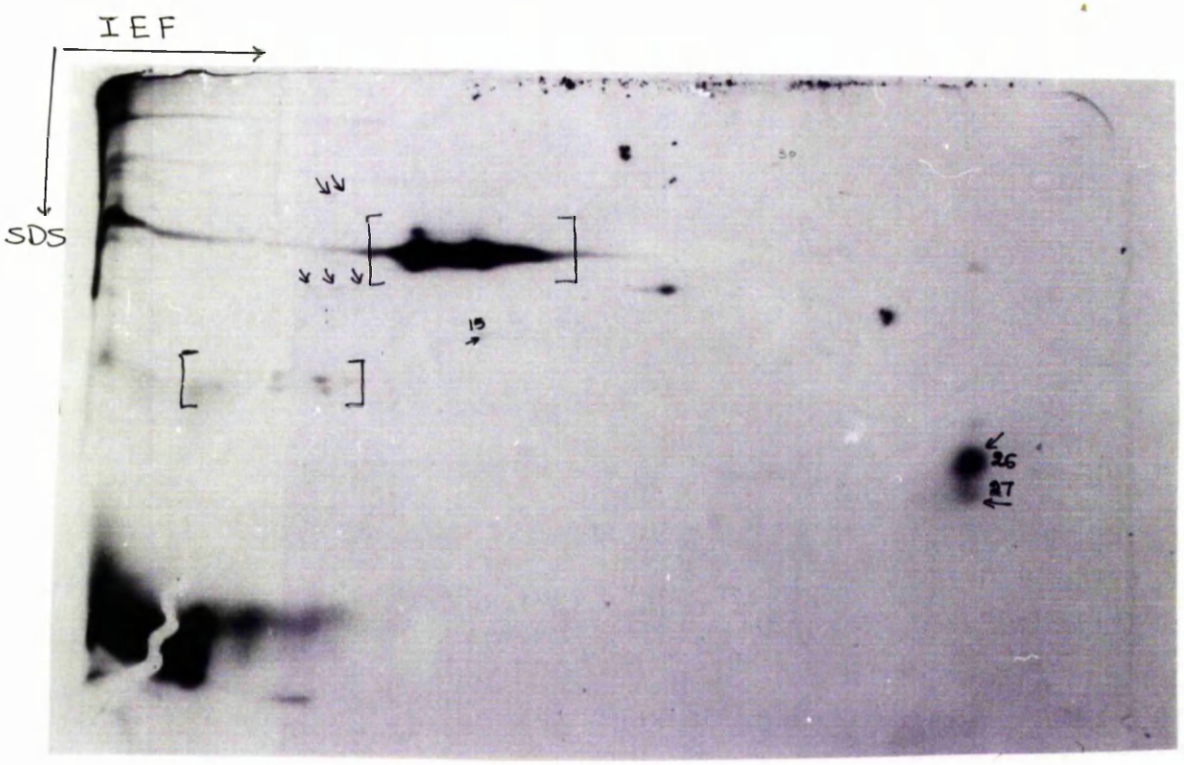
Polysomal poly(A)$^-$ u$^+$ RNA was translated *in vitro* in a rabbit reticulocyte cell-free translation system and the products were analysed on two-dimensional gels according to the method of O'Farrell (1975). The second dimension was 12.5% polyacrylamide/SDS gel.

(a) polypeptides produced when no mRNA is added to the translation system.

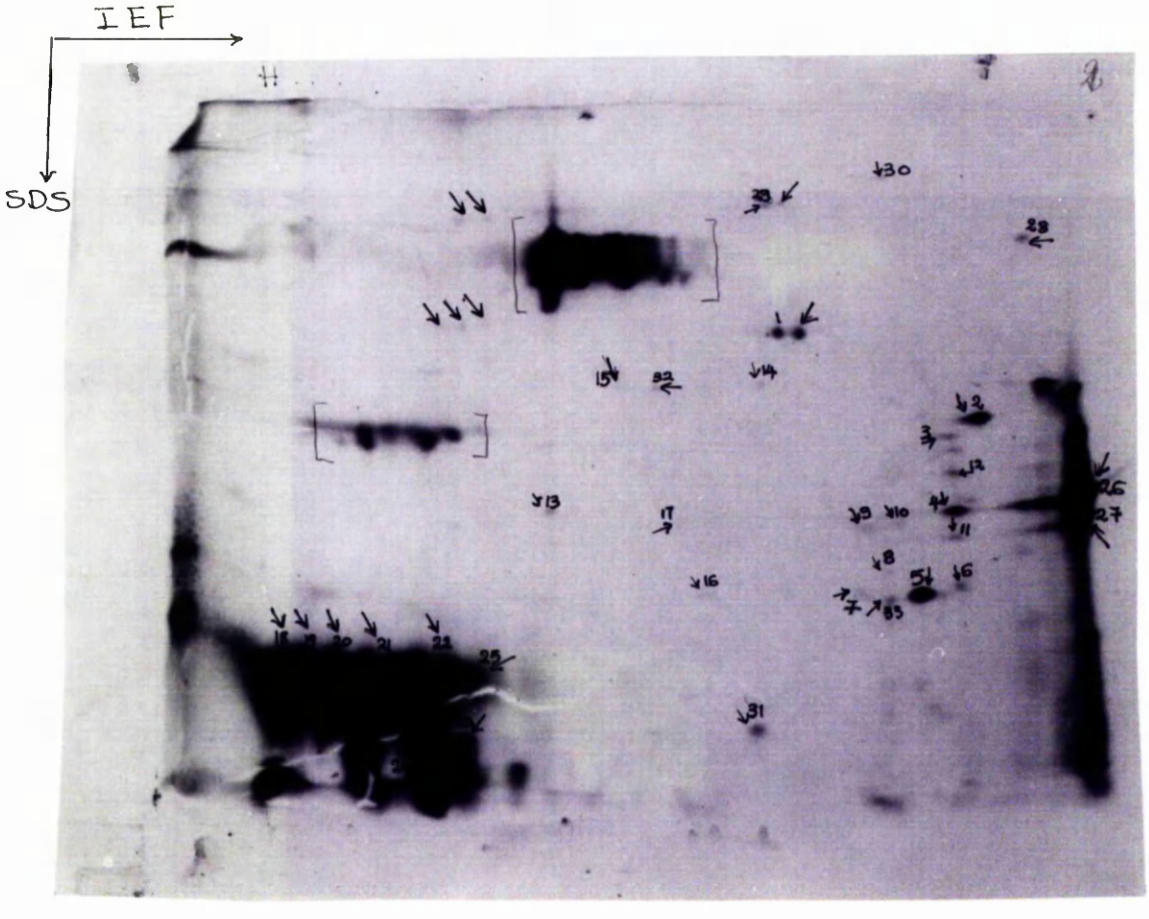
(b) Polypeptides produced when 1µg of poly(A)$^-$u$^+$ RNA was added in 25µl of translation assay.

The arrows in (A) indicate the proteins encoded by the lysate's endogenous mRNA. The arrows in (B) indicate the translation products of poly(A)$^-$u$^+$ RNA and include those shown in (A).

Fluorogram 11B was exposed to X-ray film for a period three times longer than the period of exposure of the fluorogram shown in 11A, even though both gels contained the same amount of cpm. This was done in order to detect all the polypeptide spots encoded by poly(A)$^-$u$^+$ RNA. It is quite possible that this is the reason why some of the spots detected among the lysate's endogenous mRNA products are so much enriched among the poly(A)$^-$u$^+$ RNA's products.



(A)



(B)

TABLE 6

In vitro incorporation of [^{35}S]-methionine into polypeptides directed by cytoplasmic poly(A)$^+$, poly(A)$^-$u$^+$ or poly(A)$^-$u$^-$ RNA from HeLa cells

Cytoplasmic RNA assayed	Amounts µg/25 µl	[^{35}S]-methionine incorporation cpm/µl assay
poly(A)$^-$u$^+$	1.0	750
poly(A)$^+$	1.0	62,500
poly(A)$^-$u$^-$	5.0	28,500

Assay mixtures (25µl) containing various amounts of cytoplasmic poly(A)$^+$ or poly(A)$^-$u$^+$ or poly(A)$^-$u$^-$ RNA and [^{35}S]-methionine were incubated for 60 min at 37^{o}C under standard conditions. Incorporated radioactivity was estimated as described in Methods (Section 13.2.). Incorporation due to endogenous messenger activity was subtracted in each case.

FIGURE 12

Fluorograms of two-dimensional polyacrylamide gels of [^{35}S]-methionine polypeptides resulting from the translation of polysomal poly(A)$^+$ or poly(A)$^-$u$^+$ RNA from HeLa cells

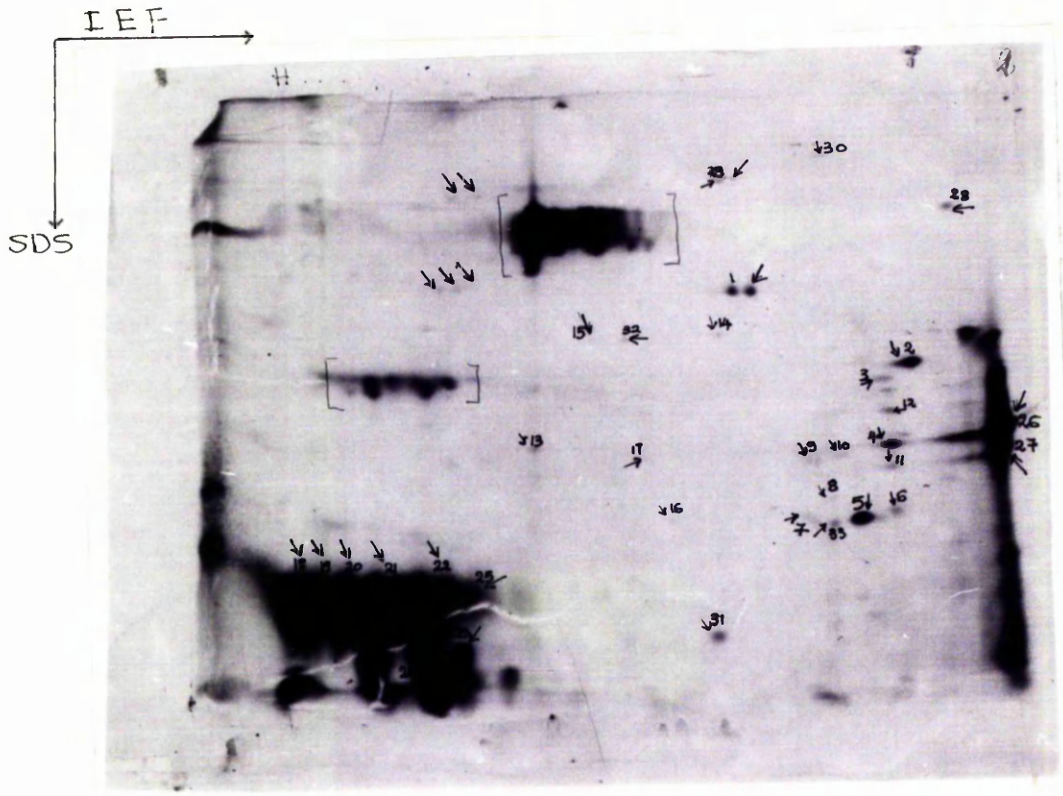
Polysomal poly(A)$^+$ or poly(A)$^-$u$^+$ RNA was translated *in vitro* in a rabbit reticulocyte cell-free translation system and the products were analysed on two-dimensional gels according to the method of O'Farrell (1975). The second dimension was 12.5% polyacrylamide/SDS gel.

(a) polypeptides encoded by polysomal poly(A)$^-$u$^+$ RNA.

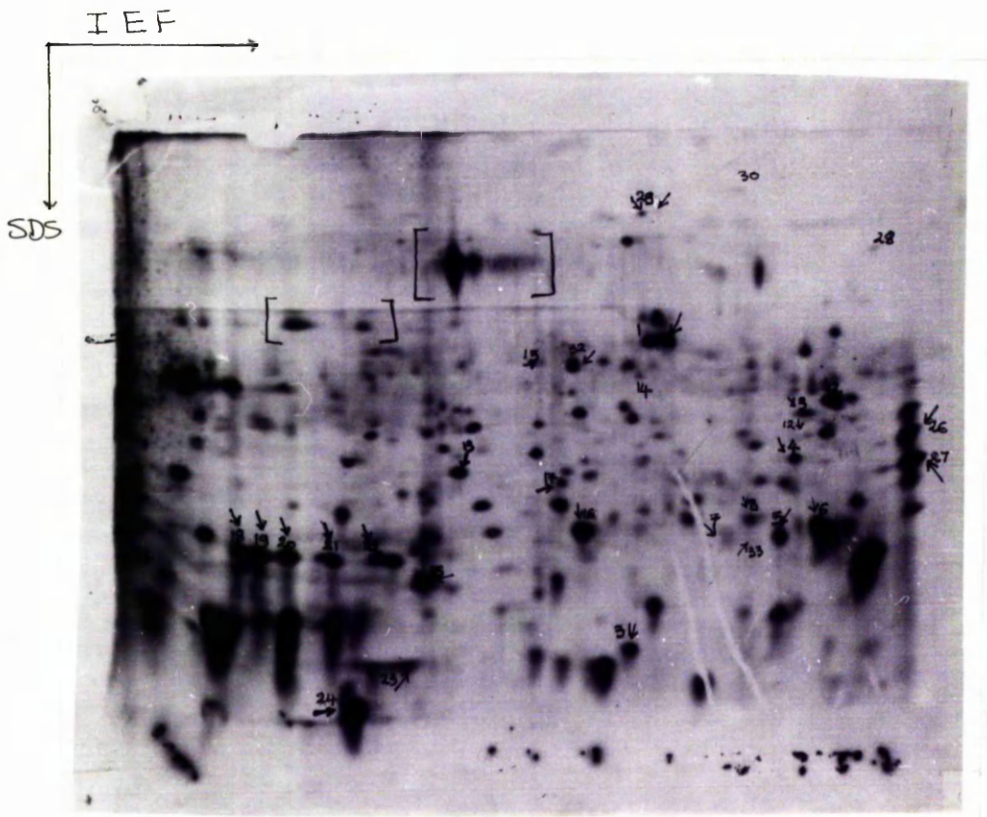
(b) Polypeptides encoded by polysomal poly(A)$^+$ RNA.

All the polypeptide spots detected among the translation products of polysomal poly(A)$^-$u$^+$ RNA (Figure 12A) are indicated by arrows and numbers. Some of the spots in Figure 12A are detected among the products of the lysate's endogenous mRNA (see Figure 11A).

Numbered and arrowed spots in Figure 12A indicate the location of corresponding poly(A)$^-$u$^+$ RNA's products among the products of poly(A)$^+$ RNA.



(A)



(B)

analysis of the products showed that very few protein bands are encoded by poly(A)$^-$u$^+$ RNA and again all are found among the translation products of cytoplasmic poly(A)$^+$ RNA (see Figure 13, bands 1, 2, 3, 4, and 5).

At the same time, comparison of the translation products of polysomal poly(A)$^+$ or poly(A)$^-$u$^-$ RNA with those of cytoplasmic poly(A)$^+$ or poly(A)$^-$u$^-$ RNA (see Figure 14) showed that most of the abundant poly(A)$^+$ or poly(A)$^-$u$^-$ sequences from total cytoplasm are also represented among the polysomal abundant mRNA population in HeLa cells. There is only one exception: band A is encoded by cytoplasmic poly(A)$^+$ RNA, but not by polysomal poly(A)$^+$ RNA.

2.4. The effect of prior heating on the translational efficiency of cytoplasmic poly(A)$^+$ or poly(A)$^-$u$^+$ RNA from HeLa cells

Milcarek (1979) has recently reported the existence of internally located oligo(A) sequences in non-polyadenylated mRNAs from HeLa cells. Since oligo(u) sequences could form intramolecular duplex with such oligo(A) sequences, the question arose whether poly(A)$^-$u$^+$ RNA's secondary structure was responsible for its poor translational activity. In order to answer this question, total cytoplasmic poly(A)$^+$, poly(A)$^-$u$^+$ or poly(A)u$^-$ RNA was heated up at 70^{o}C for 5 mins, cooled rapidly on ice and then added to the rabbit reticulocyte cell-free translation mixture and incubated at 37^{o}C for 60 mins. *In vitro* translation of RNAs which were not

FIGURE 13

Fluorogram of [^{35}S]-methionine labelled polypeptides resulting from the in vitro translation of cytoplasmic poly(A)$^-$u$^+$and poly(A)$^+$ RNA from HeLa cells

Cytoplasmic poly(A)$^-$u$^+$ or poly(A)$^+$ RNA was translated *in vitro* in a rabbit reticulocyte cell-free system under the conditions described for translation of polysomal RNA. The products were analysed on 8.75% polyacrylamide/SDS gel.

Lane 1 Translation products of the lysate's endogenous mRNA.

Lane 2 Translation products of 1µg cytoplasmic poly(A)$^-$u$^+$ RNA.

Lane 3 Translation products of 1µg cytoplasmic poly(A)$^+$ RNA.

Bands 1, 2, 3, 4 and 5 are those encoded by poly(A)$^-$u$^+$ and not by the lysate's endogenous mRNA.

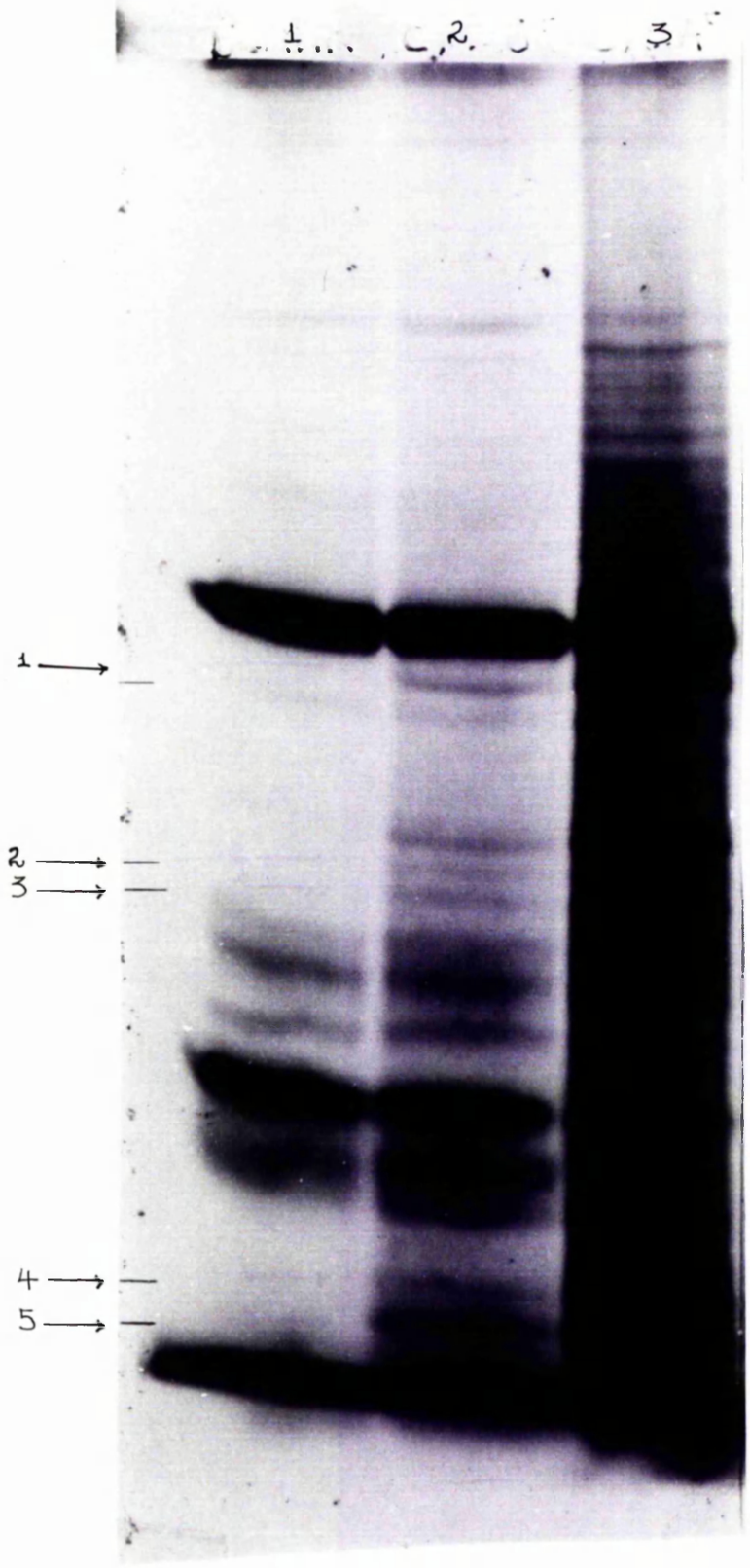

1
2
3
1
2
3
4
5

Figure 14

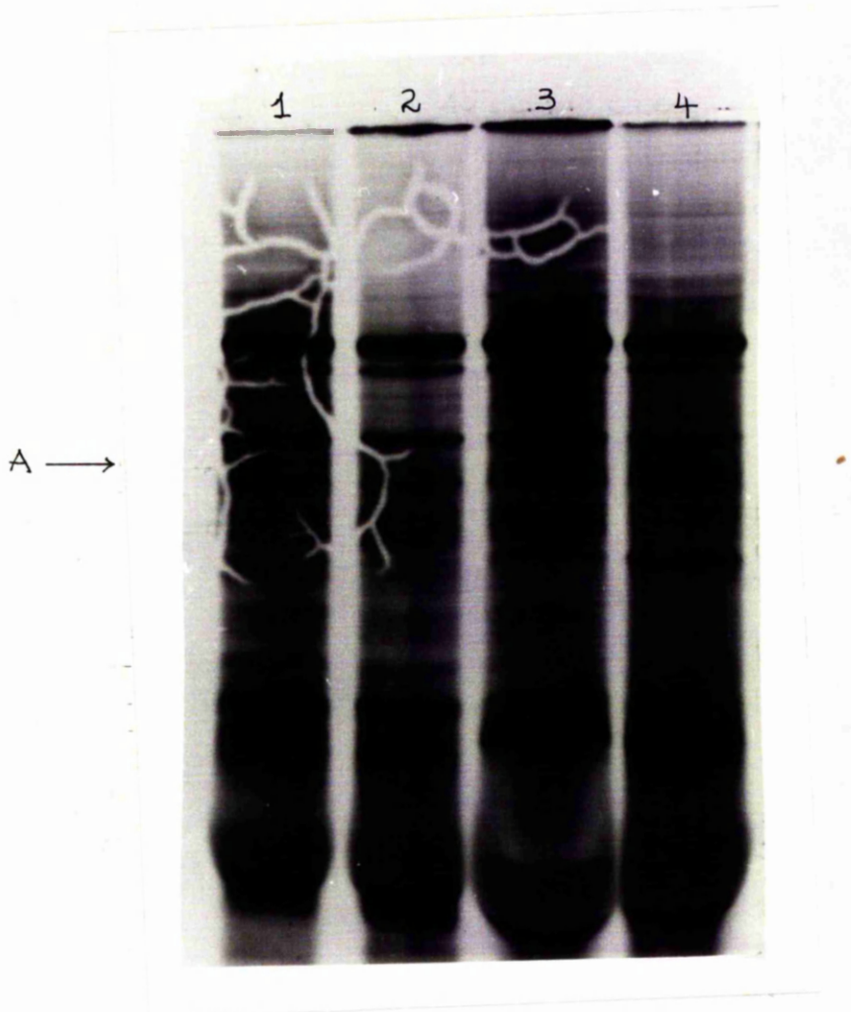
Fluorogram of [^{35}S]-methionine labelled polypeptides resulting from the translation of polysomal and total cytoplasmic poly(A)$^{+}$ and poly(A)$^{-}$u^{-} RNA from HeLa cells

Lane 1 cytoplasmic poly(A)$^{+}$ RNA added to the translation system at a concentration of 1µg/25µl.

Lane 2 Polysomal poly(A)$^{+}$ RNA added to the translation system at a concentration of 1µg/25µl.

Lane 3 Cytoplasmic poly(A)$^{-}$u^{-} RNA added at a concentration of 5µg/25µl.

Lane 4 Polysomal poly(A)$^{-}$u^{-} RNA added at a concentration of 5µg/25µl.


1
2
3
4
A

preheated was also carried out at the same time. The results shown in Table 7 show that preheating at 70°C for 5 mins eliminated the translational activity of poly(A)$^-$u$^+$ RNA, while the translational activity of both poly(A)$^+$ and poly(A)$^-$u$^-$ RNA was not much affected.

2.5. Conclusion

The results presented in this section have shown, that in HeLa cells poly(A)$^-$u$^+$ RNA molecules show little translation activity. However, more than one polypeptides was detected among its translation products and all of which were also encoded by poly(A)$^+$ mRNA. So, to some extent our initial objective of isolating poly(A)$^-$ mRNA sequences coding for a limited number of polypeptides which are also encoded by poly(A)$^+$ mRNAs was achieved. The next step would be to prepare cDNA from these RNA molecules, clone it into the appropriate plasmid and use the cDNA clones for comparisons with poly(A)$^+$ RNA molecules coding for the same proteins. However, when this part of the work was underway, results published by Molloy (1980) showed that some intramolecular duplex structures of oligo(u) sequences with 3' poly(A) can only be disrupted completely with formaldehyde (HCHO) treatment, (which unfortunately renders the mRNA molecules untranslatable (Lodish (1970)). This raised the possibility that the poly(A)$^-$u$^+$ RNA molecules we had isolated were actually polyadenylated RNA molecules which had failed to bind to poly-(U) Sepharose because of the duplex structures formed between the poly(A) tail and internal oligo(u) sequences but which were nonspecifically retained by poly(A) Sepharose.

Thus, the HeLa cell poly(A)$^-$ RNA exhibiting affinity for poly(A) Sepharose seemed a doubtful candidate for extensive comparisons with poly(A)$^+$ mRNA.

3. Protein synthesis in normal and heat-shocked HeLa cells

As an alternative means of exploring the relationship between specific poly(A)$^+$ and poly(A)$^-$ mRNAs in HeLa cells, the mRNAs encoding the specific polypeptides induced after heat shock were examined. When

TABLE 7

In vitro incorporation of [^{35}S]-methionine into polypeptides directed by preheated or unheated poly(A)$^+$, or poly(A)$^-$u$^+$ or poly(A)$^-$u$^-$ RNA fractions from HeLa cells.

		A	B
Cytoplasmic RNA assayed	Amounts µg/25µl	[^{35}S]-methionine incorporation cpm/µl assay	[^{35}S]-methionine incorporation cpm/µl assay
poly(A)$^+$	1.0	37,000	39,000
poly(A)$^-$u$^+$	1.0	0	0
poly(A)$^-$u$^-$	5.0	27,000	25,000

Cytoplasmic poly(A)$^+$, poly(A)$^-$u$^+$ or poly(A)$^-$u$^-$ RNA preheated or unheated were translated in a rabbit reticulocyte cell-free protein synthesising system.

(A) [^{35}S]-methionine incorporation resulting from translation of unheated RNAs.

(B) [^{35}S]-methionine incorporation resulting from translation of RNAs which had been preheated at 70°C for 5 min and cooled rapidly on ice before being added to the translation mixture.

Drosophila larvae, or their excised tissues, are incubated at an elevated temperature (for example, 40 mins at 37°C, the normal culture temperature being 25°C) puffs are induced at several specific polytene chromosome bands (Ritossa, 1964; Ashburner and Bonner, 1979). The induction of the puffs occurs within 1 min of the increase in temperature and the puffs continue to increase in size for 30-40 mins at 37°C before regressing. The maximum size of the puffs are a function of the severity of the temperature shock (Ashburner, 1970).

The heat shock also results in the production of a set of RNAs transcribed from the specific chromosome puffs. Some of these RNAs are preferentially translated into a set of polypeptides known as heat shock polypeptides (Tissieres et al., 1974). Unlike the puffing, the synthesis of the heat shock polypeptides is detected 10 mins after the start of the heat shock and continues for several hours afterwards (Lewis et al., 1975). In general there appears to be at least in Drosophila, no tissue specificity in the number and sizes of the induced proteins even though some charge heterogeneity and size polymorphism has been reported. This may be due to post-translational protein modification and/or aberrant transcription at the elevated temperature (Sondermeijei and Lubsen, 1978; Mirault et al., 1978).

In order to find out whether HeLa cells respond to heat shock treatment in the same way, we studied protein synthesis in normal and heat shocked HeLa cells.

3.1. Electrophoretic analysis of proteins from normal and heat shocked HeLa cells

HeLa cells were heat shocked at 45°C for 5 mins, returned to 37°C for 2 hrs and then labelled for 1 hr with [^{35}S]-methionine. Normal cells were also labelled with [^{35}S]-methionine for 1 hr. Lysates of both control and heat shocked cells were then analysed on one-dimensional acrylamide/SDS gels. Comparison of the protein pattern obtained from

heat shocked cells to that of normal cells (see Figure 15) shows the following:

1) All the protein bands obtained from normal HeLa cells are also obtained from heat shocked cells.

2) There is, however, a noticeable increase in the synthesis of three particular bands in the heat shocked cell lysate. These protein bands have molecular weights in the region of 100,000, 72-74,000 and 37,000 daltons (Figure 15, see arrows a, b and c respectively).

Since each of the heat induced protein bands could well result from a mixture of different polypeptides of the same or similar molecular weights, two-dimensional gel analysis was carried out according to the method of O'Farrell (1975). Figure 16 presents fluorograms of two-dimensional analyses of proteins from control (A) and heat shocked cells (B). Comparison of figure 16A with Figure 16B firstly confirms the observations made in the one-dimensional gel analysis. Secondly, the 72,000-74,000 molecular weight band was resolved into two groups. The more basic one comprised two polypeptide spots (Figure 16, two arrows near group b) and was found to have a pI value around 7.3. The more acidic one probably comprised at least seven polypeptides (Figure 16, group b), whose pI values ranged from 7.1 - 6.5. Thirdly, the 100,000 and 37,000 molecular weight bands were resolved into at least two polypeptide spots each (Figure 16, polypeptides a′ and c′). Comparison of Figure 16A with Figure 16B also reveals some polypeptides (not seen on one-dimensional gels which appear more strongly labelled after heat shock (Figure 16B, spots 1, 2).

3.2. Time course of heat shock protein synthesis

In order to find out how soon and for how long HeLa cells produce increased amounts of heat shocked proteins, cells were incubated in normal medium at 45°C for 5 mins and at various times thereafter

FIGURE 15

Fluorogram of an SDS/polyacrylamide slab gel of [^{35}S]-methionine labelled proteins from normal and heat-shocked HeLa cells

(A) Proteins from cells cultured at 37^{o}C and labelled for 1 hr with [^{35}S]-methionine.

(B) Proteins from cells heat-shocked at 45^{o}C for 5 mins and transferred to 37^{o}C for 2 hrs prior to labelling for 1 hr with [^{35}S]-methionine.

The arrows a, b and c refer to heat-shock protein bands in the molecular weight regions of 100,000, 72,000 - 74,000 and 37,000 daltons.

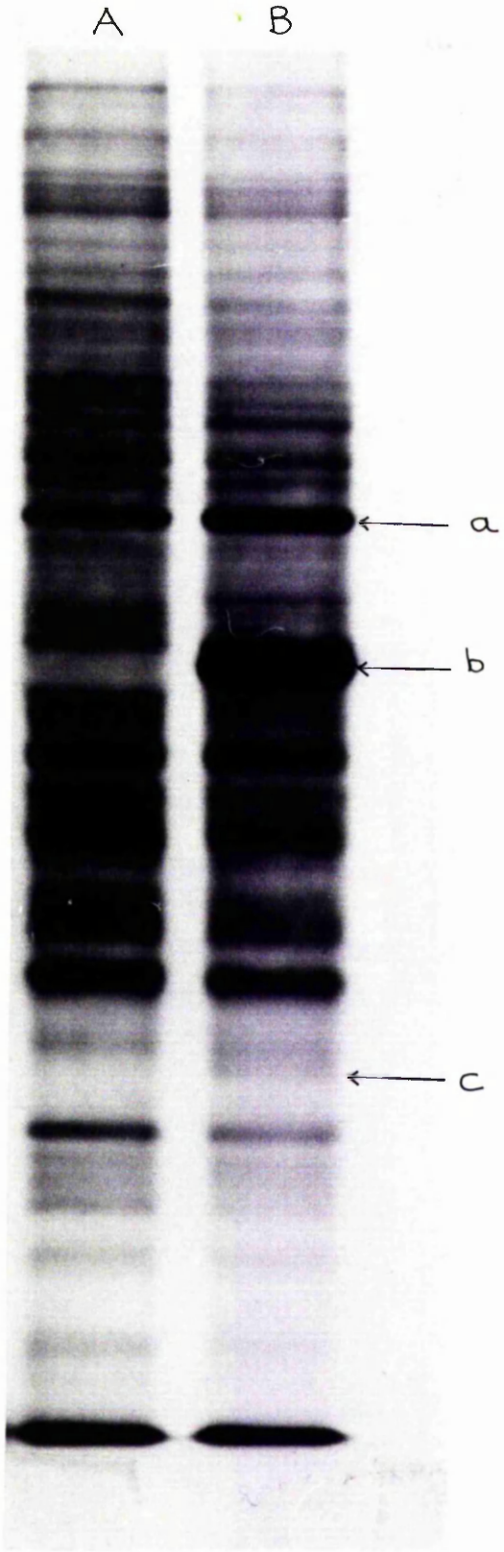

A
B
a
b
c

FIGURE 16

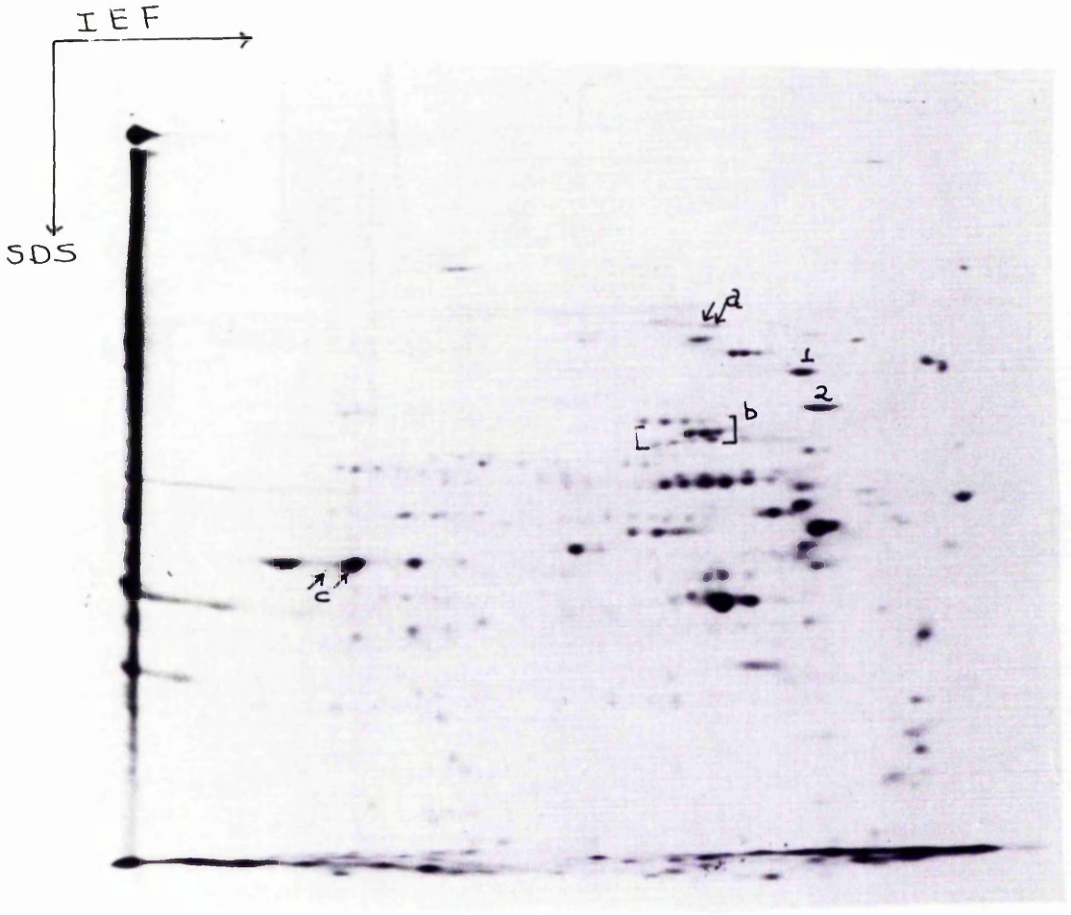
Fluorogram of two-dimensional polyacrylamide gels of [^{35}S]-methionine labelled proteins from control and heat-shocked HeLa cells

(A) Proteins from cell culture at 37°C and labelled with [^{35}S]-methionine for 1 hr at 37°C.

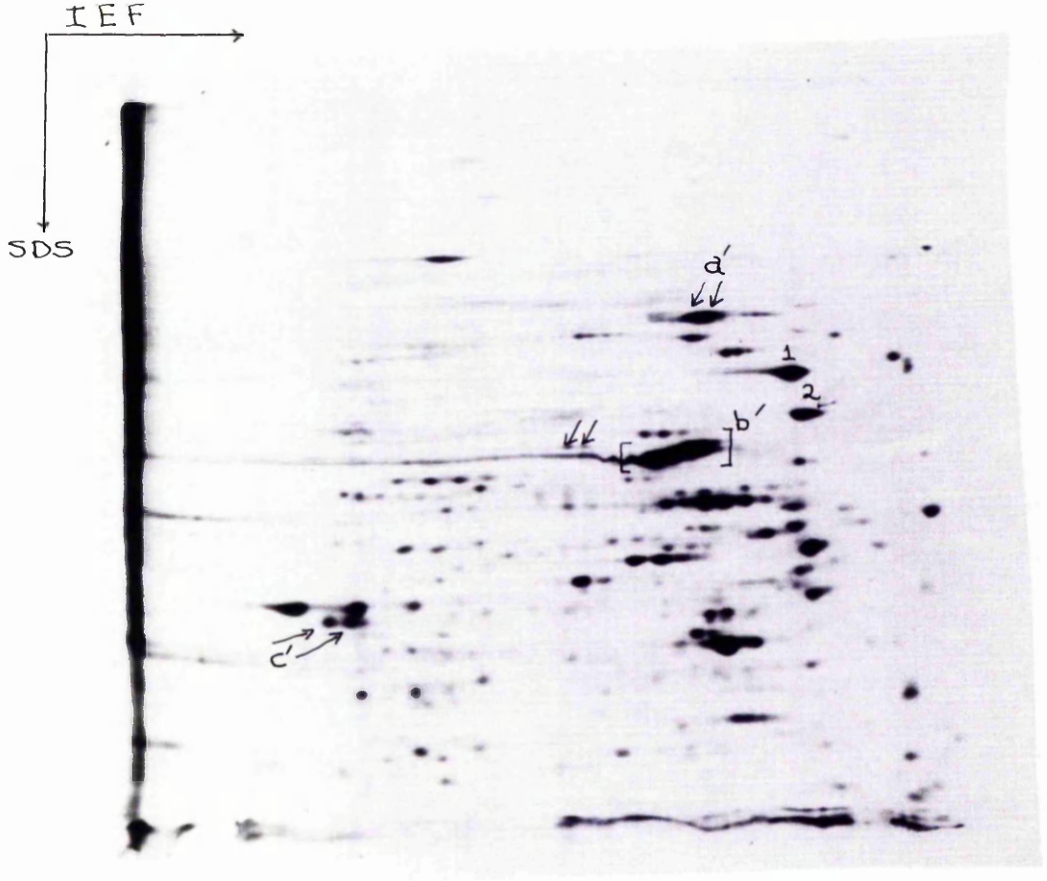
(B) Proteins from cells heat-shocked at 45°C for 5 mins, transferred to 37°C for 2 hrs and then labelled for 1 hr with [^{35}S]-methionine.

The arrows a, b and c refer to the low level of synthesis of the 100,000, 72,000 - 74,000 and 37,000 molecular weight proteins at 37°C, whilst a′, b′ and c′ refer to the increased synthesis of these proteins after heat-shock.

Spots 1 and 2 refer to other polypeptide spots which appear stronger after heat-shock.



(A)



(B)

samples were labelled for 1 hr and the pattern of protein synthesised examined by one-dimensional gel analysis. In order to quantify changes in protein synthesis following the heat treatment, densitometric analysis was applied to the fluorographic pattern of [^{35}S]-labelled proteins separated in these gels. The relative fraction of the heat shock protein bands was calculated as a measure of the area under the individual peak divided by the total area under the scan that included all the protein bands in a particular gel track (Kelley and Schlesinger, 1978). In this way it was found that in the case of proteins with molecular weight of 100,000 and 72,000 - 74,000, synthesis reaches a maximum by 2 hrs after return to normal growth temperature (37^{o}C) but thereafter the level of synthesis declines (Figure 17).

3.3. Is RNA synthesis necessary for the increased synthesis of the heat shock proteins in heat shocked HeLa cells?

In order to find out whether the increased synthesis of heat shocked proteins in heat shocked HeLa cells was due to some control exercised at the transcriptional rather than the translational level HeLa cells were treated with actinomycin D (1µg/ml), heat shocked at 45^{o}C for 5 mins, allowed to recover for 2 hrs at 37^{o}C and then labelled with [^{35}S]-methionine at 37^{o}C for 1 hr. Normal cells were also treated with actinomycin D (1µg/ml) and labelled with [^{35}S]-methionine at 37^{o}C for 1 hr. As it is shown in Figure 18 most of the protein bands labelled *in vivo* in normal HeLa cells (Figure 18, Lane 3) are also labelled *in vivo* in normal HeLa cells treated with actinomycin D (Figure 18, Lane 1). However, when actinomycin D is added to HeLa cells prior to heat shock treatment and during the recovery and labelling period, the absence of increased synthesis of proteins in the 72,000 - 74,000 daltons region was particularly noticeable (Figure 18, compare Lane 2 and Lane 4). The effect on the 100,000 and 37,000 daltons regions was the same but less easy to see.

FIGURE 17

Relative levels of the heat-shock proteins synthesised in 1 hr at 37°C at various times after 5 mins heat-shock at 45°C

Each point shows the relative amount of [^{35}S]-methionine incorporated into proteins in 1 hr at 37°C after heat-shock. The values were determined after scanning with the Joyce-Loebl densitometer and the relative percentage calculated as a measure of the area under an individual peak divided by the total area under the scan that included all the protein in a particular track (Kelley and Schlesinger, 1978).

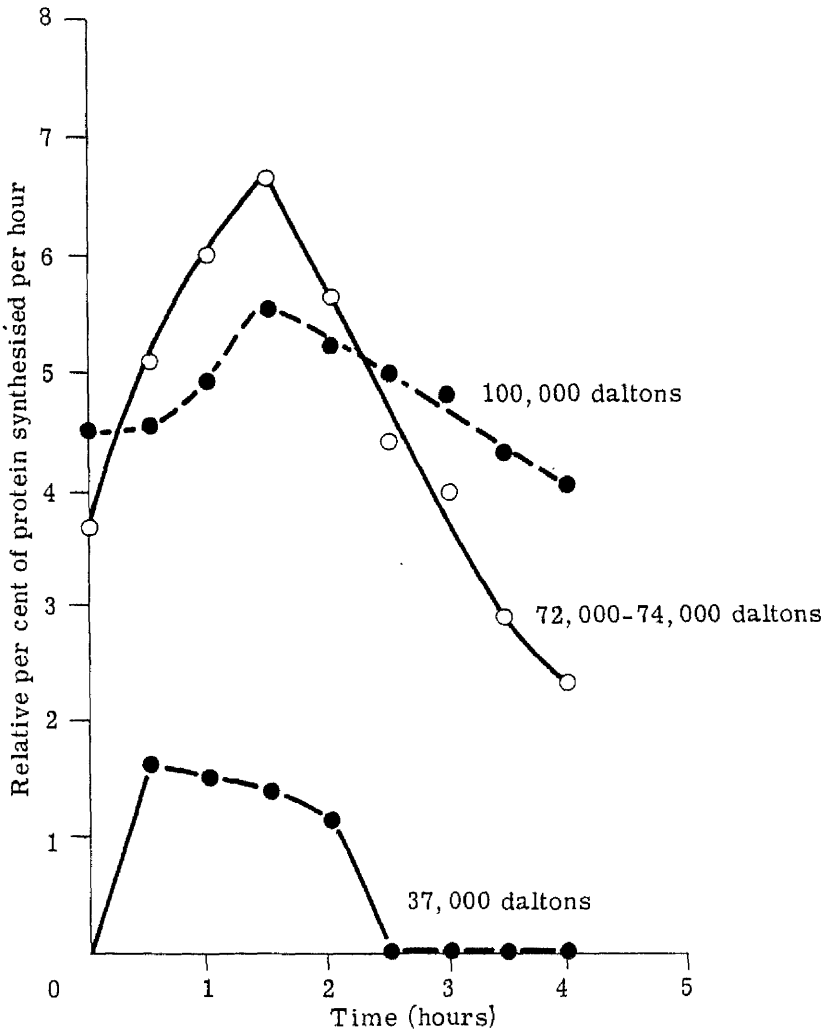

Relative per cent of protein synthesised per hour
8
7
6
5
4
3
2
1
0
1
2
3
4
5
Time (hours)
100, 000 daltons
72, 000-74, 000 daltons
37, 000 daltons

FIGURE 18

Fluorogram of an SDS/polyacrylamide slab gel of [^{35}S]-methionine-labelled proteins from normal and heat-shocked HeLa cells treated with actinomycin D

All lanes show *in vivo* labelled polypeptides from HeLa cells treated in the following way:

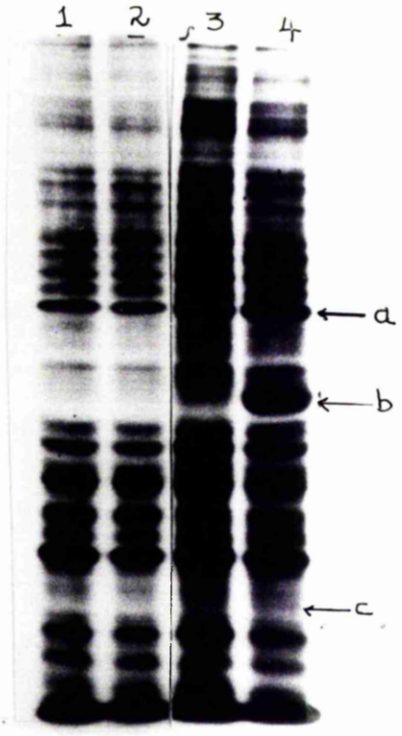
Lane 1 HeLa cells grown at 37°C were treated with actinomycin D (1μg/ml) for 2.5 hrs and then labelled with [^{35}S]-methionine for 1 hr at 37°C in the presence of actinomycin D.

Lane 2 HeLa cells were incubated with actinomycin D (1μg/ml) for 0.5 hrs and then heat-shocked at 45°C for 5 mins. After a recovery period of 2 hrs at 37°C they were labelled with [^{35}S]-methionine at 37°C for 1 hr in the presence of actinomycin D.

Lane 3 HeLa cells grown at 37°C were labelled with [^{35}S]-methionine for 1 hr at 37°C.

Lane 4 HeLa cells were heat-shocked at 45°C for 5 mins, allowed to recover at 37°C for 2 hrs and then labelled with [^{35}S]-methionine for 1 hr at 37°C.

Arrow a indicates the position of 100,000 dalton heat-shock protein, arrow b the position of the 72,000 - 74,000 dalton heat-shock protein and arrow c the position of the 37,000 dalton heat-shock protein.


1
2
3
4
a
b
c

These results suggest that the increased levels of heat shocked proteins in heat shocked HeLa cells may be due to the increased synthesis of heat shock specific mRNAs.

3.4. Conclusion

The results presented in this section have shown that HeLa cells, like Drosophila cells, respond to heat shock treatment by synthesising increased amounts of a small set of polypeptides (hsp's) which are possibly encoded by newly transcribed mRNA molecules. However, the response of HeLa cells differs from that of Drosophila in many aspects.

Firstly, unlike the situation in Drosophila, where maximum synthesis of hsp's takes place about 1 hr after the start of the heat shock treatment (Ashburner and Bonner, 1979), in HeLa cells maximum synthesis occurs 2 hrs after the heat shock treatment and while the cells are recovering at 37°C. Secondly, in HeLa cells there is no evident decrease in the synthesis of pre-heat shock proteins, while in Drosophila heat shock results in preferential translation of heat shock proteins and "shut off" of the synthesis of almost all pre-heat shock polypeptides (Ashburner and Bonner, 1979). Thirdly, the number of heat shock specific polypeptides of HeLa cells is much smaller than that of Drosophila. For example, while in HeLa cells there are three heat shock induced protein bands, in Drosophila melanogaster there are eight major ones. Two of the HeLa hsp's: the one of 72,000 - 74,000 dalton and the other of 37,000 dalton seem to have counterparts among the hsp's of Drosophila melanogaster. Indeed, the major hsp band in Drosophila is 70,000 - 72,000 dalton and it has also been shown to consist of several individual polypeptides (Mirault et al., 1978), while a 36,000 dalton hsp has been detected among the eight hsp's (Ashburner and Bonner, 1979).

4. Are HeLa heat shock proteins encoded by poly(A)$^-$ mRNA molecules?

Poly(A)$^-$ mRNA may be of significance in cells which have to respond rapidly to both external and internal changes.

There is an abundance of poly(A)$^-$ mRNA molecules in early embryonic stages (Ruderman and Pardue, 1977; James and Tata, 1980). A very rapid response of cells to a certain stimulus may well involve poly(A)$^-$ mRNA, since the very act of polyadenylation in animal cells takes about 5-120 mins (Herman and Penman, 1977; Bachellerie et al., 1978; Bastos and Aviv, 1977; Gilmore and Wall, 1979). Furthermore, poly(A)$^-$ mRNAs might be more rapidly removed from the cytoplasm once they are of no further use to the cells. Indeed, histone mRNAs which are being translated only during S-phase of the cell cycle and which disappear rapidly at the end of S-phase are primarily in a poly(A)$^-$ form (Borun et al., 1975; Gallwitz, 1975; Borun et al., 1977). Thus, a reasonable question is: are the mRNA molecules produced as a result of the heat shock treatment mainly non-polyadenylated or not?

To examine this question we tested the coding potential of both poly(A)$^+$ and poly(A)$^-$ mRNA from heat shocked cells in a rabbit reticulocyte cell-free protein synthesising system.

4.1. The adenylation status of mRNAs in HeLa cells following heat shock treatment

To decide whether the mRNAs transcribed during and after the heat shock treatment are non-polyadenylated or not, HeLa cells were treated with low doses of actinomycin D (0.04µg/ml) for 30 mins in order to suppress synthesis of rRNA (Milcarek et al., 1974) prior to labelling with [^{3}H]-Uridine. Then half were heat shocked at 45^{o}C for 5 mins while the rest were allowed to remain at 37^{o}C. Two hours after the heat shock treatment all the cells (control and heat shocked) were harvested.

The results of specific activity determination presented in Table 8 show a decrease in the total amount of RNA transcribed during the 2 hrs recovery from the heat shock treatment. However, the ratio of $\frac{\text{cpm in (A)}^+ \text{ molecules}}{\text{cpm in (A)}^- \text{ molecules}}$ remains the same for both control and heat shocked

TABLE 8

Incorporation of [^{3}H]-Uridine in poly(A)$^+$ and poly(A)$^-$ RNA molecules in the presence of low doses of actinomycin D in normal and heat shocked HeLa cells

Type of HeLa cells	Fraction of RNA	Total cpm	Ratio $\frac{\text{cpm in (A)}^+}{\text{cpm in (A)}^-}$	Specific Activity cpm/µg
CONTROL	poly(A)$^-$	1,096,460	0.47	342.64
	poly(A)$^+$	523,240		7,267.2
HEAT SHOCKED	poly(A)$^-$	537,160	0.42	206.6
	poly(A)$^+$	232,300		5,234.09

Six 80 oz Winchester bottles of HeLa cells, each containing about 10^8 cells were treated with actinomycin D (0.04 µg/ml) for 30 mins prior to labelling. Then [^{3}H]- Uridine was added and 10 mins later half the burlers were heat shocked at 45°C for 5 mins and then allowed to recover at 37°C for 2 hrs (heat shocked cells). The other half remained at 37°C throughout the labelling period (control cells). Total cytoplasmic RNA was extracted from both cell populations as already described in Methods (Section 4.2.). The RNAs were fractionated into poly(A)$^+$ and poly(A)$^-$ by passing it twice through a poly-(U) Sepharose column. The quantity of the RNA was estimated from the optical density readings at 260 nm. Radioactivity was determined as described in Methods (Section 13.1.).

HeLa cells. This suggests that the production of poly(A)$^+$ mRNA molecules is not favoured as a result of the heat shock.

4.2. The 72,000 - 74,000 dalton heat shock protein is encoded by both poly(A)$^+$ and poly(A)$^-$ RNA

In order to find out whether the mRNAs coding for the heat shock proteins exist in both adenylated or non-polyadenylated forms total cytoplasmic RNA was isolated from HeLa cells which had been heat shocked at 45^{o}C for 5 mins and then allowed to recover at 37^{o}C for 2 hrs. The 2 hrs recovery period was chosen because this is the time when maximum synthesis of heat shock proteins occurs (see Results, Section 3.2.). Total cytoplasmic RNA was also isolated from control cells grown at 37^{o}C. Poly(A)$^+$ and poly(A)$^-$ RNA from both control and heat shocked HeLa cells was translated in vitro in a rabbit reticulocyte cell-free protein synthesising system. The products were analysed on one-dimensional acrylamide/SDS gels. The conclusions drawn from the examination of the gel are the following:

1) Comparison of the electrophoretic mobilities of the translation products of cytoplasmic poly(A)$^+$ RNA from control and heat shocked cells showed that most of the protein bands made from cytoplasmic poly(A)$^+$ RNA from heat shocked cells appear to be present in the products translated from the cytoplasmic poly(A)$^+$ RNA from control cells. However, two protein bands were prominent amongst the translation products of cytoplasmic poly(A)$^+$ RNA from heat shocked cells (Figure 19, Lanes 2 and 3). These particular bands have electrophoretic mobilities on SDS/polyacrylamide gel that are similar to the 100,000 and 72,000 - 74,000 daltons heat shock proteins (Figure 19, Lane 7). These heat shock proteins are not made in large amounts in control cells cultured at 37^{o}C (Figure 19, Lane 6), nor were they translated in large amounts in vitro from the poly(A)$^+$ mRNAs from normal HeLa cells cultured at 37^{o}C (Figure 19, Lane 2).

FIGURE 19

Fluorogram of [^{35}S-methionine labelled polypeptides resulting from the translation of total cytoplasmic poly(A)$^+$ and poly(A)$^-$ RNA from control and heat-shocked cells

Total cytoplasmic poly(A)$^+$ and poly(A)$^-$ RNAs were extracted from HeLa cells cultured at 37^{o}C and cells heat-shocked at 45^{o}C for 10 mins, followed by 2 hr at 37^{o}C. 1µg of poly(A)$^+$ amd 5µg poly(A)$^-$ RNA from control and heat-shocked cells were translated in 25µl of a rabbit reticulocyte cell-free system using [^{35}S]-methionine as radioactive label. Aliquots (not exceeding 10µl of translation assay) corresponding to equal amounts of hot TCA precipitable radioactivity were treated as described in Methods (Section 7.1.) and loaded on Lanes 2-7. 10µl of translation assay were treated as in Methods (Section 7.1.) and loaded on Lane 1.

Lane 1 Translation products of lysate's endogenous mRNA.

Lane 2 Translation products of poly(A)$^+$ RNA from control HeLa cells.

Lane 3 Translation products of poly(A)$^+$ RNA from heat-shocked cells.

Lane 4 Translation products of poly(A)$^-$ RNA from control cells.

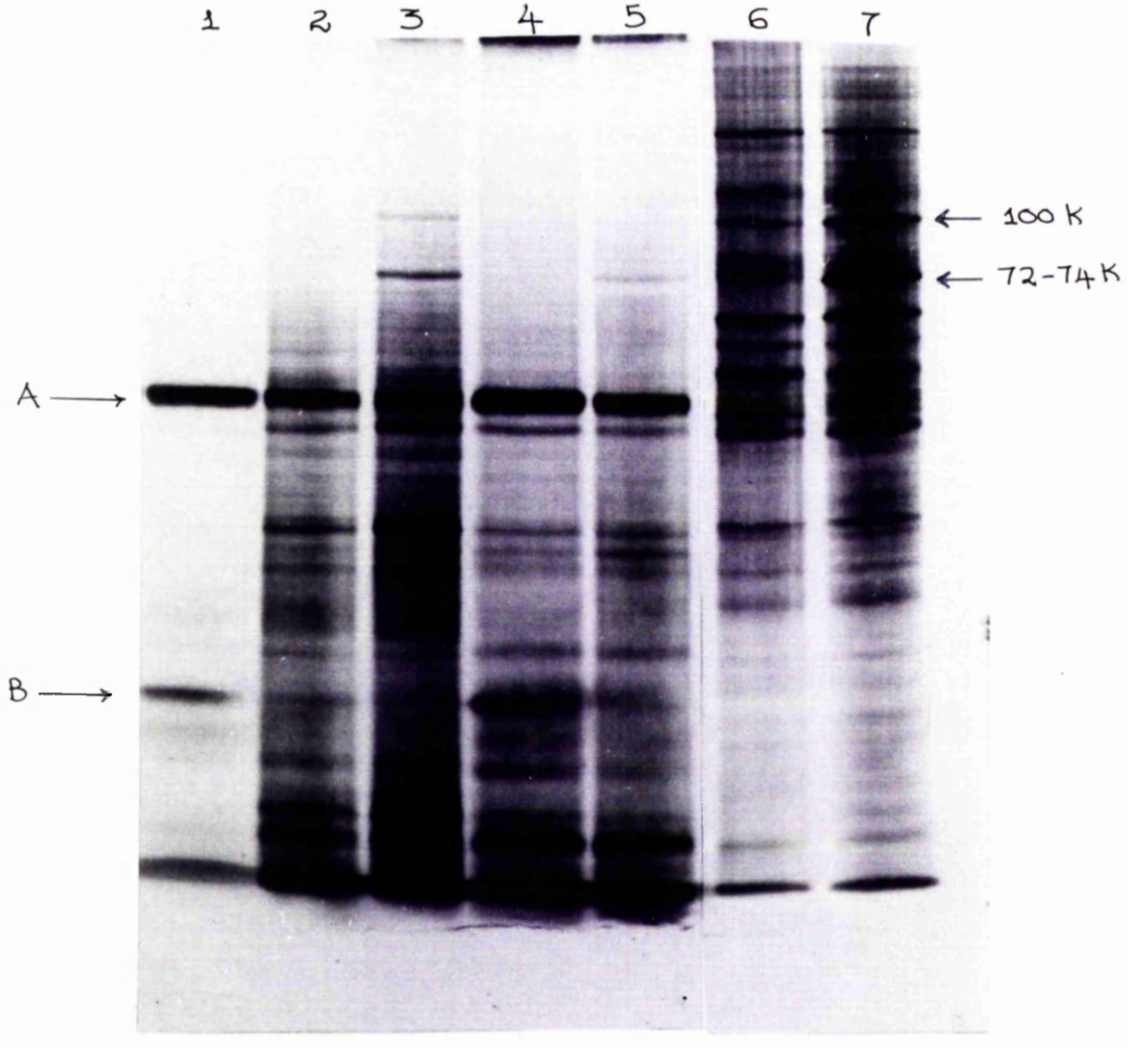
Lane 5 Translation products of poly(A)$^-$ RNA from heat-shocked cells.

Lane 6 Proteins labelled in vivo by incubating intact HeLa cells with [^{35}S]-methionine for 1 hr at 37^{o}C.

Lane 7 Proteins labelled in vivo by incubating heat-shocked cells with [^{35}S]-methionine for 1 hr after 2 hrs recovery.

Arrows indicate the migration of proteins in the 100,000 and 72,000 - 74,000 dalton size classes respectively.

Bands A and B always appear amongst the products when the lysates are used without added mRNAs. They may be the result of translation of endogenous mRNA or the outcome of covalent attachment of methionine to endogenous proteins (Morch and Benicourt, 1980).


1
2
3
4
5
6
7
100 K
72-74 K
A
B

2) Comparison of the electrophoretic mobilities of translation products of cytoplasmic poly(A)$^-$ mRNA from control and heat shocked cells showed that similar to the situation with cytoplasmic poly(A)$^+$ RNA most of the proteins products made from total cytoplasmic poly(A)$^-$ RNA from heat shocked cells appear to be present in the products translated from the cytoplasmic poly(A)$^-$ RNA from control cells except for an enrichment only in the 72,000 - 74,000 dalton region (Figure 19, Lanes 4 and 5).

Comparison of the translation products of heat shocked cell poly(A)$^+$mRNA (see Figure 19, Lane 3) with those of heat shocked cell poly(A)$^-$ mRNA (see Figure 19, Lane 5) shows that the 72,000 - 74,000 dalton heat shock protein is much more enriched among the products of heat shocked cell poly(A)$^+$ mRNA. Since the amount of poly(A)$^-$ mRNA molecules is only about 1% of the total poly(A)$^-$ RNA (Kaufmann et al., 1977; Milcarek et al., 1974) it is likely that in 5 µgs of poly(A)$^-$ RNA there is only 0.05 µg of poly(A)$^-$ mRNA. Thus, the difference in the intensity of the 72,000 - 74,000 among the products of poly(A)$^+$ and poly(A)$^-$ mRNA might be due to the fact that the concentration of poly(A)$^+$ mRNA used is almost 20 times greater than that of poly(A)$^-$ mRNA.

In order to investigate this possibility heat shocked cell poly(A)$^+$ RNA was translated in vitro at various subsaturating concentrations and the products of each translation assay were analysed on one-dimensional polyacrylamide/SDS gels. As it is shown in Figure 20 (Lane 5) when the concentration of heat shocked cell poly(A)$^+$ RNA is close to the actual concentration of poly(A)$^-$ mRNA the intensity of the 72,000 - 74,000 dalton heat shock protein is diminished. This suggests that the difference in the intensity of the 72,000 - 74,000 dalton heat shock protein among the translation products of poly(A)$^+$ and poly(A)$^-$ RNA (Figure 19) may be due to the difference in the amounts of mRNA translated in each case. It is of interest that while the production of most proteins is decreasing with

FIGURE 20

Fluorogram of [^{35}S-methionine labelled polypeptides resulting from the translation of various amounts of heat-shocked cell poly(A)$^+$ RNA

Various amounts of heat-shocked cell poly(A)$^+$ RNA were translated in a rabbit reticulocyte cell-free protein synthesising system under standard conditions and the products were analysed on an 8.75% polycrylamide/SDS gel by loading aliquots corresponding to equal amounts hot TCA precipitable radioactivity

Lane 1 Translation products of 1μg poly(A)$^+$ heat-shocked cell RNA.

Lane 2 Translation products of 0.5μg poly(A)$^+$ heat-shocked cell RNA.

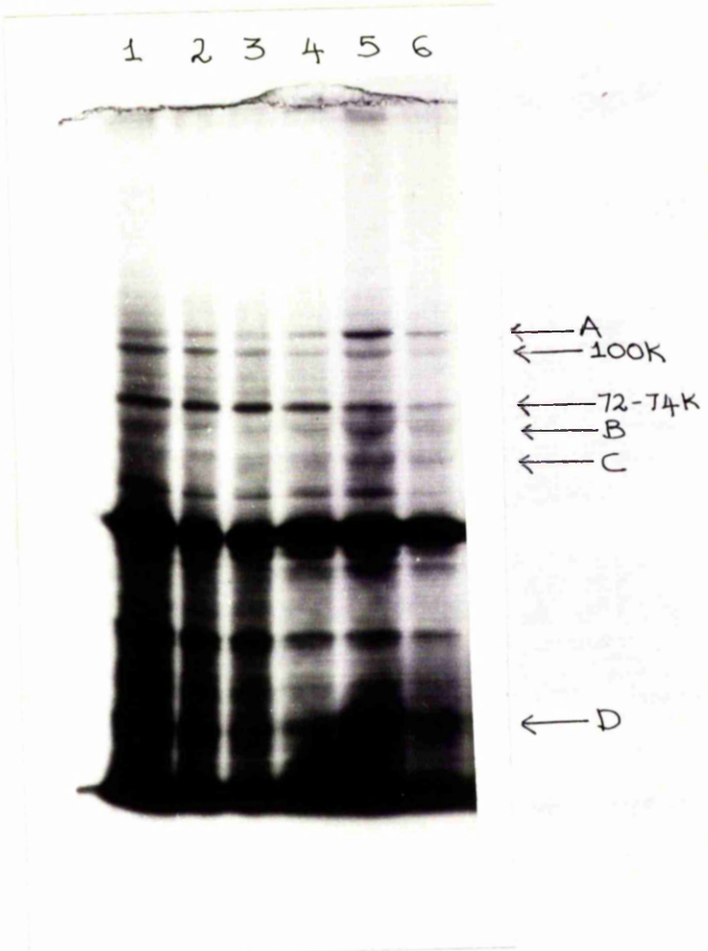
Lane 3 Translation products of 0.25μg poly(A)$^+$ heat-shocked cell RNA.

Lane 4 Translation products of 0.12μg poly(A)$^+$ heat-shocked cell RNA.

Lane 5 Translation products of 0.06μg poly(A)$^+$ heat-shocked cell RNA.

Lane 6 Translation products of 0.03μg poly(A)$^+$ heat-shocked cell RNA.

The position of 100,000 and 72,000 -74,000 heat-shock proteins is indicated. Bands a, b, c, and d are those which are increasing with lower concentrations of poly(A)$^+$ RNA.


1 2 3 4 5 6
A
100K
72-74K
B
C
D

decreasing concentrations of poly(A)$^+$ RNA, the production of some proteins are increased (see Figure 20, bands A, B, C and D).

4.3. Separation of poly(A)$^-$ mRNAs coding for the 72,000 - 74,000 dalton heat shock protein from the bulk of poly(A)$^-$ RNAs

As an initial step in comparing the poly(A)$^-$ and poly(A)$^+$ mRNAs which code for the 72,000 - 74,000 dalton heat shock protein, a partial purification of the 72,000 - 74,000 hsp coding poly(A)$^-$ mRNAs from the bulk of poly(A)$^-$ RNA was carried out. Poly(A)$^-$ RNA from heat shocked cells (which has been shown to yield translation products enriched in 72,000 - 74,000 dalton heat shok protein) was dissolved in 1mM EDTA, 10mM Hepes, pH 7.5, heated at 65^{o}C for 10 mins, cooled rapidly on ice and analysed on 5-20% sucrose linear gradients under the conditions described in Methods (Section 5.2.) 27 fractions were collected. RNA from fractions corresponding to sizes >4S was alcohol precipitated and translated in vitro in a rabbit reticulocyte cell-free protein synthesising system. One-dimensional analysis of the products revealed that 4 fractions contained mRNAs coding for the 72,000 - 74,000 dalton heat shock protein (Figure 21, fractions 6-9). The sedimentation values of these poly(A)$^-$ mRNA molecules was calculated to range between 21S and 24S.

When total cytoplasmic poly(A)$^+$ RNA from heat shocked HeLa cells was analysed on 5-20% sucrose gradients under identical conditions with those described for poly(A)$^-$ RNA and the RNA from each fraction of the gradient was translated in vitro in a rabbit reticulocyte cell-free protein synthesising system, one-dimensional analysis of the translation products revealed a broader size range for the 72,000 - 74,000 dalton hsp coding poly(A)$^+$ mRNAs. As it is shown in Figure 22 poly(A)$^+$ mRNAs coding for the 72,000 - 74,000 dalton group sedimented in fractions 1-10 (approximately between 28S and 18S), whilst the mRNA coding for the 100,000 dalton heat shock polypeptide sedimented between fractions 8 to 10 (approximately 21S

FIGURE 21

Fluorogram of [^{35}S]-methionine labelled polypeptides resulting from the translation of poly(A)$^-$ RNA from heat-shocked cells which was fractionated on 5-20% sucrose gradients

Cytoplasmic poly(A)$^-$RNA from heat-shocked cells was fractionated on 5-20% sucrose linear gradients as described in Methods (Section 5.2.). RNA from the first (from the bottom of the gradient) 19 fractions was alcohol precipitated, quantitated, alcohol precipitated again and the *in vitro* translated by adding equal amounts of RNA (1µg) from each fraction to 25µl of translation mixture. The products were analysed on 8.75% acrylamide/SDS gel.

Lanes 1-19 Present in *in vitro* translation products of RNA from fractions 1-19 of the gradient (counting from the bottom).

Lane 20 Proteins labelled *in vivo* in normal HeLa cells (see Figure 15).

Lane 21 Proteins labelled *in vivo* in heat-shocked cells (see Figure 15).

The arrow indicates the position of 72,000 - 74,000 dalton heat-shock protein.

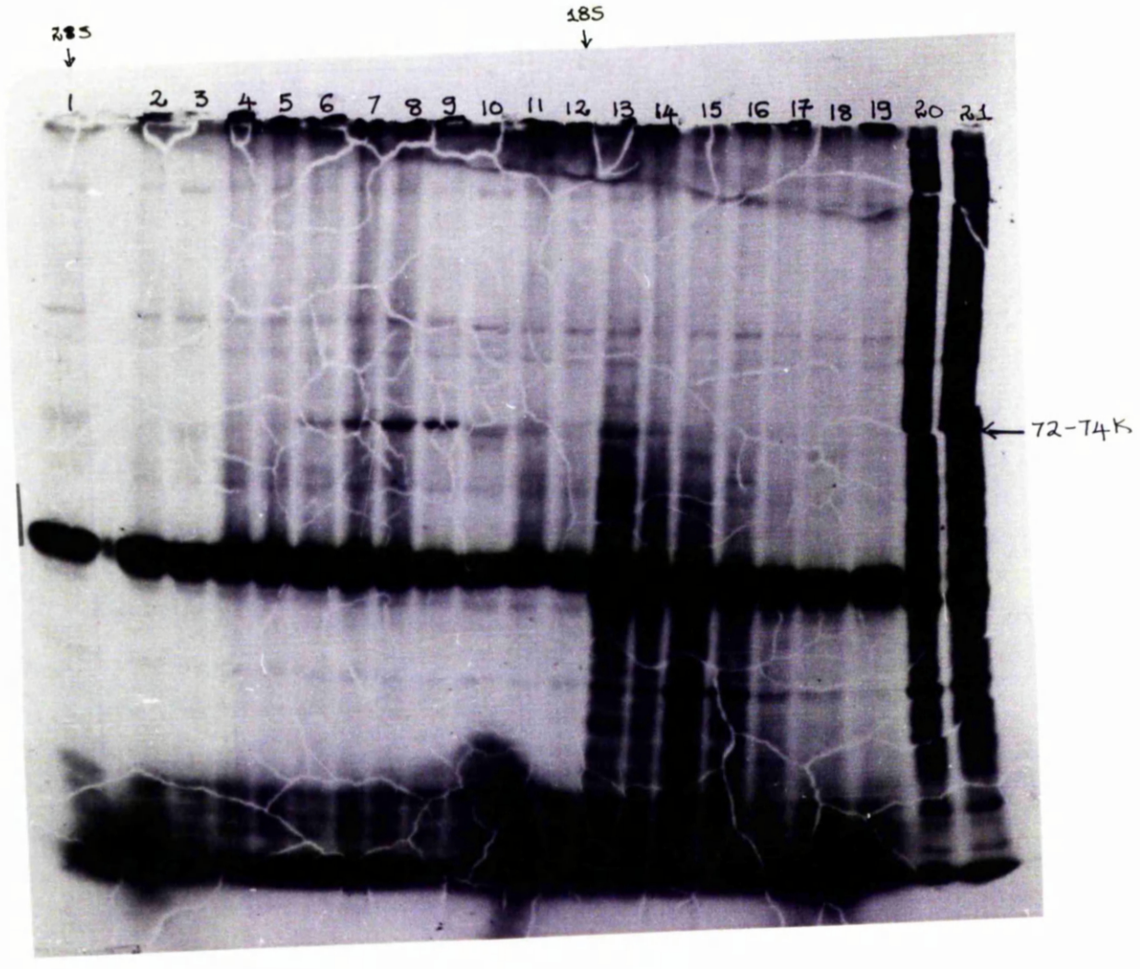

28S
18S
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
72-74K

FIGURE 22

Fluorogram of [^{35}S]-methionine labelled polypeptides resulting from the translation of poly(A)$^+$ RNA from heat-shocked cells which was fractionated on 5-20% sucrose gradients

Cytoplasmic poly(A)$^+$ RNA from heat-shocked cells was fractionated on 5-20% sucrose linear gradients as described in Methods (Section 5.2.). RNA from the first (from the bottom of the gradient) 18 fractions was alcohol precipitated and *in vitro* translated by adding equal amounts of RNA (1µg) from each fraction to 25µl of translation mixture. The products were analysed on 8.75% polyacrylamide/SDS gel.

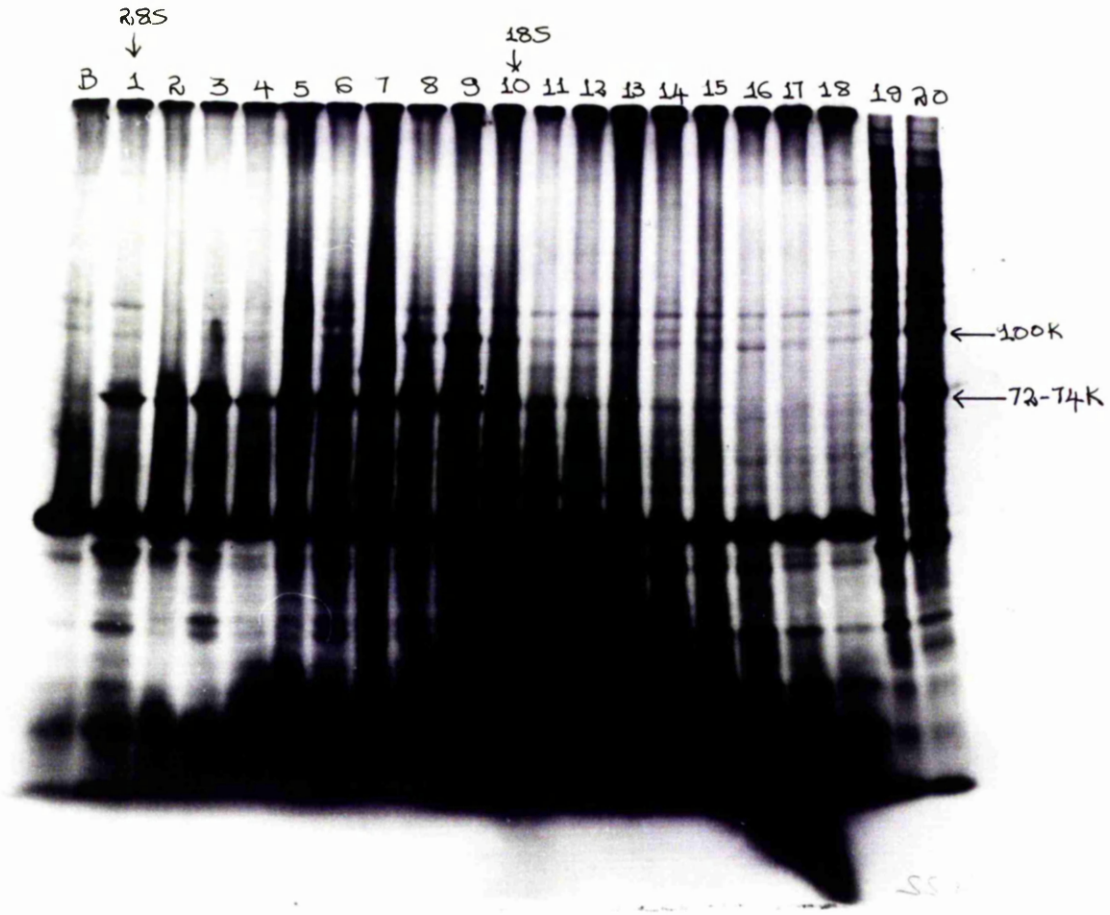
Lanes 1-18 Present *in vitro* translation products of RNA from fractions 1-18 of the gradient.

Lane designated as B shows one-dimensional analysis of proteins encoded by lysate's endogenous mRNA.

Lane 19 Proteins labelled *in vivo* in normal HeLa cells (see Figure 15).

Lane 20 Proteins labelled *in* vivo in heat-shocked cells (see Figure 15).

Arrows indicate the positions of 100,000 and 72,000 - 74,000 dalton heat-shock proteins.


28S
18S
B 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
100K
72-74K

to 18S). The existence of poly(A) tails in the poly(A)$^+$ mRNAs coding for the 72,000 -74,000 dalton proteins can not be the only possible reason for their broad size range compared to that of poly(A)$^-$ mRNAs coding for the same proteins. It is possible, though, that the surprisingly broad sedimentation profile of these poly(A)$^+$ mRNAs may be a consequence of secondary structures adopted by these RNAs during centrifugation in what were non-denaturing gradients.

4.4. Are the poly(A)$^-$ mRNAs which code for the 72,000 - 74,000 hsp actually poly(A)$^+$ mRNAs?

In order to show that poly(A)$^-$ mRNAs coding for the 72,000 - 74,000 dalton heat shock protein are not just poly(A)$^+$ mRNA molecules which for some reason failed to be retained by poly-(U) Sepharose, 1µg of partially purified poly(A)$^-$ mRNA (from fraction 7 of the sucrose gradient presented in Figure 21), which has been shown to code for the 72,000 - 74,000 dalton heat shock protein was partially cleaved with OH^- end-labelled with [^{32}P]-labelled γ-ATP as described in Methods (Section 11). 1µg poly(A)$^+$ RNA from heat shocked HeLa cells was also labelled in vitro under the same conditions after the same treatment. Both samples, dissolved in binding buffer (0.4M NaCl, 10mM EDTA, 0.2% N-lauroyl sarcosine, 10mM Tris, pH 7.4), were denatured at 70°C for 5 mins, cooled rapidly on ice and applied twice to separate poly-(U) Sepharose columns. The columns were then washed with 6 mls of (10mM EDTA, 0.2% N-lauroyl sarcosine, 10mM Tris, ph 7.4) buffer. The bound material was eluted with 12 ml of 90% formamide in (0.5% N-lauroyl sarcosine, 10mM EDTA, 10mM Tris, pH 7.4).

The data presented in Table 9 show that while only 0.786% of the total radioactivity incorporated into poly(A)$^-$ RNA molecules is retained by poly-(U) Sepharose, 14.58% of the total radioactivity of poly(A)$^+$ mRNAs is bound to the column under these conditions. This suggests that even though some poly(A) sequences exist in our poly(A)$^-$ mRNA population, these sequences may be few or short compared to those found in poly(A)$^+$ mRNAs.

TABLE 9

Binding of in vitro [^{32}P]-labelled poly(A)$^-$ RNAs (coding for the 72,000 - 74,000 dalton heat-shock protein) and poly(A)$^+$ RNAs to poly-(U) Sepharose

RNA examined	Total amount applied cpm	Retained by poly-(U) Sepharose cpm	% of applied radioactive RNA bound to poly-(U) Sepharose
(i) [^{32}P]-poly(A)$^-$ RNA	4,118,725	32,363	0.786
(ii) [^{32}P]-poly(A)$^+$ RNA	4,008,861	584,779	14.58

1µg of partially purified poly(A)$^-$ RNA from fraction 7 of the sucrose gradient presented in Figure 21, or 1µg of poly(A)$^+$ RNA from heat-shocked cells was subjected to mild alkaline hydrolysis by heating at 90^{o}C for 5 mins and then labelled in vitro with [^{32}P]-labelled γ-ATP at a specific activity of 16 x 10^6 cpm/µg following the method described in Methods (Section 11). Both samples dissolved in 0.5 ml of [0.4 M Nacl, 10 mM EDTA, 0.2% N-lauroyl sarcosine, 10 mM Tris, pH 7.4), denatured at 70^{o}C for 5 mins, cooled rapidly on ice and applied twice to separate poly-(U) Sepharose columns. The columns were washed with 6 mls of [10 mM EDTA, 0.2% N-lauroyl sarcosine, 10 mM Tris, pH 7.4] buffer and the bound material was eluted with 12 ml of 90% formamide in [0.5% N-lauroyl sarcosine, 20 mM EDTA, 10 mM Tris, pH 7.4].

Trichloroacetic acid-precipitable radioactivity was determined as described in Methods (Section 13.1.).

Indeed, comparison of the elution profile of poly(A)$^-$ RNA retained by the column with that of poly(A)$^+$ RNA (see Figure 23), shows that the low level of purified end-labelled poly(A)$^-$ RNA initially retained by the column however, elutes very readily with formamide. The large level of label bound in the case of poly(A)$^+$ RNA, however, elutes less quickly suggesting that in the latter case the poly(A)$^+$ segments are more tightly bound and thus probably longer.

Whilst this experiment is not definitive it does suggest that some of the poly(A)$^-$ mRNAs do contain at least low levels of possibly short oligo(A) tracts. To examine the situation further, with particular emphasis on the mRNA for the 72,000 - 74,000 heat shock protein, the effect of multiple cycles of affinity chromatography at various temperatures was carried out.

4.5. Poly(A)$^-$ mRNAs coding for the 72,000 - 74,000 heat shock protein are not retained by poly-(U) Sepharose after three cycles of chromatography

Since some of the poly(A)$^-$ mRNAs coding for the 72,000 - 74,000 heat shock protein might contain short poly(A) regions three successive cycles of chromatography on poly-(U) Sepharose were carried out and the ability of the unbound RNA fraction to code for the 72,000 - 74,000 dalton heat shock protein was tested in each case.

Total cytoplasmic heat shocked cell RNA was subjected to chromatography on poly-(U) Sepharose as described in Methods (Section 5.1.3.). Poly(A)$^+$ and poly(A)$^-$ RNA was subjected to a second chromatography cycle under the conditions described before.

The material which was not retained by poly-(U) Sepharose during the second cycle was subjected to a third chromatography cycle on a separate column. As it is shown in Table 10 the RNAs which failed to be retained by poly-(U) Sepharose after the first chromatography cycle but were retained after a second or third cycle did not stimulate the *in vitro*

FIGURE 23

Elution profiles of in vitro [^{32}P]-labelled "purified" heat-shock specific poly(A)$^-$ RNA or heat-shocked cell poly(A)$^+$ RNA bound to poly-(U) Sepharose column

In vitro [^{32}P]-labelled "purified" poly(A)$^-$ or total cytoplasmic poly(A)$^+$ RNA from heat-shocked HeLa cells was chromatographed on poly-(U) Sepharose (see Table 9).

Fractions 1-6 contain the material washed off the column during the washing step.

Fractions 7-18 contain the bound material which was eluted with 90% formamide in [0.5% N-Lauroyl sarcosine, 10 mM EDTA, 10 mM Tris, pH 7.4].

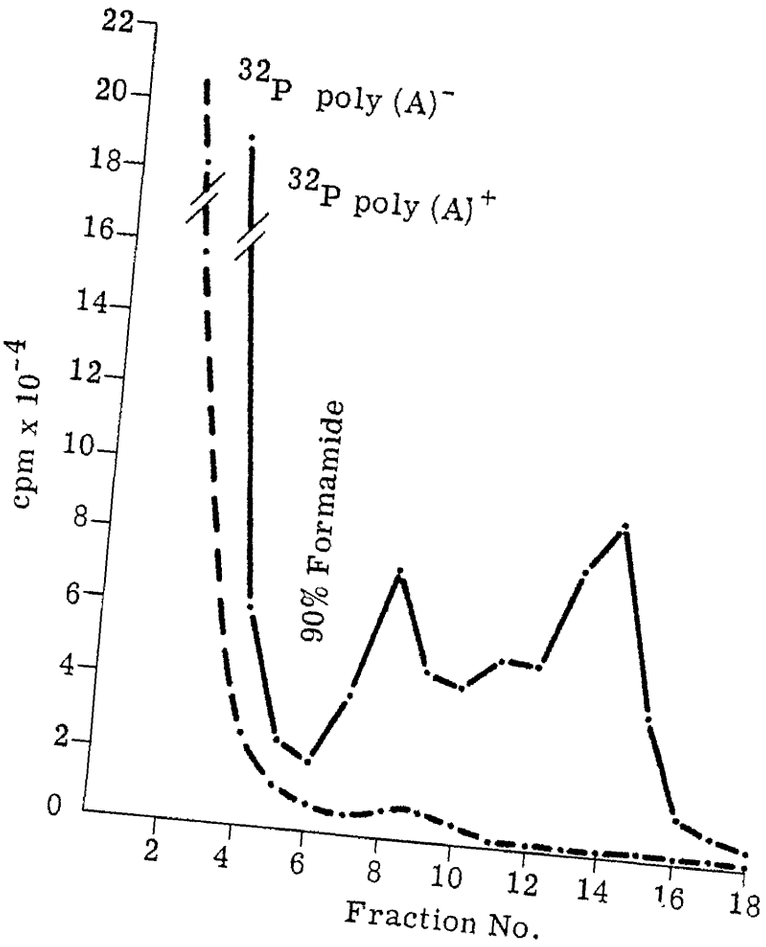

22
20
18
16
14
12
10
8
6
4
2
0
cpm x 10^{-4}
^{32}P poly (A)$^-$
^{32}P poly (A)$^+$
90% Formamide
2
4
6
8
10
12
14
16
18
Fraction No.

TABLE 10

In vitro incorporation of [^{35}S]-methionine into polypeptides directed by various fractions of cytoplasmic RNA from heat-shocked cells after three chromatography cycles on poly-(U) Sepharose

RNA assayed	Amounts µg/25µl assay	[^{35}S]-methionine incorporation cpm/µl assay
poly(A)$^-_1$ RNA	5.0	21,400
poly(A)$^-_2$ RNA	5.0	19,600
poly(A)$^-_3$ RNA	5.0	17,600
poly(A)$^+_2$ RNA	1.0	0
poly(A)$^+_3$ RNA	1.0	0

Assay mixtures (25µl) containing various amounts of RNA fractionated by successive cycles of chromatography of poly-(U) Sepharose were incubated at 60 mins at 37°C under standard conditions. Incorporated radioactivity was estimated as described in Methods (Section 13.2.). Incorporation due to endogenous messenger activity was subtracted in each case.

poly(A)$^-_1$ RNA: RNA which was not retained by poly-(U) Sepharose after one chromatography cycle.

poly(A)$^-_2$ RNA: RNA not retained by poly-(U) Sepharose after two chromatoghraphy cycles.

poly(A)$^-_3$ RNA: RNA not retained by poly-(U) Sepharose after three chromatography cycles.

poly(A)$^+_2$ RNA: RNA not retained after one chromatography cycle, but retained during the second cycle.

poly(A)$^+_3$ RNA: RNA not retained after two chromatography cycles, but retained during the third cycle.

incorporation of [^{35}S]-methionine into polypeptides. Since these RNAs show no messenger activity, they could possibly be rRNA molecules retained by poly-(U) Sepharose because of their secondary structure. When the in vitro translation products of the various RNA fractions were analysed on one-dimensional acrylamide/SDS gels (see Figure 24) it was shown that the mRNAs which failed to get retained by poly-(U) Sepharose after three chromatography cycles code, for the 72,000 - 74,000 dalton heat shock proteins (see Figure 24, Lane 4), while the ability of this RNA fraction to code for some other protein bands (Figure 24, Lane 4, bands A and B) is substantially reduced. Thus, even though some of the mRNAs fractionated as poly(A)$^-$ mRNAs after one chromatography cycle are retained by poly-(U) Sepharose after three successive cycles, the majority of the mRNAs coding for the 72,000 - 74,000 heat shock protein is not.

This result suggests that most of the particular mRNAs for the 72,000 - 74,000 hsp have either no poly(A) tails, or oligo(A) tails shorter than the minimum length required in order to be retained by poly-(U) Sepharose (see next section).

4.6. Poly(A)$^-$ mRNAs coding for the 72,000 - 74,000 dalton heat-shock protein are not retained by poly-(U) Sepharose after a second chromatography cycle carried out at 4°C

Poly - (U) Sepharose retains poly(A)$^+$ mRNAs with a poly(A) sequence larger than about 10-15 nucleotides long (Dubroff and Nemer, 1975; Humphries et al., 1976), while oligo(dT)-cellulose larger than 20 nucleotides long (Gorski et al., 1974; Groner et al., 1974). However, when oligo (dT) cellulose chromatography is carried out at 4°C poly(A) sequences as short as 6-10 nucleotides long are retained (Morrison et al., 1979; Levenson and Marcu, 1976).

In order to improve the ability of poly-(U) Sepharose to retain shorter poly(A) tails than usual, the following was done.

FIGURE 24

Fluorogram of [^{35}S]-methionine labelled polypeptides resulting from the translation of unbound or bound to poly-(U) Sepharose cytoplasmic RNAs from heat-shocked HeLa cells after successive cycles of poly-(U) Sepharose chromatography

Assay mixtures (25µl) containing various amounts of RNA fractionated by successive cycles of chromatography on poly-(U) Sepharose were incubated at 37°C for 60 mins under standard conditions. aliquots containing equal amounts of radioactivity from each assay were analysed on 8.75% polyacrylamide/SDS gel.

Lane 1 No RNA added to the translation system.

Lane 2 5µg of RNA which remained in the unbound fraction after one chromatography cycle.

Lane 3 5µg of RNA which remained in the unbound fraction after two chromatography cycles.

Lane 4 5µg of RNA which remained unbound after three chromatography cycles.

Lane 5 1µg of RNA which was not retained by poly-(U) Sepharose after one chromatography cycle but was retained during the second cycle.

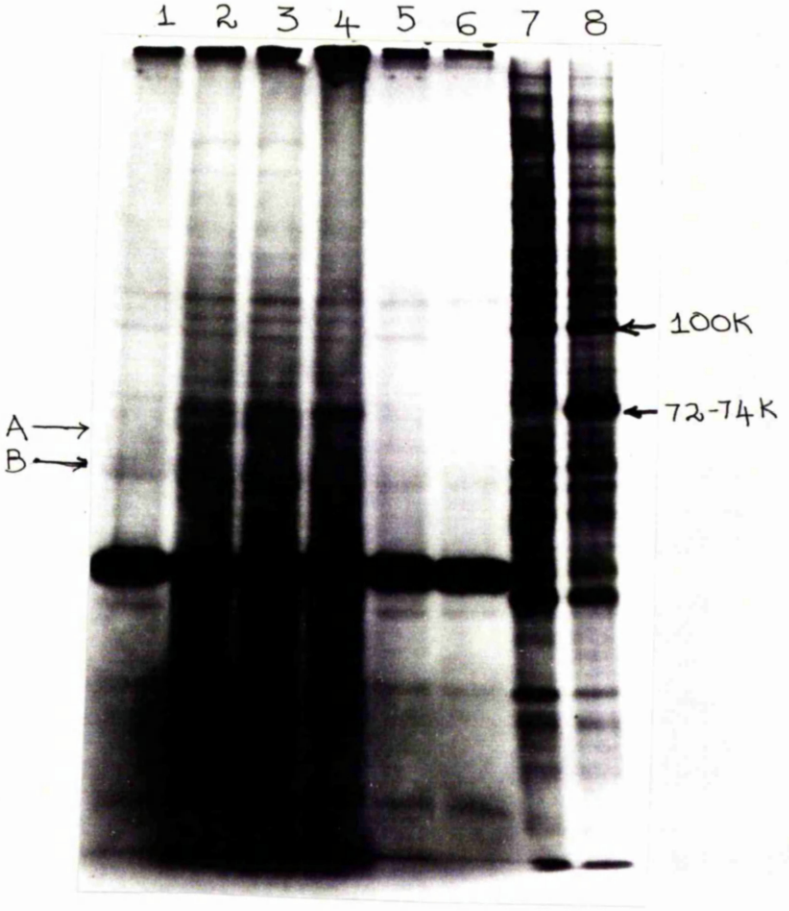
Lane 6 1µg of RNA not retained by poly-(U) Sepharose after two chromatography cycles but was retained during the third cycle.

Lane 7 In vivo labelled proteins from normal cells.

Lane 8 In vivo labelled proteins from heat-shocked HeLa cells.

The arrows indicate the position of the 100,000 and 72,000 - 74,000 dalton heat-shock proteins.

Protein bands A and B are those encoded by mRNAs which failed to get bound to poly-(U) Sepharose during the first and second chromatography cycle, but were retained during the third cycle.


1 2 3 4 5 6 7 8
100K
72-74K
A
B

Heat-shocked cell cytosplasmic RNA was subjected to one cycle of poly-(U) Sepharose chromatography at room temperature (~ 20°C). The RNA fraction which was not retained by the column (poly(A)$^-$ RNA) was pooled, ethanol precipitated and subjected to a second chromatography cycle at 4°C.

As it is shown in Table 11, 1.8 % of the total RNA is retained by poly-(U) Sepharose during the first chromatography cycle at 20°C, while an increased amount (4.6% of the total RNA) is retained after a second chromatography cycle at 4°C. When appropriate amounts of all RNA fractions were translated in vitro in a rabbit reticulocyte cell-free translation system the RNAs retained by poly-(U) Sepharose after the second chromatography cycle at 4°C stimulated protein synthesis (Table 12).

Thus, unlike the situation after a second chromatography cycle at 20°C, when no translational activity was detected in the RNA fraction retained by poly-(U) Sepharose during the second cycle (see Table 10 and Figure 24, Lane 5), a decrease of the temperature during the second cycle seems to favour the retention of some more mRNA molecules. Since the percentage of total RNA retained by poly-(U) Sepharose at 4°C is 4.6% compared to 1.8% at 20°C (see Table 10), it is quite possible that some of these RNAs are also rRNA molecules. The decrease in the translational activity of the mRNA retained at 4°C (compared with that of the RNA retained at 20°C) might be due to the presence of this increased amount of rRNA.

Analysis of the translation products on one-dimensional polyacrylamide/SDS gels showed that some of the poly(A)$^-$ mRNAs coding for the 72,000 - 74,000 dalton heat-shock protein band are retained by poly-(U) Sepharose at 4°C (Figure 25, Lane 3) even though the bulk of this poly(A)$^-$ mRNA population remains unbound (see Figure 25, Lane 5).

TABLE 11

Quantitation of RNA fractionated after one chromatography cycle at 20°C followed by a second one at 4°C

RNA Assayed	Amount µgs	% of the total RNA
RNA unbound after one chromatography cycle at 20°C and a second one at 4°C	816	93.5
RNA retained by poly-(U) Sepharose after one chromatography cycle at 20°C	16	1.8
RNA not retained after the first cycle at 20°C, but retained after the second one at 4°C	40	4.6

Cytoplasmic RNA from heat-shocked HeLa cells was fractionated by poly-(U) Sepharose chromatography at 20°C as described in Methods (Section 5.1.3.). An aliquot of the unbound material was kept while the rest was ethanol precipitated, dissolved in binding buffer (see Methods, Section 5.1.3.), denatured at 70°C for 5 mins and applied twice to poly-(U) Sepharose at 4°C. The washing step was also carried out at 4°C, while the bound material was eluted at 20°C. RNA from all fractions was ethanol precipitated, collected by centrifugation, dissolved in H_2O and the optical density was measured at 260 nm in UV light. The amount of RNA was calculated taking in account that 1 O.D. unit corresponds to 40µg of RNA.

TABLE 12

In vitro incorporation of L-[^{35}S]- methionine into polypeptides directed by various fractions of cytoplasmic RNA from heat-shocked HeLa cells after one chromatography cycle at 20°C followed by a second cycle at 4°C

RNA Assayed	Amounts µg/25µl	[^{35}S]- methionine Incorporation cpm/µl assay
RNA bound to poly-(U) Sepharose at 20°C	1.0	37,600
RNA unbound to poly-(U) Sepharose at 20°C	5.0	17,450
RNA which fails to get bound at 20°C, but does get bound at 4°C	1.0	21,862
RNA which remains unbound at 20°C and 4°C	5.0	13,071

Assay mixtures (25µl) containing various amounts of RNA fractionated by one chromatography cycle on poly-(U) Sepharose at 20°C followed by a second cycle at 4°C, were incubated for 60 mins at 37°C under standard conditions. Incorporated radioactivity was estimated as described in Methods (Section 13.2.). Incorporation due to endogenous mRNA activity was subtracted in each case.

FIGURE 25

Fluorogram of [^{35}S]-methionine labelled polypeptides resulting from the translation of various RNA fractions resulting from one chromatography cycle at 20°C followed by a second cycle at 4°C

Translation products of the various RNA products were analysed on one-dimensional 8.75% polyacrylamide/SDS gel.

The polypeptide pattern shown was obtained from the translation of the following RNA samples.

Lane 1 Without added mRNA.

Lane 2 1µg of heat-shocked cell RNA retained by poly-(U) Sepharose after one chromatography cycle at 20°C.

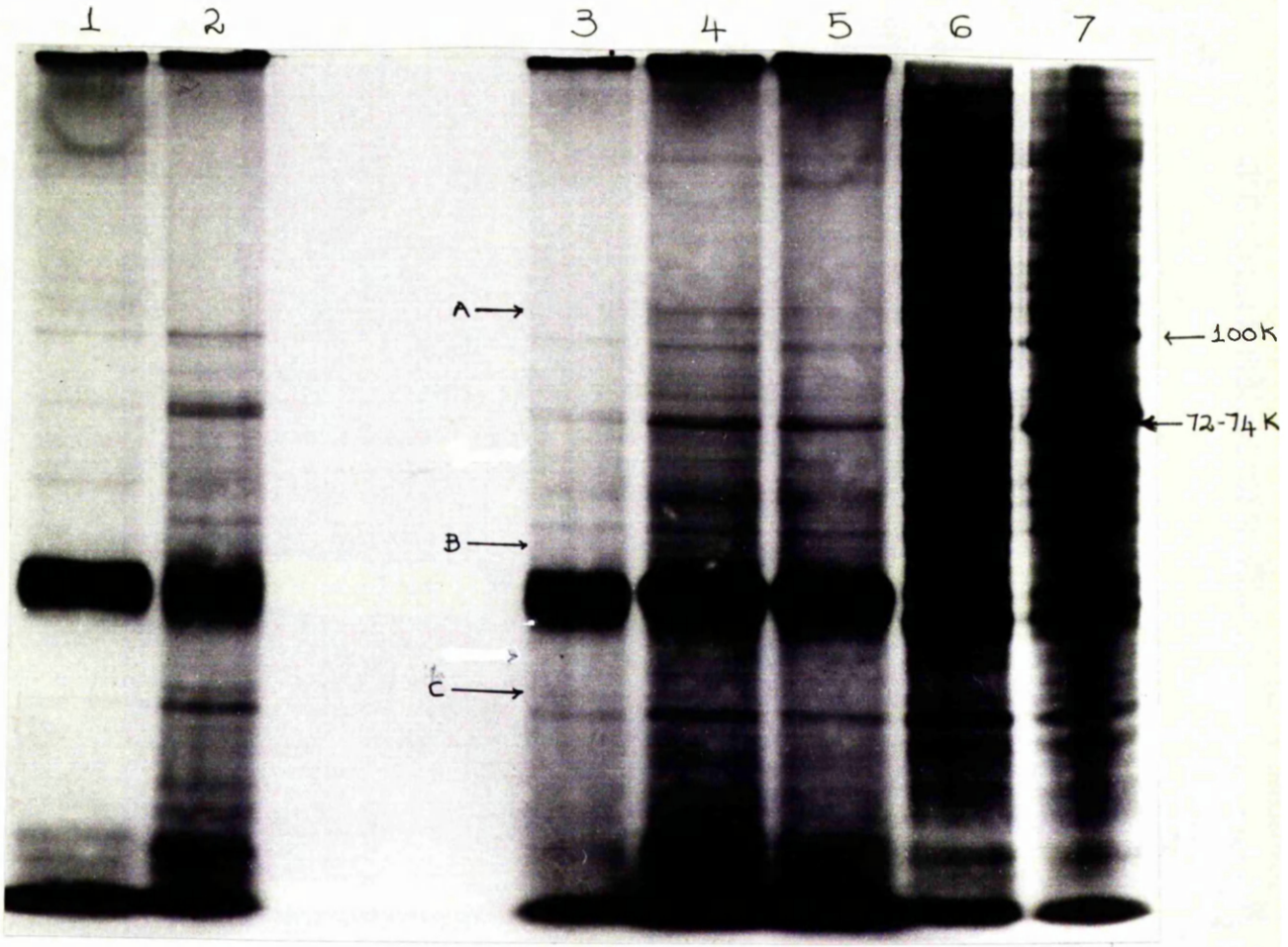
Lane 3 1µg of heat-shocked cell mRNA which was not retained during the first cycle but during the second one at 4°C.

Lane 4 5µgs of heat-shocked cell mRNA unretained after the first chromatography cycle at 20°C.

Lane 5 5µgs of heat-shocked cell RNA unretained by poly-(U) Sepharose after one chromatography cycle at 20°C followed by a second one at 4°C.

The arrow indicates the position of the 72,000 - 74,000 dalton heat-shock protein.

The RNA which was not retained by poly-(U) Sepharose after a second chromatography cycle at 4°C has a decreased ability to code for protein bands A, B and C (Lane 5).


1
2
3
4
5
6
7
A
B
C
100K
72-74 K

Comparison of the pattern of proteins encoded by poly(A)$^-$ mRNAs after the first chromatography cycle at 20°C (Figure 25, Lane 4) with those encoded by RNAs which did not get bound to poly-(U) Sepharose after a second chromatography cycle at 4°C (Figure 25, Lane 5), shows that some of the protein bands are decreased among the products of the latter. (Figure 25, Lane 5, Bands A, B and C).

All these results show that even when more efficient separation of poly(A)$^+$ and poly(A)$^-$ mRNAs is achieved after a second chromatography cycle at 4°C, the 72,000 - 74,000 dalton heat-shock protein is still one of the two major in vitro translation products of the RNA fraction which is not retained by poly-(U) Sepharose (poly(A)$^-$mRNA).

4.7. Two-dimensional analysis of the in vitro translation products of the "partially purified" poly(A)$^-$ mRNA coding for the 72,000 - 74,000 dalton heat-shock protein

Two-dimensional electrophoretic analysis has shown that the 100,000 dalton heat-shock protein is made up of at least two polypeptide species, whilst the 72,000 - 74,000 dalton class may comprise several. In order to investigate whether all these heat-shock polypeptides result directly from mRNA translation and whether poly(A)$^+$ and poly(A)$^-$ code for the same or different polypeptide spots, a comparison of the in vitro translation products of cytoplasmic poly(A)$^+$ and poly(A)$^-$ RNA was carried out using the two-dimensional electrophoretic separation system of O'Farrell (1975). An initial requirement was to identify the heat-shock polypeptides amongst the polypeptide pattern resulting from the translation of either poly(A)$^+$ or poly(A)$^-$ mRNA. To this end a two-dimensional analysis of the products resulting from the translation of "partially purified" poly(A)$^-$ mRNA for the 72,000 - 74,000 groups (from fraction 8 of Figure 21) was carried out. As it is shown in Figure 26

seven polypeptide products appeared in the region expected for the 72,000 - 74,000 heat-shock proteins and these are designated (α, α', β, γ, δ, ε,ζ). The position of these polypeptides in the following fluorograms of the two-dimensional analysis of the polypeptides from in vitro translation of either poly(A)$^+$ or poly(A)$^-$ mRNA was then determined by comparison of each fluorogram with the fluorogram presented in Figure 26. Also, to facilitate comparison of gels it should be pointed out that all two-dimensional gels of in vitro translation products include 7 polypeptides and a group of polypeptides (Figure 27, spots 1-7 and a group of spots in brackets) which are always detected when the lysate is incubated in the absence of exogenous mRNA and can be used as additional reference markers.

4.8. Two-dimensional analysis of the in vitro translation products of poly(A)$^+$ RNA from control and heat-shocked HeLa cells

As it has already been shown the 72,000 - 74,000 heat-shock protein is encoded by poly(A)$^+$ RNA from control cells, but at minimal amounts (Figure 19, Lane 2).

Since the 72,000 - 74,000 heat-shock protein encoded by poly(A)$^-$ RNA from heat-shocked cells comprises seven polypeptides (Figure 25, α, α', β, γ, δ,ε, ζ), two questions arose. Firstly, do poly(A)$^+$ mRNAs from control cells code for all the seven polypeptides and secondly, do poly(A)$^+$ mRNAs from heat-shocked cells code for an identical set of polypeptides.

In order to answer these questions the in vitro translation products of total poly(A)$^+$ mRNA from both control and heat-shocked cells were next analysed on two-dimensional gels following the procedure of O'Farrell (1975). Comparing the products of poly(A)$^+$ mRNA from control cells with those of poly(A)$^+$ mRNA from heat-shocked cells

FIGURE 26

Fluorogram of two-dimensional analysis of [^{35}S]-methionine labelled in vitro translation products of the partially purified poly(A)$^-$ cytoplasmic RNA from heat-shocked cells that yields proteins of the 72,000 - 74,000 dalton size class

1μg poly(A)$^-$ RNA from fraction 8 of the 5-20% sucrose density gradient (see Figure 21) was translated *in vitro* in a rabbit reticulocyte cell-free translation system and the products analysed by the two-dimensional gel system of O'Farrell (1975). The separated proteins of the 72,000 -74,000 dalton size class are designated α, α′, β, γ, δ, ε and ζ.

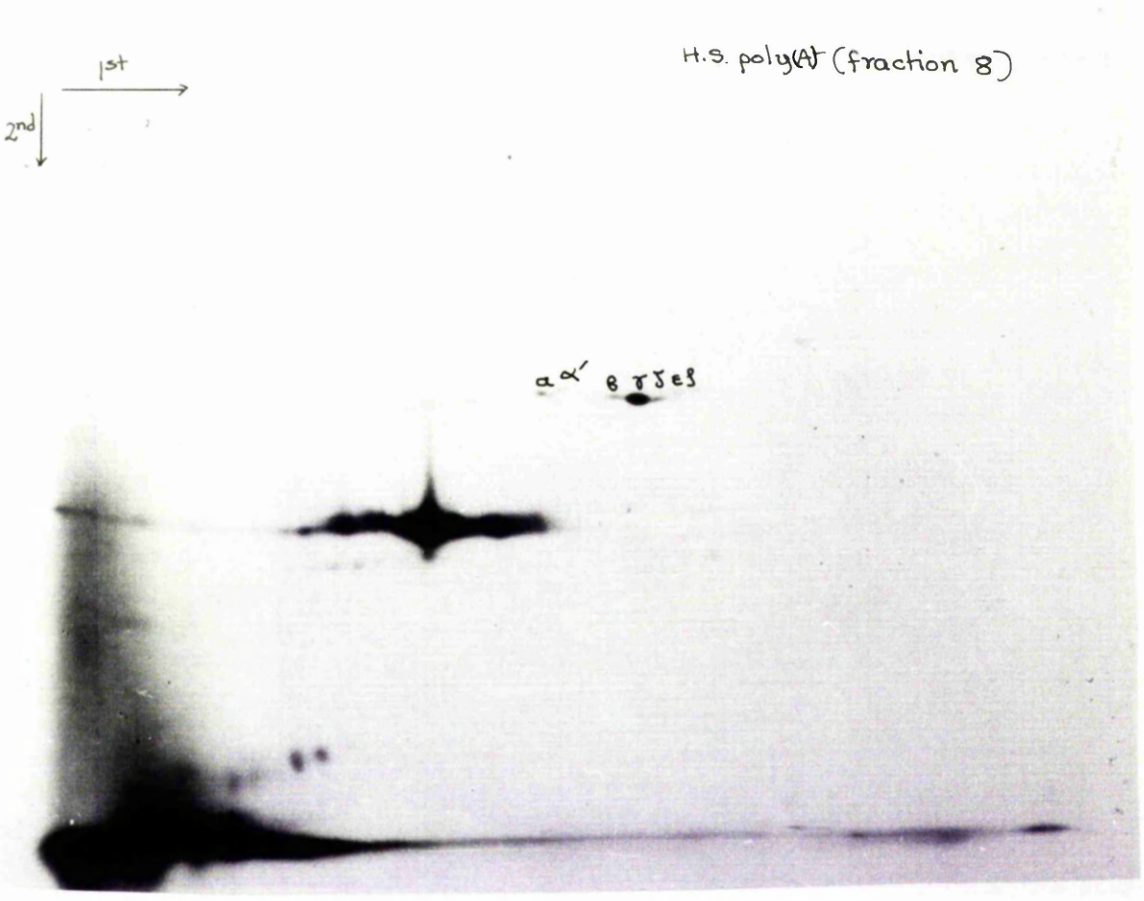
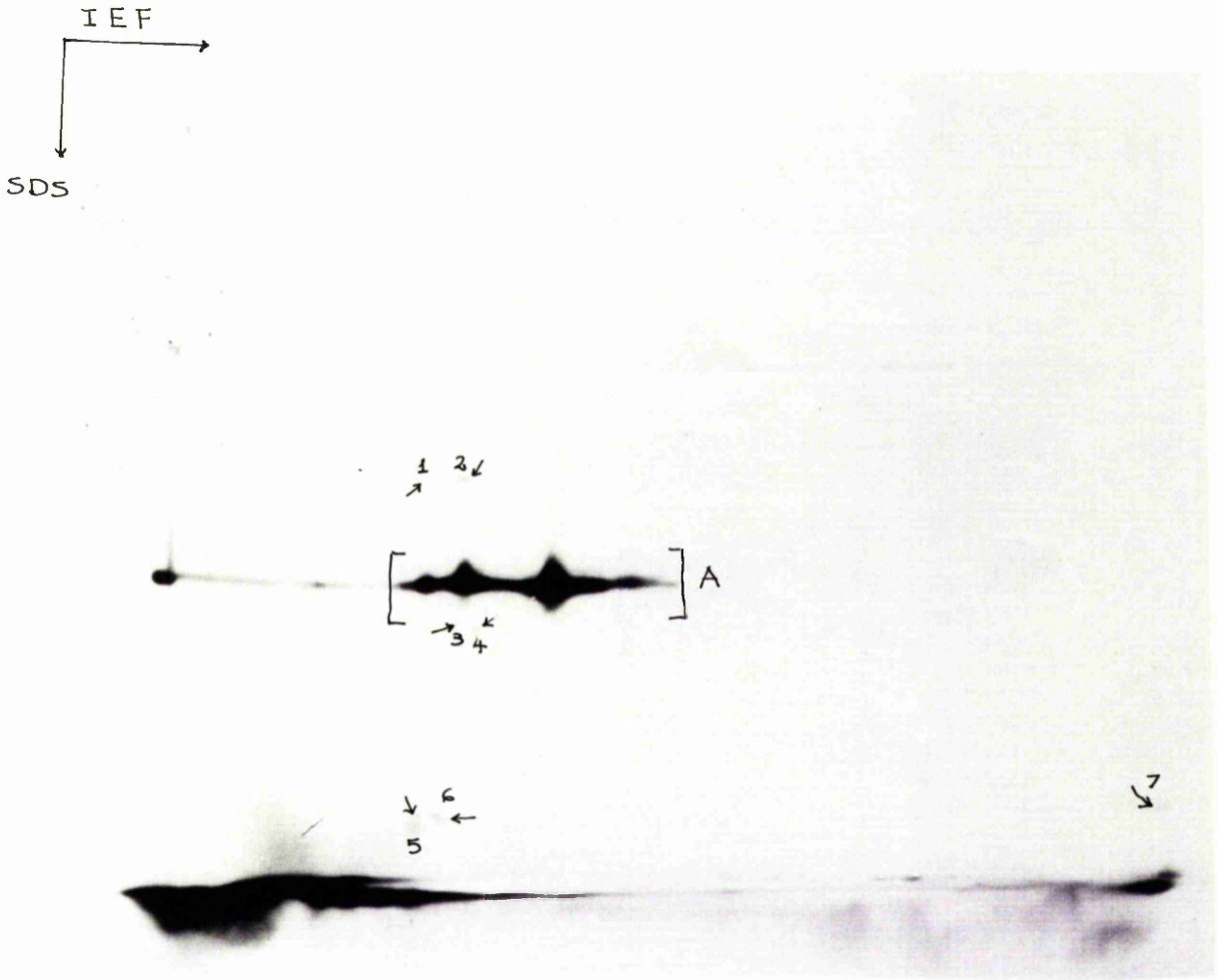

1st
2nd
H.S. polyAt (fraction 8)
α α' β γ δ ε ζ

FIGURE 27

Fluorogram of two-dimensional analysis of [^{35}S]- methionine labelled polypeptides detected when rabbit reticulocytes lysate is incubated in the absence of exogenous mRNA

25µl of rabbit reticulocyte cell-free protein synthesising system to which no exogenous RNA was added was incubated at 37^{o}C for 60 mins. The labelled polypeptides were analysed using the two-dimensional system of O'Farrell (1975).

Seven polypeptides (1-7) and a group of polypeptides (A) were detected in this fluorogram.


IEF
SDS
1
2
A
3
4
5
6
7

the following are concluded:

1) Some polypeptide spots in the fluorogram of the translation products of poly(A)$^+$ RNA from control cells were not observed in the pattern of translation products of poly(A)$^+$ RNA from heat-shocked cells (such polypeptides are indicated by arrows in Figure 28A).

2) Whereas in the area of the 72,000 - 74,000 dalton class of heat-shock proteins seven polypeptide spots (α, α', β, γ, δ, ε, ζ) are encoded by the poly(A)$^+$ RNA from heat-shocked cells only three (γ, ε, and ζ) seem to be coded by poly(A)$^+$ RNA from control cells.

3) Only one strong polypeptide spot was found in the position expected of the 100,000 dalton heat-shock proteins after translation of cytoplasmic poly(A)$^+$ RNA from heat-shocked cells (Figure 28B). This polypeptide is also formed but with slightly reduced intensity in the translation products of the poly(A)$^+$ RNA from control cells (Figure 28A).

4) Although it is not always easy to see the polypeptide migrating in the 37,000 dalton region two-dimensional analysis of products from heat-shocked cell poly(A)$^+$ RNA translation showed a single spot in this region which is not present in the translation products from control cell poly(A)$^+$ RNA (Figure 28B). This polypeptide may be a 37,000 dalton heat-shock protein.

4.9. Two-dimensional analysis of the in vitro translation products of poly(A)$^-$ mRNA from control and heat-shocked HeLa cells

When the translation products of poly(A)$^-$ mRNA from control and heat-shocked HeLa cells were analysed in two-dimensional gels (O'Farrell, 1975) the following was observed:

1) A number of polypeptide spots are made by poly(A)$^-$ RNA from control cells but which are not encoded in by the poly(A)$^-$ RNA from heat-shocked cells. Some of these "missing" polypeptides are

FIGURE 28

Fluorogram of the two-dimensional electrophoretic separation of [^{35}S]-methionine labelled in vitro translation products of cytoplasmic poly(A)$^+$ RNA from control and heat-shocked HeLa cells

Total cytoplasmic poly(A)$^+$ RNA was translated *in vitro* using a rabbit reticulocyte cell-free translation system and the translation products were analysed by two-dimensional electrophoresis.

(A) Translation products of 1µg poly(A)$^+$ RNA from control HeLa cells.

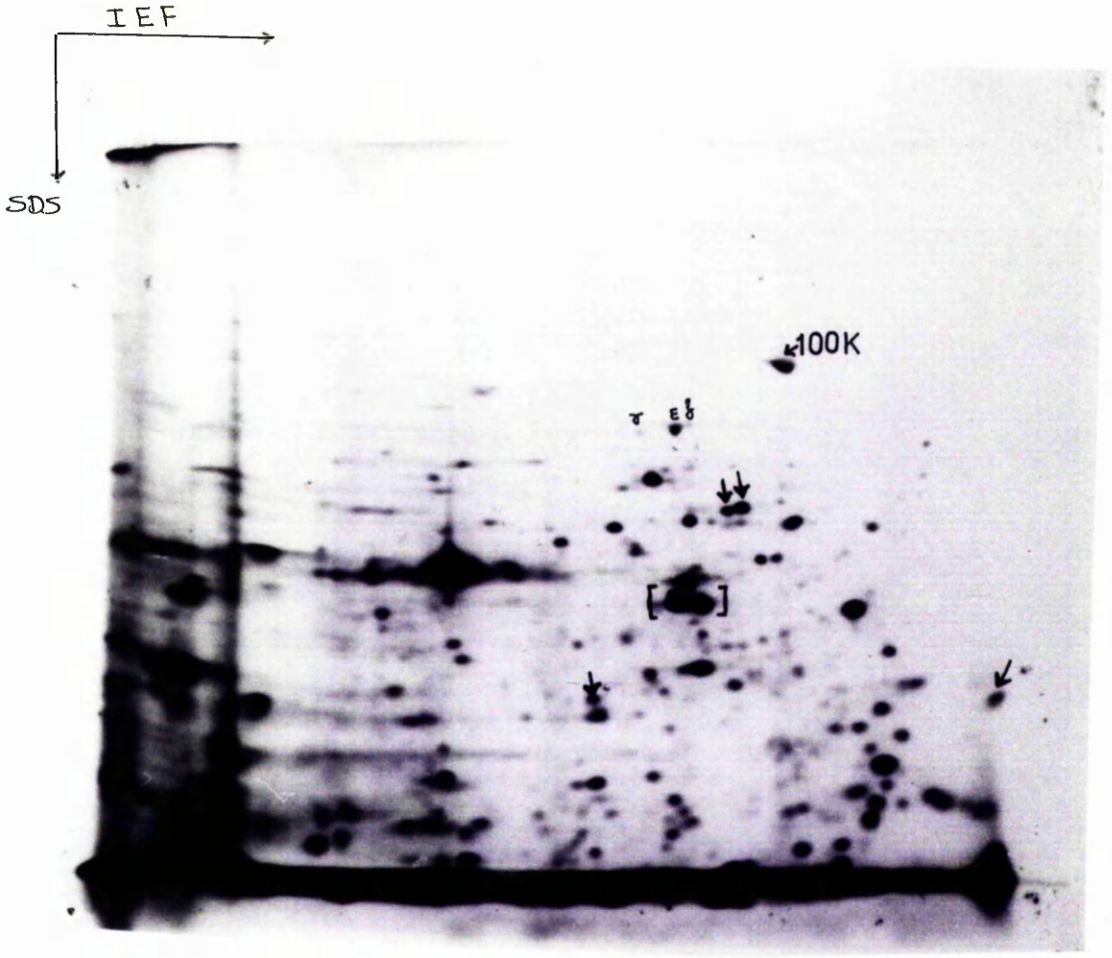
(B) Translation products of 1µg poly(A)$^+$ RNA from HeLa cells heat-shocked at 45°C for 5 mins and then allowed to recover at 37°C for 2 hrs.

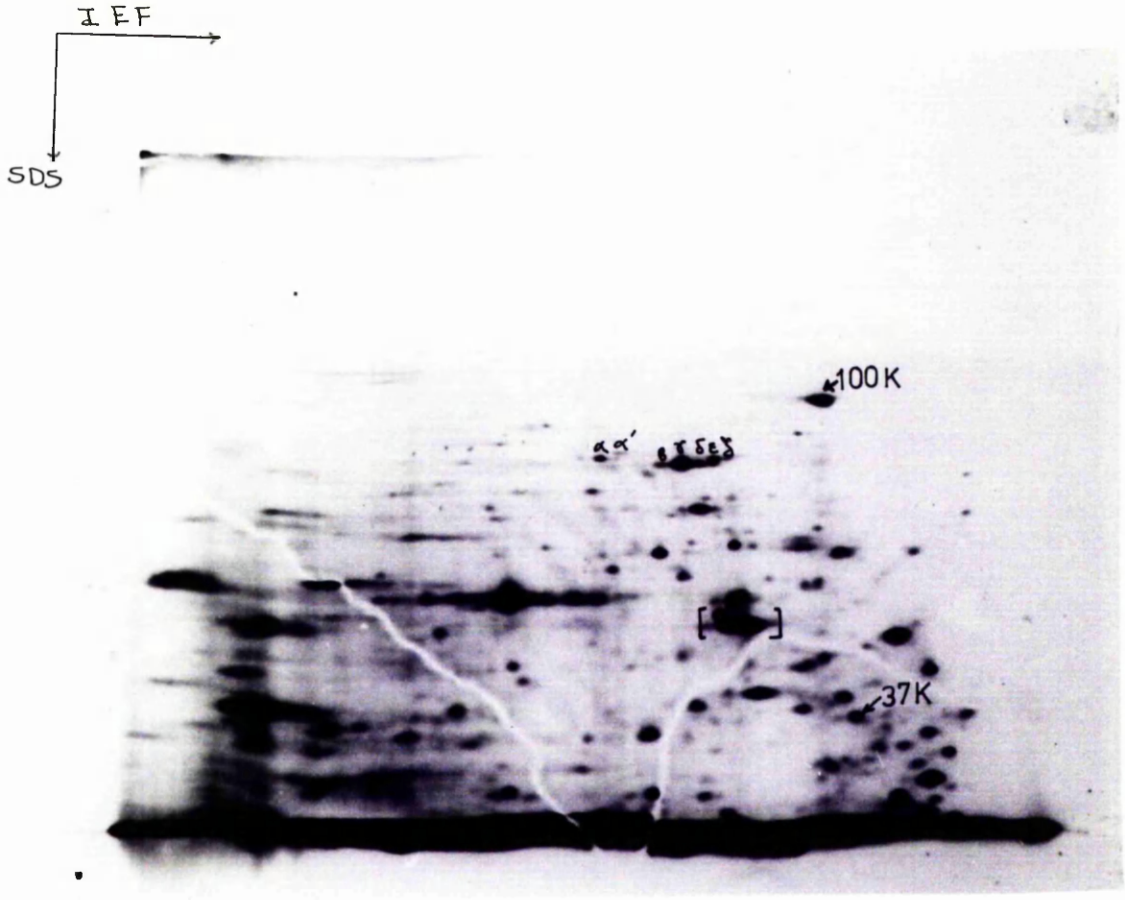
The brackets indicate the spots possibly corresponding to the multiple forms of actin.

α, α', β, γ, δ, ε and ζ refer to the proteins of the 72,000-74,000 molecular weight.

100K indicates the 100,000 daltons HeLa heat-shock protein. Similarly 37K is likely to be the 37,000 daltons HeLa heat-shock protein.

The arrows in A point to the proteins which do not appear among the translation products of the poly(A)$^+$ RNA from heat-shocked cells (B).


IEF
SDS
100K
γ ε δ


IEF
SDS
100K
α α' β γ δ ε β
37K

indicated by arrows in Figure 29A and those that are exclusively poly(A)$^-$ translation products are both arrowed and numbered (see Figure 29A).

2) Similar to the situation with poly(A)$^+$ RNA, only three proteins of the 72,000 - 74,000 dalton class (γ, ε ,ζ) are translated from cytoplasmic poly(A)$^-$ RNA from control cells (see Figure 29A), whilst all seven (α, α', β, γ, δ, ε, ζ) were encoded by cytoplasmic poly(A)$^-$ RNA from heat-shocked cells (Figure 29B).

3) A 100,000 dalton protein spot was also detected among the translation products of poly(A)$^-$ RNA from both control and heat-shocked HeLa cells, but it did not seem to be particularly enriched among the translation products of poly(A)$^-$ mRNA for heat-shocked cells (compare Figures 29A and 29B). A polypeptide migrating in a position expected of the 37,000 dalton heat-shock protein was also detected among the translation products of poly(A)$^-$ mRNA from heat-shocked cells (see Figure 29B). So, it appears that despite possible differences in relative abundances both cytoplasmic poly(A)$^+$ and poly(A)$^-$ mRNAs from heat-shocked cells code for the same overall variety of heat-shock proteins in the 72,000 - 74,000 dalton size classes (and possibly in the 37,000 dalton and 100,000 size classes as well).

4.10. Conclusion

The results presented in this section have shown that both poly(A)$^+$ and poly(A)$^-$ mRNAs from heat-shocked HeLa cells code for the 72,000 - 74,000 heat-shock protein. The poly(A)$^-$ mRNAs which code for this particular protein band do not seem to contain poly(A) tails of the length found in poly(A)$^+$ mRNAs. However, one can not exclude the possibility of small oligo(A) tracts, either internally or externally located. The 72,000 - 74,000 dalton hsp *in vitro* encoded by both heat-shocked cell poly(A)$^+$ and poly(A)$^-$ mRNAs consists of seven polypeptides

FIGURE 29

Fluorogram of the two-dimensional electrophoretic separation of [^{35}S] - methionine labelled in vitro translation products of cytoplasmic poly(A)$^-$ RNA from control and heat-shocked HeLa cells

Total cytoplasmic poly(A)$^-$ RNA was translated in vitro using a rabbit reticulocyte cell-free translation system and the translation products were analysed by two-dimensional electrophoresis.

(A) Translation products of 5µg poly(A)$^-$ RNA from control HeLa cells.

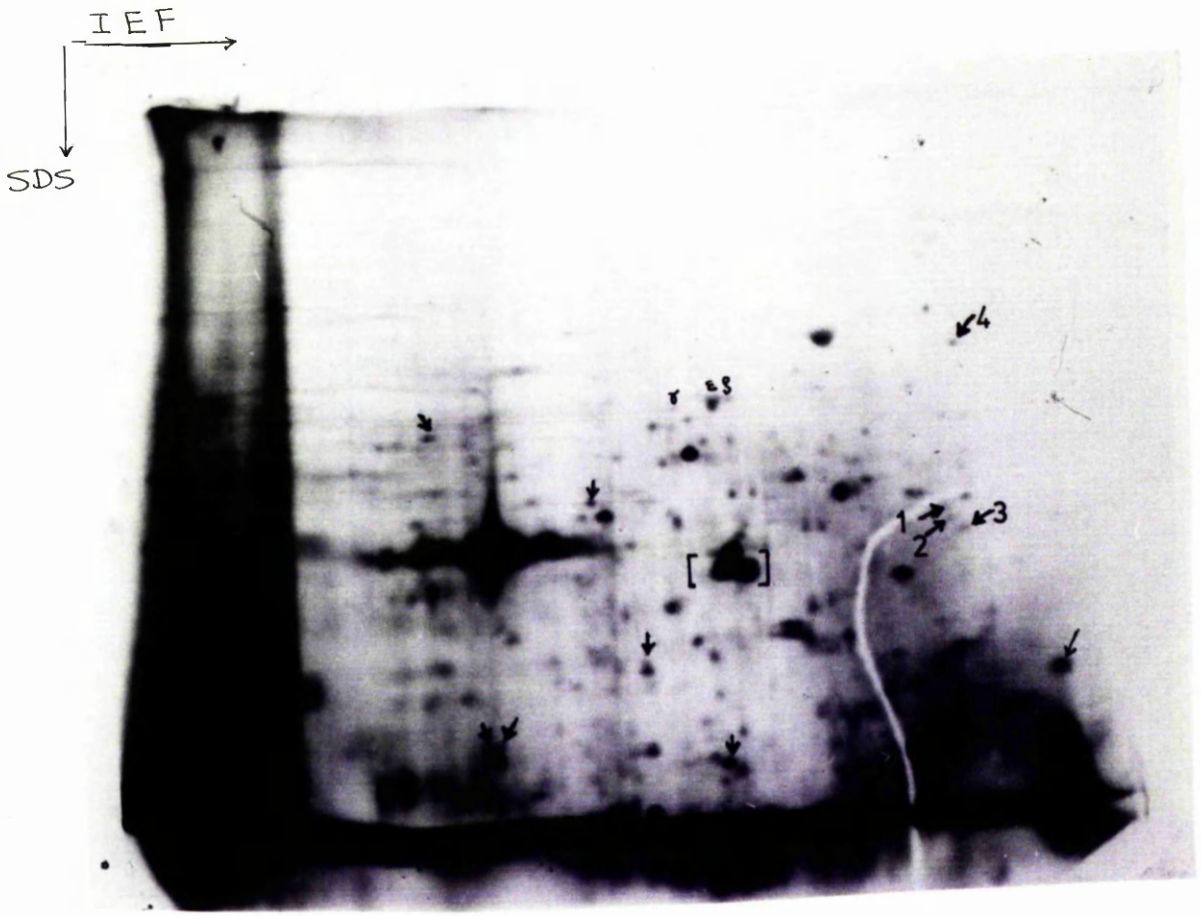
(B) Translation products of 5µg poly(A)$^-$ RNA from HeLa cells heat-shocked at 45°C for 5 mins and then allowed to recover at 37°C for 2 hrs.

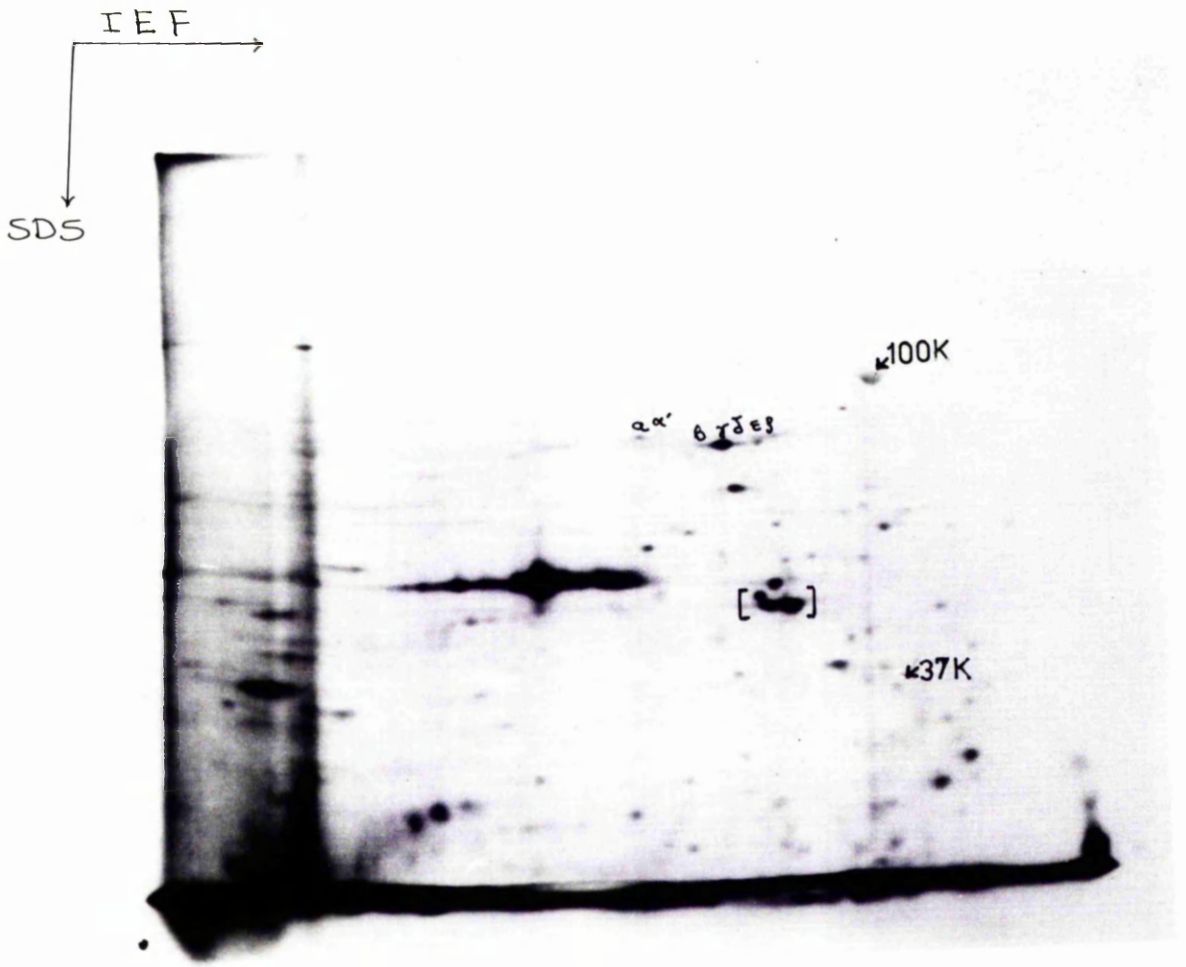
The brackets indicate the spots possibly corresponding to the multiple forms of actin.

α, α', β, γ, δ, ε and ζ refer to the proteins of the 72,000-74,000 molecular weight.

100K indicates the 100,000 daltons HeLa heat-shock protein. Similarly 37K is likely to be the 37,000 daltons HeLa heat-shock protein.

The arrows in (A) point to the proteins which do not appear among the translation products of the poly(A)$^-$ RNA from heat-shocked cells (B).


IEF
SDS
4
1
2
3


IEF
SDS
100K
37K

(α, α', β, γ, δ, ε, ζ). However, poly(A)$^+$ and poly(A)$^-$ mRNAs from normal HeLa cells code for only three of these polypeptides (γ, ε and ζ).

5. Effect of heat-shock treatment on the polysomal profile

A very rapid response of Drosophila cultured cells to heat-shock treatment is the breakdown of pre-existing polysomes (McKenzie et al., 1975; Biessman et al., 1978). Polysomes reappear some 30 mins after the start of the heat-shock treatment and have been shown to be loaded with heat-shock specific mRNAs (Lindquist-McKenzie et al., 1975; McKenzie and Meselson, 1977; Spradling et al., 1977; Mirault et al., 1978; Moran et al., 1978). However, when actinomycin D is added before heat-shock treatment polysomes disaggregate but fail to reappear 30 mins later (Lindquist-McKenzie et al., 1975). On the other hand, later experiments have shown that when heat-shocked Drosophila cells are transferred to normal temperature normal polysomal profile is restored even if the synthesis of heat-shocked RNA has been blocked with actinomycin D (Storti et al., 1980).

Since the conditions of heat-shock treatment in HeLa cells are quite different from those in Drosophila cells, the effect of heat-shock upon the polysomal profile of HeLa cells was investigated.

5.1. Polysomal profile of HeLa cells after the heat-shock treatment

HeLa cells were heat-shocked at 45°C for 5 mins and harvested immediately after the heat-shock treatment or allowed to recover at 37°C for o.5, 1 or 2 hrs before harvesting. Cytoplasmic extracts from all the samples (see Methods, Section 3.1.1.) were analysed on 15-30% sucrose gradients (see Methods, Section 3.2.1.) and harvested by pumping through a Gilford 2,000 recording spectrophotometer set at 260 nm.

As it is shown in Figure 30B polysomes disappear after heat-shock treatment at 45°C for 5 mins and are being converted to monosomes.

FIGURE 30

Polysomal profile of normal HeLa cells or heat-shocked HeLa cells harvested 0, 0.5, 1 or 2 hrs after the heat-shock

Cytoplasmic extract was prepared from normal cells or HeLa cells which had been heat-shocked at 45°C for 5 mins and then allowed to recover at 37°C for 0, 0.5, 1 or 2 hrs, as described in Methods (Section 3.1.1.). The cytoplasmic extract was then made up to 0.5% with respect to Brij-58 and 0.5% with respect to deoxycholate and layered onto 15-30% (w/v) sucrose gradients in RSB (see Methods, Section 3.2.1.). After centrifuging at 27,000 rpm for 110 mins at 4°C the gradients were harvested by pumping through a Gilford 2,000 recording spectophotometer set at 260 nm.

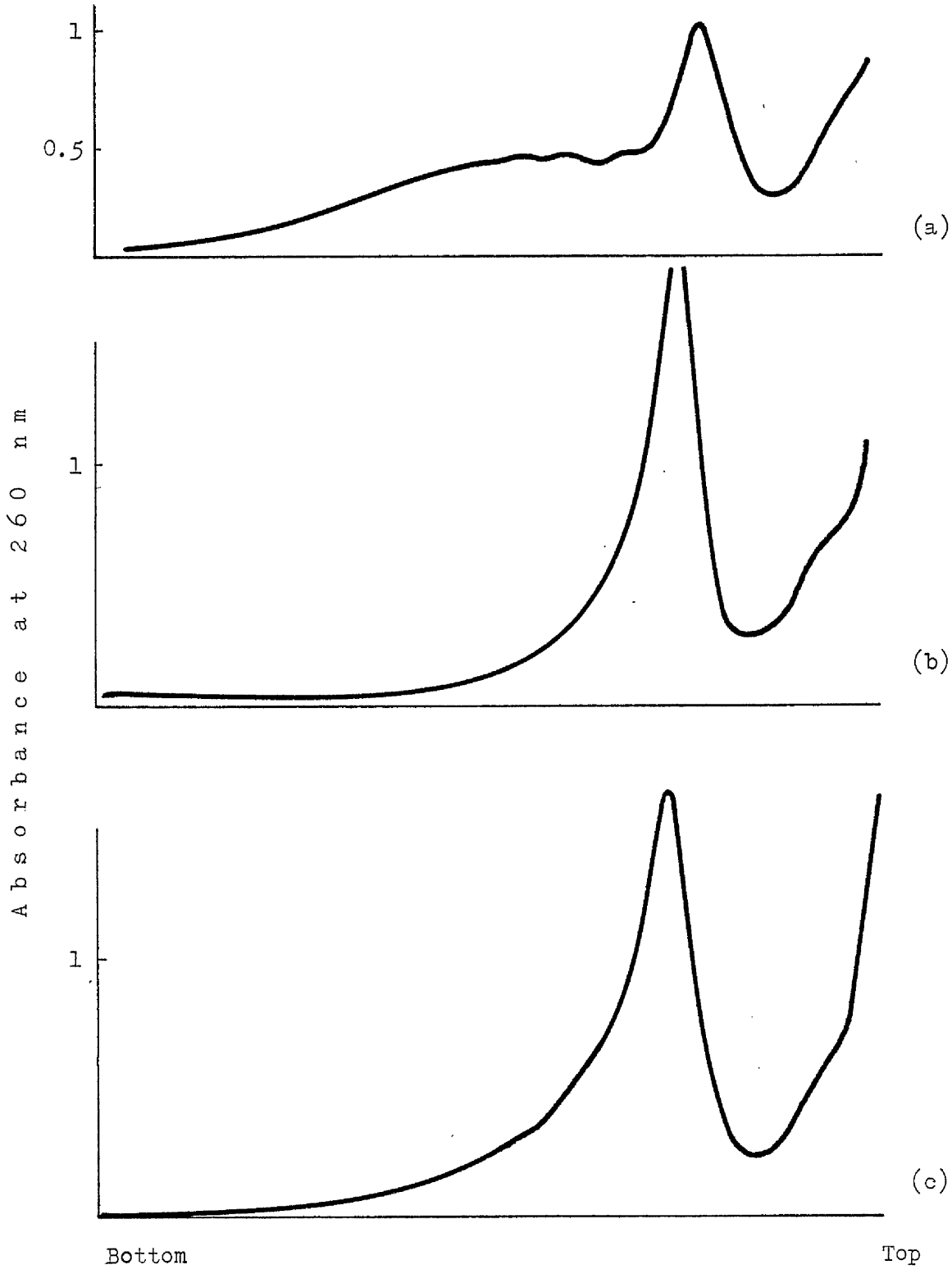
(A) Polysomal profile of normal HeLa cells.

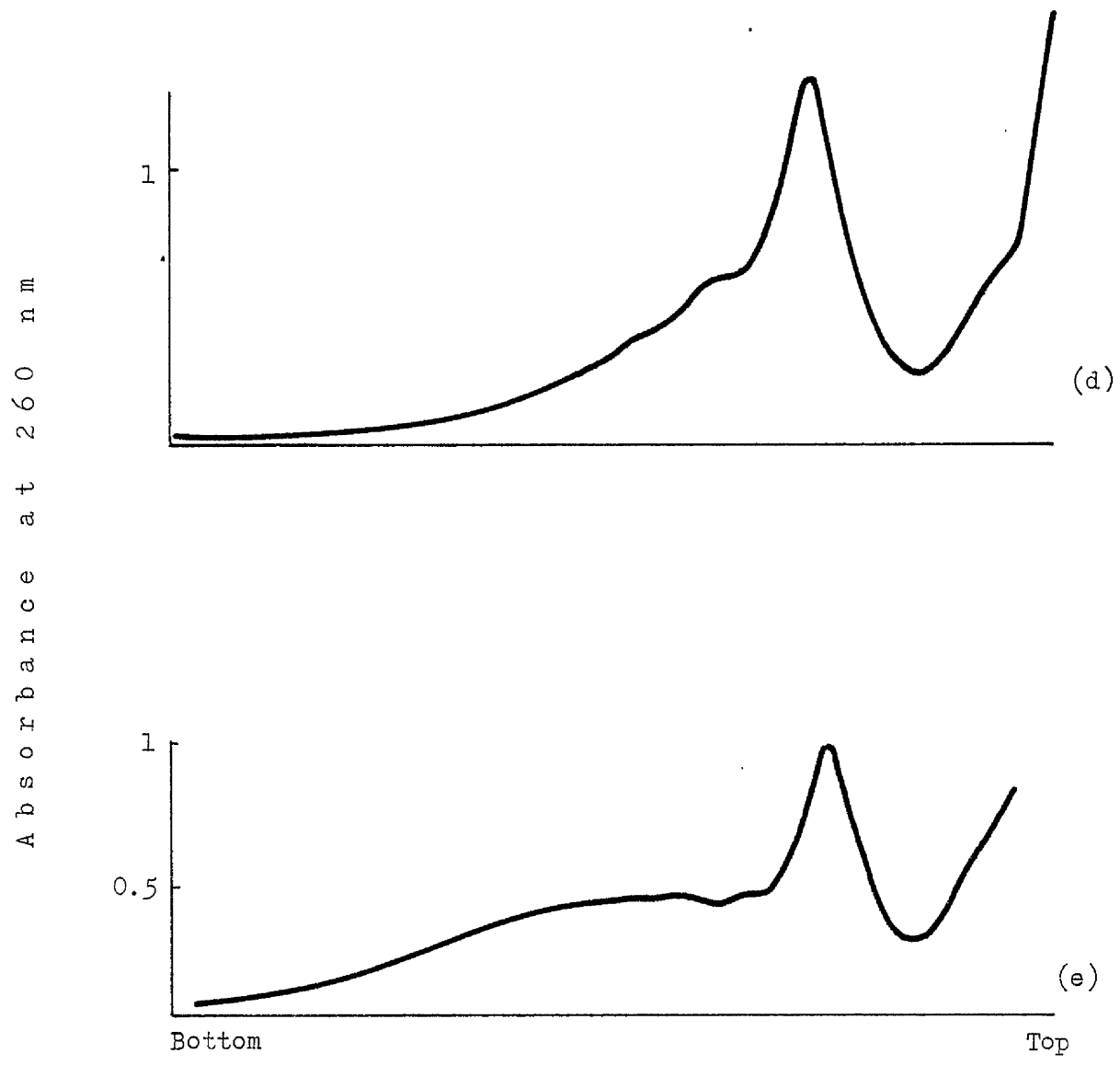
(B) Polysomal profile of HeLa cells harvested immediately after heat-shock.

(C) Polysomal profile of HeLa cells harvested 0.5 hrs after heat-shock.

(D) Polysomal profile of HeLa cells harvested 1 hr after heat-shock.

(E) Polysomal profile of HeLa cells harvested 2 hrs after heat-shock.


1
0.5
(a)
1
(b)
1
(c)
Absorbance at 260 nm
Bottom
Top
Fraction number


Absorbance at 260 nm
1
(d)
1
0.5
(e)
Bottom
Top
Fraction number

When the cells are allowed to recover at 37°C for 0.5 hrs after heat-shock treatment, there is a small decrease of the monosomal peak while disomes and trisomes are building up (see Figure 30C). 1 hr after the heat-shock treatment normal polysomal profile has not yet been restored. Even though there is a substantial decrease of the monosomal peak, most of the mRNAs seem to be engaged in light polysomes (see Figure 30D). It takes 1.5 - 2 hrs recovery at 37°C to obtain a normal polysomal profile (see Figure 30E and compare it with Figure 30A).

5.2. Is the production of heat-shock RNA required for the appearance of normal polysomal profile two hours after the heat-shock?

Since normal polysomal profile is obtained in HeLa cells 2 hrs after the heat-shock treatment when accumulation of heat-shock mRNAs sequences occurs in the nucleus (Burdon, 1982) a question arises whether the production of heat-shock mRNAs is required in order to have normal polysomal profile restored.

To answer this, we incubated HeLa cells with actinomycin D at a concentration 1 µg/ml of medium for 30 mins. Then half the cells were heat-shocked at 45°C for 5 mins and afterwards transferred at 37°C for 2 hrs (heat-shocked cells), while the other half were kept at 37°C all the time (normal cells). Cytoplasmic extracts were prepared from both normal and heat-shocked cells (see Methods, Section 3.1.1.), analysed on 15-30% sucrose gradients (see Methods, Section 3.2.1.) and harvested by pumping through a Gilford 2,000 recording spectophotometer set at 260 nm.

As it is shown in Figure 31, the polysomal profile of heat-shocked cells incubated with actinomycin D (1µg/ml) for 2.5 hrs (see Figure 31B) is identical to the polysomal profile of normal cells treated in the same way (see Figure 31A). However, comparison of

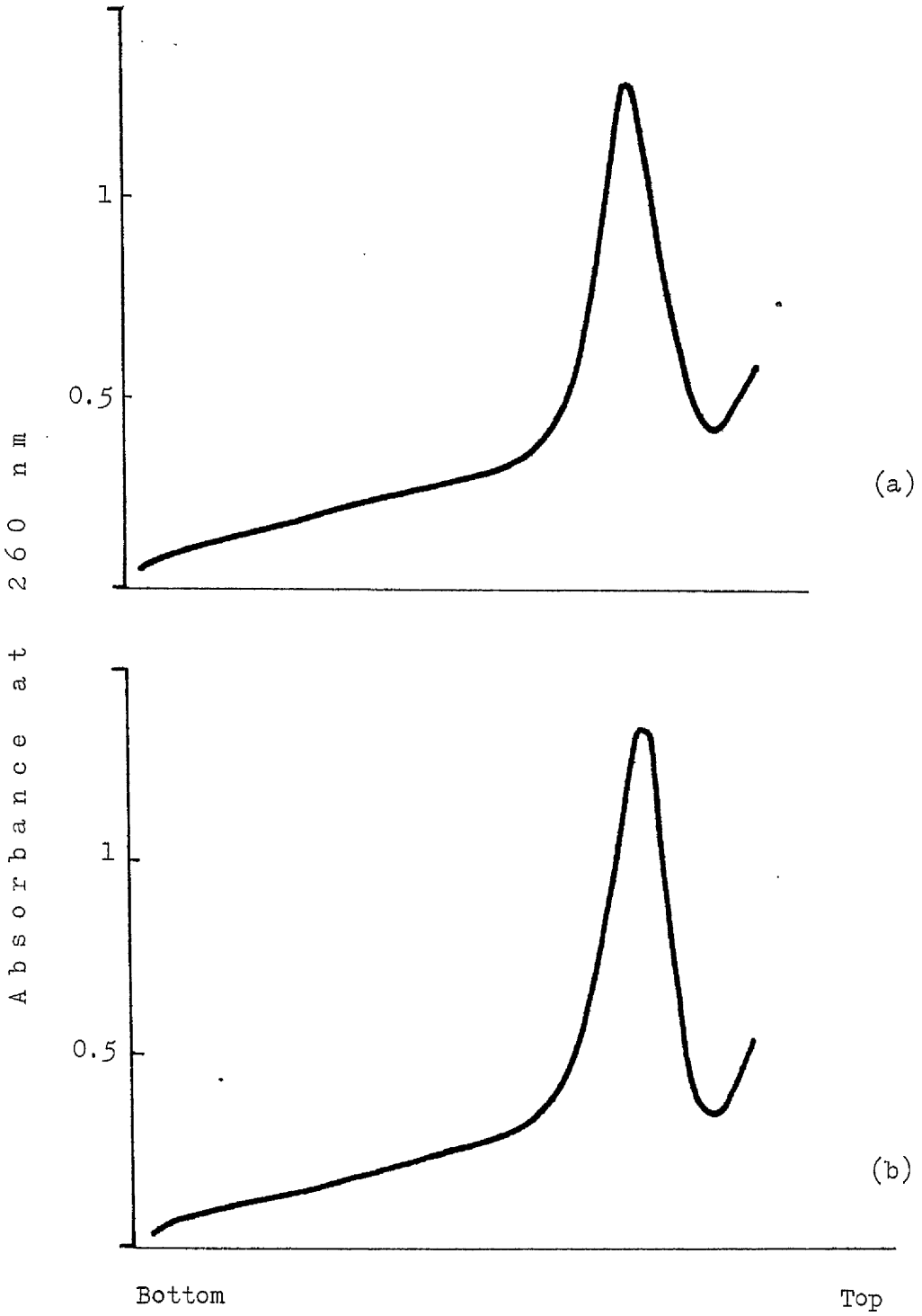
FIGURE 31

Polysomal profile of normal HeLa cells or heat-shocked HeLa cells incubated with actinomycin D (1μg/ml) for 2.5 hrs.

HeLa cells were incubated with actinomycin D (1μg/ml) for 30 mins. Then half of the cells were heat-shocked at 45°C for 5 mins and transferred at 37°C for 2 hrs, while the rest were at 37°C throughout the whole period (normal cells). Cytoplasmic extracts were prepared from both normal and heat-shocked cells and analysed on 15-30% (w/v) sucrose gradients (see Legend of Figure 30).

(A) Polysomal profile of normal HeLa cells incubated with actinomycin D for 2.5 hrs.

(B) Polysomal profile of heat-shocked HeLa cells incubated with actinomycin D for a total of 2.5 hrs.


Absorbance at 260 nm
1
0.5
(a)
1
0.5
(b)
Bottom
Top
Fraction number

Figures 30A and 31A shows that incubation of HeLa cells with actinomycin D has affected the polysomal profile of control HeLa cells. Since actinomycin D is known to affect itself the polysomal profile of polysomes (Singer and Penman, 1972), it is not possible to draw any conclusions from this experiment.

5.3. Conclusion

The results presented in this section have shown that heat-shock treatment of HeLa cells results in breakdown of polysomes. Even though the cells are then transferred to 37°C, normal polysomal profile is only obtained 1-2 hrs after the heat-shock treatment. It has not been possible to determine whether production of heat-shock specific mRNAs is required for the polysomal profile to be restored, because of the side effects of actinomycin D.

6. Stability of the mRNAs coding for the 72,000 - 74,000 dalton heat-shock protein

6.1. Are the mRNAs coding for the 72,000 - 74,000 heat-shock proteins degraded once the rate of synthesis of these proteins declines?

As it has already been shown synthesis of the heat-shock proteins in HeLa cells is increasing during the 2 hrs following the heat-shock treatment. Afterwards, synthesis of these particular proteins decreases and 4 hrs after the heat-shock treatment reaches the level at which they are found in normal HeLa cells (see Figure 17). The decrease in the synthesis of the heat-shock proteins 4 hrs after the heat-shock treatment might be coupled to degradation of the mRNAs coding for the heat-shock proteins, as it happens in the case of histone mRNAs, in HeLa cells, once histone synthesis is not required any more (Gallwitz, 1975). Since the 72,000 - 74,000 dalton heat-shock proteins are encoded by both poly(A)$^+$ and poly(A)$^-$ mRNAs (see Figure 19) and given that specific nonpolyadenylated mRNAs have been reported to be more easily susceptible to degradation

(Huez et al., 1974; Marbaix et al., 1975; Gallwitz, 1975), a question arising is whether the poly(A)$^-$ mRNAs coding for the 72,000 - 74,000 dalton heat-shock proteins are the first to be degraded once the synthesis of hsp's is substantially decreased.

In order to explore these possibilities we isolated total cytoplasmic poly(A)$^+$ and poly(A)$^-$ RNA immediately, 2hrs, 4 hrs and 6 hrs after the heat-shock treatment and tested the ability of each RNA fraction to code for the heat-shock proteins. One-dimensional analysis of the translation products (see Figure 32) revealed that the 72,000 - 74,000 heat-shock protein is one of the major products of the poly(A)$^+$ and poly(A)$^-$ mRNA not only 2 hrs after the heat-shock treatment (see Figure 32, Lanes 3 and 4) but also 4 hrs and 6 hrs after the heat-shock treatment (see Figure 32, Lanes 5, 6, 7 and 8). Comparison of the relative intensity of the 72,000 - 74,000 dalton hsp amongst the translation products of the various RNA fractions reveals that the ability of both poly(A)$^+$ and poly(A)$^-$ mRNA to code for the 72,000 - 74,000 dalton heat-shock protein remains almost the same during the first 6 hrs after the heat-shock treatment.

So, it seems that neither poly(A)$^+$ nor poly(A)$^-$ mRNA coding for the 72,000 - 74,000 hsp are degraded once they stop being translated in vivo.

6.2. Two-dimensional analysis of the in vitro translation products of poly(A)$^+$ and poly(A)$^-$ mRNA isolated 6 hrs after the heat-shock treatment

As it has already been shown, the 72,000 - 74,000 dalton heat-shock protein is encoded by seven different mRNA populations (see Figures 28B and 29B). A question arising was whether all seven poly(A)$^+$ and poly(A)$^-$ mRNA species are present in the cytoplasm 6 hrs after the heat-shock treatment. To answer this question two-dimensional analysis of

FIGURE 32

Fluorogram of [^{35}S]-methionine labelled polypeptides resulting from the translation of poly(A)$^+$ and poly(A)$^-$ mRNAs isolated 0, 2, 4 and 6 hrs after the heat-shock

HeLa cells were heat-shocked at 45°C for 5 mins and cytoplasmic RNA was isolated immediately or after 2, 4 and 6 hrs recovery at 37°C. The RNAs were fractionated into poly(A)$^+$ and poly(A)$^-$ by passing twice through a poly-(U) Sepharose column. All fractions were *in vitro* translated in a rabbit reticulocyte cell-free protein synthesising system for 60 mins at 37°C. The products were analysed on an 8.75% polyacrylamide/SDS gel.

Lane 1 Translation products of 1µg poly(A)$^+$ RNA isolated immediately after the heat-shock.

Lane 2 Translation products of 5µg poly(A)$^-$ RNA isolated immediately after the heat-shock.

Lane 3 Translation products of 1µg poly(A)$^+$ RNA isolated after 2 hrs recovery at 37°C.

Lane 4 Translation products of 5µg poly(A)$^-$ RNA isolated after 2 hrs recovery at 37°C.

Lane 5 Translation products of 1µg poly(A)$^+$ RNA isolated after 4 hrs recovery at 37°C.

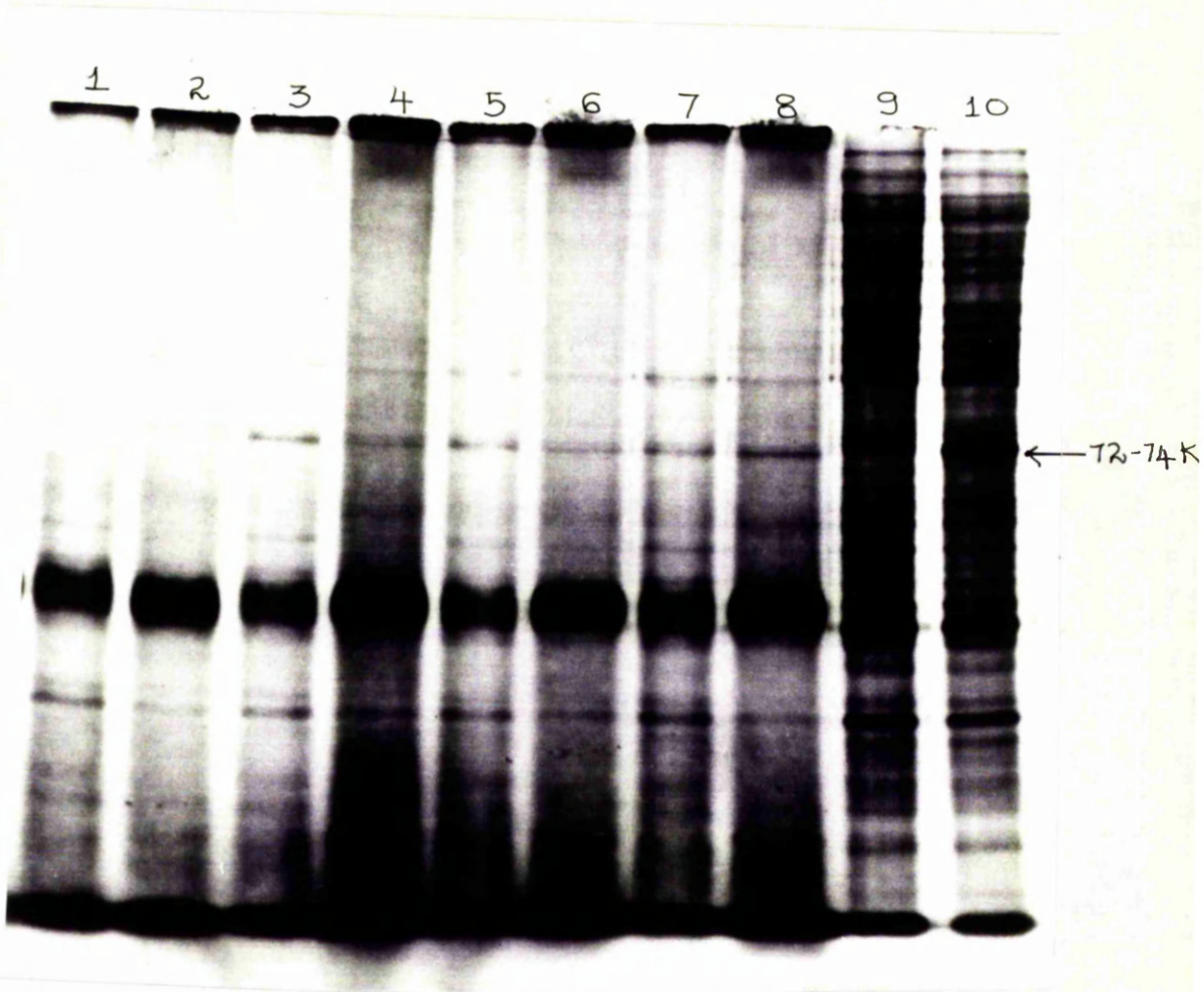
Lane 6 Translation products of 5µg poly(A)$^-$ RNA isolated after 4 hrs recovery at 37°C.

Lane 7 Translation products of 1µg poly(A)$^+$ RNA isolated after 6 hrs recovery at 37°C.

Lane 8 Translation products of 5µg poly(A)$^-$ RNA isolated after 6 hrs recovery at 37°C.

Lane 9 Proteins labelled *in vivo* by incubating intact HeLa cells with [^{35}S]- methionine for 1 hr at 37°C

Lane 10 Proteins labelled *in vivo* by incubating heat-shocked cells with [^{35}S]- methionine for 1 hr after 2 hrs recovery at 37°C. The arrow indicates the position of the 72,000 - 74,000 dalton heat-shock protein.


1
2
3
4
5
6
7
8
9
10
72-74K

the in vitro translation products of the poly(A)$^+$ and poly(A)$^-$ mRNAs isolated 6 hrs after the heat-shock treatment was carried out.

Figure 33 shows that all seven polypeptides (α, α', β, γ, δ, ε, and ζ) are found amongst the translation products of cytoplasmic poly(A)$^+$ mRNA isolated 6 hrs after the heat-shock, even though the α, α' polypeptide spots are substantially diminished. It should be noted that the polypeptide spot believed to be the 37,000 dalton hsp (see Figure 28B) is missing from the pattern of Figure 33. This raises the possibility that poly(A)$^+$ mRNAs coding for this particular polypeptide are less stable than those coding for the 72,000 - 74,000 dalton hsps. However, one can not exclude the possibility that these mRNAs were degraded during the RNA isolation procedure.

Two-dimensional analysis of the in vitro translation products of poly(A)$^-$ mRNA isolated 6 hrs after the heat-shock treatment revealed that five out of the seven poly(A)$^-$ mRNA species coding for the 72,000 - 74,000 hsps are still in the cytoplasm 6 hrs after the heat-shock treatment (see Figure 34, spots β, γ, δ, ε and ζ).

These results suggest that most of the steady state poly(A)$^-$ mRNAs coding for the major hsp's in HeLa cells are as stable as their polyadenylated counterparts.

6.3. Polysomal profile of HeLa cells isolated 6 hrs after the heat-shock treatment

The fact that the heat-shock specific mRNAs (both poly(A)$^+$ and poly(A)$^-$) are still in the cytoplasm 6 hrs after the heat-shock treatment when in vivo synthesis of hsp's has substantially decreased (see Figure 17), raises the possibility that for some reason the majority of these mRNAs may not be engaged in polysomes. In order to find out whether there is any change in the polysomal profile of HeLa cells 6 hrs

FIGURE 33

Fluorogram of two-dimensional analysis of $[^{35}S]$- methionine labelled in vitro translation products of heat-shocked cell poly(A)$^+$ mRNA isolated 6 hrs after the heat-shock

HeLa cells were heat-shocked at 45^{o}C for 5 mins and allowed to recover for 6 hrs at 37^{o}C before harvesting. Cytoplasmic RNA was isolated and fractionated by affinity chromatography on poly-(U) Sepharose. 1µg of poly(A)$^+$ RNA was translated in a rabbit reticulocyte cell-free protein synthesising system and the products were analysed on two-dimensions following the procedure described by O'Farrell (1975).

Polypeptides α, β, γ, δ, ε and ζ are six of the seven 72,000-74,000 heat-shock proteins. The position of the 100,000 hsp is also indicated.

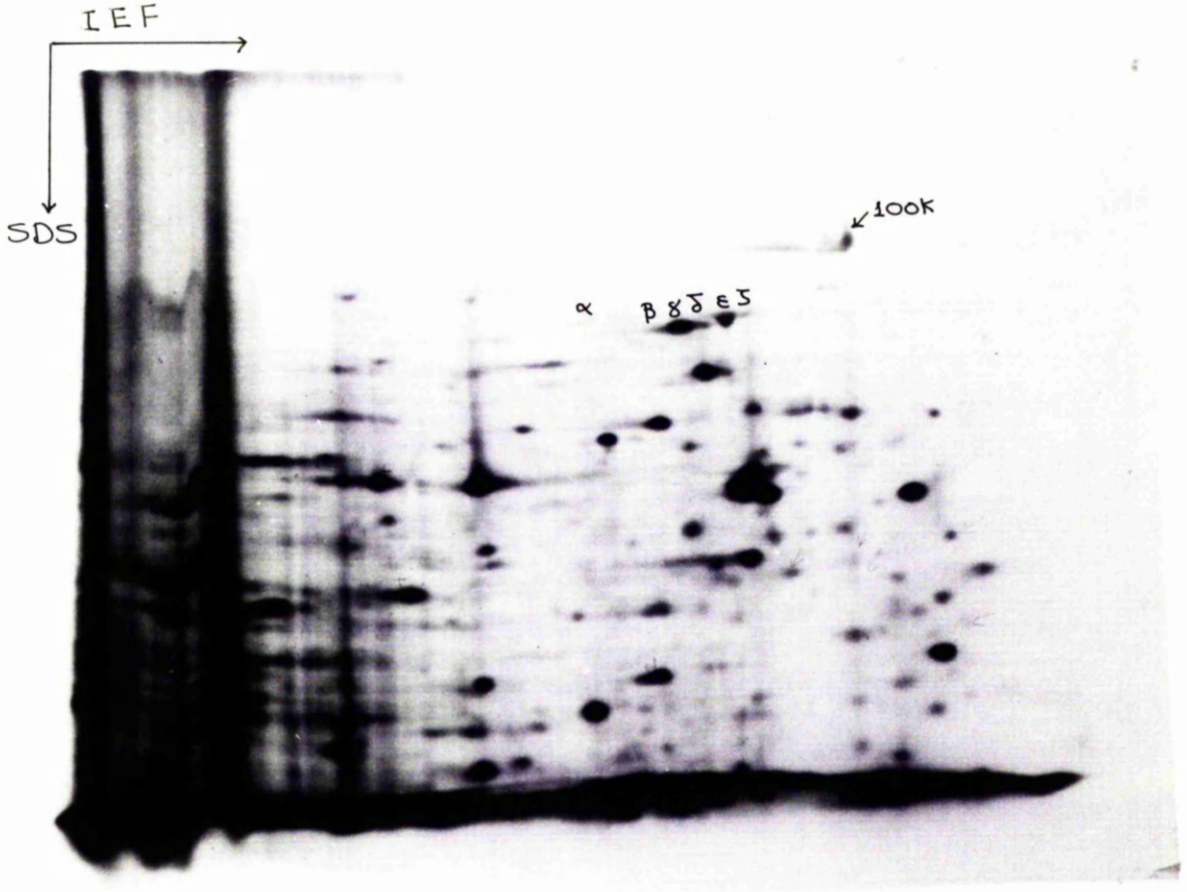
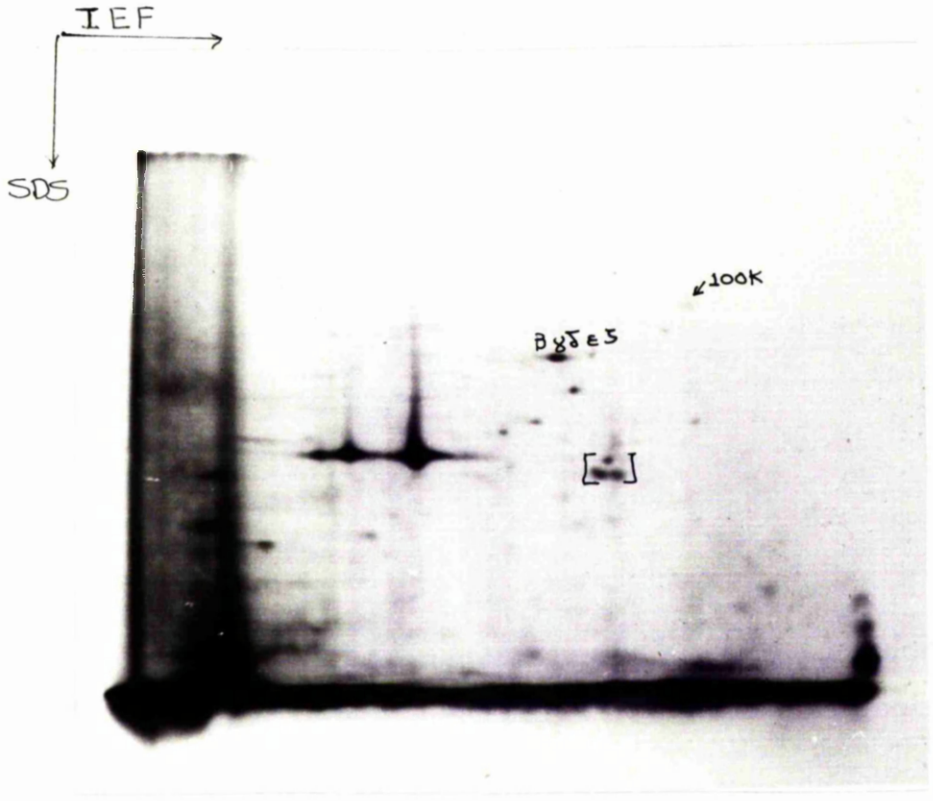

IEF
SDS
100K
α
β γ δ ε ζ

FIGURE 34

Fluorogram of two-dimensional analysis of [^{35}S]-methionine labelled in vitro translation products of heat-shocked cell poly(A)$^-$ RNA isolated 6 hrs after the heat-shock

HeLa cells were heat-shocked at 45°C for 5 mins and allowed to recover for 6 hrs at 37°C. Cytoplasmic RNA was isolated and fractionated by affinity chromatography on poly-(U) Sepharose. 5µgs of poly(A)$^-$ RNA was translated in 25µl of a rabbit reticulocyte cell-free protein synthesising system and the products were analysed on two-dimensions following the procedure of O'Farrell (1975).

Spots β, γ, δ, ε and ζ are five of the seven 72,000 - 74,000 dalton heat-shock proteins. The spots in brackets are possibly the multiple forms of actin. The position of the 100,000 dalton heat-shock polypeptide is also shown.


IEF
SDS
100K
Bγδε3

after the heat-shock, total cytoplasmic extracts from HeLa cells isolated 6 hrs after the heat-shock treatment were fractionated into polysomal and post-polysomal fractions (see Methods, Section 3.2.1.) and the RNA extracted from each fraction was quantitated. For comparisons, RNA was also extracted and quantitated from polysomal and post-polysomal fractions of normal cells and cells harvested 2 hrs after the heat-shock treatment. As it is shown in Figure 35 the polysomal profile of cells isolated 6 hrs after the heat-shock is similar to that of normal cells and cells isolated 2 hrs after heat-shock (for comparisons see Figures 30A and 30E). Also, the ratio $\frac{\text{polysomal RNA}}{\text{post-polysomal RNA}}$ calculated from the amount of total RNA found in polysomal or post-polysomal fractions of HeLa cells isolated 6 hrs after the heat-shock was very similar to the calculated ratio from normal HeLa cells (see Table 13).

6.4. Are the heat-shock specific mRNAs enriched in the post-polysomal fraction 6 hrs after the heat-shock?

To find out whether the mRNAs coding for the 72,000 - 74,000 dalton heat-shock proteins are enriched in the post-polysomal fraction 6 hrs after the heat-shock treatment, RNA from both polysomal and post-polysomal fractions from HeLa cells harvested 6 hrs after the heat-shock was translated *in vitro* and the products were analysed in two-dimensional gels according to the method of O'Farrell (1975). To provide a means of comparison the same was done with polysomal and post-polysomal RNA from HeLa cells isolated 2 hrs after the heat-shock treatment.

Examination of Figures 36A and 36B shows that while most of the 72,000 - 74,000 polypeptides are amongst the most abundant polypeptides encoded *in vitro* by mRNA in polysomes 2 hrs after the heat-shock, only polypeptides γ, ε and ζ are detectable amongst the translation products of mRNA in polysomes 6 hrs after the heat-shock treatment. These results

FIGURE 35

Polysomal profile of HeLa cells harvested 6 hrs after heat-shock treatment

HeLa cells were heat-shocked at 45°C for 5 mins and then allowed to recover at 37°C for 6 hrs before harvesting. Cytoplasmic extract prepared as described in Methods (Section 3.1.1.) was made up to 0.5% with respect to Brij-58 and 0.5% with respect to deoxycholate and layered onto 15-30% (w/v) sucrose gradients in RSB. After centrifuging at 27,000 rpm for 110 mins at 4°C the gradients were harvested by pumping through a Gilford 2,000 recording spectophotometer set at 260 nm. Polysomal and post-polysomal fractions are as indicated in the diagram.

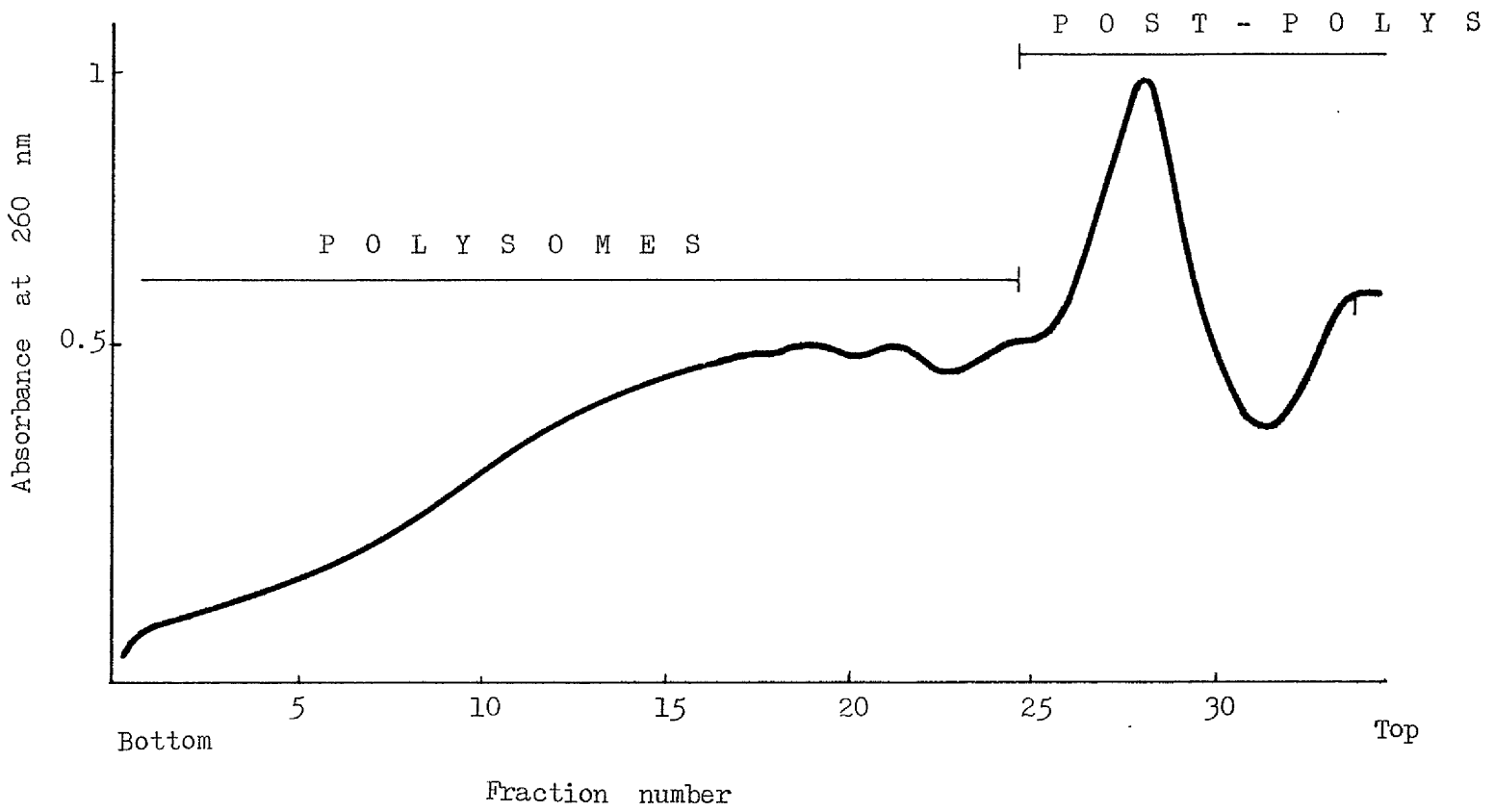

P O S T - P O L Y S
P O L Y S O M E S
Absorbance at 260 nm
1
0.5
5
10
15
20
25
30
Bottom
Top
Fraction number

TABLE 13

Quantitation of RNA of polysomal or post-polysomal fractions from normal HeLa cells or cells isolated 2 hrs or 6 hrs after the heat-shock treatment

Type of cells	Fraction	RNA Assayed µgs	µgs of total polysomal RNA / µgs of total post-polysomal RNA
Normal HeLa cells	polysomal	502	0.95
	post-polysomal	529	
HeLa cells heat-shocked at 45°C for 5 mins and allowed to recover at 37°C for 2 hrs.	polysomal	774	0.86
	post-polysomal	901	
HeLa cells heat-shocked at 45°C for 5 mins and allowed to recover at 37°C for 6 hrs.	polysomal	782	0.98
	post-polysomal	800	

Polysomal and post-polysomal fractions (see Figure 35) from normal or cells which had been heat-shocked at 45°C for 5 mins and then allowed to recover at 37°C for 2 hrs or 6 hrs were pooled separately and the RNA from each fraction was ethanol precipitated, collected by centrifugation, phenol-chloroform extracted and re-precipitated by the addition of 2 volumes of ethanol at -20°C. The quantity of the RNA was estimated from the optical density readings at 260 nm.

FIGURE 36

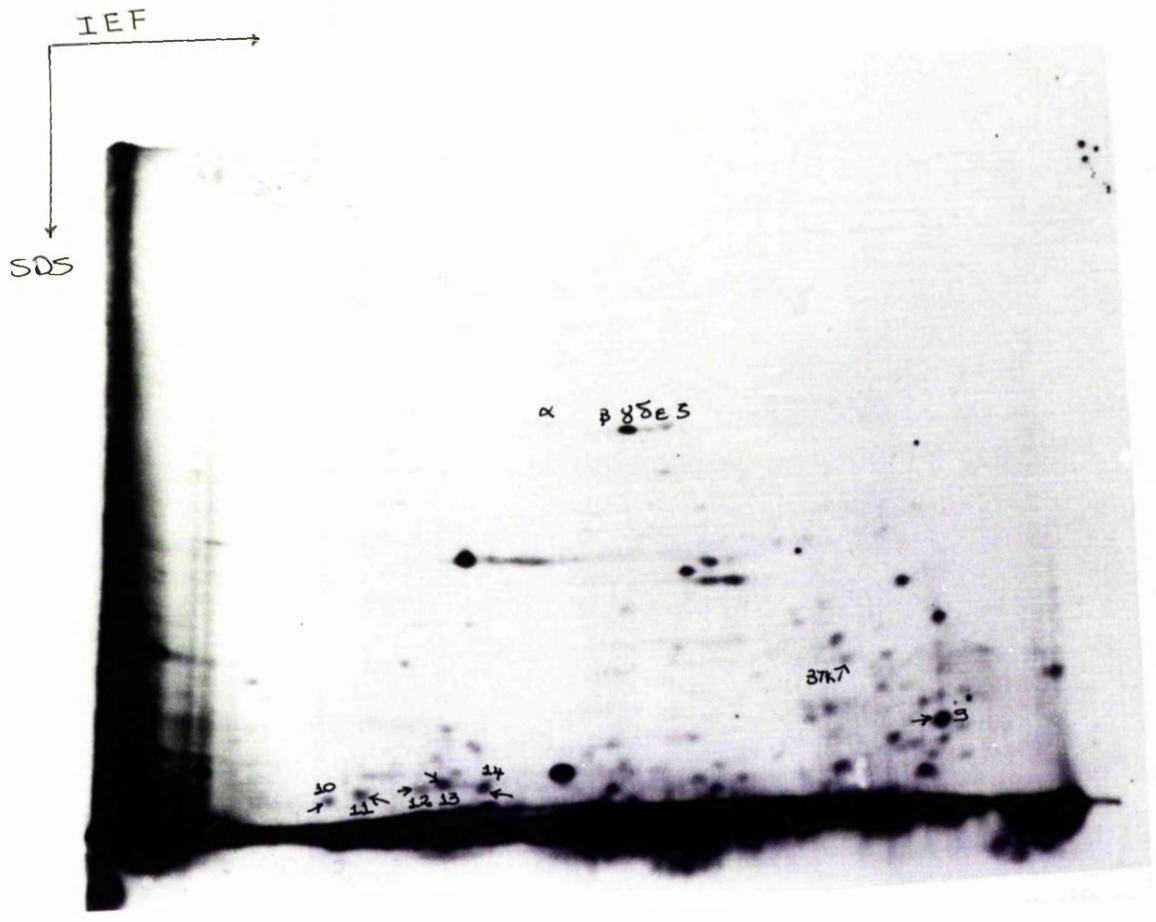
Fluorogram of two-dimensional analysis of [^{35}S]-methionine labelled in vitro translation products of HeLa cell polysomal RNA isolated 2 hrs or 6 hrs after the heat-shock

Polysomal fractions were prepared from HeLa cells which had been heat-shocked at 45°C for 5 mins and then allowed to recover at 37°C for 2 or 6 hrs as described in Methods (Section 3.2.1.). RNA was extracted from polysomal fractions essentially as described in Methods (Section 4.2.). 5µg of polysomal RNA was translated in a rabbit reticulocyte cell-free protein synthesising system and the products were analysed on two dimensions following the procedure described by O'Farrell (1975).

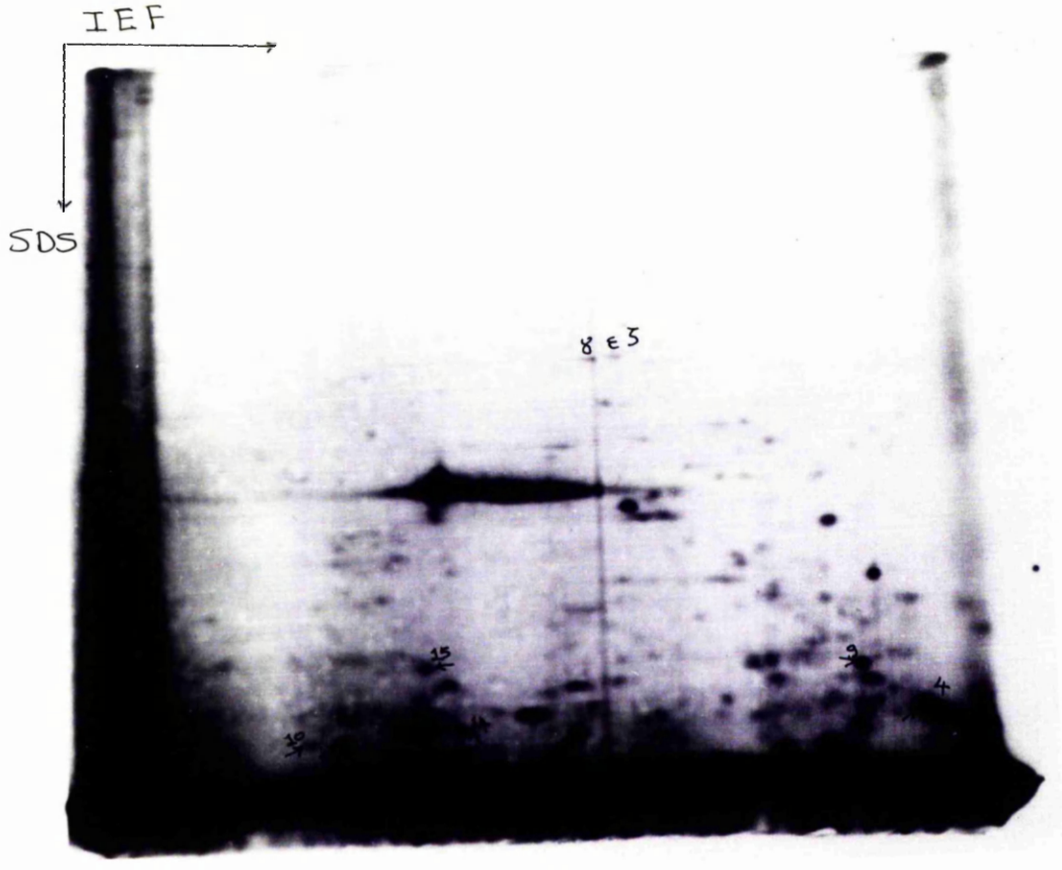
(A) Translation products of 5µg polysomal RNA isolated 2 hrs after the heat-shock.

(B) Translation products of 5µg polysomal RNA isolated 6 hrs after the heat-shock.

Numbered polypeptides are those enriched amongst the translation products of the polysomal RNA fractions as compared with the translation products of the corresponding post-polysomal RNAs (shown in Figure 37).



(A)



(B)

may suggest a decrease in the number and type of the 72,000 - 74,000 coding mRNAs in polysomes 6 hrs after the heat-shock. Figure 37B shows that γ, ε and ζ were also detected amongst the translation products of post-polysomal RNA isolated 6 hrs after the heat-shock. Comparison of Figures 36B and 37B also shows that polypeptides γ, ε and ζ are more enriched amongst the translation products of post-polysomal rather than polysomal RNA isolated 6 hrs after the heat-shock. However, this enrichment is not sufficient enough to suggest that the mRNAs coding for polypeptides γ, ε and ζ are preferentially found in the post-polysomal fraction 6 hrs after the heat-shock. It was also unexpected that none of the polypeptides α, α′, β and ζ were detected in the fluorograms presented in Figures 36B and 37B, especially since all the 72,000 - 74,000 hsp's were detected amongst the translation products of cytoplasmic RNA isolated 6 hrs after the heat-shock (see Figure 33). It is possible that the mRNAs coding for polypeptides α, α′, β and ζ were degraded during the isolation of polysomal and post-polysomal RNA especially since the procedure followed in this case is more lengthy than that followed for the isolation of cytoplasmic RNA (see Methods). It seems that limited degradation of mRNA has indeed taken place during the isolation of polysomal and post-polysomal fractions, since the 100,000 dalton hsp was not defected amongst the *in vitro* translation products of either fractions (see Figures 36A, B, and 37 A, B).

Furthermore, comparison of Figures 36A and 37A shows that 2 hrs after the heat-shock a substantial amount of the mRNAs coding for the 72,000 - 74,000 and 37,000 polypeptides are also found in a free form in the cytoplasm. These free-mRNAs may be in equilibrium with those engaged in polysomes (see Introduction).

FIGURE 37

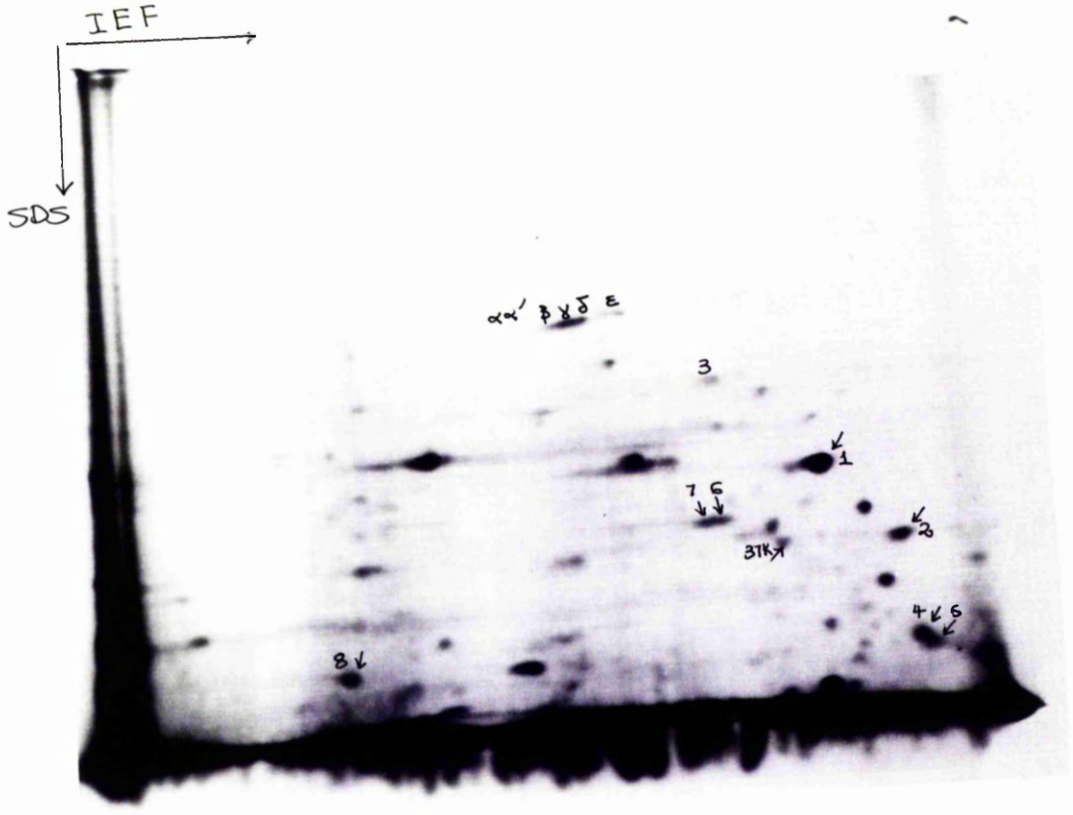
Fluorogram of two-dimensional analysis of [^{35}S]-methionine labelled in vitro translation products of HeLa cells post-polysomal RNA isolated 2 or 6 hrs after the heat-shock

Sub-polysomal and cytosolic fractions were isolated from HeLa cells harvested 2 or 6 hrs after the heat-shock as described in Methods (Section 3.2.1.). RNA was extracted from these fractions as described in Methods (Section 4.2.). 5µgs of post-polysomal RNA was translated in a rabbit reticulocyte cell-free protein synthesising system and the products were analysed on two dimensions following the procedure described by O'Farrell (1975).

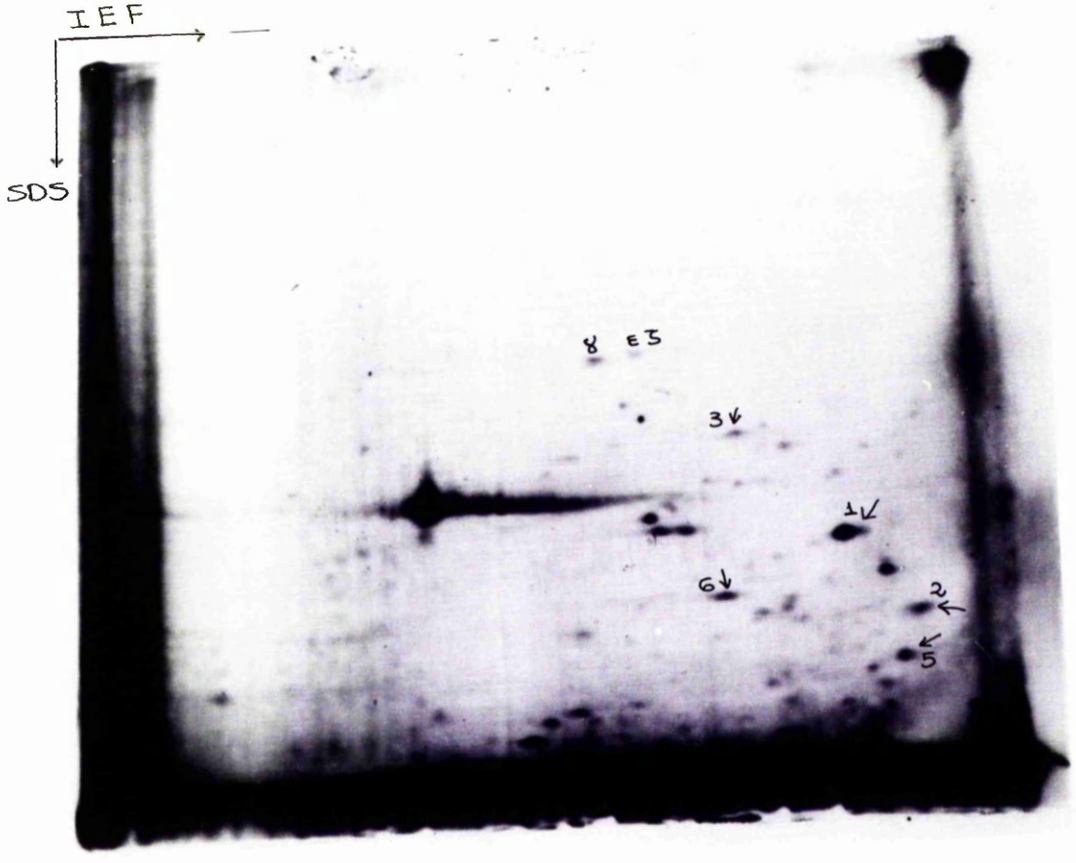
(A) Translation products of 5µg post-polysomal RNA isolated 2 hrs after the heat-shock.

(B) Translation products of 5µg post-polysomal RNA isolated 6 hrs after the heat-shock.

Numbered polypeptides are those enriched amongst the translation products of post-polysomal RNA as compared with the translation products of the corresponding polysomal RNA (shown in Figure 36).



(A)



(B)

Comparison of the in vitro translation products of polysomal and post-polysomal RNA isolated 2 hrs or 6 hrs after the heat-shock (compare Figures 36A and 37A, 36B and 37B respectively), shows that the same mRNAs species are detected in both polysomal and post-polysomal fractions. There are, however, quantitative differences. For example, polypeptides 1-8 are more enriched in the translation products of post-polysomal RNA isolated 2 hrs after the heat-shock, while polypeptides 9-14 are enriched among the translation products of polysomal RNA isolated 2 hrs after the heat-shock (see Figures 37A and 36A). These enrichments may represent enrichment of the corresponding mRNAs in one of the two fractions. On the whole, the pattern obtained from in vitro translation of polysomal and post-polysomal RNA isolated 6 hrs after the heat-shock is very similar to the pattern obtained from in vitro translation of the same fractions isolated 2 hrs after the heat shock. There is, however, one difference: polypeptide number 4, which is enriched in the translation products of post-polysomal RNA isolated 2 hrs after the heat-shock, is found enriched amongst the translation products of polysomal RNA isolated 6 hrs after the heat-shock (compare Figures 36B and 37A). Whether this represents a migration of the corresponding mRNA from the post-polysomal fraction, where it was primarily found 2 hrs after the heat-shock, to the polysomal fraction 6 hrs after the heat-shock it is not known. To answer this question together with any others concerning possible enrichment of particular mRNAs in polysomes or in free-cytoplasm, the development of an in vitro translation of cell-free system from extracts of HeLa cells would be required.

6.5. Conclusion

The results presented in this section have shown that while in vivo synthesis of the 72,000 - 74,000 hsp's declines 2 hrs after the heat-shock treatment, the corresponding mRNAs, both poly(A)$^+$ and poly(A)$^-$, can still be detected in the cytoplasm of heat-shocked HeLa

cells 6 hrs after the heat-shock. *In vitro* translation of both polysomal and post-polysomal RNA fractions from cells harvested 6 hrs after the heat-shock, failed however, to detect any dramatic increase of these mRNAs in the post-polysomal fractions.

7.1. Molecular cloning of cDNA sequences derived from cDNA sequences derived from heat-shocked cell poly(A)$^+$ RNA

In order to find out whether poly(A)$^+$ and poly(A)$^-$ mRNAs coding for the 72,000 - 74,000 dalton heat-shock proteins have similar sequences, a specific probe derived from one of the two mRNA populations is required. In order to isolate such a probe molecular cloning of cDNA sequences was carried out as described by Cato *et al.*, (1981). Figure 4 presents a diagram of the various steps. Briefly, cytoplasmic poly(A)$^+$ RNA was isolated from HeLa cells two hours after the heat-shock treatment and reverse transcribed using oligo(dT) as a primer. The resulting cDNA was made double-stranded by incubation with the Klenow fragment of *E.coli* polymerase I. The covalent links between the two strands was destroyed by S_1 nuclease treatment and the double stranded cDNA molecules were tailed with poly(dC) without prior determination of the sizes of these molecules. Poly(dG) was added to the Pstl cleaved pBR-322 and the linearised plasmid and cDNA were annealed slowly in dilute solution to form circular molecules. The annealed plasmids were used in the transformation of *Escherichia coli* HB101 cells. The plasmid pBR-322 normally confers ampicillin and tetracycline resistance to its host cell. However, insertion of DNA into the Pst 1 site destroys the ampicillin resistance and the recombinant clones may be recognised at tet^r amp^s (Bolivar *et al.*, 1977). 319 tet^r amp^s colonies were screened by *in situ* colony hybridisation technique on replica filters. One set of filters was hybridised with [^{32}P] - labelled cytoplasmic poly(A)$^+$ RNA from control HeLa

cells and the other set of filters with [^{32}P]-labelled cytoplasmic poly(A)$^+$ RNA isolated 2 hrs after HeLa cells had been heat-shocked for 5 mins at 45^{o}C. Figure 38 shows an autoradiogram of two replica filters with 41 out of 319 clones after such separate hybridisation with the two probes. This autoradiogram shows that most of the 41 clones hybridise with similar intensity to both probes, but some (those in circles around them) show stronger hybridisation to the labelled RNA from heat-shocked cells than to the labelled RNA from control cells. 13 such clones were isolated from the initial 319 recombinants but only six of these clones were studied further and these are designated pHS1, pHS2, pHS3, pHS4, pHS5 and pHS6 (see Figure 38).

7.2. Characterisation of the clones

7.2.1. One-dimensional analysis of the translation products of the heat-shocked cell cytoplasmic RNA which hybridised to clones pHS1 - pHS6

In order to establish that the pHS recombinant plasmids are indeed cDNA clones derived from mRNAs coding for HeLa heat-shock proteins, a procedure which is extension of a technique described by Stark and Williams (1979) was used. The pHS plasmids were each covalently bound to separate DBM-paper discs and hybridised with total unfractionated cytoplasmic RNA isolated 2 hrs after a 5 min heat-shock of HeLa cells at 45^{o}C (see Methods, Section 10.3.). Unbound RNA was washed away and the bound RNA was eluted, ethanol precipitated twice and then translated in a rabbit reticulocyte cell-free system. The products of translation were analysed on one-dimensional gel (Figure 39). Both pHS2 and pHS6 gave a single translation product that was similar in electrophoretic mobility to the *in vivo* labelled 72,000 - 74,000 daltons HeLa cell heat-shock protein band (Figure 39, band A). Plasmid pHS3 hybridised to mRNA that upon *in vitro* translation gave a protein band that migrated on

FIGURE 38

Autoradiogram showing the hybridisation of 41 cDNA clones to [^{32}P]-labelled poly(A)$^{+}$ RNA from (a) heat-shocked and (b) control HeLa cells

This autoradiogram shows only 41 out of 319 clones screened by the differential colony hybridisation procedure. 319 bacterial colonies were isolated from master plates and transferred to replica filters using sterile toothpicks. After overnight growth, the colonies were lysed and hybridised with 10^{6} cpm per filter of [^{32}P] labelled mRNAs from (left hand panel (a)) heat-shocked cell and (right hand panel (b)) control cells. After 16 hrs hybridisation at 65°C, the filters were washed and autoradiographed for 48 hrs. The circled colonies in panel (a) show hybridisation with greater intensity than their counterpars in panel (b). These therfore represent clones containing cDNA sequences complementary to mRNAs whose synthesis is increased after heat-shock. The 6 clones studied in detail are the ones circled and numbered.

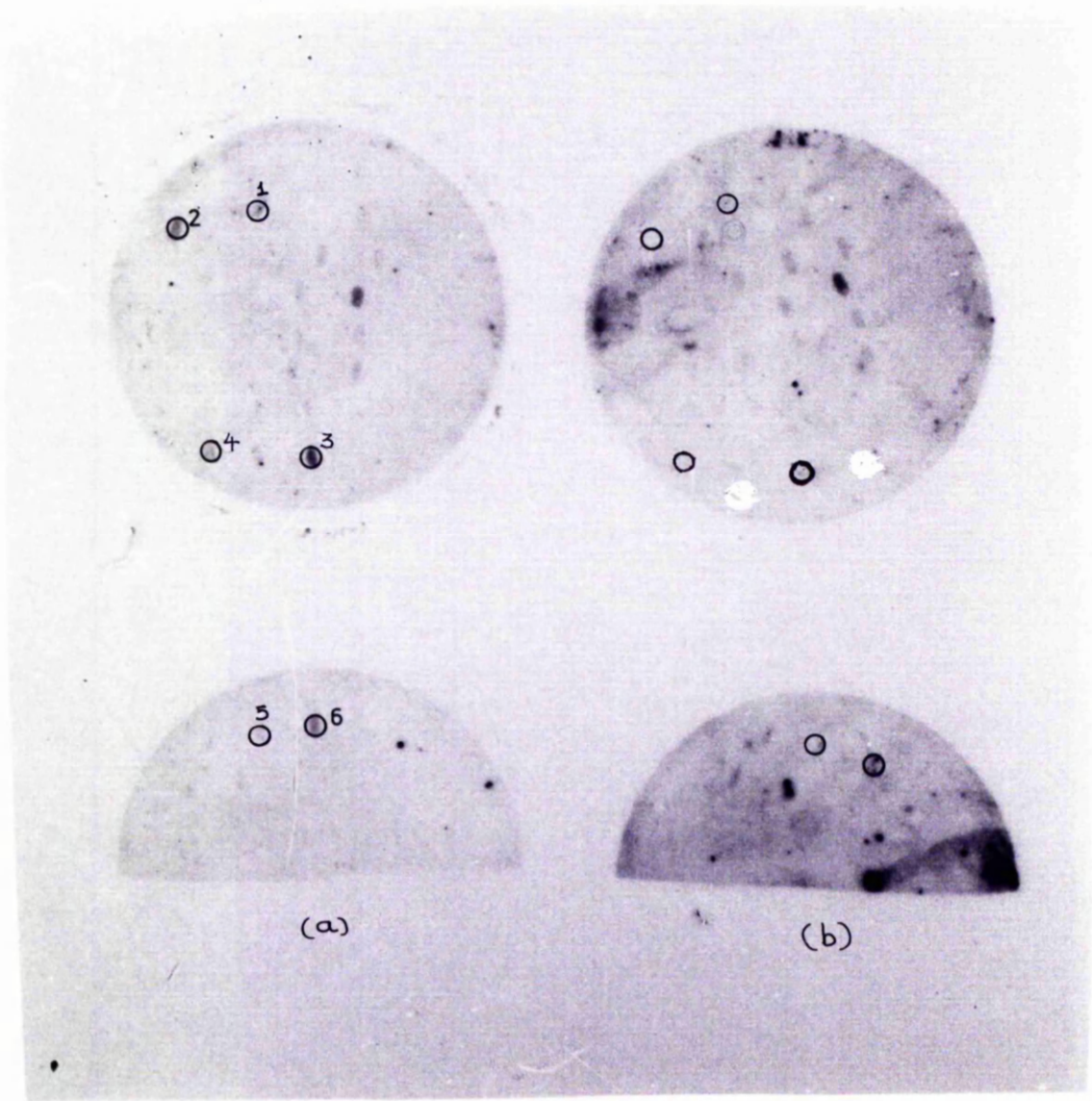
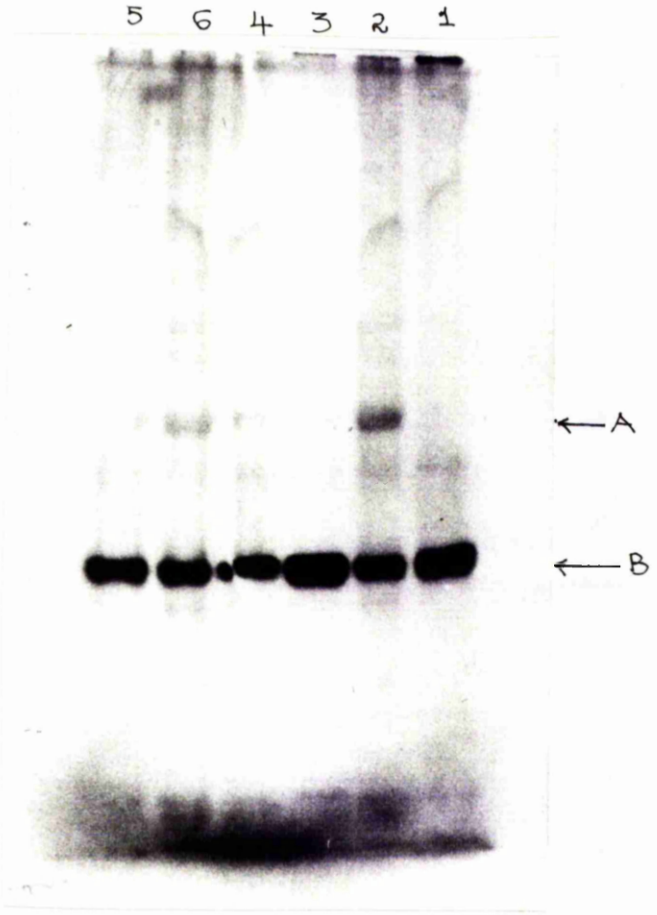

1
2
3
4
5
6
(a)
(b)

FIGURE 39

Fluorogram of SDS polyacrylamide gel analysis of [^{35}S]- methionine labelled in vitro translation products of mRNAs hybridising to recombinant DNA bound to DBM filters

10µg of plasmid DNA from clones pHS1 (Lane 1), pHS2 (Lane 2), pHS3 (Lane 3), pHS4 (Lane 4), pHS5 (Lane 5), pHS6 (Lane 6) were bound separately to DBM-filter discs (see Methods, Section 10.2.) and hybridised to total unfractionated cytoplasmic RNA from heat-shocked HeLa cells. The mRNA that hybridises to each filter was eluted and translated in a rabbit reticulocyte cell-free system (see Methods, Section 10.3.). This is a fluorogram of the translation products electrophoresed on SDS/polyacrylamide gel. The arrows A and B point to the position of the major HeLa cell heat-shock protein in the 72,000 - 74,000 molecular weight class and the position of the protein band made *in vitro* by the endogenous RNA in the translation system.


5
6
4
3
2
1
A
B

SDS/polyacrylamide gel to a position just below the protein band made by the endogenous RNA in the translation system (Figure 39, Lane 3).

7.2.2. Two-dimensional analysis of the translation products of the heat-shocked cell cytoplasmic RNA which hybridises to clones pHS2, pHS3 and pHS6

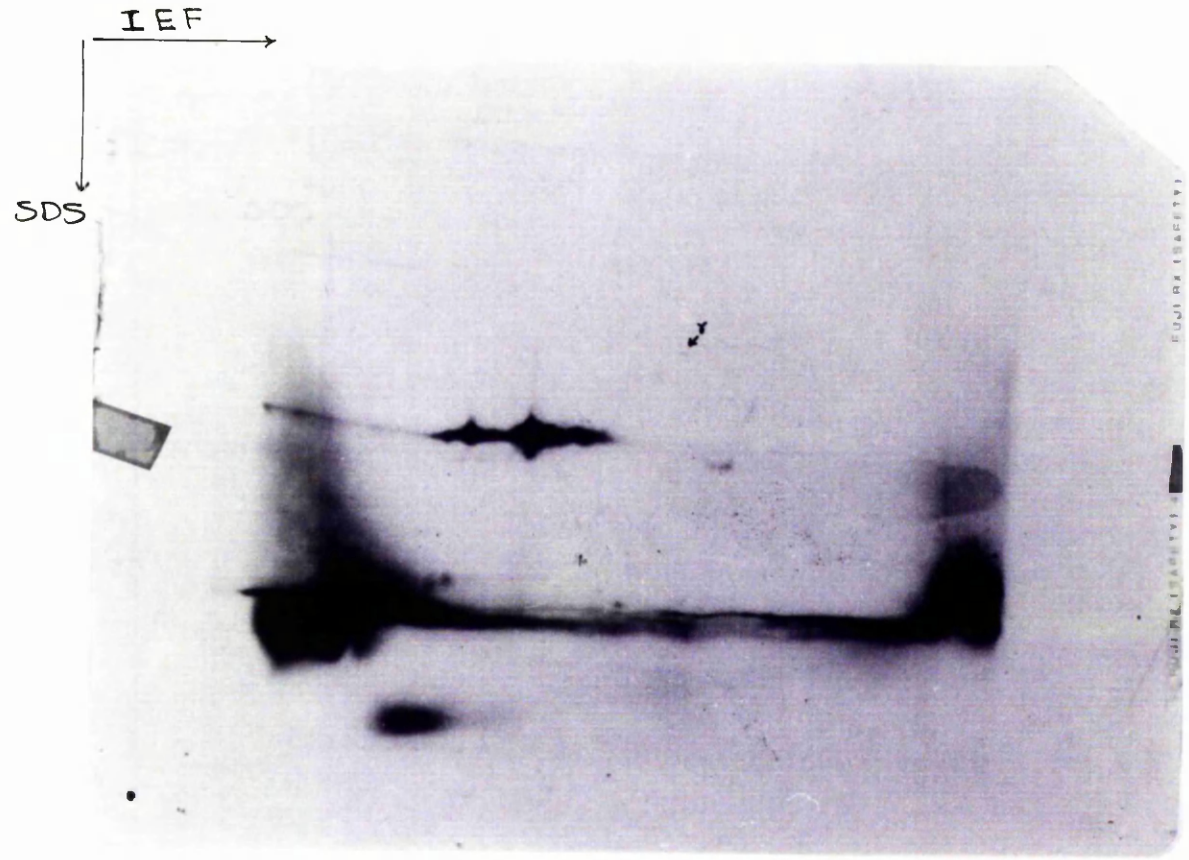
It has already been shown that the 72,000 - 74,000 daltons HeLa heat-shock protein band consists of seven polypeptides designated α, α', β, γ, δ, ε, and ζ and these are most probably encoded by mRNAs with different sequences. In order to investigate whether the inserted fragments correspond to different sequences in different mRNA species or whether they correspond to different regions of the same mRNA, two-dimensional analysis of the translation products shown in Figure 39 is needed. The whole procedure described in Results (Section 7.2.1.) was repeated, but this time the translation products were analysed by the two-dimensional gel system of O'Farrell (1975). The mRNAs which hybridised to plasmid pHS1, pHS4, pHS5 gave no more polypeptide spots than those detected when rabbit reticulocyte lysate is incubated in the absence of exogenous mRNA (Figure 27). The mRNAs which hybridised to plasmid pHS2 code for a single polypeptide spot (γ) in the 72,000 -74,000 molecular weight region (Figure 40A), while pHS6 hybridises with mRNAs that code for three heat-shock polypeptides (β, δ, and ε) in the 72,000 - 74,000 molecular weight class (see Figure 40B). Two-dimensional analysis of the mRNAs arrested by pHS3 plasmid did not show any polypeptide spots below the major endogenous mRNA product, but a single polypeptide spot at the 72,000 - 74,000 dalton region, which is quite possibly the polypeptide β (Figure 41). The fact that in Figure 39 the pHS3 arrested mRNA did not show any translation product in the 72,000 - 74,0000 dalton region might be due to degradation of the mRNA during the procedures.

The fact that recombinant plasmid pHS6 hybridises to mRNAs that

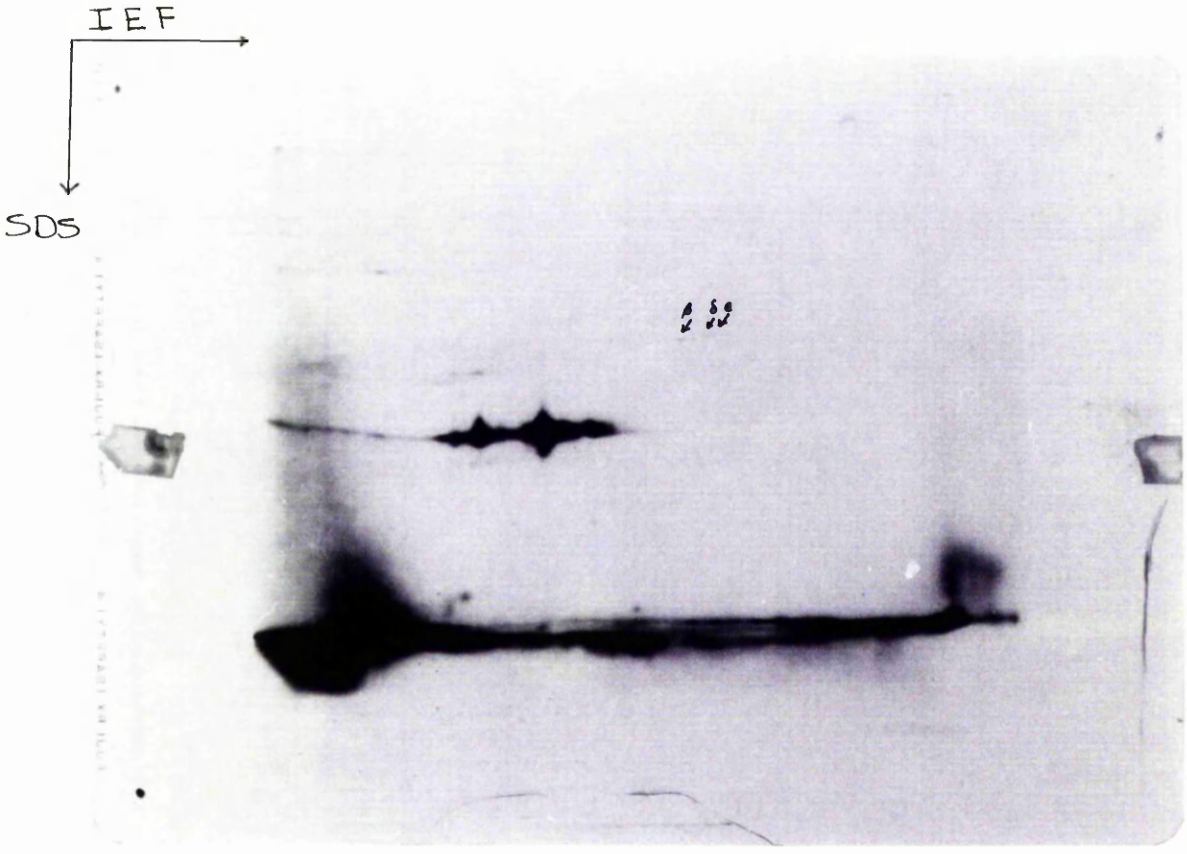
FIGURE 40

Fluorogram of two-dimensional electrophoretic analysis of [^{35}S]-methionine labelled in vitro translation products of mRNAs hybridising to recombinant plasmid DNA bound to DBM-filter discs

10μg of plasmid DNA from clones pHS2 (A) and pHS6 (B) were bound to DBM filters as described in Methods (Section 10.2.) and hybridised to total unfractionated cytoplasmic RNA from heat-shocked HeLa cells (see Methods, Section 10.3.). The mRNA which hybridised to the filters was eluted and translated in a rabbit reticulocyte cell-free system. These are fluorograms of the translation products analysed by two-dimensional gel system of O'Farrell (1975). The arrows point to the position of the β, γ, δ and ε polypeptides of the 72,000 - 74,000 dalton class.



(A)

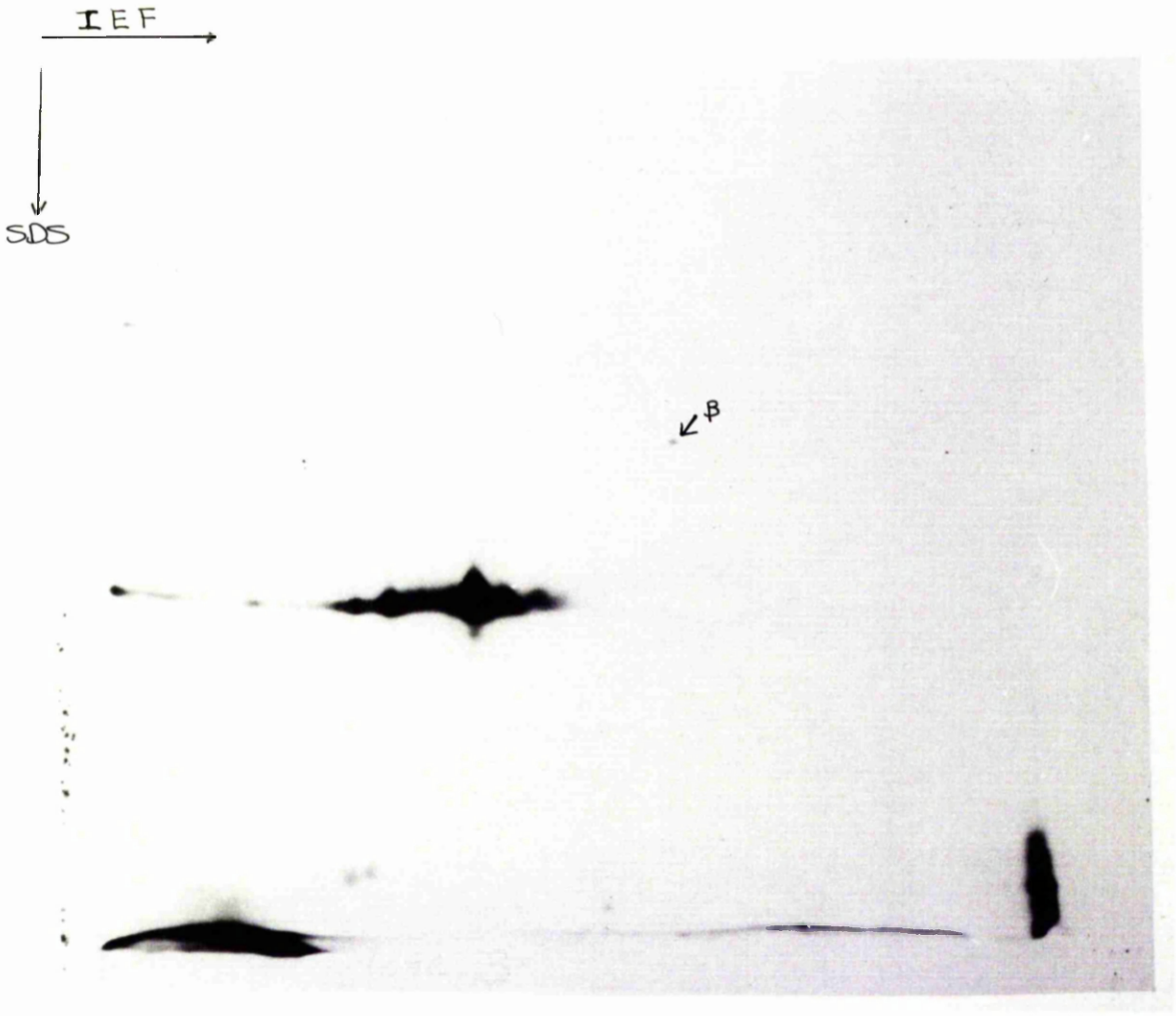


(B)

FIGURE 41

Fluorogram of two-dimensional electrophoretic analysis of [^{35}S]-methionine labelled in vitro translation products of mRNA hybridising to pHS3 recombinant plasmid DNA bound to DBM-discs

10µg of plasmid DNA from clone pHS3 was bound to DBM-filter and hybridised to total unfractionated cytoplasmic RNA from heat-shocked HeLa cells (as described in Methods, Section 10.3.). The mRNA which hybridised to the filter was eluted, ethanol precipitated and translated in a rabbit reticulocyte cell-free translation system. The products were subjected to two-dimensional electrophoretic analysis (O'Farrell, 1975). The second dimension was an 8.75% polyacrylamide/SDS gel. The spot designated as β is the β polypeptide of the 72,000-74,000 dalton group and was identified by superimposing this fluorogram with that shown in Figure 26).


IEF
SDS
β

code for three different proteins β, δ,and ε (Figure 40B) indicates that a portion or possibly the whole cDNA insert may be homologous with certain conserved regions of the different poly(A)$^+$ heat-shock mRNAs. However, plasmid pHS3 seems to contain sequences which are not conserved among the poly(A)$^+$ mRNAs and are specific for poly(A)$^+$ mRNAs coding for β polypeptide.

7.3. Hybridisation of [^{32}P]- labelled control and heat-shocked cell poly(A)$^+$ RNA with recombinant plasmid DNA immobilised on DBM-filters

Since only three of the isolated clones (i.e. pHS2, pHS3 and pHS6) were fully characterised, we used the method of spot hybridisation to further establish that all the six recombinants contained sequences enriched among heat-shocked cell poly(A)$^+$ RNA.

10 µgs of plasmid DNA from nontransformed pBR322 as well as from transformants pHS1, 2, 3, 4, 5 and pHS6 was bound at various points of DBM paper strip (see Methods, Section 10.2.). Heat-shock cell poly(A)$^+$ RNA, end-labelled at a specific activity 10^6 cpm/µg was hybridised to the DBM paper strip as described in Methods, (Section 12). Figure 42A shows that heat-shock cell poly(A)$^+$ RNA hybridises with all but one transformant: pHS4. So, it seems that PHS4 was a false "positive". Indeed, as shown in Figure 38, pHS4 had not shown very strong hybridisation to heat-shocked cell poly(A)$^+$ RNA, When control cell poly(A)$^+$ RNA, end labelled at a specific activity of 10^7 cpm/µg was hybridised to the same strip, the following was shown (Figure 42B).

1) There is quite strong hybridisation with pHS6 plasmid DNA. Since pHS6 insert has been shown to be derived from poly(A)$^+$ RNAs coding for β, δ and ε polypeptides, this is an expected result. Polypeptide ε is indeed encoded by control cell poly(A)$^+$ RNA (see Figure 28A).

FIGURE 42

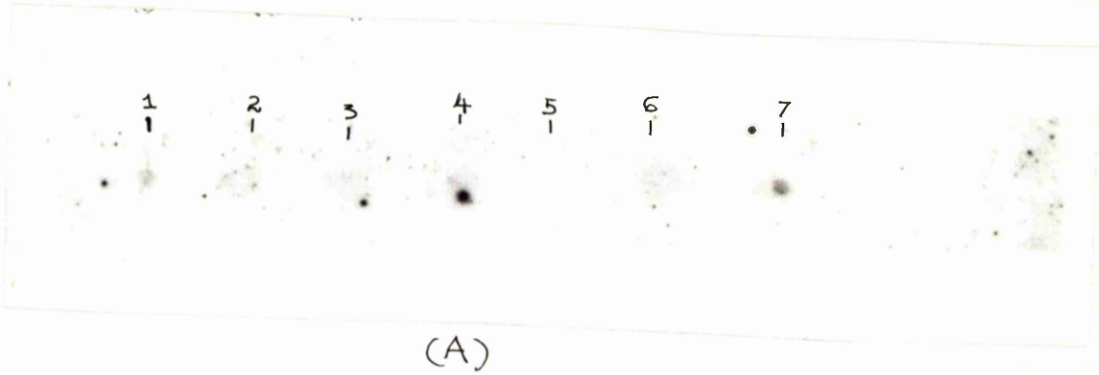
Hybridisation of [^{32}P]- labelled control and heat-shocked cell poly(A)$^+$ RNA to recombinant plasmid DNA immobilised on DBM-filters

10µgs of plasmid DNA from nontransformed pB322 (1), as well as from transformants pHS1 (2), pHS2 (3), pHS3 (4), pHS4 (5), pHS5 (6), pHS6 (7) was bound at various points of DBM paper strip as described in Methods (Section 10.2.). The DBM paper strip was hybridised to:

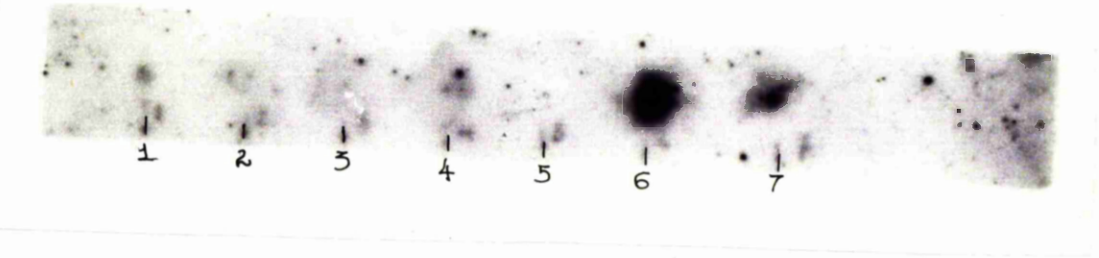
(A) ^{32}P-labelled heat shocked cell poly(A)$^+$RNA (specific activity 10^6 cpm/µg).

(B) [^{32}P]- labelled control cell poly(A)$^+$ RNA (specific activity 10^7 cpm/µg).

[^{32}P]- labelled heat-shocked cell poly(A)$^+$ RNA does not show very strong hybridisation to pHS2 or pHS3 and pHS6 as it might be expected, because the specific activity of the probe is very low compared to that of control poly(A)$^+$ RNA (B).



(A)



(B)

2) There is weak hybridisation with pHS2 plasmid DNA. This is also expected since polypeptide γ is encoded by control cell poly(A)$^+$ RNA (Figure 28A).

3) Even though pHS5's insert has not been shown to be derived from RNA molecules with messenger activity, control cell poly(A)$^+$ RNA hybridises very strongly with pHS5.

These data further establish that pHS1, pHS2, pHS3, pHS5 and pHS6 contain sequences which are enriched among heat-shocked cell poly(A)$^+$ RNA but which are also found in poly(A)$^+$ RNAs from control HeLa cells. Since the RNAs which hybridised to pHS1 and pHS5 have not been shown to code for any particular protein (see Figure 39), it is possible that these sequences are derived from RNAs which play some other role (possibly regulatory).

7.4. Hybridisation of [^{32}P]- labelled control and heat-shocked cell poly(A)$^-$ RNA to recombinant plasmid DNA immobilised on DBM filters

In order to find out whether the poly(A)$^-$ mRNAs coding for the 72,000 - 74,000 dalton heat-shock proteins share similar sequences with the poly(A)$^+$ RNAs coding for the same proteins, the following was done:

Partially purified heat-shocked cell poly(A)$^-$ RNA from fraction 8 of the sucrose gradient presented in Figure 21 was end labelled (see Methods, Section 11) at a specific activity of 10^7 cpm/μg and then hybridised to the DBM-paper strip shown in Figure 42.

The autoradiogram shown in Figure 43A shows the following:

1) Strong hybridisation of the purified heat-shocked cell poly(A)$^-$ RNA to pHS2 plasmid DNA. Since the insert in pHS2 is shown to be derived from poly(A)$^+$ mRNAs coding for the γ polypeptide spot, this result suggests that poly(A)$^+$ and poly(A)$^-$ mRNAs coding for this particular polypeptide

FIGURE 43

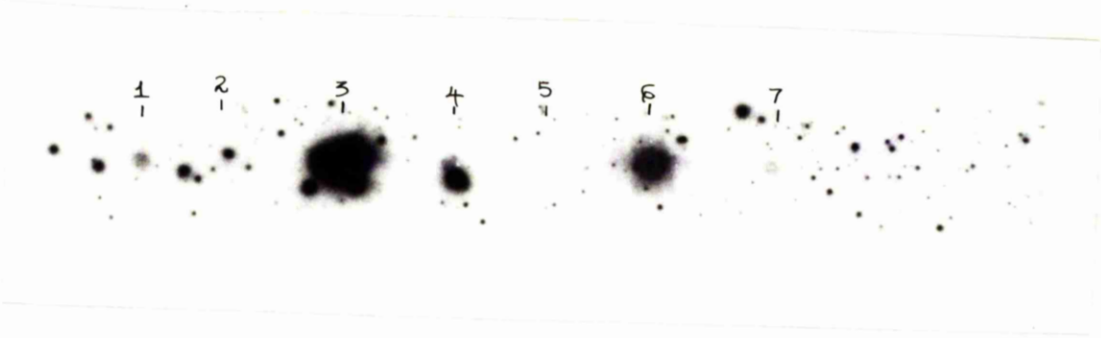
Hybridisation of [^{32}P]- labelled control and partially purified heat-shocked cell poly(A)$^-$ RNA to recombinant plasmid DNA immobilised on DBM-filters

10µgs of plasmid DNA from nontransformed pBR322 (1), as well as from transformants pHS1 (2), pHS2 (3), pHS3 (4), pHS4 (5), pHS5 (6) and pHS6 (7) was bound at various points of DBM paper strip as described in Methods (Section 10.2.). The DBM strip was hybridised to:

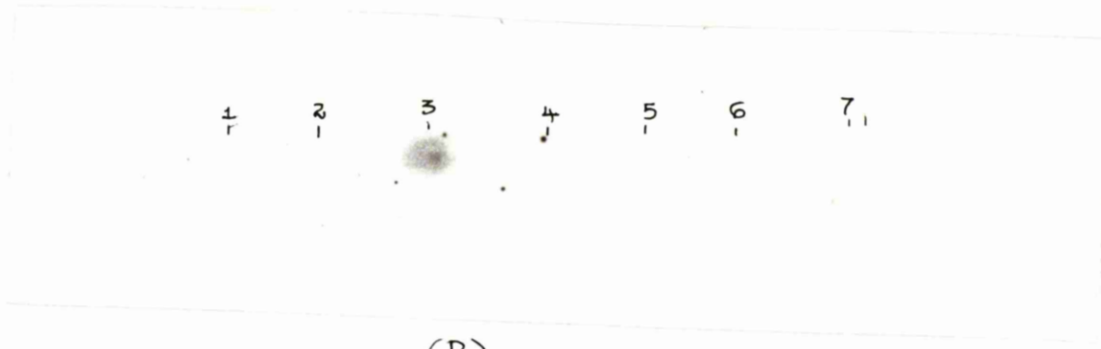
(a) [^{32}P]- labelled partially purified heat-shocked cell poly(A)$^-$ RNA from fraction 8 of the sucrose gradient shown in Figure 21 (specific activity 10^7 cpm/µg).

(B) [^{32}P]- labelled control cell poly(A)$^-$ RNA (specific activity 10^7 cpm/µg).

The hybridisation which is shown at point (4) in (A) is an artefact due to radioactive grain.



(A)



(B)

share some sequence homology. Since it is not known whether the pHS2 insert is derived from the 5' or 3' end of the poly(A)$^+$ mRNA molecules (Cato et al., 1981) one can not say which part of the mRNA molecule is involved in the sequence homology observed.

2) "Purified" heat-shocked cell poly(A)$^-$ RNA hybridises strongly to pHS5 plasmid DNA (see Figure 43A). As it is shown in Figure 43B poly(A)$^-$ RNA from control cells also hybridises to pHS5. So it seems that the sequences inserted in pHS5 are derived from RNAs which are fractionated both as poly(A)$^+$ and poly(A)$^-$ and which exist in both normal and heat-shocked cells.

Spradling et al., (1977) has reported that, in Drosophila cells, poly(A)$^+$ mRNAs coding for the major heat-shock protein are fractionated on sucrose gradients together with another class of poly(A)$^+$ RNAs which have been shown to hybridise to 87C heat-shock puff but no protein seemed to be encoded by this class of RNAs (Livak et al., 1978; Lis et al., 1978).

So, it is quite possible that the insert of pHS5 is derived from a similar class of RNAs in HeLa cells. The results suggest that, in HeLa cells, these RNAs exist in both poly(A)$^+$ and poly(A)$^-$ RNAs. One though, can not exclude the possiblity that some of these RNAs bind to poly-(U) Sepharose non specifically or due to their affinity for other RNA molecules.

3) Poly(A)$^-$ RNA from control cells hybridised to pHS2. Indeed, poly(A)$^-$ mRNA from control cells has been shown to code for γ polypeptide (see Figure 29A).

4) Even though poly(A)$^-$ mRNAs coding for the γ polypeptide seem to share at least some sequences with the corresponding poly(A)$^+$ mRNAs, purified poly(A)$^-$ RNA from heat-shocked cells did not hybridise to pHS3 and pHS6. This raises the possibility that poly(A)$^-$ mRNAs coding for β, δ and ε polypeptides do not have extensively similar sequences with those of poly(A)$^+$ mRNAs.

7.5. Conclusion

The results in this section have shown that the poly(A)$^+$ mRNAs coding for the γ heat-shock polypeptide share at least some sequences with the poly(A)$^-$ mRNAs coding for the same protein. It seems, however, that the poly(A)$^+$ mRNAs coding for the β, δ and ε polypeptides do not share any sequences with their non-polyadenylated counterparts.

DISCUSSION

1. Poly(A)$^+$ and poly(A)$^-$ mRNAs coding for the same protein

A number of polypeptides in various eukaryotic cells have been shown to be encoded by both poly(A)$^+$ and poly(A)$^-$ mRNA, poly(A)$^-$ mRNA being by definition that fraction of mRNA which fails to be retained by either oligo-(dT) cellulose or poly-(U) Sepharose. For example, the structural protein actin is synthesised by both poly(A)$^+$ and poly(A)$^-$ mRNA in Friend cells (Minty and Gros, 1980), HeLa cells (Kaufmann et al., 1977), mouse sarcoma 180 ascites cells (Geoghegan et al., 1978) calf muscle cells (Whalen and Gros, 1978), and Dictyostelium discoideum (Palatnik et al., 1979). Histones have also been shown to be encoded by both poly(A)$^+$ and poly(A)$^-$ mRNA in amphibian oocytes (Levenson and Marcu, 1976; Ruderman and Pardue, 1978), sea urchins (Ruderman and Pardue, 1978) and clams (Gabrielli and Baglioni, 1975). In HeLa cells, the mRNAs coding for some of the histones are believed to contain small poly(A) tracts (Borun et al., 1977). On the other hand, histone mRNA in yeast is mainly polyadenylated while a fraction of the H2A and H2B mRNA also exist in the poly(A)$^-$ mRNA fraction (Fahrner et al., 1980). Similarly, the majority of myosin heavy chain mRNAs in L6E9 rat myotubes fails to bind to oligo (dT)-cellulose (Benoff and Nadal-Ginard, 1979), while albumin and α-Fetoprotein mRNAs in rat liver have been shown to exist in both poly(A)$^+$ and poly(A)$^-$ fractions (Sala-Trepat et al., 1979). In the increasing list of polypeptides encoded by both poly(A)$^+$ and poly(A)$^-$ mRNA is the 70,000 dalton hsp in Drosophila (Lengyel et al., 1980; Storti et al., 1980.

Apart from individual polypeptides shown to be encoded by both poly(A)$^+$ and poly(A)$^-$ mRNAs, in vitro translation experiments have revealed that a substantial number of polypeptides are encoded by both poly(A)$^+$ and poly(A)$^-$ mRNA in mouse kidney cells (Quellette and Ordahl, 1981), rat brain (Chikaraishi, 1979) and neuroblastoma cells (Croall

and Morrison, 1980). One-dimensional analysis of the *in vitro* translation products of poly(A)$^+$ and poly(A)$^-$ polysomal mRNA from HeLa cells (see Figure 7) showed that both mRNA fractions had qualitatively the same translation products, even though some quantitative differences were detected. Two-dimensional analysis of the translation products showed that whilst several polypeptides result from the translation of either poly(A)$^+$ or poly(A)$^-$ mRNA a considerable number of mRNAs appear to exist in both poly(A)$^+$ and poly(A)$^-$ forms (see Figure 8). It seems, however, that the situation is not the same in all mammalian cells. For example, in Friend cells the majority of the polypeptides result from the translation of both poly(A)$^+$ and poly(A)$^-$ mRNA (see Figure 9).

Despite the fact that some abundant mRNA sequences seem to exist in both poly(A)$^+$ and poly(A)$^-$ RNA, experiments employing the method of saturation hybridisation have shown that in mouse liver, mouse brain and rat brain there is not much homology between poly(A)$^+$ and poly(A)$^-$ mRNAs (Grady *et al.*, 1978; Van Ness *et al.*, 1979; Chikaraishi, 1979). By the use of kinetic measurements, however, it has been shown that in Friend cells cDNA transcribed from poly(A)$^+$ mRNA hybridises to the same extent to both poly(A)$^+$ and poly(A)$^-$ mRNAs (Minty and Gros, 1980). Similar results have been obtained in mouse kidney cells (Quellette and Ordahl, 1981). Due to the limitations of the two methods employed (see Introduction, Section 2), a question arising is whether both poly(A)$^+$ and poly(A)$^-$ forms of an mRNA species are transcribed from the same gene. To answer this, an initial approach was to isolate from the bulk of poly(A)$^-$ RNA (which mainly contains rRNA and tRNA) poly(A)$^-$ mRNA sequences coding for proteins also encoded by poly(A)$^+$ mRNA. Thus a subclass of poly(A)$^-$ RNA sequences was isolated due to its affinity for poly-(A) Sepharose (poly(A)$^-$u$^+$ RNA). Poly(A)$^-$ RNA containing oligo-(u) sequences and exhibiting properties of mRNA species had been earlier reported in HeLa cells (Korwek *et al.*, 1976). Poly(A)$^-$u$^+$ mRNA has also been reported

in Friend cells (Katinakis and Burdon, 1981). By selecting poly(A)$^-$u$^+$ mRNAs on poly(A)-Sepharose columns, we found that this RNA fraction represents a minor fraction of the total mRNA population in HeLa cells (see Tables 3 and 4). When the coding potential of these poly(A)$^-$u$^+$ RNA molecules was tested by in vitro translation in rabbit reticulocyte lysates, these RNAs, at least from HeLa cells, did not seem to have much stimulating effect on in vitro protein synthesis (see Table 5). In Friend cells, however, poly(A)$^-$u$^+$ RNAs have been reported to stimulate in vitro protein synthesis. As it was shown by one-dimensional and two-dimensional analysis of the translation products, all the polypeptides encoded by poly(A)$^-$u$^+$ RNA from HeLa cells were also encoded by poly(A)$^+$ RNA (see Figures 10 and 11). Even though our initial objective of isolating poly(A)$^-$ mRNA coding for a limited number of polypeptides which are also encoded by poly(A)$^+$ mRNA was achieved, results published by Molloy (1980) while this work was underway showed that intramolecular duplex structures of oligo-(u) sequences with 3' poly(A) can only be disrupted completely with formaldehyde (HCHO) treatment. Employing this method Molloy (1980) showed that most of the oligo-(u) sequences are found in poly(A)$^+$ mRNAs in HeLa cells. These results have been lately confirmed by Wood and Edmonds (1981) who showed that poly(A)$^+$u$^+$ RNAs can be selected on poly(A)-Sepharose only after removal of the poly(A) tails. These poly(A)$^+$u$^+$ RNAs were also shown to be larger than the poly(A)$^+$ mRNAs in HeLa cells (Wood and Edmonds, 1981). This combined with the fact that these poly(A)$^+$u$^+$ RNAs had a higher ratio of cap I: cap II structures as compared with poly(A)$^+$ RNAs from the same cells (Wallace et al., 1981) raises the possibility of nuclear contamination in their preparations. At the same time, no information about the translational activity of these poly(A)$^+$u$^+$ RNAs from HeLa cells was given.

In any case, these data raised the possibility that the poly(A)$^-$u$^+$ RNA molecules we had isolated could actually be polyadenylated RNA molecules which had failed to bind to poly-(U)-Sepharose because of the duplex structures formed between the poly(A) tail and internal oligo-(u) sequences but which were nonspecifically retained by poly(A)-Sepharose.

The prevalence of poly(A)$^-$ mRNAs in cells which are not highly differentiated (Ruderman and Pardue, 1977; James and Tata, 1980) has also raised the question whether poly(A)$^-$ mRNAs are vital to cells which have to respond rapidly to external or internal changes (Katinakis and Burdon, 1980). Since heat-shock treatment of cells provides a system in which the cells respond rapidly to an external stimulus, we raised the incubation temperature of HeLa cells for a limited period and studied the mRNAs which code for the heat-shock induced proteins in these cells. Our results show that the 72,000 -74,000 heat-shock protein and possibly the 37,000 and 100,000 heat-shock proteins are encoded by both poly(A)$^+$ and poly(A)$^-$ mRNAs (Kioussis et al., 1981). It is of interest that the 70,000 dalton heat-shock protein in Drosophila cells is also encoded by both poly(A)$^+$ and poly(A)$^-$ mRNAs (Lengyel et al., 1980). The poly(A)$^-$ mRNAs coding for the 72,000 -74,000 dalton hsp do not seem to contain long poly(A) tails as judged by their inability to be retained by poly-(U)-Sepharose after three successive cycles of affinity chromatography (see Figure 24), or after affinity chromatography cycle carried out at 4^{o}C (see Figure 25). A small proportion of poly(A)$^-$ mRNA coding for the 72,000 - 74,000 dalton hsp does bind to poly-(U)-Sepharose (see Table 9), but its elution profile is distinct from that of poly(A)$^+$ mRNAs (see Figure 23), suggesting that some poly(A)$^-$ mRNAs coding for the 72,000 - 74,000 dalton hsp may contain small oligo(A) sequences. Indeed, Milcarek (1979) has reported the existence of small internally located oligo(A) sequences in poly(A)$^-$ mRNA molecules from HeLa cells. It was

not possible to determine what proportion of the 72,000 - 74,000 coding mRNAs existed in the poly(A)$^-$ form by making use of the *in vitro* translation technique, especially since the amounts of poly(A)$^+$ and poly(A)$^-$ mRNAs translated differed markedly (see Figure 20). It would be interesting, however, to determine that by making use of the clone pHS2 which is derived from mRNA coding for polypeptide γ of the 72,000 - 74,000 heat-shock proteins and which hybridises to both poly(A)$^+$ and poly(A)$^-$ mRNAs (see Figures 42 and 43).

Even though it has been proposed that cells which have to respond rapidly to an external stimulus might preferentially synthesise poly(A)$^-$ mRNAs (see above), we did not detect any decrease in the production of poly(A)$^+$ mRNAs in heat-shocked HeLa cells (see Table 8). Minty and Gros (1980) have shown that during differentiation of Friend cells by induction with dimethylsulphoxide the percentage of globin mRNA sequences in the poly(A)$^+$ form does not change.

It has been proposed that poly(A)$^-$ mRNAs may be degraded more rapidly especially since polyadenylation is known to increase the stability of some mRNAs (Huez *et al.*, 1978). However, our *in vitro* translation experiments have shown that even though synthesis of heat-shock proteins declines 4 hrs after the heat-shock, both poly(A)$^+$ and poly(A)$^-$ mRNAs coding for the 72,000 - 74,000 heat-shock polypeptides can be detected in the cytoplasm of HeLa cells 6 hrs after the heat-shock (see Figures 32, 33 and 34). It would be interesting, however, by making use of the clone pHS2 to determine the ratio of the steady state poly(A)$^+$:poly(A)$^-$ mRNAs coding for the polypeptide γ 2 and 6 hrs after the heat-shock. Croall and Morrison (1980) have detected no difference in the average poly(A) size between polysomal and non-polysomal mRNA populations in neuroblastoma cells.

Storti and colleagues (1980) by translating poly(A)$^+$ and poly(A)$^-$ mRNA from Drosophila cells kept at elevated temperatures for 1 hr showed that most of the translation activity is found in the poly(A)$^-$ mRNA fraction, suggesting loss of the poly(A) tail as a result of the heat-shock. Brandt and Milcarek (1980) have recently proposed that possibly half of the pre-existing mRNA molecules lose their poly(A) tails as a result of the heat-shock. The loss of poly(A) tail from pre-existing molecules may be of significance especially since these mRNAs are not translated in vivo during the heat-shock. (Ashburner and Bonner, 1979; Storti et al., 1980). However, the fact that 83,000 hsp mRNAs induced after the heat-shock are mainly non-polyadenylated argues against a translational discrimination in favour of poly(A)$^+$ mRNAs (Storti et al., 1980). The change in the polyadenylation status of the 83,000 hsp coding mRNAs is most probably due to a change in polyadenylation of the same transcript, especially since only one hsp 83,000 gene is as yet known in Drosophila (O'Connor and Lis, 1981).

In HeLa cells, however, a similar pattern of polypeptides is obtained by in vitro translation of both poly(A)$^+$ and poly(A)$^-$ mRNAs from normal and heat-shocked HeLa cells (Kioussis et al., 1981, also see Figures 28 and 29). A few mRNA species seemed to have disappeared or to be reduced in heat-shocked cells, but this was observed in both poly(A)$^+$ and poly(A)$^-$ mRNAs. One can not exclude the possibility that limited degradation of poly(A) tails takes place in HeLa cells as a result of the heat-shock and it would be interesting to determine the distribution of poly(A) tails before and after heat-shock.

The poly(A)$^-$ mRNAs coding for the 72,000 - 74,000 dalton protein were shown to sediment between 18-21S on sucrose gradients (see Figure 21), exhibiting a narrower sedimentation range from that of the corresponding poly(A)$^+$ mRNAs (18S-28S), (see Figure 22). However, when

the size of these poly(A)$^-$ mRNAs was determined by making use of the Northern blotting technique, it was shown that the poly(A)$^-$ mRNAs coding for the heat-shock polypeptide γ had the same size with the corresponding poly(A)$^+$ mRNAs (i.e. 6,300 bases long). Similarly, the poly(A)$^+$ and poly(A)$^-$ mRNAs coding for heat-shock polypeptides β, δ and ε were shown to be of the same size (Cato, personal communication). Indeed, in HeLa cells the mean size of poly(A)$^-$ mRNAs has been known to be similar to that of poly(A)$^+$ mRNAs (Milcarek et al., 1974).

Poly(A)$^+$ and poly(A)$^-$ mRNAs coding for γ polypeptide seem to share at least some sequence homology as shown by spot hybridisation experiments (see Figure 43). It is possible that both forms of γ coding mRNAs are derived from the same gene. This, however, would be determined by the isolation of genomic clones. On the other hand, poly(A)$^-$ mRNAs coding for β, δ and ε polypeptides do not seem to share any sequences with the corresponding polyadenylated mRNAs (see Figure 43). This result may suggest that the two forms of an mRNA species in some cases may be derived from different genes. It remains to be established, by making use of genomic clones, that there are more than one gene coding for each of the polypeptides β, δ and ε and that poly(A)$^-$ and poly(A)$^+$ mRNAs originate from different genes.

Most of the protein coding primary transcripts detected so far have been shown to be polyadenylated (see Introduction) and it has even been proposed that poly(A) tail may play a role in splicing (Bina et al., 1980). Even though there is at present no known case of a spliced mRNA lacking poly(A) when it is newly made, it has recently been shown by Zeevi and colleagues (1981) that the primary transcripts from adenovirus early regions 2 and 1b can be properly spliced even if polyadenylation is blocked by 3'-deoxyadenosine. Since there is evidence that the poly(A)$^+$ mRNAs coding for γ, β, δ and ε polypeptides may be derived from

longer transcripts by splicing (see below), it would be interesting to determine whether the same is true for the corresponding poly(A)$^-$ mRNAs.

2. The heat-shock phenomenon

It is known for some time now that incubation of Drosophila cells at 37^{o}C (25^{o}C being the normal growth temperature), results in almost complete shut-off of normal protein synthesis and preferential production of a small number of heat-shock polypeptides (hsp s) (Tissieres et al., 1974; Lewis et al., 1975; Lindquist-McKenzie et al., 1975; Koninkx, 1976; Ashburner and Bonner, 1979). In Drosophila melanogaster there are eight heat-shock induced protein bands of molecular weights 82,000, 70,000, 68,000, 36,000, 27,000, 26,000, 23,000 and 22,000, the 70,000 protein band being the major heat-shock protein (Ashburner and Bonner, 1979). In Drosophila, heat-shock proteins are not tissue specific (Tissieres et al., 1974; Lewis et al., 1975). However, some charge heterogeneity and size polymorphism in some of the proteins has been reported. This may be due to post-translational protein modifications and/or aberrant transcription at elevated temperatures (Sondermeijei and Lubsen, 1978; Mirault et al., 1978).

Synthesis of all eight hsp's starts within 10 mins following an increase in the temperature of Drosophila melanogaster and maximum synthesis is reached about 1 hr later by which time hsp's account for about 50% of total precursor incorporation. The rates of hsp synthesis then declines so that by 3 hr (at elevated temperature) they are half maximal. If the cells are transferred back to 25^{o}C, the rate of hsp synthesis declines much faster (Ashburner and Bonner, 1979). With continuous high temperature treatment the hsp's accumulate since they have long half-lives, so that by 6-8 hrs they comprise some 10% of the cell's total proteins (Moran et al., 1978).

HeLa cells, unlike Drosophila, can only be subjected to 45°C for a very short period (5-10 mins) without suffering serious damage, even though they can be subjected to 41°C-42°C for long periods (Slater et al., 1981). While HeLa cells kept at normal growth medium at 45°C for 1 hr do not show any gross morphological changes under light microscope, it has been shown that their viability is seriously affected (Slater et al., 1981). Even treatment at 45°C for 20 mins results in a 9% loss in viability of the cells (Slater et al., 1981).

Unlike the situation in Drosophila, heat-shock treatment of HeLa cells does not appear to affect normal protein synthesis (see Figure 15). All the proteins synthesised in normal cells are also synthesised in heat-shocked cells, while the production of some proteins (heat-shock specific proteins) is substantially increased. Similar results have been reported for chicken fibroblasts, baby hamster kidney cells (BHK cells) and mouse L-cells (Kelley and Schlesinger, 1978), while normal protein synthesis has been shown to be affected to some extent in Chinese hamster ovary cells (Bouche et al., 1979) and yeast Saccharomyces cerevisiae (Miller et al., 1979).

There are three heat-shock induced protein bands in HeLa cells which have molecular weights of 100,000, 72,000 - 74,000 and 37,000 daltons (see Figure 15). The 72,000 - 74,000 daltons protein is the major hsp in HeLa cells (see Figure 15). Despite the contradicting results concerning the molecular weights of hsp's in various cell lines it seems that the major hsp in chicken embryo fibroblasts, mouse L-cells and baby hamster kidney cells (Kelley and Schlesinger, 1978) are also protein bands of molecular weights around 70,000.

Two-dimensional analysis of the proteins revealed that the 100,000 and 37,000 dalton protein bands comprise of two polypeptides each (Figure 16B). Since only one of them in each case is detected amongst

the in vitro translation products of mRNA from heat-shocked cells (Figures 28B and 29B), it is possible that one of the two results from post-translational modification. It is of interest that the in vivo labelled 37,000 polypeptides (Figure 16B) are much more basic than the polypeptide believed to result from in vitro translation of the 37,000 coding mRNA (Figures 28B and 29B). This could be due to post translational elimination of negative charges as it happens upon methylation of carboxyl groups. That some of the heat-shock proteins in avian cells are methylated has, indeed, been reported (Wang et al., 1981). Similar results have been reported for the 89,000 and 27,000 proteins which are induced in chicken fibroblasts by sodium arsenite. These proteins are believed to be identical to the hsp's in the same cells (Johnston et al., 1980) and it has been shown that each of them is resolved into two polypeptides. In the case of the 89,000 protein only one of the two polypeptides is detected amongst the in vitro translation products of mRNA (Johnston et al., 1980).

The 72,000 - 74,000 daltons hsp in HeLa cells comprises of two polypeptide groups of different molecular weights and different isoelectric points (Figure 16B). The more basic one, of molecular weight 74,000 comprises of two polypeptides and has isoelectric point around 7.3 (Figure 16B, polypeptides indicated by arrows near the brackets). These two polypeptides are also detected amongst the in vitro translation products of mRNA from heat-shocked HeLa cells (Figure 28B, polypeptides α, α'). The second group, of molecular weight 72,000 comprises of several polypeptides whose isoelectric points range from 7.1 - 6.4 (Figure 16B, polypeptides in brackets). Since five of them are detected amongst the in vitro translation products of mRNA from heat-shocked cells (Figures 28B and 29B, polypeptides β, γ, δ, ε and ζ), it is possible that the rest of them result from post-translational

modifications. The 70,000 dalton protein induced in BHK cells by arsenite (which is believed to be identical to the 70,000 hsp in the same cells) has also been resolved into two polypeptide groups of different isoelectric points and different molecular weights (Wang et al., 1981). The isoelectric points of the two protein groups are, however, quite different (5.95 and 5.7) from those we have estimated for the two corresponding groups in HeLa cells. Similarly, the 70,000 daltons heat-shock protein in Drosophila cells has also been resolved into two components (Storti et al., 1980). In chicken fibroblasts the arsenite induced 73,000 dalton protein has been resolved by two-dimensional electrophoretic analysis into two groups of different isoelectric points but identical molecular weights (Johnston et al., 1980). Data from partial peptide mapping of the acidic group of the "70,000" proteins from avian and mammalian cells have suggested that group is conserved in different species (Wang et al., 1981). Comparative analysis of the two forms of the "70,000" protein in chicken fibroblasts and BHK cells by one-dimensional peptide "mapping" revealed homologies and differences, suggesting that these two groups may be evolutionally related (Wang et al., 1981). That the two 72,000 - 74,000 groups are not products of the same genes in HeLa cells, is proposed by the fact that both groups arise from in vitro translation of mRNA (Figures 28B and 29B).

The heat-shock proteins are also found in normal HeLa cells but at much lower amounts (Figure 16). Even though the 37,000 daltons hsp band was not detected in one-dimensional analysis of proteins from normal HeLa cells (Figure 15), two-dimensional analysis showed that the two 37,000 polypeptides existed in normal cells but at very low amounts (Figure 16A). Maximum synthesis of hsp's occurs 2 hrs after the heat-shock treatment in HeLa cells and while the cells are recovering at 37^{o}C

(Figure 17). After that hsp synthesis declines, reaching the normal levels after 4 hrs (Figure 17). However, even though HeLa cells have lost their ability for elevated synthesis of heat-shock proteins by 4 hrs, the proteins synthesised in response to heat-shock are metabolically stable (Slater et al., 1981). It is known for some time now, that a variety of reagents such as uncouplers of oxidative phosphorylation, inhibitors of electron transport, hydrogen acceptors and inhibitors of various enzymes and various other functions induce, in Drosophila, a set of puffs identical to that induced by heat-shock (Ashburner and Bonner, 1979). It has recently been reported that several transition series metals (copper, cadmium, zinc and mercury), the sulphydryl reagent sodium arsenite and diamide enhance the synthesis of specific proteins in chick embryo cells and human foreskin cells in culture (Levinson et al., 1980). Partial peptide mapping has shown that these proteins are similar, if not identical, to the proteins induced by heat-shock in the same cells (Levinson et al., 1980). Proteins of molecular weights similar to those of the heat-shock proteins are also induced in chicken embryo cells when treated with amino acid analogues (Kelley and Schlesinger, 1978; Levinson et al., 1980).

A question arising is by which mechanism such a variety of agents induces the synthesis of the same set of proteins since it is known that in most cases transcription of the corresponding genes is involved (see below). Since many of the inducing agents appear to have as targets mitochondrial electron transport and oxidative phosphorylation, it is not evident how the effects of these inducers can be transmitted to the genome. Data showing that supernatant from isolated heat-shocked mitochondria can induce heat-shock puffs when injected into the cytoplasm of salivary gland cells (Sin, 1975), have proposed that inducers may bring about a change in the conformation of the subcellular

compartmentalisation of specific pre-existing molecules (possibly proteins), which then induce transcription of heat-shock genes (Ashburner and Bonner, 1979). It should be mentioned, however, that a number of inhibitors of electron transport and oxidative phosphorylation do not induce heat-shock proteins in HeLa cells (Burdon et al., 1982).

Since the transition metals and arsenite are known to bind to sulfhydryl groups, it has also been proposed that the repressor for the sequences coding for the induced proteins is a sulfhydryl containing molecule which is inactivated by the binding of the transition metals and arsenite (Levinson et al., 1980).

Since diamide, a reagent known to cause oxidation of glutathione (Kosower et al., 1969), has been reported to induce hsp's and given that addition of oxidised, glutathione in rabbit reticulocytes results in phosphorylation of eIF-2, it is possible that oxidised glutathione may phosphorylate the repressor of the heat-shock genes, thus inactivating it. Heat shock treatment of rabbit reticulocytes has been shown to result in phosphorylation of eIF-2(Bonanou-Tzedaki et al., 1981), thus raising the possibility that heat shock may induce transcription of the heat-shock genesin the same way.

3. Intracellular localisation and possible function of the heat-shock proteins

To determine what is the role of the heat-shock proteins, it is important to know where these proteins migrate once they are synthesised. Cytological studies have shown that in Drosophila melanogaster a major part (about 80%) of the small molecular weight hsp's and about 30% of the 68,000 and 70,000 proteins migrate to the nucleus where they are found associated in chromatin and nucleoli preparations, while the rest of the 68,000 and 70,000 hsp's with most of the 83,000 dalton protein are found in the cytoplasm (Arrigo et al., 1980). These results have

been confirmed by cell fractionation data (Velazquez et al., 1980) and it has been recently reported that nuclear hsp's of Drosophila melanogaster are not found significantly associated with DNA or histones (Sinibaldi and Morris, 1981). It has even been proposed that Drosophila melanogaster nuclear hsp's become components of the nuclear scaffold and that their function may be stoichiometric rather than catalytic (Sinibaldi and Morris, 1981). This suggestion is further supported by a recent data suggesting that the major hsp of chicken fibroblasts may be component of chicken skeletal myofibrils (Wang et al., 1981).

Results from our laboratory have shown that 30% of the 72,000 - 74,000 dalton protein are found in the nucleus of heat-shocked HeLa cells (Burdon et al., 1982). Data from protein "blotting" experiments have suggested that these nuclear hsp's are not DNA binding proteins (Burdon et al., 1982). Even though one can not exclude a possible role of these proteins in transcriptional regulation, it may be that HeLa cell 72,000 - 74,000 heat-shock protein is part of the cytoskeleton. This hypothesis is supported by a recent report which claims that the 66,000 - 68,000 dalton hsp of HeLa cells copurifies with HeLa microtubules (Wang et al., 1981). This 66,000 - 68,000 dalton hsp seems to correspond to our 72,000 - 74,000 dalton hsp.

None of the hsp's found in the cytoplasm of heat-shocked Drosophila melanogaster seem to be associated with mitochondria (Arrigo et al., 1980; Sinibaldi and Morris, 1981). A similar conclusion has also been drawn for HeLa hsp's located in the cytoplasm (Burdon et al., 1982). So, even though it had been postulated that heat-shock proteins may function in cellular respiration (Ashburner and Bonner, 1979), this seems rather unlikely on the basis of intracellular distribution of heat-shock proteins and on data which show that several inhibitors of electron transport and oxidative phosphorylation (e.g. sodium azide, KCN, atractyloside, dinitrophenol and sodium arsenate) fail to induce heat-

shock proteins in HeLa cells (Burdon et al., 1982).

It has also been suggested (Kelley and Schlesinger, 1978) that hsp's may be proteins of the cell membrane and possibly involved in hexose transport. Even though the 83,000 dalton hsp of Drosophila melanogaster may be of membranous origin (Sinibaldi and Morris, 1981), analysis of plasma membrane fractions from heat-shocked HeLa cells did not reveal any enrichment with heat-shock proteins (Burdon et al., 1982). Other lines of evidence also suggest that hsp's are unlikely to be associated with the membrane. Studies on the in vitro translation of heat-shock protein mRNAs indicate that HeLa hsp's are not initially synthesised as larger molecular weight precursors (Figure 19). Also, treatment of HeLa cells with tunicamycin has revealed no effect on the electrophoretic mobility of hsp's, indicating no extensive modification with carbohydrate side chains (Burdon et al., 1982). Even though a role of hsp's in hexose metabolism is possible, treatment of HeLa cells with 2-deoxyglucose or sodium fluoride does not induce heat-shock proteins (Burdon et al., 1982).

A number of other possible functions have been suggested for the heat-shock proteins by various investigators. The synthesis of heat-shock proteins has been correlated with the acquisition of heat resistance in yeast (McAlister and Filkelstein, 1979) and the development of thermotolerance in human cells (Burdon et al., 1982). Data showing that prior heat treatment of Drosophila cells at 35^{o}C affects the time of recovery of both heat-shock protein synthesis and normal protein synthesis after a second heat-shock treatment have also suggested that hsp's may be involved in the regulation of protein synthesis following heat-shock (Petersen and Mitchell, 1981). It has also been suggested that the heat-shock proteins in HeLa cells may be involved in the in vivo modulation of $Na^{+}K^{+}$ ATPase activity (Burdon and Cutmore, 1982).

4. RNA synthesis in heat-shocked cells

4.1. Transcriptional control in heat-shocked cells

At the level of RNA synthesis, there are at least three distinct responses of Drosophila tissues to heat-shock: the induction of the synthesis of a class of heat-shock RNAs some of which, although not all, are translated into hsp's; the suppression of the synthesis of most other messages, although not those coding for the histones or those coded by mitochondrial genome; and the disruption of the normal processing of the primary transcription products of the 5S and 18S + 28S ribosomal RNA cistrons (Ellgaard and Clever, 1971; Lengyel and Pardue, 1975; Rubin and Hogness, 1975; Jacq et al., 1977).

The changes in RNA synthesis resulting from heat-shock have been shown in several ways: Autoradiography of the polytene chromosomes after incorporation of [^{3}H]-uridine into RNAs shows that the heat-shock puffs are newly induced sites of RNA synthesis and that as a consequence of the heat-shock pre-existing puffs cease to incorporate precursors (Ritossa, 1964; Berendes, 1968; Tissieres et al., 1974; Belayena and Zhimulev, 1976; Bonner and Pardue, 1976). The changing pattern of transcription resulting from heat-shock is also reflected in a change in the distribution of RNA polymerase II and other chromosomal proteins detected immunochemically. A marked migration of these components into the heat-shock puff sites has been reported (Holt, 1970; Plagens et al., 1976; Silver and Elgin, 1977; Jamrich et al., 1977a; Elgin et al., 1978; Spruill et al., 1978). Heat-shock has also been shown to result in the depletion of RNA polymerase II from non-heat-shock puff chromosomal regions (Greenleaf et al., 1978) and an increase in the level of fluorescent staining for this enzyme in the nucleoplasm (Jamrich et al., 1977b). The accumulation of specific heat-shock RNA sequences at the heat-shock

puff sites in Drosophila has been demonstrated by cytological hybridisation of polysomal heat-shock poly(A)$^+$ RNA complementary DNA sequences to the puff sites (Livak et al., 1978).

Labelling experiments have shown that in HeLa cells kept at 43^{o}C tRNA and 5S RNA synthesis remains unaffected, hnRNA and mRNA are still produced but at reduced rates, while rRNA synthesis is totally inhibited (Zieve et al., 1977). Our labelling experiments have shown that during the first 2 hrs of recovery following a 5 mins at 45^{o}C heat-shock treatment, HeLa cells synthesise slightly decreased amounts of poly(A)$^+$ RNA as compared to HeLa cells not subjected to heat-shock. There is also a slight decrease in the synthesis of poly(A)$^-$ RNA which under our labelling conditions (in the presence of low amounts of actinomycin D) includes poly(A)$^-$ mRNA species, and possibly tRNA and 5S RNA (see Table 8).

That the appearance of heat-shock proteins in HeLa cells 2 hrs after the heat-shock might be due, as in the case of Drosophila, to some type of transcriptional control was initially suggested by experiments with actinomycin D (see Figure 18). Further evidence was obtained from the in vitro translation experiments which revealed that the mRNAs coding for the heat-shock polypeptides α, α', β and δ can only be detected by in vitro translation of mRNA isolated 2 hrs after the heat-shock (Figures 28B and 29B). Also, the increased amounts of hsp γ synthesised by in vitro translation of mRNA isolated 2 hrs after the heat-shock treatment suggested that increase in the concentration of the corresponding mRNA may have taken place during the 2 hrs recovery period (Figures 28A, B and 29A, B). More evidence has been obtained from "Northern" blotting experiments which showed that 1-2 hrs after the heat-shock treatment high molecular weight nuclear RNA hybridising to DNA from recombinants pHS2 and pHS6 (see Results, Section 7) accumulate in the nucleus (Burdon, 1982).

In vitro translation experiments have also revealed that some polypeptides encoded by mRNA from normal HeLa cells are not detected amongst the translation products of mRNA from heat-shocked cells (see Figures 28 and 29). These mRNAs may have been degraded as a result of the heat-shock, but a question arising is whether their degradation is coupled by reduction or even "shut-off" of their synthesis. To answer this, however, specific probes would be required.

4.2. Heat-shock specific RNAs

Sucrose gradient analysis of poly(A)$^+$ polysomal RNA from heat-shocked Drosophila cells has revealed that heat-shock induced RNA fractionates into two major size classes, one at 20S and the other at 12S (McKenzie and Meselson, 1977; Spradling et al., 1977; Mirault et al., 1978; Moran et al., 1978). Further fractionation of heat-shock polysomal poly(A)$^+$ RNA by electrophoresis under denaturing conditions revealed four species of 20S RNA (Spradling et al., 1977). The largest one, (A_1) has been shown to hybridise in situ to puff 63BC, the second largest (A_2) to both puffs 87A and 87C and the smallest (A_3) to puff 95D (Ashburner and Bonner, 1979). The minor species (A_4) has been shown to hybridise preferentially to 87C (Livak et al., 1978). The development of cloned DNA sequences complementary to the various loci and the use of hybrid-arrested translation technique has allowed the correlation of particular puffs with particular hsp's. So, it is established now that 63BC puff codes for 83,000 hsp and it seems that there is only hsp 83,000 gene at this locus (Holmgren et al., 1979; O'Connor and Lis, 1981). Similarly, 87A and 87C loci in Drosophila melanogaster code for hsp 70,000 (Schedl et al., 1978; Livak et al., 1978). The total number of hsp 70 genes in Drosophila varies from five to about eight according to the genotype, there being two copies at locus 87A7 and three to five copies at 87C1 (Ish-Horowicz et al., 1979b; Mirault et al., 1979; Holmgren et al., 1979). The gene for hsp 68,000 is at locus 95D (Ashburner and Bonner, 1979),

while the genes for hsp's 27,000, 26,000, 23,000 and 22,000 are located at 67B locus of Drosophila melanogaster (Wadsworth et al., 1980; Craig and McCarthy, 1980). (A_4) RNA species, however, has not been shown to code for any particular protein (Livak et al., 1978). It has also been shown that Drosophila melanogaster heat-shock puff site 93D hybridises strongly to cytoplasmic poly(A)$^-$ RNA, whose function is not established yet (Lengyel et al., 1980).

Our in vitro translation experiments suggest that there is probably only one mRNA species coding for each of the 100,000 and 37,000 heat-shock protein in HeLa cells (Kioussis et al., 1981; also see Figures 28 and 29). There is also evidence that the 100,000 hsp is primarily encoded by poly(A)$^+$ RNA (see Figure 19), whose sedimentation range estimated on non-denaturing sucrose gradient seems to be around 18S-21S (see Figure 22). The 72,000 - 74,000 dalton hsp is encoded by seven mRNA species which have been shown to exist in both poly(A)$^+$ and poly(A)$^-$ forms (Kioussis et al., 1981). Sucrose gradient analysis revealed a wide sedimentation range (18S-28S) for the mRNAs coding for the 72,000 - 74,000 hsp's (see Figure 22). Since this broad sedimentation profile could be a consequence of secondary structures adopted by these RNAs during centrifugation in non-denaturing gradients or of different sizes of the seven mRNAs coding for the individual proteins of the 72,000 - 74,000 dalton group, Burdon and colleagues (1982) made use of the cDNA clones (see Results, Section 7) to establish the size of four particular mRNAs. Using the "Northern" blotting technique they showed that the poly(A)$^+$ mRNA coding for the γ polypeptide is about 6,300 bases long, while the mRNAs coding for polypeptides β, δ and ε are around 1,900 nucleotides long which is the range expected of an mRNA coding for a protein 72,000 - 74,000 daltons.

The large size of the mRNA coding for the γ polypeptide could be due to the presence of rather long 3'- and 5'- untranslated regions. Long 3'-untranslated regions have indeed been detected in the SV40 small t antigen (Fiers et al., 1978) and chicken ovalbumin mRNA (O'Hare et al., 1979). Whilst there is nothing unusual about the 3'-untranslated regions of the Drosophila heat-shock mRNAs, the 5'untranslated regions of small heat-shock RNAs encoded by the 67B Drosophila locus range from 111 to 253 bases (Ingolia and Craig, 1981), while the 5' non-coding region for the 70,000 gene is 244 bases long (Ingolia et al., 1980). So, it seems that in general, the 5'-non-coding regions of Drosophila heat-shock genes are much longer than other known genes (Ingolia and Craig, 1981). Since the 5'-noncoding regions of mRNAs may be involved in the initiation of translation (see Introduction), it is possible that differences in the length of the 5'-untranslated regions may result in differential translation-initiation rates. Two different tissue-specific mouse α-amylase mRNAs differing in the 5'-noncoding regions have been shown to originate from the same gene and it has been proposed that differences in the length of the 5'-noncoding region may influence the mRNA stability in the two tissues they are expressed (Young et al., 1981).

In chick embryo fibroblasts, however, there is a small heat-shock protein (hsp 22,000) which is coded for by a large mRNA and it has been shown by immunoprecipitation studies that this protein is synthesised initially as a higher molecular weight precursor of about two times the size of the matured protein (Kelley et al., 1980). Although there is no similar hsp in HeLa cells, the possibility that the γ polypeptide, unlike the other 72,000 - 74,000 dalton polypeptides arises from a larger precursor can not be ruled out. However, the in vivo labelling (see Figure 16) and in vitro mRNA translation data (see Figures 28 and 29) makes it seem unlikely.

As it has already been mentioned, the possible precursors of the β, γ, δ and ε mRNAs accumulate in the nucleus 1-2 hrs after the heat-shock (Burdon, 1982). Northern blotting experiments showed that cDNA sequences from recombinant pHS2 (which contains insert from γ-coding mRNA, see Results, Section 7) hybridises to nuclear RNA 15,800 and 6,300 bases long (Burdon, 1982.). At the same time cDNA sequences from recombinants pHS3 and pHS6 (which contain inserts from β, δ and ε mRNAs) hybridised to nuclear RNA 15,800, 2,500 and 1,900 bases long (Burdon, 1982). A question arising is whether β, γ, δ and ε polypeptide coding mRNAs arise from the same precursor. It is also not known whether the 2,500 bases long nuclear RNA is a processing intermediate. To answer these questions, the isolation of the suitable genomic sequences would be helpful.

5. Translational control in heat-shocked cells

One rapid response of Drosophila cells to heat-shock treatment is the breakdown of pre-existing polysomes (Lindquist-McKenzie et al., 1975). A different population of polysomes reappears later on with at least twice as many ribosomes attached per message (Lindquist-McKenzie et al., 1975; Ashburner and Bonner, 1979). Even though there is a "shut-off" of normal protein synthesis in Drosophila cells kept at elevated temperatures (Tissieres et al., 1974; Lewis et al., 1975; Koninkx et al., 1976), pre-existing messages are not degraded and can be detected in the cytoplasm by in vitro translation (Mirault et al., 1978; Storti et al., 1980). It has recently been shown by in vitro translation in rabbit reticulocytes that normal messages are found associated with polysomes in heat-shocked Drosophila cells (Kruger and Benecke, 1981). However, in vitro translation of polysomal mRNA in a cell-free system prepared from heat-shocked Drosophila cells showed that normal messages even though associated with polysomes, are poorly translated in this lysate (Kruger and Benecke, 1981).

In HeLa cells, heat-shock also results in breakdown of polysomes (see Figure 30B) and even though the cells are then transferred at 37°C (normal culture temperature), it takes 1-2 hrs to have normal polysomal profile restored (see Figure 30). While in Drosophila cells kept at elevated temperatures normal polysomal profile is not obtained in the absence of heat-shock mRNA synthesis, transfer of the cells to normal temperature results in restoration of normal polysomal profile even at the absence of new transcription (Storti et al., 1980). Heat-shock has also been shown to result in polysomes breakdown in L-cells (Schochetman and Perry, 1972) and soybean (Key et al., 1981). In higher plants, in particular, polysomal profile is restored 4 hrs after the start of heat-shock and while the cells are kept at elevated temperature (Key et al., 1981). It is not known why during the first hour of HeLa cells recovery at 37°C the new polysomes appear with only a few ribosomes attached per message. It could be that the heat-shock has some reversible effect on the translation apparatus which is overcome 1-2 hrs after the heat-shock. For example, heat-treatment of rabbit reticulocyte lysates has been shown to activate a kinase which appears to inactivate the small subunit of the initiation factor eIF-2 by phosphorylation (Bonanou-Tzedaki et al., 1981).

Unlike the situation in Drosophila cells where there is discrimination in favour of heat-shock mRNAs (Storti et al., 1980; Kruger and Benecke, 1981; Lindquist, 1981), a similar phenomenon is not suggested by in vivo labelling of proteins in HeLa cells (see Figure 16), which has shown that all messages are translated together with the heat-shock mRNAs. Even though there is no indication of some type of translational control during the 4 first hours of recovery, our results suggest that this may happen 6 hrs after the heat-shock. Even though there is not increased in vivo synthesis of hsp's 6 hrs after the heat-

shock, increased amounts of 72,000 - 74,000 hsp coding mRNAs can be detected in the cytoplasm by *in vitro* translation in rabbit reticulocytes (see Figure 32). Some of these mRNAs are found in both polysomal and post-polysomal fractions with a slight enrichment in the post-polysomal fraction (see Figures 36B and 37B). *In vitro* translation in a cell-free system prepared from HeLa cells 2 hrs and 6 hrs after the heat-shock would be necessary to prove that there is translational control at this stage. Unfortunately, attempts to prepare cell-free translation system from HeLa cells have been unsuccessful. It would also be interesting to find out how these mRNAs are prevented from being translated 6 hrs after the heat-shock. It could be that proteins feed back to polysomes, thus preventing translation of the corresponding mRNAs.

It should also be mentioned that full recovery from heat-shock does not seem to involve in general, any mobilisation of mRNAs from the polysomal to the post-polysomal fraction (see Figures 36 and 37). Only one mRNA species seems to be transferred from the post-polysomal fraction where it was 2 hrs after the heat-shock to the polysomal fraction 6 hrs after the heat-shock. Whether this is of any importance is not known. It should, however, be confirmed by *in vitro* translation in cell-free extracts from HeLa cells.

Translational control has been also suggested to operate in heat-shocked plant cells (Key *et al.*, 1981). In these cells, however, there is evidence that heat-shock mRNAs are degraded once they stop being translated (Key *et al.*, 1981).

Even though translational control seems to operate at some stage in some heat-shocked cells, it is not fully understood which factor(s) is responsible for this. Translational control in favour of heat-shock mRNAs may be due to the fact that heat-shock mRNAs exhibit high affinity for ribosomes. This could result from structural or even the primary sequence of the heat-shock mRNAs. For example, it may be of importance that the 5' end of both the 70,000 hsp's

and histones in Drosophila are so A-rich compared to other mRNA molecules (Ingolia and Craig, 1981). Messenger RNAs with high affinity for ribosomes would not be affected by a partial depletion of the translational machinery (such as inactivation of eIF-2 by phosphorylation), while other mRNAs would be affected and this could explain the high initiation rates of Drosophila heat-shock mRNAs reported by Lindquist (1980).

Because the ability to discriminate against the normal mRNA population in Drosophila is not transferable to 25°C cell-free lysates by particle-free cytoplasm from heat-shocked cells, it has been proposed that the responsible factor may be associated with ribosomes (Kruger and Benecke, 1981). Scott and Pardue (1981) have, indeed, succeeded in rescuing the translation of normal messages in 37°C lysates by the addition of a crude ribosome fraction from a 25°C lysate, and Glover (1982) has very recently reported a difference between the ribosomes of control and heat-shocked Drosophila cells. One ribosomal protein (possibly the ribosomal protein S6) is heavily phosphorylated in Drosophila cells grown at normal temperatures but it is completely dephosphorylated in heat-shocked cells (Glover, 1982).

It should be mentioned, however, that translational control is not the only way of promoting translation of heat-shock proteins. For example, in yeast this is achieved by degradation of pre-existing mRNAs (Lindquist, 1981). Our in vitro experiments showed that some of the mRNAs found in normal HeLa cells are not detected in heat-shock HeLa cells (see Figures 28B and 29B). However, this is more likely to be due to degradation of the corresponding mRNAs during the isolation procedure, especially since no inhibition of specific protein synthesis has been shown in HeLa cells (see Figure 16).

REFERENCES

Abelson, J. (1979). Ann. Rev. Biochem. 48, 1035-1069.

Adams, J.W., Kaufman, R.E., Kretschner, P.J., Harrison, M. and Nienhuis, A-W. (1980). Nucl. Acid. Res. 8, 6113-6128.

Alestrom, P., Akusjarri, G., Perricaudet, M., Mathews, M.B., Kleissig, D.F. and Petterson, U. (1980). Cell 19, 671-681.

Alwine, J.c., Kemp, D.J. and Stark, G.R. (1977). Proc. Natl. Acad. Sci. U.S.A. 74, 5350-5354.

Angerer, R.C., Davidson, E.H. and Britten, R.J. (1975). Cell 6, 141-155.

Arrigo, P-A., Fakan, S. and Tissieres, A. (1980). Dev. Biol. 78, 86-103.

Artavanis-Tsakonas, S., Schedl, P., Mirault, M-E. and Moran, L. (1979). Cell, 9-18.

Ashburner, M. (1970). Chromosoma 31, 356-376.

Ashburner, M. and Bonner, J.J. (1979). Cell 17, 241-254.

Aviv, H., Volock, Z., Bastas, R. and Levy, S. (1976). Cell 8, 495-503.

Avvedimento, V.E., Vogeli, G., Yamada, Y., Maizel, J.V. Jr., Pastan, I. and de Crombrugghe, B. (1980). Cell 21, 689-696.

Axel, R., Feigelson, P. and Schutz, G. (1976). Cell 7, 247-254.

Bachellerie, J.P., Amalric, F. and Caboche, M. (1978). Nucl. Acid. Res. 5, 2927-2943.

Baer, B.W. and Kornberg, R. (1980). Proc. Natl. Acad. Sci. U.S.A. 77, 1890-1892.

Baker, C.C., Herisse, J., Courtois, G., Galibert, F. and Ziff, E. (1979). Cell 18, 569-580.

Banerji, J., Rusconi, S. and Schaffner, W. (1981). Cell 27, 299-308.

Bantle, J.A. and Hahn, W. (1976). Cell 8, 139-150.

Baralle, F.E. (1977). Cell 10, 549-558.

Baralle, F.E. and Brownlee, G.G. (1978). Nature 274, 84-87.

Barrieux, A., Ingraham, H.A., David, D.N. and Rosenfeld, M. (1975). Biochemistry 14, 1815-1820.

Barrieux, A. and Rosenfeld, M.A. (1977). J. Biol. Chem. 252, 392-398.

Barrieux, A. and Rosenfeld, M.A. (1978). J. Biol. Chem. 253, 6311-6314.

Bastos, R.N. and Aviv, H. (1977). Cell 11, 641-650.

Bastos, R.N., Voloch, Z. and Aviv, H. (1977). J. Mol. Biol. 110, 191-203.
Belayeva, E.S. and Zhimulev, I.F. (1976). Cell Differentiation 4, 415-427.

Bell, G.I., Pictet, R.L., Rutter, W.J., Cordell, B., Tischer, E. and Goodman, H.M. (1980). Nature 284, 26-32.

Beltz, G.A. and Flint, S.J. (1979). J. Mol. Biol. 131, 353-373.

Benoff, S. and Nadal-Ginard, B. (1979). Proc. Natl. Acad. Sci. U.S.A. 76, 1853-1857.

Benoist, C., O'Hare, K., Breathnach, R. and Chambon, P. (1980). Nucl. Acid Res. 8, 127-142.

Benoist, C. and Chambon, P. (1981). Nature 290, 304-310.

Berendes, H.D. (1968). Chromosoma 24, 418-437.

Berger, S.L. and Cooper, ;H.L. (1975). Proc. Natl. Acad. Sci. U.S.A. 72, 3873-3877.

Berget, S.M. and Sharp, P.A. (1979). J. Mol. Biol. 129, 547-565.

Berns, A.J.M., Van Kraaikamp, M., Bloemendal, H. and Lane, C.D. (1972). Proc. Natl. Acad. Sci. U.S.A. 69, 1606-1609.

Bester, A.J., Kennedy, D.S. and Heywood, S.M. (1975). Proc. Natl. Acad. Sci. U.S.A. 72, 1523-1527.

Biessmann, H., Wadswort, S., Levy-W.B. and McCarthy, B.J. (1977). Cold Spring Harbor Symp. Quant. Biol. XLII, 829-834.

Biessmann, H., Levy, B.W. and McCarthy, B.J. (1978). Proc. Natl. Acad. Sci. U.S.A. 75, 759-763.

Bina, M., Feldmann, R.J. and Deeley, R.G. (1980). Proc. Natl. Acad. Sci. U.S.A. 77, 1278-1282.

Birnie, G.D., MacPhail, E., Young, B.D., Getz, M.J. and Paul, J. (1974). Cell Differentiation 3, 221-232.

Bishop, J.O., Rosbash, M. and Evans, D. (1974). J. Mol. Biol. 85, 75-86.

Bitter, G.A. and Roeder, R.G. (1978). Biochemistry 17, 2198-2205.

Blanchard, J-M., Weber, J., Jelinek, W. and Darnell, J.E. (1978). Proc. Natl. Acad. Sci. U.S.A. 75, 5344-5348.

Blobel, G. (1973. Proc. Natl. Acad. Sci. U.S.A. 70, 924-928.

Bloom, K.S. and Anderson, J.N. (1978). Cell 15, 141-150.

Bogdanovsky, D., Hermann, N. and Shapira, G. (1973). Biochem. Biophys. Res. Commun. 54, 25-29.

Borun,T.W.,Scharff,M. and Robbins,E.(1967).Proc. Natl. Acad. Sci. U.S.A.,58,1977-2019.

Bolivar, F., Rodriguez, R., Greene, P., Betlach, M., Heynecker, H. and Boyer, H.W. (1977). Gene 2, 95-113.

Bonanou-Tzedaki, S.A., Sohi, M., and Arnstein, H.R.V. (1981). Eur. J. Biochem. 114, 69-77.

Bonner, W. and Laskey, R.A. (1974). Eur. J. Biochem. 46, 83-88.

Bonner, J.J. and Pardue, M.L. (1976). Cell 8, 43-50.

Borun, T.W., Gabarielli, F., Ajizo, K., Zweidler, A. and Baglioni, C. (1975). Cell 4, 59-67.

Borun, T.W., Ajizo, K., Zweidler, A., Dolby, T.W. and Ralph, E. (1977). J. Biol. Chem. 252, 173-180.

Bouche, G., Almaric, F., Caizergues-Ferrer, M. and Zalta, J.P. (1979). Nucl. Acid Res. 7, 1739-1747.

Brakel, C. and Kates, J.R. (1974). J. Virol. 14, 724-732.

Brandhorst, B.P. and Humphreys, T. (1972). J. Cell Biol. 53, 474-483.

Brandt, C. and Milcarek, C. (1980). Biochemistry 19, 6152-6158.

Breathnach, R., Benoist, C., O'Hare, K., Gannon, F. and Chambon, P. (1978). Proc. Natl. Acad. Sci. U.S.A. 75, 4853-4857.

Breathnach, R. and Chambon, P. (1981). Ann. Rev. Biochem. 50, 349-383.

Britten, R.J. and Kohne, D.E. (1968). Science 165, 349-357.

Buckingham, M.K., Caput, D., Cohen, A., Whalen, R.G. and Gross, F. (1974). Proc. Natl. Acad. Sci. U.S.A. 71, 4467-4471.

Burdon, R.H. (1982). Cold Spring Harbor on Induction of heat shock proteins (in press).

Burdon, R.H. and Cutmore, C.M.M. (1982). FEBS Lett. 140, 45-48.

Burdon, R.H., Shenkin, A., Douglas, J. and Smellie, G. (1976). Biochim. Biophys. Acta 474, 254-267.

Burdon, R.H., Slater, A., McMahon, M. and Cato, A.C.B. (1982). British Journal of Cancer. In press.

Busslinger, M., Portmann, R., Irminger, J.C. and Birnstiel, M.L. (1980). Nucl. Acid. Res. 8, 957-977.

Calabretta, B., Robberson, D.L., Maizel, A.L. and Saunders, G.F. (1981). Proc. Natl. Acad. Sci. U.S.A. 78, 6003-6007.

Calvet, J.P. and Pederson, T. (1978). J. Mol. Biol. 122, 361-378.

Carlock, L. and Jones, N.C. (1981). Nature 294, 572-574.

Caskey, C. Th. (1980). Trends Biochem. Sci., 5, 234-237.

Cato, A.C.B., Sillar, G.M., Kioussis, J. and Burdon, R.H. (1981). Gene 16, 27-34.

Cavalieri, R., Havell, E.A., Vilcek, J. and Pestka, S. (1977). Proc. Natl. Acad. Sci. U.S.A. 74, 4415-4419.

Chambon, P. (1975). Ann. Rev. Biochem. 44, 613-638.

Chantrenne, H., Burny, A. and Marbaix, G. (1967). Prog. Nucl. Acid. Res. Mol. Biol. 7, 173-194.

Cheng, T-C. and Kazazian, H.H. Jr. (1977). J. Biol. Chem. 252, 1758-1763.

Chikaraishi, D.M. (1979). Biochemistry, 18, 3249-3256.

Clarke, L. and Carbon, J. (1976). Cell 9, 91-99.

Clark, B. (1980). Trends Biochem. Sci. 5, 234-237.

Clemens, M.J., Safer, B., Merrick, W.C., Anderson, W.F. and London, I.M. (1975). Proc. Natl. Acad. Sci. U.S.A. 72, 1286-1290.

Cooper, J.A. and Moss, B. (1979). Virology, 96, 368-380.

Corces, V., Pelliger, A., Axel, R. and Meselson, M. (1981). Proc. Natl. Acad. Sci. U.S.A. 78, 7038-7042.

Cordell, B., Bell, G., Tischer, E., DeNoto, F.M., Ullrich, A., Pictet, R., Rutter, W.J. and Goodman, H.M. (1979). Cell 18, 533-543.

Corden, J., Wasylyk, K.B., Buchwalder, A., Sassone-Corsl, P. and Vedinger, L. (1980). Science 209, 1406-1414.

Cozens, P.J., Cato, A.C.B. and Jost, J-P. (1980). Eur. J. Biochem. 112, 443-450.

Craig, E.A., McCarthy, B.J. and Wadsworth, S.C. (1979). Cell 16, 575-588.

Craig, E.A. and McCarthy, B.J. (1980). Nucl. Acid Res. 8, 4441-4457.

Craine, B.L. and Kornberg, T. (1981a). Biochemistry 20, 6584-6588.

Craine, B.L. and Kornberg, T. (1981b). Cell 25, 671-681.

Croall, D.E. and Morrison, M.R. (1980). J. Mol. Biol. 140, 549-564.

Curtis, P.J. and Weissmann, C. (1976). J. Mol. Biol. 106, 1061-1075.

Curtis, P.J., Mantel, N., Van den Berg, J. and Weissmann, C. (1977). Proc. Natl. Acad. Sci. U.S.A. 74, 3184-3188.

Dagert, M. and Ehrlich, S.D. (1979). Gene 6, 23-28.

Darnell, J.E. (1968). Bacteriol. Rev. 32, 262-290.

Darnell, J.E., Philipson, L., Wall, R. and Adesnik, M. (1971). Science 174, 507-510.

Darnell, J.E., Jelinek, W.R. and Molley, G.R. (1973). Science 181, 1215-1221.

Davidson, J.N. (1969). The Biochemistry of the Nucleic Acids. Chapman and Hall.

Davidson, E.R. and Britten, R.J. (1973). Quart. Rev. Biol. 48, 565-613.

Davidson, E.R., Hough, B.R., Amenson, C.S. and Britten, R.J. (1973). J. Mol. Biol. 77, 1-24.

De Haro, C., Datta, A. and Ochoa, S. (1978a). Proc. Natl. Acad. Sci. U.S.A. 75, 243-247.

De Haro, C. and Ochoa, S. (1978b). Proc. Natl. Acad. Sci. U.S.A. 75, 2713-2716.

Delaunay, J.C., Ranu, R.S., Lewin, D.H., Ernst, V. and London, L.M. (1977). Proc. Natl. Acad. Sci. U.S.A. 74, 2264-2269.

Derman, E. and Darnell, J.E. (1974). Cell 3, 255-264.

De Robertis, E.M. and Mertz, J.E. (1977). Cell 12, 175-182.

Desphande, A.K., Chatterjee, B. and Roy, A.K. (1979). J. Biol. Chem. 254, 8937-8942.

Dickson, R.C., Abelson, J., Barnes, W.M. and Reznikoff, W.S. (1975). Science 187, 27-34.

Diez, J. and Brawerman, G. (1974). Proc. Natl. Acad. Sci. U.S.A. 71, 4091-4095.

Dubroff, L.M. and Nemer, N. (1975). J. Mol. Biol. 95, 455-476.

Dubroff, L.M. (1977). Proc. Natl. Acad. Sci. U.S.A. 74, 2217-2221.

Dugaiczik, A., Woo, S.L.C., Lai, E.C., Mace, M.L., McReynolds, L. and O'Malley, B.W. (1978). Nature 274, 328-333.

Duncan, C., Biro, P.A., Choudary, P.V., Elder, J.T., Wang, R.C., Forget, B.G., De Riel, J.K. and Weissman, S.R. (1979). Proc. Natl. Acad. Sci. U.S.A. 76, 5098-5099.

Early, P., Rogers, J., Davis, M., Calame, K., Bond, M., Wall, R. and Hood, R. (1980). Cell 20, 313-319.

Edmonds, M. and Abrams, R. (1960). J. Biol. Chem. 235, 1142-1149.

Edmonds, M. and Caramela, M.G. (1969). J. Biol. Chem. 244, 1314-1324.

Edmonds, M., Vaughan, M.H. and Nakazato, H. (1971). Proc. Natl. Acad. Sci. U.S.A. 68, 1336-1340.

Efstratiadis, A., Posakony, J.W., Maniatis, T., Lawn, R.M., O'Connell, C., Spritz, R.A., DeRiel, J.K., Forget, B.G., Weissman, S.M., Slightom, J.L., Blechl, A.E., Smithies, O., Baralle, F.E., Shoulders, C.C. and Proudfoot, N.J. (1980). Cell 21, 653-668.

Elgin, S.C.R., Serunian, L.A. and Silver, L.M. (1978). Cold Spring Harbor Symp. Quant. Biol. 42, 839-850.

Ellgaard, E.G. and Clever, U. (1971). Chromosoma 36, 60-78.

Engel, J.N., Cunning, P.W. and Kedes, L. (1981). Proc. Natl. Acad. Sci. U.S.A. 79, 4674-4678.

Fahrner, K., Yarger, J. and Hereford, L. (1980). Nucl. Acid Res. 8, 5725-5737.

Farrell, P.J., Balkow, K., Hunt, T. and Jackson, R.J. (1977). Cell 11, 187-200.

Felsenfeld, G. (1978). Nature 271, 115-122.

Fiers, W., Contreras, R., Haegeman, G., Rogiers, R., van de Voorde, A., van Heuverswyn, H., van Herreweghe, J., Volckaert, G. and Ysebaert, M. (1978). Nature 273, 113-119.

Fillipowicz, E. and Haemis, A-L. (1979). Proc. Natl. Acad. Sci. U.S.A. 76, 3111-3115.

Firtel, R.A. and Kindle, K. (1975). Cell 5, 401-411.

Foe, V.E., Wilkinson, L.E. and Laird, C.D. (1976). Cell 9, 131-146.

Ford, J.P. and Hsu, M-T. (1978). J. Virol. 28, 795-801.

Freedman, M.L., Geraghty, M. and Rosman, J. (1974). J. Biol. Chem. 249, 7290-7294.

Friederici, K., Kaehler, M. and Rotman, F. (1976). Biochemistry 15, 5234-5240.

Fritsch, E.F., Lawn, R.M. and Maniatis, T. (1980). Cell 19, 959-972.

Fromson, D. and Verma, D.P.S. (1976). Proc. Natl. Acad. Sci. U.S.A. 73, 148-151.

Fyrberg, E.A., Kindle, K.L. and Davidson, N. (1980). Cell 19, 365-378.

Gabrielli, F. and Baglioni, C. (1975). Dev. Biol. 43, 254-263.

Gage, L.P. and Manning, R.F. (1976). J. Mol. Biol. 101, 327-348.

Galau, G.A., Britten, R.J. and Davidson, E.H. (1974). Cell 2, 8-20.

Gallwitz, D. (1975). Nature 257, 247-248.

Gander, E.S., Stewart, A.G., Morel, C.M. and Scherrer, K. (1973). Eur. J. Biochem. 38, 443-452.

Gannon, F., O'Hare, K., Perrin, F., Le Pennec, J.P., Benoist, C., Cochet, M., Breathnach, R., Royal, A., Garapin, A., Cami, B. and Chambon, P. (1979). Nature 278, 428-434.

Garapin, A.C., Cami, B., Roskam, W., Kourilsky, P., Le Pennec, J.P., Perrin, F., Getlinger, P., Cochet, M. and Chambon, P. (1978). Cell 14, 629-639.

Garel, A. and Axel, R. (1976). Proc. Natl. Acad. Sci. U.S.A. 73, 3966-3970.

Garel, A., Zolan, M. and Axel, R. (1977). Proc. Natl. Acad. Sci. U.S.A. 74, 4867-4871.

Geller, A.I. and Rich, A. (1980). Nature 283, 41-46.

Gey, G.O., Coffman, W.D. and Kibicek, M.T. (1962). Can. Res. 12, 264-265.

Geoghegan, T.E., Sonenschein, G.E. and Brawerman, G. (1978). Biochemistry 17, 4200-4207.

Gilmore-Herber, M. and Wall, R. (1978). Proc. Natl. Acad. Sci. U.S.A. 75, 342-345.

Gilmore-Herbert, M., Hercules, K., Komaromy, M. and Wall, R. (1978). Proc. Natl. Acad. Sci. U.S.A. 75, 6044-6048.

Gilmore, H. and Wall, R. (1979). J. Mol. Biol. 135, 879-891.

Gilmour, R.S., Harrison, P.R., Windess, J.D., Afforia, N.A. and Paul, J. (1974). Cell. Differ. 3, 9-17.

Goldenberg, S., Vincent, A. and Scherrer, K. (1980). Nucl. Acid Res. 8, 5057-5070.

Goldberg, S., Schwartz, H. and Darnell, J.E. Jr. (1977). Proc. Natl. Acad. Sci. U.S.A. 74, 4520-4523.

Goldschmidt-Clermont, M. (1980). Nucl. Acid Res. 8, 235-252.

Goldstein, J.L., Beaudet, A.L. and Caskey, C.T. (1970). Proc. Natl. Acad. Sci. U.S.A. 67, 699- .

Golini, F., Thach, S.S., Birge, C.H., Safer, B., Merrick, W.C. and Thach, R.E. (1976). Proc. Natl. Acad. Sci. U.S.A. 73, 3040-3044.

Gorski, J., Morrison, M.R., Merkel, C. and Lingrel, J.B. (1974). J. Mol. Biol. 86, 363-371.

Grady, L.J. and Campbell, W.P. (1975). Nature 254, 356-358.

Grady, L.J., North, A.B. and Campbell, W.P. (1978). Nucl. Acid Res. 5, 697-711.

Greenberg, J.R. (1977). J. Mol. Biol. 108, 403-416.

Greenleaf, A.L., Plagens, U., Jamrich, M. and Bautz, E.K.F. (1978). Chromosoma 65, 127-136.

Glover, C.V.C. (1982). Proc. Natl. Acad. Sci. U.S.A. 79, 1781-1785.

Groner, B., Hynes, N. and Philips, T. (1974). Biochemistry, 13, 5378-5383.

Grosschedl, R. and Birnstiel, M.L. (1980). Proc. Natl. Acad. Sci. U.S.A. 77, 1432-1436.

Grosveld, G.C., Schewmaker, C.K., Jat, P. and Flavell, R-A. (1981). Cell 25, 215-226.

Grunstein, M. and Hogness, D.S. (1975). Proc. Natl. Acad. Sci. U.S.A. 72, 3961-3965.

Gruss, P. and Khoury, G. (1980). Nature 286, 634-637.

Guilfoyle, T.J., Lin, C.Y., Chen, Y.M. and Key, J.L. (1976). Biochim. Biophys. Acta 418, 344-357.

Hahn, W.E., Van Ness, J. and Maxwell, I.H. (1980). Nature 283, 601-603.

Hall, B.D. and Spiegelman, S. (1961). Proc. Natl. Acad. Sci. U.S.A. 47, 137-146.

Hamada, H., Igarashi, T. and Murumatsu, M. (1980). Nucl. Acid Res. 8, 587-599.

Hamer, D.H. and Leder, P. (1979). Cell 27, 737-747.

Hamer, D.H. and Sommer, R. (1980). Nature 283, 693-694.

Hames, B.D. and Perry, R.P. (1977). J. Mol. Biol. 109, 437-453.

Harpold, M.M., Dobner, P.R., Evans, R., Bancroft, F.C. and Darnell, J.E. (1979). Proc. Natl. Acad. Sci. U.S.A. 76, 3133-3144.

Harris, S.E., Means, A.R., Mitchell, W.M. and O'Malley, B.W. (1973). Proc. Natl. Acad. Sci. U.S.A. 70, 3776-3780.

Heckle, W.L. Jr., Fenton, R.G., Wood, T.G., Merkel, C.G. and Lingrel, J.B. (1977). J. Biol. Chem. 252, 1764-1770.

Heilig, R., Perrin, F., Gannon, F., Mandel, J.L. and Chambon, P. (1980). Cell 20, 625-637.

Henshaw, E.C., Revel, M. and Kiatt, H.H. (1965). J. Mol. Biol. 14, 241-256.

Hereford, L.M. and Rosbash, M. (1977). Cell 10, 455-462.

Herman, R.C., Williams, J.G. and Penman, S. (1976). Cell 7, 429-438.

Herman, R.C. and Penman, S. (1977). Biochemistry 16, 3460-3465.

Hersh ey, A.D., Dixon, J. and Chase, M. (1953). J. Gen. Physiol. 36, 777-789.

Heywood, S.M., Kennedy, D.S. and Bester, A.J. (1975). Eur. J. Biochem. 58, 587-593.

Hickey, E.D., Weber, L.A. and Baglioni, C. (1976). Proc. Natl. Acad. Sci. U.S.A. 73, 19-23.

Hodgson, C.P., Siegal, G.P. and Getz, M.J. (1979). J. Cell Biol. 83, 189a.

Holley, R.W., Apgar, J. and Merrill, S.H. (1961). J. Biol. Chem. 236, 42-43.

Holmgren, R., Livak, K., Morimoto, R., Freund, R. and Meselson, M. (1979). Cell 18, 1359-1370.

Holt, Th.K.H. (1970). Chromosoma 32, 428-435.

Houghton, M., Jackson, I.J., Porter, A.G., Doel, S.M., Gatlin, G.H., Barber, C. and Carey, N.H. (1981). Nucl. Acid Res. 9, 247-266.

House, W. and Wildy, P. (1965). Lab. Practice 14, 594-595.

Hu, S-L. and Manley, J. (1981). Proc. Natl. Acad. Sci. U.S.A. 78, 820-824.

Huez, G., Marbaix, G., Hubert, E., Leclercq, M., Nudel, U., Soreq, H., Salomon, R., Lelleu, B., Revel, M. and Littauer, V. (1974). Proc. Natl. Acad. Sci. U.S.A. 71, 3143-3146.

Huez, G., Marbaix, G., Hubert, E., Cleuter, Y., Leclercq, M., Chantrenne, H., Devos, R., Soreq, H., Nudel, U. and Littauer, U.Z. (1975). Eur. J. Biochem. 59, 589-592.

Huez, G., Marbaix, G., Gallwitz, D., Weineberg, E., Devos, R., Hubert, E. and Cleuter, Y. (1978). Nature 271, 572-573.

Humphries, P., Old, R., Coggins, L.W., McShane, T., Watson, C. and Paul, J. (1978). Nucl. Acid. Res. 5, 905-924.

Humphries, S., Windass, J. and Williamson, R. (1976). Cell 7, 267-277.

Hunt, T. (1980). Trends Biochem. Sci. 5, 178-181.

Hunter, T. and Garrels, J.I. (1977). Cell 12, 767-781.

Ilan, J. (1973). J. Mol. Biol. 77, 437-448.

Ingolia, T., Craig, E. and McCarthy, B. (1980). Cell 21, 669-679.

Ingolia, T-D. and Craig, E.A. (1981). Nucl. Acid Res. 9, 1627-1642.

Irwin, D., Kumar, A. and Malt, R.A. (1975). Cell 4, 157-166.

Ish-Horowicz, D., Holden, J. and Gehring, W.J. (1977). Cell 12, 643-652.

Ish-Horowicz, D., Pinchin, S.M., Gausz, J., Gyurkovics, H., Bencze, G., Goldschmidt-Clermont, M. and Holden, J.J. (1979a). Cell 17, 565-571.

Ish-Horowicz, D., Pinchin, S.M., Schedl, P., Artavanis-Tsakonas, S. and Mirault, M-E. (1979b). Cell 18, 1351-1358.

Ish-Horowicz, D. and Pinchin, S.M. (1980). J. Mol. Biol. 142, 231-245.

Jacq, B., Jourdan, R. and Jourdan, B.R. (1977). J. Mol. Biol. 117, 785-795.

Jacob, F. and Monod, J. (1961). J. Mol. Biol. 3, 318-356.

Jacob, S.T. (1973). Prog. Nucl. Acid Res. Mol. Biol. 13, 93-126.

Jacob, E., Malacinski, G. and Birnstiel, M.L. (1976). Eur. J. Biochem. 69, 45-54.

Jain, S.K. and Sarkar, S. (1979). Biochemistry 18, 745-753.

James, T.C. and Tata, J.R. (1980). Cell Differentiation. 16, 9-22.

Jamrich, A., Greenleaf, A.L. and Bautz, E.K.F. (1977a). Proc. Natl. Acad. Sci. U.S.A. 74, 2079-2083.

Jamrich, M., Haars, R., Walt, E., and Bautz, E.K.F. (1979b). Chromosoma 64, 319-326.

Jelinek, W. and Darnell, J.E. (1972). Proc. Natl. Acad. Sci. U.S.A. 69, 2537-2541.

Jelinek, W.R., Molloy, G., Fernandez-Munoz, R., Salditt, M. and Darnell, J.E. (1974). J. Mol. Biol. 82, 361-370.

Jelinek, W.R. (1977). J. Mol. Biol. 115, 591-602.

Jelinek, W. and Leinwand, L. (1978). Cell 15, 205-214.

Jelinek, W.R., Toomey, T.P., Leinwand, L., Duncan, C.H., Biro, P.A., Choidary, A.V., Weissman, S.M., Rubin, C.M., Houck, C.M., Deininger, W.F. and Schmid, C.W. (1980). Proc. Natl. Acad. Sci. U.S.A. 77, 1398-1402.

Jendrisak, J.J. and Burgess, R.B. (1977). Biochemistry 16, 1959-1964.

Johnston, D., Oppermann, H., Jackson, J. and Levinson, W. (1980). J. Biol. Chem. 255, 6975-6980.

Kabat, D. and Chappel, M.R. (1977). J. Biol. Chem. 252, 2684-2690.

Kafatos, F.C., Efstratiades, A., Forget, B.G. and Weissman, S.M. (1977). Proc. Natl. Acad. Sci. U.S.A. 74, 5618-5622.

Kates, J. (1970). Cold Spring Harbor Symp. Quant. Biol. 35, 743-752.

Katinakis, P.K. and Burdon, R.H. (1980). FEBS Lett. 116, 1-7.

Katinakis, P.K. and Burdon, R.H. (1981). Biochim. Biophys. Acta 653, 27-38.

Kaufmann, Y., Milcarek, C., Berissi, H. and Penman, S. (1977). Proc. Natl. Acad. Sci. U.S.A. 74, 4801-4805.

Kedes, L.H. (1979). Ann. Rev. Biochem. 48, 837-870.

Keene, M.A., Corces, V., Lowenhaupt, V. and Elgin, S.C.R. (1981). Proc. Natl. Acad. Sci. 78, 143-146.

Kelley, P.M. and Schlesinger, M.J. (1978). Cell 15, 1277-1286.

Kosower, N.S., Kosower, E.N. and Wertheim, B. (1969) Biochem. Biophys. Res. Comm. 37, 593-597.

Kelley, P.M., Aliperti, G. and Schlesinger, M.J. (1980). J. Biol. Chem. 255, 3230-3233.

Kerr, I.M. and Brown, R.E. (1978). Proc. Natl. Acad. Sci. U.S.A. 75, 256-260.

Key, J.L., Lin, C.Y. and Chen, Y.M. (1981). Proc. Natl. Acad. Sci. U.S.A. 78, 3526-3530.

Khoury, G., Gruss, P., Dhar, R. and Lai, C-J. (1979). Cell 18, 85-92.

Kimchi, A., Zilberstein, A., Schmidt, A., Schulman, L. and Revel, M. (1979). J. Biol. Chem. 254, 9846-9853.

Kioussis, J., Cato, A.C.B., Slater, A. and Burdon, R.H. (1981). Nucl. Acid Res. 9, 5203-5214.

Kindle, K.L. and Firtel, R.A. (1978). Cell 15, 763-778.

Kinniburgh, A.J., Mertz, J.E. and Ross, J. (1978). Cell 14, 681-693.

Kinniburgh, A.J. and Ross, J. (1979). Cell 17, 915-921.

Kish, V.M. and Pederson, T. (1976). J. Biol. Chem. 251, 5888-5894.

Kish, V.M. and Pederson, T. (1977). Proc. Natl. Acad. Sci. U.S.A. 74, 1426-1430.

Kleinman, L., Birnie, G.D., Young, B.D. and Paul, J. (1977). Biochemistry 16, 1218-1223.

Konecki, D., Aune, K.C., Tate, W. and Cashey, C.T. (1977). J. Biol. Chem. 252, 4514-4520.

Koninkx, J.F.J.G. (1976). Biochem. J. 158, 623-628.

Korwek, E.L., Nakazato, H., Venkatesan, S. and Edmonds, M. (1976). Biochemistry 15, 4643-4649.

Kozak, M. (1978). Cell 15, 1109-1123.

Kronenberg, H.M., Roberts, B.E. and Efstratiadis, A. (1979). Nucl. Acid Res. 6, 153-166.

Kruger, C. and Benecke, B-J. (1981). Cell 23, 595-603.

Kumar, A. and Pederson, T. (1975). J. Mol. Biol. 96, 353-366.

Kwan, S-P., Wood, T.G. and Lingrel, J.B. (1977). Proc. Natl. Acad. Sci. U.S.A. 74, 178-182.

Lai, C-J. and Khoury, G. (1979). Proc. Natl. Acad. Sci. U.S.A. 76, 71-75.

Laird, C.D., Wilkinson, L.E., Foe, V.E. and Chooi, W.Y. (1976). Chromosoma 58, 169-192.

Lamb, M.M. and Daneholt, B. (1979). Cell 17, 835-848.

Lane, c.D., Marbaix, G. and Gurdon, J.B. (1971). J. Mol. Biol. 61, 73-91.

Langberg, S.R. and Moss, B. (1981). J. Biol. Chem. 256, 10054-10060.

Le Stourgeon, W. and Beyer, A.L. (1977). Methods Cell Biol. 16, 387-406.

Lengyel, J.A. and Pardue, M.L. (1975). J. Cell Biol. 67, 240a.

Lengyel, J.A. and Penman, S. (1977). Dev. Biol. 57, 243-253.

Lengyel, J.A., Ransom, L.J., Graham, M.L. and Pardue, M.L. (1980). Chromosoma 80, 237-252.

Lerner, M.R., Boyle, J.A., Mount, S.M., Wolin, S.L. and Steitz, J.A. (1980). Nature 283, 220-224.

Levenson, R.G. and Marcu, K. (1976). Cell 9. 311-322.

Levinson, W., Opperman, H. and Jackson, J. (1980). Biochim. biophys. Acta 606, 170-180.

Levy, W.B. and Rizzino, A. (1977). Exp. Cell. Res. 106, 377-380.

Lewin, B. (1975). Cell 4, 77-93.

Lewin, B. (1980). Gene Expression 2. John Wiley and Sons, New York.

Lewis, M., Helmsing, P.J. and Ashburner, M. (1975). Proc. Natl. Acad. Sci. U.S.A. 72, 3604-3608.

Liautard, J.P., Setyono, B., Spindler, E. and Kohler, O. (1976). Biochim. Biophys. Acta 425, 373-383.

Lindquist-McKenzie, S., Henikoff, S. and Meselson, M. (1975). Proc. Natl. Acad. Sci. U.S.A. 72, 1117-1121.

Lindquist, S. (1980). J. Mol. Biol. 137, 151-158.

Lindquist, S. (1981). Nature 293, 311-314.

Lis, J.T., Prestidge, L. and Hogness, D.S. (1978). Celll 14, 901-919.

Little, P.F.R., Flavell, R.A., Kooter, J.M., Annison, G. and Williamson, R. (1979). Nature 278, 227-230.

Livak, K.J., Freund, R., Schweber, M., Wensink, P.C. and Meselson, M. (1978). Proc. Natl. Acad. Sci. U.S.A. 75, 5613-5617.

Lockard, R.E. and Raj Bhandary, U.L. (1976). Cell 9, 747-760.

Lodish, H.F. (1970). J. Mol. Biol. 50, 689-702.

Lodish, H.F. (1971). J. Biol. Chem. 246, 7131-7138.

Lodish, H.F. and Jacobsen, M. (1972). J. Biol. Chem. 247, 3622-3629.

Lodish, H., Jacombson, A., Firtel, R., Alton, T. and Tuchman, J. (1974). Proc. Natl. Acad. Sci. U.S.A. 71, 5103-5108.

Lohr, D. and Ide, G. (1979). Nucl. Acid Res. 6, 1909-1927.

Lomedico, R., Rosenthal, N., Efstratiadis, A., Gilbert, W., Kolodner, R. and Lizard, R. (1979). Cell 18, 345-358.

Lowenhaupt, K. and Lingrel, J.B. (1978). Cell 14, 337-344.

Lowenhaupt, K. and Lingrel, J.B. (1979). Proc. Natl. Acad. Sci. U.S.A. 76, 5173-5177.

Luse, D.S. and Roeder, R.G. (1980). Cell 20, 691-699.

Mandel, J-L., Breathnach, R., Gerlinger, P., Le Meur, M., Gannon, F. and Chambon, P. (1978). Cell 14, 641-653.

Manning, J.E., Schmid, C.W. and Davidson, N. (1975). Cell 4, 141-155.

Marbaix, G. and Lane, C.D. (1972). J. Mol. Biol. 67, 517-524.

Marbaix, G., Huez, G., Burny, A., Cleuter, Y., Hubert, E., Leclercq, M., Chantrenne, H., Soreq, H., Nudel, U. and Littauer, U. (1975). Proc. Natl. Acad. Sci. U.S.A. 72, 3065-3067.

Margolskee, J.P. and Lodish, H.F. (1980a). Dev. Biol. 74, 37-49.

Margolskee, J.P. and Lodish, H.F. (1980b). Dev. Biol. 74, 50-64.

Mayrand, S., Setyono, B., Greenberg, J.R. and Pederson, T. (1981). J. Cell Biol. 90, 380-384.

Mathews, M.B., Osborn, M., Berns, A.J.M. and Bloemendal, J. (1972). Nature New Biol. 236, 5-7.

Mathews, M.B. (1973). in Essay in Biochemistry (Campbell, P.N. and Dickens, F. eds.) 9, pp.59-102, Academic Press, London and New York.

Mathews, M.B. (1980). Nature 285, 575-577.

Mathis, D., Oudet, P. and Chambon, P. (1980). Prog. Nucl. Acid Res. Mol. Biol. 24, 2-55.

Mathis, D.J. and Chambon, P. (1981). Nature 290, 310-315.

Maziels, N. (1976). Cell 9, 431-438.

Mellon, P., Parker, V., Gluzman, V. and Maniatis, T. (1981). Cell 27, 279-288.

Mendecki, J., Lee, S.Y. and Brawerman, G. (1972). Biochemistry 11, 792-798.

Mendelsohn, S.L. and Young, D.A. (1978). Biochim. Biophys. Acta 519, 461-473.

Merkel, C.G., Kwan, S-P. and Lingrel, J.B. (1975). J. Biol. Chem. 250, 3725-3728.

Metz, D.H. (1975). Cell 6, 429-439.

Milcarek, C., Price, R. and Penman, S. (1974). Cell 3, 1-10.

Milcarek, C. (1979). Eur. J. Biochem. 102, 467-476.

Miller, M.J., Xuong, N-H, and Geiduschek, P. (1979). Proc. Natl. Acad. Sci. U.S.A. 76, 5222-5225.

Minty, A.J. and Gros, F. (1980). J. Mol. Biol. 139, 61-83.

Mirault, M.E., Goldschmidt-Clermont, M., Moran, L., Arrigo, A.P. and Tissieres, A. (1978). Cold Spring Harbor Symp. Quant. Biol. 42, 819-827.

Mirault, M-E., Goldschmidt-Clermont, M., Artavanis-Tsakonas, S. and Schedl, P. (1979). Proc. Natl. Acad. Sci. U.S.A. 76, 5254-5258.

Molloy, G., Thomas, W.L. and Darnell, J. (1972). Proc. Natl. Acad. Sci. U.S.A. 69, 3684-3688.

Molloy, G.R., Jelinek, W., Salditt, M. and Darnell, J.E. (1974). Cell 1, 43-54.

Molloy, G.R. (1980). J. Biol. Chem. 255, 10375-10381.

Moran, L., Mirault, M-E., Arrigo, A.P., Goldschmidt-Clermont, M. and Tissieres, A. (1978). Phil. Trans. Roy. Soc. Lond. B. 283, 391-406.

Moran, L., Mirault, M-E., Tissieres, A., Lis, J., Schedl, P., Atravanis-Tsakonas, S. and Gehring, W.J. (1979). Cell 17, 1-8.

Morris, N.R. (1976). Cell 9, 627-632.

Morrison, M.R., Brodeur, R., Pardue, S., Baskin, F., Hall, G.L. and Rosenberg, R.N. (1979). J. Biol. Chem. 254, 7675-7683.

Moss, B., Rosenblum, N. and Paoletti, E. (1973). Nature New Biol. 245, 59-63.

Munroe, S.H. and Pederson, T. (1981). J. Mol. Biol. 147, 437-449.

Murray, V. and Holliday, R. (1979a). FEBS Lett. 106, 5-7.

Murray, V. and Hollidlay, R. (1979b). Genet. Res. 34, 173-188.

McAlister, L., Strausberg, S., Kulaga, A. and Finkelstein, D.B. (1979). Current Genet. 1, 63-74.

McAlister, L. and Filkelstein, D.B. (1980). Biochem. Biophys. Res. Commun. 93, 819-824.

McDowell, M.J., Joklik, W.K., Villa-Kamaroff, L. and Lodish, H.F. (1972). Proc. Natl. Acad. Sci. U.S.A. 69, 2649-2653.

McGhee, J.D. and Felsenfeld, G. (1980). Ann. Rev. Biochem. 49, 1115-1156.

McKenzie, S.L. and Meselson, M. (1977). J. Mol. Biol. 117, 279-283.

McKeown, M., Taylor, W.C., Kindle, K.C., Firtel, R.A., Bender, W. and Davidson, N. (1978). Cell 15, 789-800.

McKnight, S.L. and Miller, O.L. (1976). Cell 8, 305-319.

McKnight, S.L., Sullivan, N.L. and Miller, O.L. (1976). Prog. Nucl. Acid Res. Mol. Biol. 19, 313-334.

McReynolds, L., O'Malley, B.W., Nisbet, A.D., Fathergill, J.E., Girol, D., Fields, S., Robertson, M. and Brownlee, G.G. (1978). Nature 273, 723-728.

Nagata, S., Mantei, N. and Weissmann, C. (1980). Nature 287, 401-408.

Nakazato, H., Edmonds, M. and Kopp, D.W. (1974). Proc. Natl. Acad. Sci. 71, 200-204.

Nemer, M., Graham, M. and Dubroff, L.M. (1974). J. Mol. Biol. 89, 735-754.

Nemer, M., Dubroff, L.M. and Graham, M. (1975). Cell 6, 171-178.

Nevins, J.R. and Darnell, J.E. (1978). Cell 15, 1477-1493.

Nevins, J.R., Blanchard, J-M. and Darnell, J.E. Jr. (1980). J. Mol. Biol. 144, 377-386.

Nevins, J.R. and Wilson (1981). Nature 290, 113-118.

Nishioka, Y. and Leder, P. (1979). Cell 18, 865-873.

Nokin, P., Burny, A., Huez, G. and Marbaix, G. (1976). Eur. J. Biochem. 68, 431-436.

Nordstrom, J.L., Roop, D.R., Tsai, M-J. and O'Malley, B.W. (1979). Nature 278, 328-331.

Nunberg, J.H., Kaufman, R.J., Chang, A.C.Y., Cohen, S.N. and Schimke, R.T. (1980). Cell 19, 355-364.

O'Connor, D. and Lis, J.T. (1981). Nucl. Acid Res. 9, 5075-5092.

O'Farrell, P.H. (1975). J. Biol. Chem. 250, 4007-4021.

O'Hare, K., Breathnach, R., Benoist, C. and Chambon, P. (1979). Nucl. Acid Res. 7, 321-334.

Ohno, T., Cozens, P.J., Cato, A.C.B. and Jost, J-P. (1980). Biochim. Biophys. Acta 606, 34-36.

Old, J.M., Clegg, J.B., Weatherall, D.J., Ottolenghi, S., Comi, P., Giglioni, B., Mitchell, J., Tolstoshev, P. and Williamson, R. (1976). Cell 8, 13-18.

Ottolenghi, S., Lanyon, W.G. and Williamson, R. (1976). Cell 8, 13-18.

Palatnik, C.M., Storti, R.V. and Jacobson, A. (1979). J. Mol. Biol. 128, 371-395.

Palatnik, C.M., Storti, R.V., Capone, A.K. and Jacobson, A. (1980). J. Mol. Biol. 141, 99-118.

Palmiter, R.D. and Carey, N.H. (1974). Proc. Natl. Acad. Sci. U.S.A. 71, 2357-2361.

Paterson, M. and Bishop, J.O. (1977). Cell 12, 751-765.

Paul, J. (1982). Bioscience Reports 2, 63-76.

Paule, M.R. (1981). Trends. Biochem. Sci. 6, 128-131.

Peebles, C.L., Ogden, R.C., Knapp, G. and Abelson, J. (1979). Cell 18, 27-35.

Pelham, H.R.B. and Jackson, R.J. (1976). Eur. J. Biochem. 67, 247-256.

Pelham, H.R.B. (1978). Nature 272, 469-471.

Penman, S. (1966). J. Mol. Biol. 17, 117-130.

Penman, S. (1969) in Fundamental Techniques in Virology, Academic Press (Habel, K. and Salzman, N.P. eds.), pp.35-48.

Penman, S., Greenberg, H. and Willems, M. (1969). in Fundamental Techniques in Virology, Academic Press (Habel, K. and Salzman, N.P. eds) pp.49-58.

Penman, S., Scherrer, K., Becker, Y. and Darnell, J.E. (1963). Proc. Natl. Acad. Sci. U.S.A. 49, 654-662.

Penman, S., Vesco, C. and Penman, M. (1968). J. Mol. Biol. 34, 49-69.

Perler, F., Efstratiadis, A., Lomedico, P., Gilbert, W., Kolodner, R. and Dodgson, J. (1980). Cell 20, 555-566.

Perlman, S., Abelson, A.T. and Penman, S. (1973). Proc. Natl. Acad. Sci. U.S.A. 70, 350-353.

Perry, R.P. and Kelley, D.E. (1968). J. Mol. Biol. 35, 37-60.

Perry, R.P. and Kelley, D. (1973). J. Mol. Biol. 79, 681-696.

Perry, R.P., Kelley, D., Friderici, K. and Rottman, F. (1975). Cell 4, 387-394.

Perry, R.P. and Kelley, D.E. (1976). Cell 8, 433-442.

Perry, R.P., Bard, E., Hames, E.D., Kelley, D.E. and Schibler, U. (1976). Prog. Nucl. Acid Res. Mol. Biol. 19, 275-292. (eds. Cohn, W.E. (New York).

Petersen, N.S. and Mitchell, H.K. (1981). Proc. Natl. Acad Sci. U.S.A. 78, 1708-1711.

Peterson, D.T., Merrick, W.C. and Safer, B. (1979). J. Biol. Chem. 254, 2509-2514.

Pinphanichakarn, P., Kramer, G. and Hardesty, B. (1977). J. Biol. Chem. 252, 2106-2112.

Plagens, U., Greenleaf, A.L. and Bautz, E.K.F. (1976). Chromosoma 59, 157-165.

Pravinkumar, B.S., Soreq , H. and Tamm, I. (1978). Proc. Natl. Acad. Sci. U.S.a. 75, 5030-5033.

Preobrazensky, A.A. and Spirin, A.S. (1978). Prog. Nucl. Acid Res. Mol. Biol. 21, 2-37.

Proudfoot, N.J. and Brownlee, G.G. (1976). Nature 263, 211-214.

Proudfoot, N.J. (1977). Cell 10, 559-570.

Puckett, L., Chambers, S. and Darnell, J.E. (1975). Proc. Natl. Acad. Sci. U.S.A. 72, 389-392.

Puckett, L. and Darnell, J.E. (1976). J. Cell Physiol. 90, 521-534.

Quellette, A.J. (1980). J. Biol. Chem. 255, 2740-2746.

Quellette, A.J., Reed, S.L. and Malt, R.A. (1976). Proc. Natl. Acad. Sci. U.S.A. 73, 2609-2613.

Quellette, A.J. and Ordahl, C.P. (1981). J. Biol. Chem. 256, 5104-5108.

Reeves, R. (1978). Biochemistry 17, 4908-4915.

Richards, R.I., Shine, J., Ullrich, A., Wells, J.R.E. and Goodman, H.M. (1979). Nucl. Acid Res. 7, 1137-1146.

Ritossa, F.M. (1964). Exp. Cell Res.. 35, 601-607.

Roberts, B.E. and Paterson, B.M. (1973). Proc. Natl. Acad. Sci. U.S.A. 70, 2330-2334.

Robertson, H.D., Dickson, E. and Jelinek, W. (1977). J. Mol. Biol. 115, 571-590.

Roeder, R.G. and Rutter, W.J. (1969). Nature New Biol. 224, 234-237.

Roeder, R.G. (1974). J. Biol. Chem. 249, 249-256.

Roeder, R.G. (1976). in The Molecular Biology of Hormone Action (Papaconstantinou, ed.) pp.29-52.

Roeder, R.G. (1977). in RNA polymerase, Cold Spring Harbor Monograph Series (Losick, R., Chamberlin, M. eds.).

Rogers, J., Early, P., Carter, C., Calame, K., Bond, M., Hood, L. and Wall, R. (1980). Cell 20, 303-312.

Rogers, J. and Wall, R. (1980). Proc. Natl. Acad Sci. U.S.A. 77, 1877-1879.

Roop, D.R., Nordstrom, J.L., Tsai, S.Y., Tsai, M-J. and O'Malley, B.W. (1978). Cell 15, 671-685.

Roop, D.R., Tsai, M-J. and O'Malley, B.W. (1980). Cell 19, 63-68.

Rosenberg, M. and Court, D. (1979). Ann. Rev. Genet. 13, 319-353.

Ross, J. (1976). J. Mol. Biol. 106, 403-420.

Ross, J. and Knecht, D.A. (1978). J. Mol. Biol. 119, 1-20.

Royal, A., Garapin, A., Cami, B., Perrin, F., Mandel, J.L., Chambon, P. and Kourilsky, P. (1979). Nature 279, 125-132.

Roychoudhury, R., Jay, E. and Wu, R. (1976). Nucl. Acid Res. 3, 101-116.

Rubin, G.M. and Hogness, D.S. (1975). Cell 6, 207-213.

Ruderman, J.V. and Pardue, M. (1977). Dev. Biol. 60, 48-62.

Ruderman, J.V. and Pardue, M.L. (1978). J. Biol. Chem. 253, 2018-2025.

Ruderman, J.V., Woodland, H.R. and Sturgess, E.A. (1979). Dev. Biol. 71, 71-82.

Ryffel, G.U. and McCarthy, B.J. (1975). Biochemistry, 14, 1379-1385.

Ryffel, G.U., Wyler, T., Nuellener, D.B. and Weber, R. (1980). Cell 19, 53-61.

Ryscov, A., Takarskaya, D.V., Georgiev. G.P., Coutelle, C. and Thiele, B. (1976). Nucl. Acid Res. 3, 1487-1498.

Sala-Trepat, J.M., Dever, J., Sargent, T.D., Thomas, K., Sell, S. and Bonner, J. (1979). Biochemistry 18, 2167-2178.

Salditt-Georgieff, M., Jelinek, W., Darnell, J. and Furuichi, Y. (1976). Cell 7, 227-237.

Salditt-Georgieff, M., Harpold, M., Chen-Kiang, S. and Darnell, J.E. (1980). Cell 19, 69-78.

Schechter, I. (1974). Biochemistry 13, 1875-1885.

Schedl, P., Artavanis-Tsakonas, S., Steward, R. and Gehring, W.J. (1978). Cell 14, 921-929.

Scheer, U. (1978). Cell 13, 535-549.

Schibler, U. and Perry, R.P. (1976). Cell 9, 121-130.

Schibler, U., Marcu, K.B. and Perry, R.P. (1980). Cell 15, 1495-1509.

Schmid, C. and Deininger, P.L. (1975). Cell 6, 345-358.

Schochetman, G. and Perry, R.P. (1972). J. Mol. Biol. 63, 577-590.

Schmidt, A., Chernajovsky, L., Schielman, P., Federman, H., Berissi, H. and Revel, M. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4788-4792

Schwartz, H. and Darnell, J.E. (1976). J. Mol. Biol. 104, 833-852.

Scott, M.P. and Pardue, M.L. (1981). Proc. Natl. Acad. Sci. U.S.A. 78, 3353-3357.

Segal, S. and Khoury, G. (1979). Proc. Natl. Acad. Sci. U.S.A. 76, 5611-5615.

Segal, S., Levine, A.J. and Khoury, G. (1979). Nature 280, 335-338.

Setyono, B. and Greenberg, J.R. (1981). Cell 24, 775-783.

Shafritz, D.A., Weinstein, J.A., Safer, B., Merrick, W.C., Weber, L.A., Hickey, E.D. and Boglioni, C. (1976). Nature 261, 291-294.

Shapiro, J. and Schimke, R.T. (1975). J. Biol. Chem. 250, 1759-1760.

Shatkin, A.J. (1976). Cell 9, 645-653.

Sheiness, D. and Darnell, J.E. (1973). Nature 241, 265-268.

Shenkin, A. and Burdon, R.H. (1974). J. Mol. Biol. 85, 19-39.

Siebenlist, U., Simpson, R.B. and Gilbert, W. (1980). Cell 20, 269-281.

Silver, L.M. and Elgin, S.C.R. (1977). Cell 11, 971-983.

Sin, Y.T. (1975). Nature 258, 159-160.

Singer, M.F. (1982). Cell 28, 433-434.

Singer, R.H. and Penman, S. (1972). Nature New Biol. 240, 100-102.

Singer, R.H. and Penman, S. (1973). J. Mol. Biol. 78, 321-334.

Sinibaldi, R.M. and Morris, P.W. (1981). J. Biol. Chem. 256, 10735-10738.

Skup, D., Zarbl, H. and Millward, S. (1981). J. Mol. Biol. 151, 35-55.

Slater, A., Cato, A.C.B., Sillar, G.M., Kioussis, J. and Burdon, R.H. (1981). Eur. J. Biochem. 117, 341-346.

Slattery, E., Ghash, N., Samanta, H. and Lengyel, P. (1979). Proc. Natl. Acad. Sci. U.S.A. 76, 4778-4782.

Smith, D.F., Searle, P.F. and Williams, J.G. (1979). Nucl. Acid. Res. 6, 487-506.

Smith, K. and Lingrel, J.B. (1978). Nucl. Acid Res. 5, 3295-3301.

Sondermeijei, P.J.A. and Lubsen, N. (1978). Eur. J. Biochem. 98, 331-339.

Sonenberg, N., Rupprecht, K.M., Hecht, S.M. and Shatkin, A. (1979). Proc. Natl. Acad. Sci. U.S.A. 76, 4345-4349.

Spradling, A., Hui, H. and Penman, S. (1975). Cell 4, 131-137.

Spradling, A., Pardue, M.L. and Penman, S. (1977). J. Mol. Biol. 109, 559-587.

Spruill, W.A., Hurwitz, D.R., Lucchesi, J.C. and Steiner, A.L. (1978). Proc. Natl. Acad. Sci. U.S.A. 75, 1480-1484.

Stalder, J., Seebeck, T. and Braun, R. (1978). Eur. J. Biochem. 90, 391-396.

Stalder, J., Larsen, A., Engel, J.D., Dolan, M., Croudine, M. and Weintraub, H. (1980). Cell 20, 451-460.

Stark, G.R. and Williams, J.G. (1979). Nucl. Acid Res. 6. 195-203.

Stein, J.L., Stein, G.S. and McGuire, P.M. (1977). Biochemistry 16, 2207-2212.

Stewart, A.G., Clissold, P.M. and Arnstein, H.R.V. (1976). Eur. J. Biochem. 65, 349-355.

Stewart, A.G., Lloyd, M. and Arnstein, H.R.V. (1977). Eur. J. Biochem. 80, 453-459.

Storti, R.V., Scott, M.P., Rich, A. and Pardue, M.L. (1980). Cell 22, 825-834.

Strair, R.K., Yap, S.H. and Shafritz, D.A. (1976). Proc. Natl. Acad. Sci. U.S.A. 74, 4346-4350.

Strair, R.K., Skoultchi, A.I. and Shafritz, D.A. (1977). Cell 12, 133-141.

Surrey, S. and Nemer, M. (1976). Cell 9, 589-595.

Suzuki, Y., Gage, L.P. and Brown, D.D. (1972). J. Mol. Biol. 70, 637-657.

Taylor, J.M. and Schimke, R.T. (1974). J. Biol. Chem. 249, 3597-3607.

Teissere, M., Penon, P., Azou, Y. and Ricard, J. (1977). FEBS Lett. 82, 77-81.

Thimmappaya, B. and Schenk, T. (1979). Cell 17, 737-747.

Thoma, F. and Koller, T. (1977). Cell 12, 101-107.

Thoma, F., Koller, T. and Klugg, A. (1979). J. Cell. Biol. 83, 403-427.

Thomas, J.O. and Thompson, R.J. (1977). Cell 19, 633-640.

Tilgham, S.M., Tiemeir, D.C., Seidman, J.G., Peterlin, B.M., Sullivan, M., Maizel, J.V. and Leder, P. (1978a). Proc. Natl. Acad. Sci. U.S.A. 75, 725-729.

Tilgham, S.M., Curtis, P.J., Tiemeir, D.C., Leder, P. and Weissmann, C. (1978b). Proc. Natl. Acad. Sci. U.S.A. 75, 1309-1313.

Tissieres, A., Mitchell, H.K. and Tracy, U.M. (1974). J. Mol. Biol. 84, 389-398.

Tobin, S.L., Zulauf, E., Sanchez, F., Craig, E.A. and McCarthy, B.J. (1980). Cell 19, 121-131.

Toltoshev, P., Williamson, R., Eskdale, J., Verdier, G., Godet, J., Nigon, V., Trabuchet, G. and Benabadji, M. (1977). Eur. J. Biochem. 78, 161-166.

Torok, I. and Karch, F. (1980). Nucl. Acid Res. 8, 3105-3123.

Trachsel, H., Sonnberg, N., Shatkin, A., Rose, J.K., Lelong, K., Bergman, J.E., Gordon, J. and Baltimore, D. (1980). Proc. Natl. Acad. Sci. U.S.A. 77, 770-774.

Tsai, M-J., Ting, A.C., Nordstrom, J.F., Zimmer, W. and O'Malley, B.W. (1980a). Cell 22, 219-230.

Tsai, S.Y., Roop, D.R., Stumph, W.E., Tsai, M-J. and O'Malley, B.W. (1980b). Biochemistry, 19, 1755-1761.

Tsuda, M., Otishima, Y. and Suzuki, Y. (1979). Proc. Natl. Acad. Sci. U.S.A. 76, 4872-4876.

Vandekerchore, J. and Weber, K. (1978). Proc. Natl. Acad. Sci. U.S.A. 75, 1106-1110.

Van den Berg, J., Van Ooyen, A., Mantei, N., Schambock, A., Grosveld, G., Flavell, R.A. and Weissmann, C. (1978). Nature 276, 37-43.

Van Ness, J., Maxwell, I.H. and Hahn, W.E. (1979). Cell 18, 1341-1349.

Varley, J.M., Macgregor, H.C. and Erba, H.P. (1980). Nature 283, 686-688.

Varshavsky, A.J., Bakayev, V.V. and Georgiev, G.P. (1976). Nucl. Acid Res. 3, 477-492.

Velazquez, J.M., DiDomedico, B.J. and Lindquist, S. (1980). Cell 20, 679-689.

Venkatesan, S., Nakazato, H., Kopp, D.W. and Edmonds, M. (1979). Nucl. Acid Res. 6, 1097-1110.

Venrooij, W.J., Wagenmarkers, A.J.M., van den Oetelaar, P. and Reinder, R.J. (1981). Mol. Biol. Rep. 7, 93-99.

Vincent, A., Civelli, O., Buri, J.F. and Scherrer, K. (1977). FEBS Lett. 77, 281-286.

Vincent, A., Goldenberg, S., Standart, N., Civelli, O., Imaizumi-Scherrer, T., Maundrell, K. and Scherrer, K. (1981). Mol. Biol. Rep. 7, 71-81.

Volckaert, G., Feunteun, J., Crawford, L.V., Berg, P. and Fiers, W. (1979). J. Virol. 30, 674-682.

Volloch, V. and Housman, D. (1981). Cell 23, 509-514.

Wadsworth, S.C., Craig, E.A. and McCarthy, B.J. (1980). Proc. Natl. Acad. Sci. U.S.A. 77, 2134-2137.

Wahli, W., Dawid, I.B., Wyler, T., Jaggi, R.B., Weber, R. and Ryffel, G.U. (1979). Cell 16, 535-549.

Wahli, W., Dawid, I.B., Wyler, T., Weber, R. and Ryffel, G.U. (1980). Cell 20, 107-117.

Wallace, J.C., Wood, W.M. and Edmonds, M. (1981). Biochemistry 20, 5364-5368.

Walters, R.A., Yardell, P.M. and Enger, M.D. (1979). Biochemistry 18, 4254-4261.

Wang, C., Gomer, R.H. and Lazarides, E. (1981). Proc. Natl. Acad. Sci. U.S.A. 78, 3531-3535.

Warner, J.R., Knopf, P.M. and Rich, A. (1963). Proc. Natl. Acad. Sci. U.S.A. 49, 122-129.

Wasylyk, B., Kedinger, C., Corden, J., Brison, O. and Chambon, P. (1980). Nature 285, 367-373.

Weaver, S., Haigwood, N.L., Hutchison, C.A. and Edgell, M.H. (1979). Proc. Natl. Acad. Sci. U.S.A. 76, 1385-1389.

Weber, J., Blanchard, J-M., Ginsberg, H. and Darnell, J.E. Jr. (1980). J. Virol. 33, 286-291.

Weber, L.A., Feman, E.R. and Baglioni, C. (1975). Biochemistry 14, 5315-5321.

Weber, L.A., Feman, E.R., Hickey, E.D., Williams, M.C. and Baglioni, C. (1976). J. Biol. Chem. 251, 5657-5662.

Weil, P.A., Luse, D.S., Segall, J. and Roeder, R.G. (1979a). Cell 18, 469-484.

Weil, P.A., Segall, J., Harris, B., Ng, S-Yu. and Roeder, P.G. (1979b). J. Biol. Chem. 254, 6163-6173.

Weinberg, E.S., Birnstiel, M.L., Purdom, I.F. and Williamson, R. (1972). Nature New Biol. 240, 225-228.

Weinberg, R.A. (1973). Ann. Rev. Biochem. 42, 329-354.

Weintraub, H. and Groudine, M. (1976). Science 193, 848-856.

Weissbach, H. and Ochoa, S. (1976). Ann. Rev. Biochem. 45, 191-216.

Weisbrod, S. and Weintraub, H. (1979). Proc. Natl. Acad. Sci. U.S.A. 76, 630-634.

Wensink, P.C., Finnegan, D.J., Donelson, J.E. and Hogness, D.E. (1974). Cell 3, 315-325.

Whalen, R. and Gros, F. (1978). Biochim. Biophys. Acta 519, 372-382.

Whitlock, J.P. Jr. and Simpson, R.T. (1976). Biochemistry 15, 3307-3314.

Wild, M.A. and Sommer, R. (1980). Nature 283, 693-694.

Wilson, M.C. and Melli, M. (1977). J. Mol. Biol. 110, 511-536.

Wilson, M.C. and Darnell, J.E. Jr. (1981). J. Mol. Biol. 148, 231-251.

Wilt, F.H. (1977). Cell 11, 673-681.

Winters, M.A. and Edmonds, M. (1973). J. Biol. Chem. 248, 4763-4768.

Wood, W.M. and Edmonds, M. (1981). Biochemistry 20, 5359-5364.

Wu, G.U. (1978). Proc. Natl. Acad. Sci. U.S.A. 75, 2175-2179.

Wu, C., Wong, Y.C. and Elgin, S.C.R. (1979). Cell 16, 807-814.

Wu, C. (1980). Nature 286, 854-860.

Wu, G-J. (1980). J. Biol. Chem. 255, 251-258.

Yang, N.S., Manning, R.F. and Gage, L.P. (1976). Cell 7, 339-348.

Yogo, Y. and Wimmer, E. (1972). Proc. Natl. Acad. Sci. U.S.A. 69, 1877-1882.

Young, R.A., Hagenbuchle, O. and Schibler, U. (1981). Cell 23, 451-458.

Yu, Fu-Li. (1980). Biochem. J. 188, 381-385.

Zeevi, M., Nevins, J.R. and Darnell, J.E. Jr. (1981). Cell 26, 39-46.

Zelenka, P. and Piatigorsky, J. (1976). J. Biol. Chem. 251, 4294-4298.

Zieve, G., Benecke, B. and Penman, S. (1977). Biochemistry 16, 4520-4525.

Zieve, G.W. (1981). Cell 25, 296-297.

Ziff, E.B. and Evans, R.M. (1978). Cell 15, 1463-1475.

Ziff, E.B. (1980). Nature, 287, 491-499.