STUDIES ON RNA METABOLISM IN HELA MITOCHONDRIA

Thesis by

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ABSTRACT

The metabolism of mitochondrial ribonucleic acid was studied in the human cell line HeLa. Studies of labeling kinetics show that polyadenylated mitochondrial RNA species are turned over with half-lives ranging from a few minutes to about two hours.

Several giant RNA species transcribed from the mitochondrial DNA light strand were studied. They range in size from approximately 25% to 60% of the length of the mitochondrial genome and overlap in their mapping locations.

The kinetics of labeling of mitochondrial polyadenylic acid not covalently linked to larger RNA molecules was studied. This "free" polyadenylic acid is not derived from the breakdown of larger polyadenylated RNA molecules and may be a precursor to polyadenylic acid covalently linked to messenger RNA molecules.

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Chapter 1

Introduction

INTRODUCTION

This thesis describes studies on mitochondrial RNA metabolism in HeLa, an established human cell line. By now, the mitochondrial literature is enormous and could not be summarized briefly. This introduction is therefore restricted to various aspects of the study of mitochondria which are most relevant to the present work.

In addition to various mammalian cell lines, mitochondria of yeast are under intense investigation at present. This discussion centers on animal cell mitochondria but includes discussion of and comparison with results of studies on yeast mitochondrial function.

In addition to yeast and mammalian cells, a number of other life forms have been used for the study of mitochondrial function, for example, Xenopus, Tetrahymena, and Neurospora. A number of volumes exist (such as Sager, 1972) describing studies of mitochondrial genome function in a variety of organisms. Several symposia have recently been held and the resulting discussions are available (Bucher et al., 1976; Saccone and Kroon, 1976; Bandlow et al., 1977).

THE MITOCHONDRION: DISTRIBUTION AND FUNCTION

Mitochondria are the intracellular sites of several important biochemical pathways in eucaryotic cells, including the Krebs cycle, electron transport and oxidative phosphorylation (Lehninger, 1964). Although a lower eucaryotic parasite is known which is lacking mitochondria (Raff and Mahler, 1972), presumably as a second loss, mitochondria are essentially ubiquitous in the eucaryotes.

The morphology of mitochondria is variable, ranging from ovoid to rodlike to filamentous. Mitochondria are present at one or a few

copies per cell in some forms to several thousand copies per cell in others (reviewed by Attardi et al., 1975).

In HeLa cells, mitochondria appear to be discrete structures which may be ovoid or rodlike or, rarely, filamentous (Posakony et al., 1975). Mitochondria of animal cells typically occupy 5-14% of intracellular volume (Clayton and Bogenhagen, 1974; Posakony et al., 1977).

Mitochondria appear to "reproduce" by continuous addition of material to pre-existing mitochondria followed by division, rather than by de novo synthesis (Luck, 1963, 1965).

The image in the light microscope of mitochondria as structures about the size and shape of some bacteria led to the hypothesis as early as 1918 (Portier) that mitochondria are the result of endosymbiosis; that is, that the precursors to mitochondria were free-living bacteria which entered an earlier eucaryotic cell and maintained a symbiotic relationship with that cell. This hypothesis remains controversial. Raff and Mahler (1972) have presented an alternative hypothesis that mitochondria are derived evolutionarily from the membrane-bound respiratory apparatus present in the early eucaryote cell (see also Raff and Mahler, 1972; Mahler and Raff, 1975). Margulis (1970) and Borst and Grivell (1978) have argued in favor of the endosymbiont hypothesis.

THE EXISTENCE OF THE MITOCHONDRIAL GENOME

The existence of "cytoplasmic" and "maternal" inheritance has been recognized for many years (Sager, 1972). The modern era of mitochondrial genetics began with the demonstration by Nass and Nass (1963a, 1963b) that the mitochondria of chicken cells contain DNA molecules. Fibrous

material observed by electron microscopy was proved to contain DNA by a series of enzyme digestion and staining experiments. This work was then extended (Nass et al., 1965) by the demonstration of the existence of DNA in the mitochondria of a wide range of phyla.

Since the early experiments, an enormous literature has appeared regarding mitochondrial nucleic acids (for example, see the review by Borst, 1972). No attempt will be made here to review that literature in depth, but a few highlights should be mentioned.

The size of mitochondrial DNA molecules ranges from a molecular weight of approximately 10^7 (corresponding to 16,000 nucleotide pairs) in animal cells to 5×10^7 (corresponding to 75,000 nucleotide pairs) in yeasts and 7×10^7 in higher plants (Borst, 1977). The DNA of most forms is circular. The known exceptions which contain linear mitochondrial DNA are Paramecium (Cummings et al., 1976) and Tetrahymena (Goldback et al., 1976).

Nass (1969) has measured the sizes of mitochondrial DNAs from a variety of animal species. The DNA of all the species studied was close to 5 microns in length. The small differences (less than 10%) observed between different species are probably real differences because experiments involving the mixing of DNA from different species showed bimodal size distributions.

The mechanism of mitochondrial DNA replication in animal cells has been reviewed by Kasamatsu and Vinograd (1974). DNA is replicated by a modified Cairns (1963) mechanism, involving the production of a "D-Loop" form at the beginning of replication.

Mitochondrial DNA can be purified of contaminating nuclear DNA by cesium chloride-ethidium bromide centrifugation (Radloff et al., 1967). This has been of great benefit in the investigation of mitochondrial phenomena since the mitochondrial origin of RNA and DNA can be demonstrated unambiguously by hybridization studies.

THE EXISTENCE OF MITOCHONDRIAL TRANSCRIPTION AND TRANSLATION

The demonstration by McLean et al. (1958) of mitochondrial protein synthesis in HeLa cells was controversial at the time (discussed by Borst, 1977). By now, the existence of mitochondrial transcription and translation are well established.

The existence of mitochondrial transcription in HeLa was demonstrated (Attardi and Attardi, 1967, 1968) by means of hybridization of mitochondrial DNA to labeled RNA extracted from a cytoplasmic membrane preparation. Zylber et al. (1969) confirmed this result by showing that the labeling of RNA extracted from mitochondria was sensitive to ethidium bromide

The properties of the mitochondrial translation system were investigated in various ways. Swanson and Dawid (1970) characterized the mitochondrial ribosome of Xenopus laevis as did Attardi and Ojala (1971) for HeLa mitochondria. Polysomes were described (Ojala and Attardi, 1972) in HeLa mitochondria, but with properties somewhat different from the properties of cytoplasmic polysomes. Puromycin sensitivity was demonstrated, but EDTA sensitivity was reduced and RNase sensitivity depended on a preliminary protease treatment. The unusual results were attributed to the protection of the polysome structure by the (highly hydrophobic) nascent polypeptides.

A variety of techniques using inhibitory drugs for the differential labeling of mitochondrial and cytoplasmic macromolecules were provided by Penman's lab. The use of ethidium bromide to block mitochondrial transcription (Zylber et al., 1969) provided a tool for the identification of mitochondrial transcripts. The use of Actinomycin D (Perlman and Penman, 1970) and Camptothecin (Perlman et al., 1973) to block nuclear transcription allowed the selective labeling of mitochondrial RNAs. Mitochondrial proteins can be selectively labeled in the presence of emitine or cycloheximide or their labeling can be selectively blocked by chloramphenicol (Ojala and Attardi, 1972).

RNAs AND POLYPEPTIDES OF MITOCHONDRIAL ORIGIN: THE ORGANIZATION OF THE MITOCHONDRIAL GENOME

Studies of the organization of the mitochondrial genome and its transcription products have taken divergent paths with regard to animal cells and lower eucaryotes. The ability to isolate mitochondrial mutants of animal cells was extremely limited in the past (Mitchell et al., 1972). Although it is now possible to generate mutants of animal cell mitochondria (Wiseman and Attardi, 1978, 1979), studies have been limited up to the present.

Genetic manipulation is possible with yeast mitochondria in a way which is impossible in animal cell cultures. Yeast can grow in the absence of a functional mitochondrial genome. "Peptide" mutants missing most of the mitochondrial genome can be used in genetic cross experiments (Borst, 1972). The retention of genetic markers in petite mutants can be correlated with the physical map of yeast mitochondrial DNA by

hybridization experiments. As a result of these advantages, the study of the genome organization of yeast mitochondria has been intense and has far surpassed comparable studies in animal cells.

In particular, mutations which result in functional changes (for example, mutations of particular polypeptides of the electron transport chain, ribosomal RNAs, or the oligomycin sensitive ATPase) have been mapped genetically and physically (reviewed by Borst and Grivell, 1978; also Tzagoloff et al., 1979).

The situation with respect to animal cell mitochondrial genomes has been considerably different. In the absence of the sophisticated genetic tools available in other systems, investigations have generally concentrated on physical analyses of the products of transcription and translation. The smaller size of the animal mitochondrial genome (about 20% the size of the yeast mitochondrial genome) has been of some advantage, particularly with regard to mapping studies. The products of HeLa mitochondrial transcription have been characterized in detail (Amalric et al., 1978, submitted as Chapter 2 of this manuscript) and have been mapped onto the known restriction map of the HeLa mitochondrial genome (Ojala et al., manuscript in preparation). Earlier, the ribosomal and 4S genes were mapped (Wu et al., 1972).

Transcription mapping by means of hybridization of DNA restriction fragments to bulk RNA has been reported for mouse (Battey and Clayton, 1978) and for Xenopus (Rastle and Dawid, 1979).

The characterization of polypeptides synthesized by mammalian mitochondria has likewise concentrated on physical studies. Characterization

of cytochrome c oxidase of HeLa mitochondria is reported by Hare et al. (1979).

The polypeptides coded by the mitochondrial genome of yeast and Neurospora have been more intensively studied due to the genetic advantages of these systems. It is interesting that mitochondrial respiratory complexes can consist of mixtures of polypeptide subunits of both cytoplasmic and mitochondrial origin.

Cytochrome oxidase and the oligomycin-sensitive ATPase have been so characterized (Tzagoloff et al., 1979). One subunit, the ATPase proteolipid, is of mitochondrial origin in yeast and cytoplasmic origin in Neurospora (reviewed by Tzagoloff et al., 1979).

There are significant differences in the organization and expression of the mitochondrial genomes of yeast and animals. Yeast genomes are considerably larger, as mentioned above, but both are circular. Each genome has one copy of each of the ribosomal genes. The ribosomal genes of animal mitochondria are found to map close together. For example, in HeLa, they are separated only by a short segment (Robberson et al., 1972). In contrast, the yeast mitochondria ribosomal genes are widely separated and at least one is a "split" gene containing an intervening sequnece (reviewed by Borst and Grivell, 1978). Many of the RNAs synthesized by mammalian mitochondria are polyadenylated, containing a polyadenylic acid segment somewhat shorter than the corresponding cytoplasmic segment (Perlman et al., 1973; Ojala and Attardi, 1974a, 1974b). In contrast, most or all of yeast RNA is not polyadenylated (Moorman et al., 1978). In HeLa, tRNA genes are scattered about the genome without

clustering, whereas in yeast, there is pronounced clustering (Borst, 1977).

HeLa mitochondrial genomes are transcribed symmetrically; that is, both DNA strands are transcribed completely or almost completely (Aloni and Attardi, 1971; Murphy et al., 1975). Symmetric transcription may also occur in yeast mitochondria, but the evidence is incomplete (reviewed by Borst and Grivell, 1978).

The number of tRNA genes found on the mitochondrial genome varies widely for different life forms. Animal cells have at least 17 mitochondrial tRNA genes (Lynch and Attardi, 1976). Yeast mitochondrial genomes contain at least 30 tRNA sites (Borst and Grivell, 1978). Tetrahymena, however, have only 4 known tRNA sites on the mitochondrial genome (Chiu et al., 1974). Tetrahymena mitochondria, it would appear, probably import tRNAs synthesized elsewhere.

A preliminary transcription map of the yeast mitochondrial genome has been presented by Van Ommen and Groot (1977). Thirteen species of RNA known to be different from ribosomal and transfer RNAs were hybridized to DNA restriction fragments according to the Southern (1975) technique. The physical transcription map was found to correlate well with the positions of known genetic markers.

MITOCHONDRIAL AND NUCLEAR GENE INTERACTIONS

The limited information available regarding the interaction of the nuclear and mitochondrial genomes of HeLa has been reviewed (Attardi et al., 1977). One finding of interest is that mitochondrial transcription is dependent on cytoplasmic translation, whereas synthesis of macromolecules

in the nucleus and cytoplasm is not significantly affected by a block of mitochondrial translation (Storrie and Attardi, 1972). England et al. (1978) showed that mitochondrial protein synthesis is not significantly affected for at least 24 hours following enucleation of African Green Monkey cells.

The evidence for nuclear-mitochondrial genomic interactions in yeast and Neurospora is largely the result of genetic studies. Nuclear mutations are known which affect the expression of mitochondrial genes in a specific way (reviewed by Tzagoloff etal., 1979).

RECENT FINDINGS

Heggeness et al. (1978), using fluorescent antibody staining techniques, found that mitochondria are associated with microtubules in a variety of cultured animal cells. In contrast, mitochondria were not closely associated with actin.

There has been intense study of the "cob-box" region of the yeast mitochondrial genome recently. Borst and Grivell (1978) have reviewed this work. It appears that at least in this region, structural genes contain multiple intervening sequences which probably result in the need for post-transcriptional processing of messenger RNAs.

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Chapter 2

Fractionation of Mitochondrial RNA from HeLa Cells by High-resolution Electrophoresis under Strongly Denaturing Conditions

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Fractionation of Mitochondrial RNA from HeLa Cells by High-resolution Electrophoresis under Strongly Denaturing Conditions

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Electrophoresis through agarose slab gels in the presence of the strong denaturing agent methylmercuric hydroxide, in combination with autoradiography, has been used in the present work to resolve different species of ^{32}P-labeled mitochondrial RNA from HeLa cells. At least 18 discrete poly(A)-containing RNA components and 14 discrete components lacking poly(A) have been identified in the molecular weight range from 9×10^4 to $3{\cdot}4 \times 10^6$. Heterodisperse RNA, especially abundant in the high molecular weight region of the gel, up to about $4{\cdot}5 \times 10^6$, has also been observed. The sensitivity to ethidium bromide and the base sequence homology to mitochondrial DNA of all the discrete components and of a substantial portion of the heterogeneous RNA point to their mitochondrial DNA origin.

The number and size of the discrete poly(A)-containing RNA components transcribed from the heavy mitochondrial DNA strand are such that their sequences cannot be accommodated in the portions of the heavy strand not occupied by the ribosomal RNA and tRNA genes; this indicates either a precursor to product relationship between some of these components, or overlapping transcription of segments of the heavy strand. Furthermore, the size limit of the heterogeneous components hybridizing to the light mitochondrial DNA strand strongly suggests that this strand may be transcribed in the form of long, continuous molecules, which undergo progressive shortening as a result of processing.

1. Introduction

Recent work has demonstrated the occurrence in HeLa cell mitochondria of RNA species transcribed from mtDNA‡ which contain poly(A) stretches of about 55 nucleotides at the 3′ end (Perlman *et al.*, 1973; Attardi *et al.*, 1973; Hirsch & Penman, 1973; Ojala & Attardi, 1974*a*). A considerable difficulty in the fractionation of poly(A)-containing RNA is the great tendency of this RNA to aggregate (Ojala & Attardi, 1974*a*); this tendency is due in part to the presence of complementary sequences resulting from the symmetrical transcription of mtDNA (Aloni & Attardi,

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‡ Abbreviations used: mtDNA, mitochondrial DNA; H strand, heavy strand; L strand, light strand; rRNA, ribosomal RNA.

1971*a*; Young & Attardi, 1975; Murphy *et al.*, 1975), and in part, probably, to the poly(A) tails themselves, because of their lack of secondary structure at neutral pH (Rich *et al.*, 1961). Because of such a tendency to aggregate, denaturing conditions were introduced for the analysis of this RNA. Thus, by electrophoresis through cylindrical polyacrylamide/formaldehyde gels, formaldehyde-treated poly(A)-containing RNA isolated from the mitochondrial "polysomal"† structures could be resolved into eight discrete components hybridizable to mtDNA (Ojala & Attardi, 1974*b*). This method of analysis did not have, however, an adequate degree of resolution, and, especially, it proved unsuitable for the fractionation of the more complex mixture of RNA components present in the fastest sedimenting structures of the Triton X100 lysate, which contain the bulk of newly synthesized RNA (Ojala & Attardi, 1974*a*,*b*).

In the present work, electrophoresis through agarose slab gels in the presence of the strong denaturing agent methyl mercuric hydroxide, in combination with autoradiography, has been used to resolve different species of ^{32}P-labeled mitochondrial RNA from both the Triton X100-insoluble structures and the "polysomal" structures. A much larger number of discrete components than previously recognized, both poly(A)-containing and non-poly(A)-containing, have been identified in the molecular weight range from $9{\cdot}0 \times 10^4$ to $3{\cdot}4 \times 10^6$, and characterized in their sequence complementary to separated mtDNA strands. The number and size of the discrete poly(A)-containing RNA components transcribed from the H strand mtDNA indicate that some of these components are related to one another as precursor to product, or result from overlapping transcription of segments of HeLa cell mtDNA. Furthermore, the size limit of the heterogeneous components complementary to the L strand mtDNA strongly suggests that this strand may be transcribed in the form of long, continuous molecules, which are gradually shortened due to processing.

2. Materials and Methods

(a) *Cell growth and labeling conditions*

The method of growth of HeLa cells in suspension has been previously described (Amaldi & Attardi, 1968). For the labeling of mitochondrial RNA with [^{32}P]orthophosphate, exponentially growing cells, washed 3 times with warm modified Eagle's medium (Levintow & Darnell, 1960) containing no phosphate, were resuspended in the same medium at a concn of $1{\cdot}5 \times 10^6$/ml and incubated for 1 h at 37°C. Cold phosphate (10^{-4} M-final concn) and carrier-free [^{32}P]orthophosphate (0·25 mCi/ml) were then added, and incubation was continued for 3 h. In most experiments, camptothecin (20 μg/ml), added 20 min before addition of [^{32}P]orthophosphate, was used to inhibit high molecular weight nuclear RNA synthesis (Horwitz *et al.*, 1970; Kessel, 1971; Abelson & Penman, 1970). In some experiments, 0·1 μg actinomycin D/ml was used to inhibit the synthesis of nuclear rRNA (Perry, 1964; Dubin, 1967; Penman *et al.*, 1968).

(b) *Subcellular fractionation and RNA extraction*

A crude mitochondrial fraction was prepared by differential centrifugation, as previously described (Attardi *et al.*, 1969), except that only one cycle of low and high-speed centrifugation was used to minimize the possibility of RNA degradation. In some experiments,

† The term "polysomal" is used in this paper to indicate the Triton X100-soluble structures which sediment in a sucrose gradient in the region of mitochondrial polysomes: the low amount of heterogeneous RNA relative to the discrete components present in these structures suggests that most of the RNA labeled under the present conditions, which sediments in this region of the gradient, is associated with mitochondrial polysomes.

EDTA (0·04 M) was added to the cytoplasmic extract before the 9000 revs/min centrifugation (to reduce the contamination of mitochondria by cytoplasmic components (Attardi *et al.*, 1969)), and the RNA extracted directly from the crude mitochondrial fraction as described below. In other experiments, mitochondrial polysomes were isolated by lysing the mitochondrial fraction with Triton X100, spinning down the insoluble structures and running the cleared lysate on a sucrose gradient, as has been previously reported (Ojala & Attardi, 1974*b*). RNA was extracted from the crude mitochondrial fraction, or the Triton X100-insoluble structures, or the ethanol-precipitated "polysomal" structures, as described below.

The pelleted material was dissolved in 0·05 M-Tris buffer (pH 7·4 at 25°C), 0·01 M-EDTA, 1% sodium dodecyl SO_4 (1 ml/$1·5 \times 10^8$ cell equivalents), and incubated for 1 h at room temperature in the presence of proteinase K (100 μg/ml) (Ebeling *et al.*, 1974). After addition of NaCl (0·15 M-final concn), the RNA was extracted by shaking twice with an equal vol. of a phenol/chloroform/isoamyl alcohol mixture (50:50:1 by vol.) at room temperature; after ethanol precipitation of the aqueous phase and centrifugation, the pelleted nucleic acids were dissolved in 0·05 M-Tris buffer (pH 6·7), 0·0025 M-$MgCl_2$, 0·025 M-KCl (1 ml/$1·5 \times 10^8$ cell equivalents), and incubated for 30 to 60 min at 0°C in the presence of 100 μg DNase/ml (RNase-free, Worthington). Sodium dodecyl SO_4 (1%) and proteinase K (100 μg/ml) were then added, and, after 20 min incubation at room temperature, the solution was brought to 0·15 M-NaCl and the RNA re-extracted, as described above.

In some experiments, the EDTA-treated crude mitochondrial fraction was subjected to RNase treatment before RNA extraction, in order to degrade the extramitochondrial RNA. For this purpose, the mitochondrial fraction was resuspended in 0·25 M-sucrose, 0·01 M-Tris buffer (pH 6·7), 0·1 M-NaCl, 0·001 M-EDTA (1 ml/$1·5 \times 10^8$ cell equivalents) and treated with 10 μg RNase A/ml (preheated for 10 min at 80°C) for 10 min at 2°C. The suspension was then diluted with 36 ml of 0·25 M-sucrose, 0·01 M-Tris buffer (pH 6·7), 0·001 M-EDTA, 0·5 M-NaCl, and centrifuged at 10^4 revs/min for 10 min in an SS-34 rotor. The mitochondrial pellet was resuspended in 40 ml of the above-mentioned medium and recentrifuged. After repeating the washing procedure once more, the final pellet was solubilized with sodium dodecyl SO_4 and proteinase K, and then subjected to RNA extraction as described above.

(c) *RNA fractionation and analysis*

RNA was fractionated by 2 consecutive passages through oligo(dT)-cellulose columns (Ojala & Attardi, 1974*a*). After the first chromatography, both the bound and the unbound material, collected by ethanol precipitation and centrifugation, were dissolved in 0·001 M-Tris buffer (pH 8·0) containing 0·001 M-EDTA, and subjected to a denaturation step, by heating for 5 min at 80°C, before the second passage through oligo(dT)-cellulose. Each RNA preparation thus yielded, after the second oligo(dT)-cellulose chromatography, 4 fractions: bound–bound, bound–unbound, unbound–bound, unbound–unbound.

The RNA was fractionated under denaturing conditions by electrophoresis through agarose slab gels in the presence of methylmercuric hydroxide, a strong denaturing agent (Bailey & Davidson, 1976). For this purpose, agarose was dissolved, at a concn of 1·2 or 1·4%, in hot electrophoresis buffer (0·05 M-boric acid, 0·005 M-$Na_2B_4O_7 \cdot 10\ H_2O$, 0·01 M-sodium sulfate, 0·001 M-EDTA, pH 8·2), and the cooled (60°C) solution was made 0·005 M in CH_3HgOH just before pouring the slab gel (16 cm × 15 cm × 0·2 cm). The RNA samples were dissolved in 20 to 40 μl of 0·001 M-Tris buffer (pH 7·4), 0·001 M-EDTA, and heated for 5 min at 80°C in sealed 50-μl capillary pipets; after addition of 5 μl glycerol and 5 μl bromophenol blue (0·2%), the samples were layered onto the gel. The electrophoresis was carried out at 100 V (6·3 V/cm) for 3 to 4·5 h. For analytical purposes, the gel was dried and exposed for autoradiography at room temperature, using Kodak X-ray film (XR-5), as described by Studier (1973). For preparative purposes, 1 glass plate was removed after the electrophoresis, and the gel was soaked for 1 h at room temperature in 0·02 M-dithiothreitol in order to complex the mercurial. The wet gel was wrapped in Saran-wrap, upon which marks were made with radioactive ink, to be used for the subsequent positioning of the bands; the gel was thereafter exposed for autoradiography at 4°C for an

appropriate time. The contours of the bands and the marks appearing in the autoradiographic film were drawn on a celluloid film, which was then applied to the Saranwrap-covered gel, using the radioactive ink marks as reference points; the individual RNA bands were cut out of the gel with a scalpel. Each gel slice was extruded by means of a syringe into a plastic 10-ml pipet cut at about 15 cm from the tip and closed at the narrow end with glass wool; the tip of the pipet was connected with a dialysis bag filled with 0·04 M-Tris buffer, 0·02 M-sodium acetate, 0·002 M-EDTA (adjusted to pH 7·4 with acetic acid), 0·02 M-dithiothreitol. RNA was electro-eluted into the dialysis bag at 5 mA per tube for 12 h at 2°C, and, after addition of NaCl to 0·1 M, precipitated with 2 vol. ethanol. The recovery was in general greater than 90%, as estimated from the residual radioactivity in the gel and dialysis bag.

For molecular weight determination of the discrete RNA species analyzed here, the different RNA fractions were run through 0·8% agarose gels, using ^{32}P-labeled *Escherichia coli* 23 S and 16 S rRNAs, HeLa cell 45 S, 32 S, 28 S and 18 S rRNAs (Spohr *et al.*, 1976), and HeLa cell 16 S, 12 S and 7 S mitochondrial RNAs (Robberson *et al.*, 1971; Ojala & Attardi, 1974*b*) as molecular weight standards. At that low agarose concentration, a linear relationship between migration and logarithm of the molecular weight was observed for all the standards used.

Analysis of the size of the poly(A) stretches was carried out as previously described (Ojala & Attardi, 1974*a*).

(d) *RNA–DNA hybridization*

The preparation of [^{3}H] or [2-^{14}C]thymidine-labeled mtDNA and the separation of H and L strands of mtDNA in an alkaline CsCl density gradient have been previously described (Aloni & Attardi, 1971*b*).

The hybridizations in DNA excess were carried out as previously reported (Ojala & Attardi, 1974*a*), except that the hybridization mixtures, after annealing, were treated with 5 μg of pancreatic RNase/ml and 100 units of T_1 RNase/ml (both enzymes from Sigma Co., preheated at 80°C for 10 min) for 30 min at room temperature, and then precipitated with 5% trichloroacetic acid and collected on Millipore membranes.

3. Results

(a) *Oligo(dT)-cellulose fractionation of mitochondrial RNA*

Since the first investigations in this laboratory on poly(A)-containing RNA in HeLa cells (Ojala & Attardi, 1974*a*,*b*), it has been clear that this RNA has a great tendency to aggregate. This tendency demands the use of denaturing conditions both in the separation of poly(A)-containing and poly(A)-lacking RNA species by oligo(dT)-cellulose chromatography and in the electrophoretic fractionation of these species (Ojala & Attardi, 1974*a*,*b*).

In the present work, a qualitative and quantitative study of the fractionation of ^{32}P-labeled mitochondrial RNA by oligo(dT)-cellulose chromatography has been carried out. In these experiments, the crude mitochondrial fraction from HeLa cells labeled for 3 hours with [^{32}P]orthophosphate in the presence of 20 μg camptothecin/ml was resuspended in 2% Triton X100, and the detergent-insoluble structures and the "polysomal" structures (Ojala & Attardi, 1974*a*,*b*) were separated as described above. RNA was extracted from both types of structures and run on oligo(dT)-cellulose columns; the bound and unbound RNA fractions from each sample were then heat denatured and rerun on an oligo(dT)-cellulose column. Thus, four different fractions were obtained for both the RNA extracted from the Triton X100-insoluble components and the RNA from the "polysomal" structures. These four fractions will be referred to below as unbound–unbound, unbound–bound, bound–unbound, bound–bound.

Table 1 shows the distribution of radioactivity among the four above-mentioned

TABLE 1

Distribution of radioactivity among different fractions obtained by oligo(dT)-cellulose chromatography of ^{32}P-labeled RNA extracted from the Triton X100-insoluble components and the "polysomal" structures

Fraction	Triton X100-insoluble structures (% cts/min)†	"Polysomal" structures (% cts/min)†
Bound–bound	5·2 (3·8)	11·6 (3·1)
Bound–unbound	12·2 (9·0)	4·5 (1·2)
Unbound–bound	2·8 (2·1)	6·5 (1·7)
Unbound–unbound	80·7 (59·0)	77·4 (20·5)

† The numbers in parentheses represent the percentages relative to the sum of the radioactivities of the RNA from detergent-insoluble and the RNA from "polysomal" structures.

fractions in the RNA extracted from the Triton X100-insoluble structures and the "polysomal" structures. The RNA extracted from the latter structures appears to contain about 25% as much radioactivity as that associated with the RNA from the Triton X100-insoluble fraction: this proportion is close to that reported previously for mitochondrial RNA from cells labeled with [5-^{3}H]uridine or [8-^{3}H]adenosine for 2 hours in the presence of 0·1 μg actinomycin D/ml (Ojala & Attardi, 1974*a*). In both the RNA from the detergent-insoluble structures and the RNA from the "polysomal" structures, a substantial proportion of the material bound to oligo(dT) in the first run is not retained in a second run performed after a denaturation step (about 70% and 30%, respectively). By contrast, only a small portion of the RNA not retained after the first chromatography binds to oligo(dT) in the second run (about 8% and 3·5%, respectively, in the material from the insoluble and the "polysomal" structures). It should be noted, however, that this unbound–bound fraction represents a non-negligible amount relative to the bound–bound fraction.

Figure 1 shows the autoradiogram, after agarose/CH_3HgOH electrophoresis, of different fractions obtained by one or two cycles of oligo(dT)-cellulose chromatography of RNA extracted from the "polysomal" structures of cells labeled for three hours with [^{32}P]orthophosphate in the presence of 20 μg camptothecin/ml. Equivalent portions of all fractions were run on the gel (except for the fraction bound in the first chromatographic run, which was used in one-fifth the amount), so that it is possible to make a direct quantitative comparison between the various electrophoretic patterns. After the first oligo(dT)-cellulose chromatography, the bulk of the two mitochondrial rRNA species, 12 S and 16 S RNA, is recovered in the non-bound fraction (Fig. 1(e), arrows), in confirmation of previous findings (Perlman *et al.*, 1973; Ojala & Attardi, 1974*a*). However, as shown in Figure 1(a) (arrows), appreciable amounts of these species are present in the bound fraction, in addition to a large number of other discrete components. After the denaturation step, all the 12 S RNA and all, or almost all, of the 16 S RNA present in the bound fraction are not retained on the second oligo(dT)-cellulose column (Fig. 1(c), arrows), while all the other components bound in the first run are also bound in the second run (Fig. 1(b)); presumably, these are the poly(A)-containing RNA components. One can also see in

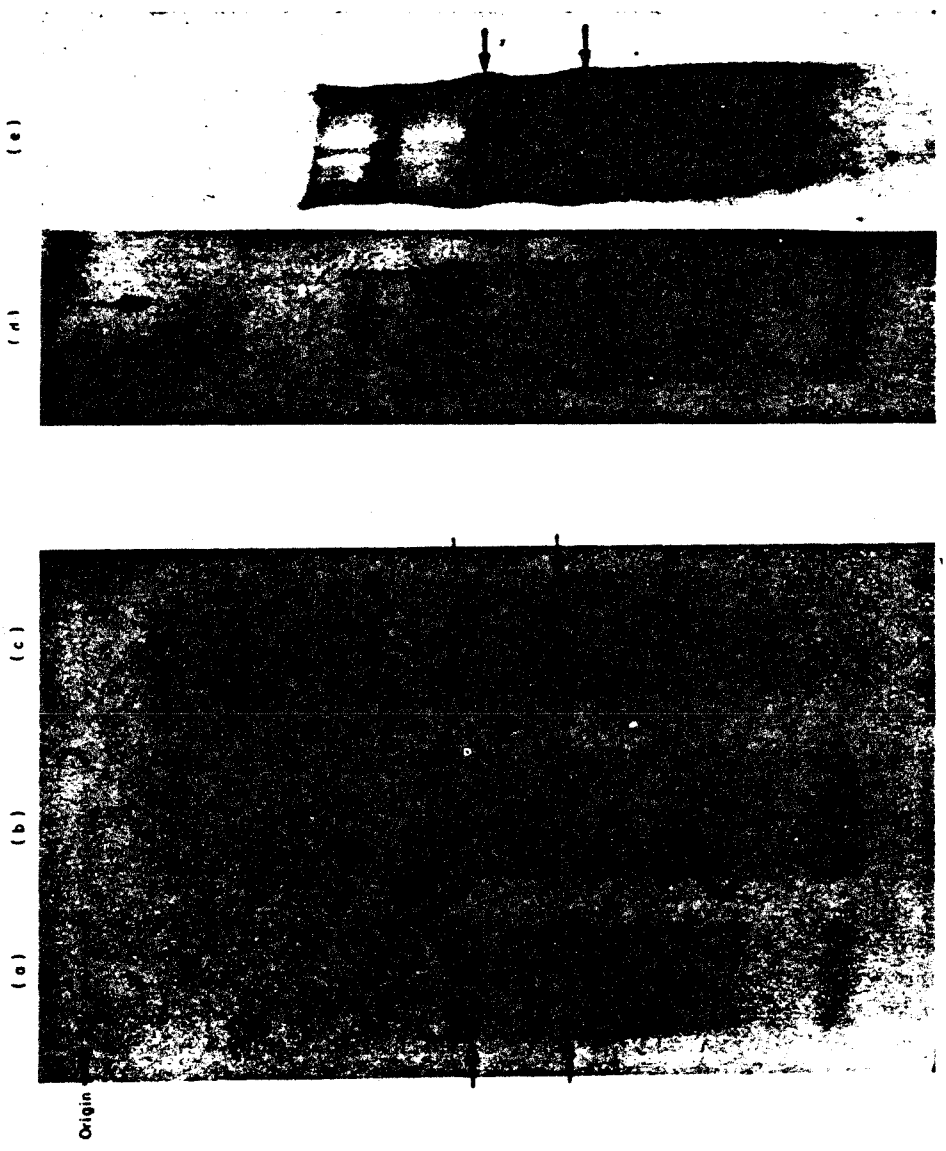

FIG. 1.

Figure 1 that the RNA fraction which was not retained in the first oligo(dT)-cellulose chromatography contains an appreciable amount of these same poly(A)-containing RNA components, which can be bound to oligo(dT)-cellulose after a denaturation step (Fig. 1(d)). Results qualitatively similar to those described above were obtained when an electrophoretic analysis was carried out on the different fractions of the first and second oligo(dT)-cellulose chromatography of RNA extracted from the Triton X100-insoluble components. These results indicate clearly that two cycles of oligo(dT)-cellulose chromatography, with a heat denaturation step before the second cycle, have the capacity of discriminating well between two different classes of mitochondrial RNA molecules, presumably poly(A)-containing and poly(A)-lacking species (see below).

(b) *Analysis by agarose/CH_3HgOH slab gel electrophoresis of mitochondrial RNA*

The electrophoretic patterns shown in Figure 1 illustrate the great resolution of mitochondrial RNA species achieved when CH_3HgOH is used as a denaturing agent during electrophoresis through agarose slab gels. Figure 2 shows the patterns obtained after longer electrophoretic runs of the RNA from the Triton X100-insoluble structures (oligo(dT) bound–bound (a) and unbound–unbound (b)) and from the "polysomal" structures (bound–bound (c) and unbound–unbound (d)) of cells labeled as in the experiment of Figure 1. In the oligo(dT)-bound RNA fraction from the Triton X100-insoluble components (Fig. 2(a)), at least 18 bands of varying intensity can be detected (designated by progressive arabic numerals in order of increasing electrophoretic mobility): band 18 corresponds to the 7 S RNA previously identified (Attardi & Ojala, 1974*b*) (see below). Furthermore, heterogeneous material can be seen as a fairly uniform background throughout the upper two-thirds of the gel. In the oligo(dT)-unbound RNA fraction from the same structures (Fig. 2(b)), 14 different bands are clearly resolved (designated by progressive roman numerals). Most prominent among these are bands VIII and XIII, which represent the two mitochondrial rRNA species 16 S and 12 S RNA (4 S RNA has run off the gel). Some of the bands appear to correspond in position to components of the oligo(dT)-bound RNA fraction; in particular, bands II to IX correspond to components 4 to 11. Other bands do not show any obvious correspondence, for example, bands XI to XIV. A large amount of high molecular weight heterogeneous RNA is present in the oligo(dT)-unbound RNA fraction. In the oligo(dT)-bound (Fig. 2(c)) and unbound (Fig. 2(d)) RNA fractions from the "polysomal" structures one can observe in general the same components as in Figure 2(a) and (b), though with some differences in the relative intensity of the bands. In particular, the high molecular weight oligo(dT)-bound discrete RNA components (1 to 3) appear to be present in much lower amounts in the polysomal structures. Also the high molecular weight heterogeneous RNA is present in reduced quantity in this fraction. The densitometric tracings shown in Figure 3 illustrate the relative proportions of the discrete and heterogeneous

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FIG. 1. Autoradiogram, after electrophoresis (3 h) through an agarose (1·4%)/CH_3HgOH slab gel, of different fractions of RNA extracted from the mitochondrial "polysomal" structures of cells labeled for 3 h with [^{32}P]orthophosphate in the presence of 20 μg camptothecin/ml. The fractions run on the gel are the once-bound to oligo(dT)-cellulose (a), the bound–bound (b), the bound–unbound (c), the unbound–bound (d), and the unbound–unbound (e). All samples analyzed here were in comparable amounts in terms of cell equivalents, except sample (a) which was in $\frac{1}{3}$ of the amount. All lanes are from the same slab gel.

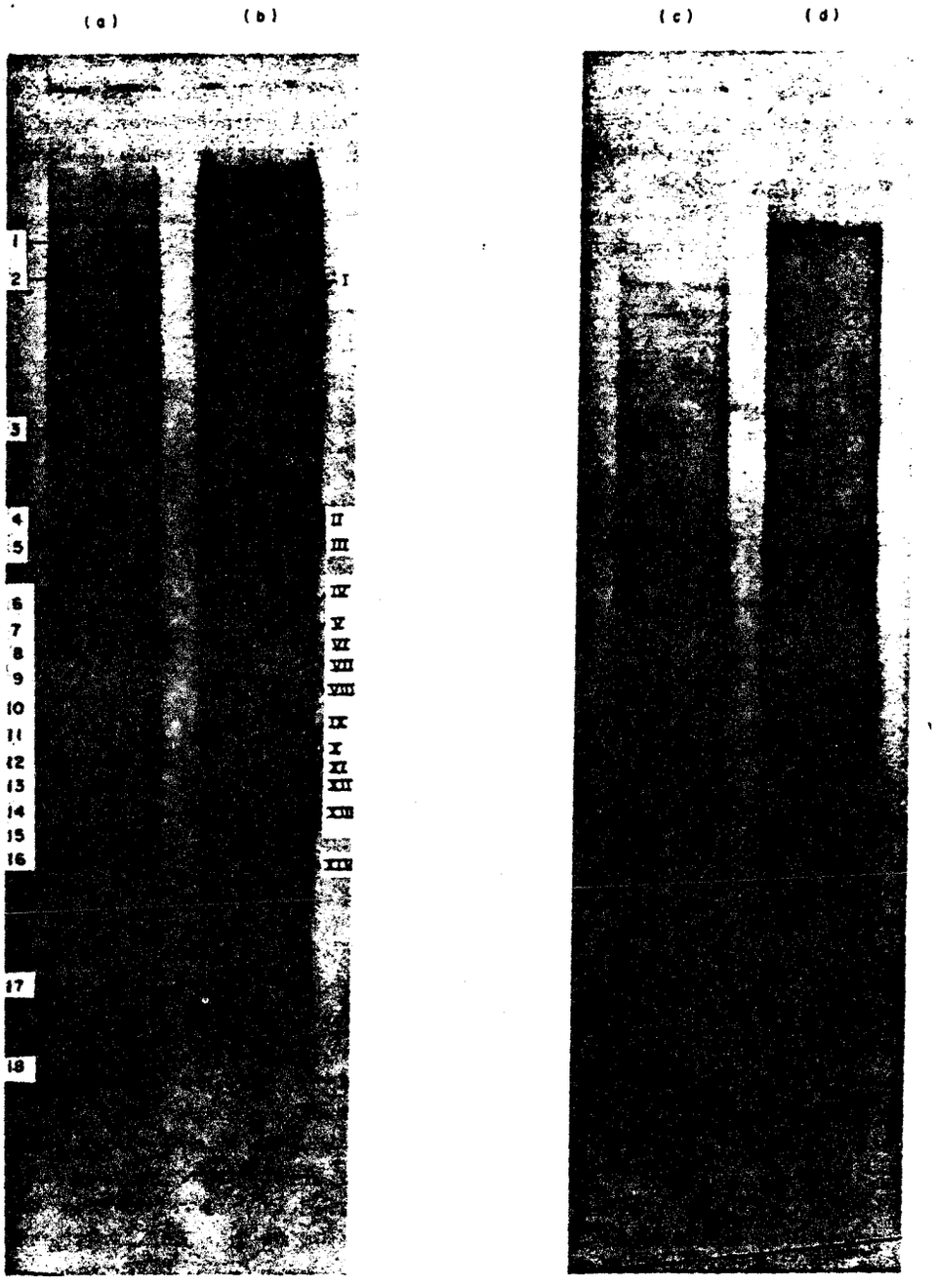

FIG. 2. Autoradiogram, after electrophoresis (4 h) through an agarose (1·2%)/CH_3HgOH slab gel, of the RNA from the Triton X100-insoluble structures (oligo(dT) bound–bound (a) and unbound–unbound (b)), and from the "polysomal" structures (bound–bound (c) and unbound–unbound (d)). The cells were labeled as in the experiment of Fig. 1. All lanes are from the same slab gel.

components in the four fractions of mitochondrial RNA analyzed in the above described experiment.

The correspondence in position of band 10 in the oligo(dT)-bound RNA fraction (Fig. 2(a)) and band VIII (16 S rRNA) in the oligo(dT)-unbound RNA fraction (Fig. 2(b)) suggested that band 10 might represent a residue of 16 S RNA still retained on oligo(dT)-cellulose after two passages. In agreement with this idea, when the bound–bound RNA fraction from the Triton X100-insoluble structures was heat-denatured in low ionic strength buffer and rerun for the third time through oligo(dT)-cellulose, substantially none of component 10 was retained (Fig. 4). (There is a very faint band in the bound RNA fraction, which moves, however, slightly slower than component 10.) Similarly, a large fraction of component 5 and most of the high molecular weight heterogeneous RNA was not retained in the third passage.

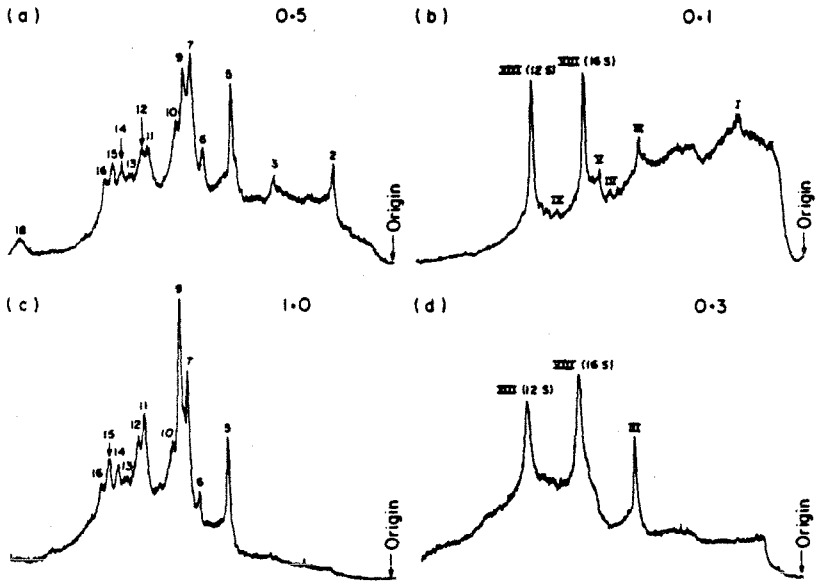

FIG. 3. Joyce-Loebl microdensitometer tracings of the autoradiograms shown in Fig. 2. The number in each panel indicates the fraction of the total sample run on gel.

(c) *Poly(A) content of the bound–bound and the unbound–unbound RNA fractions*

Previous work had shown the presence of poly(A) stretches corresponding to about 4 S_E† (~55 nucleotides) in mitochondrial oligo(dT)-bound RNA from HeLa cells (Perlman *et al.*, 1973; Attardi *et al.*, 1973; Hirsch & Penman, 1973; Ojala & Attardi, 1974*a*). In order to analyze the poly(A) content of the bound–bound and the unbound–unbound RNA fractions separated in the present work, these fractions were isolated from total mitochondrial RNA extracted by sodium dodecyl SO_4/proteinase K/phenol/chloroform from the EDTA-washed mitochondrial fraction of a mixture of cells labeled for three hours with [^{32}P]orthophosphate and cells labeled for three hours with [5-^{3}H]uridine (both in the presence of 20 μg camptothecin/ml).

† The symbol S_E is used to indicate the s value estimated from the relative electrophoretic mobility of the RNA.

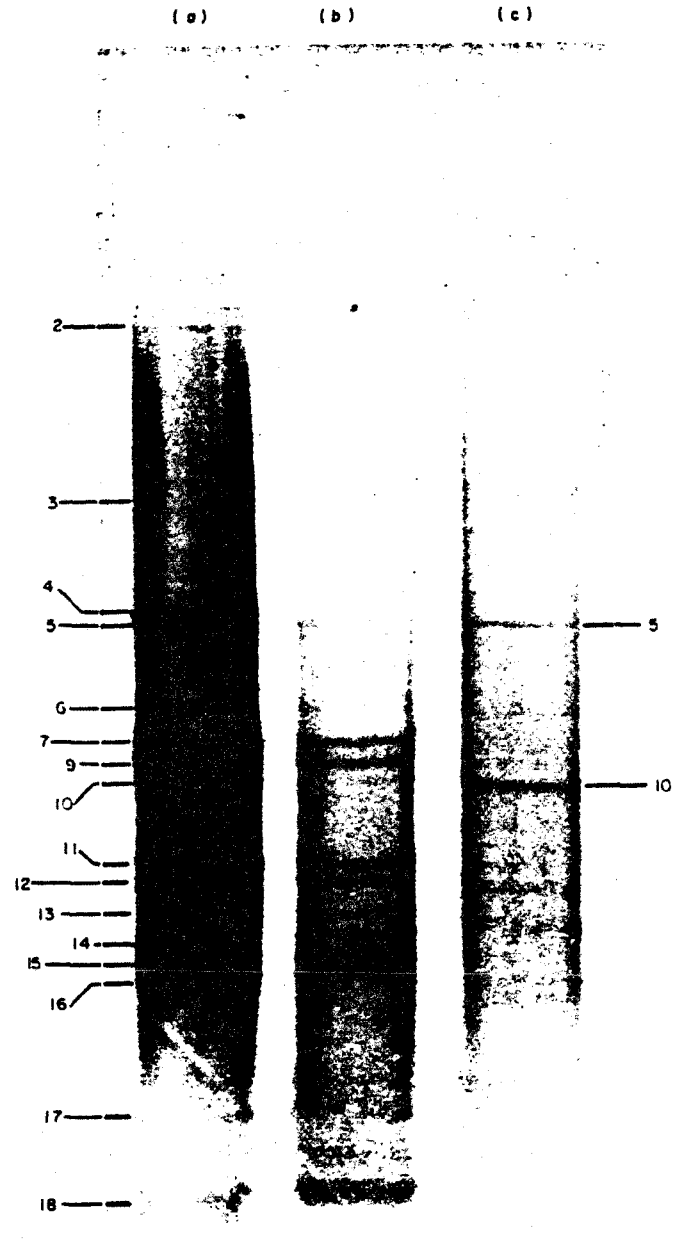

FIG. 4. Autoradiogram, after electrophoresis (4 h) through an agarose (1·2%)/CH_3HgOH slab gel, of the oligo(dT) bound–bound RNA from the Triton X100-insoluble structures of HeLa cells labeled as in the experiment of Fig. 1, before (a) and after a 3rd cycle of fractionation through oligo(dT)-cellulose ((b) bound, (c) unbound). The samples run in lanes (b) and (c) correspond to $\frac{1}{2}$ of the sample run in lane (a).

The two fractions, exhibiting a ^{32}P cts/min to ^{3}H cts/min ratio of about 2, were subjected to pancreatic and T_1 RNase digestion and polyacrylamide gel electrophoresis, as previously described (Ojala & Attardi, 1974*a*). In confirmation of previous findings, the digest from the bound–bound RNA fraction showed a relatively narrow peak of ^{32}P radioactivity migrating slightly faster than mitochondrial 4 S RNAs. There was no appreciable [5-^{3}H]uridine radioactivity in this peak; furthermore, nucleotide analysis of the 4 S_E peak after alkali hydrolysis revealed, as expected, only adenylate residues. A similar analysis performed on the unbound–unbound RNA fraction revealed ^{32}P-labeled and [5-^{3}H]uridine-labeled RNase-resistant material migrating in the gel in the region between 3 S_E and the buffer front. Nucleotide analysis of this material showed the presence of all four nucleotides in approximately equimolar amounts, suggesting strongly that it consisted of double-stranded RNA segments (Young & Attardi, 1975). Thus, this analysis failed to detect the presence of poly(A) stretches in the unbound–unbound RNA fraction.

(d) *Origin of the multiple discrete poly(A)-containing and poly(A)-lacking RNA components from the mitochondrial fraction*

The number of discrete poly(A)-containing components detected here, both in the RNA from the Triton X100-insoluble fraction and in the RNA from the "polysomal" structures, is considerably greater than the eight components previously identified by polyacrylamide/formaldehyde gel electrophoresis in the RNA from the "polysomal" structures and shown to be mtDNA coded (Ojala & Attardi, 1974*b*). In addition, a large number of discrete poly(A)-lacking RNA components was observed here. It thus became important to investigate the origin of these components.

The observation that the RNA analyzed here had been labeled with [^{32}P]orthophosphate in the presence of 20 μg camptothecin/ml, an effective inhibitor of high molecular weight nuclear RNA synthesis (Horwitz *et al.*, 1970; Kessel, 1971; Abelson & Penman, 1970), suggested that all the discrete components described above were of mtDNA origin. In order to verify the extent of inhibition of nuclear RNA synthesis by camptothecin under our experimental conditions, nuclear and post-mitochondrial cytoplasmic RNA were extracted from cells labeled with [^{32}P]orthophosphate for three hours in the presence of 20 μg camptothecin/ml: each RNA sample was then separated into an oligo(dT)-bound and a non-oligo(dT)-bound fraction by two passages through oligo(dT)-cellulose, using the standard technique employed in this work. Figure 5(a) to (c) shows the autoradiogram, after agarose/CH_3HgOH slab gel electrophoresis, of the poly(A)-containing fractions of, respectively, the nuclear, post-mitochondrial, and, for comparison, mitochondrial RNA. The labeled nuclear poly(A)-containing RNA (a) exhibits mainly high molecular weight heterogeneous components, with traces of the typical mitochondrial discrete components (resulting presumably from mitochondrial contamination of the nuclear fraction). The post-mitochondrial poly(A)-containing RNA (b) also shows, in addition to heterogeneous components, a small amount of discrete species; these, however, do not exhibit any obvious electrophoretic correspondence with the oligo(dT)-bound mitochondrial RNA components (c). Electrophoretic analysis of the labeled poly(A)-lacking RNA extracted from the nucleus revealed a heavy background of high molecular weight heterogeneous RNA with a few high molecular weight discrete components (>18 S_E) being barely discernible; the labeled post-mitochondrial poly(A)-lacking RNA showed only two weakly labeled bands at the expected positions for 18 S and 28 S rRNAs (not shown).

Thus, although there appears to be, in the presence of 20 μg camptothecin/ml, a residual synthesis in the nucleus, and transport to the cytoplasm, of RNA, this exhibits an electrophoretic pattern of discrete components very different from that of the mitochondrial RNA. These results, therefore, excluded a contamination by nuclear structures or post-mitochondrial supernatant as being responsible for the discrete components appearing in the mitochondrial fraction. They did not exclude, of course, a possible nuclear origin of some intramitochondrial RNA components or of RNA components associated with the endoplasmic reticulum which contaminates the mitochondrial fraction (Attardi *et al.*, 1969).

Strong evidence in favor of a mtDNA origin for all the discrete components recognized here came from an analysis of the effects of ethidium bromide, an effective inhibitor of mtDNA transcription (Zylber *et al.*, 1969), on the labeling of these components. As shown in Figure 5(d), the mitochondrial RNA extracted from cells exposed for three hours to [^{32}P]orthophosphate in the presence of 1 μg ethidium bromide/ml did not reveal any of the poly(A)-containing RNA components found in the absence of the drug (Fig. 5(e)), but only a small amount of low molecular weight heterogeneous RNA. Similar results were obtained for the non-poly(A)-containing RNA. Further evidence supporting the mtDNA origin of the discrete RNA components identified in this work has been provided by hybridization experiments with separated strands of mtDNA, to be described below.

In the experiments described in Figure 5, total mitochondrial RNA had been extracted with phenol/chloroform/isoamyl alcohol from the EDTA-washed mitochondrial fraction, after solubilization with sodium dodecyl SO_4 and digestion with proteinase K, as described in Materials and Methods. The electrophoretic pattern obtained for the oligo(dT) bound–bound fraction (Fig. 5(c) and (e)) is substantially identical to that observed for the corresponding RNA fraction isolated from the Triton X100-insoluble structures or from the "polysomal" structures (Fig. 2), except for the presence of an extra band migrating slightly faster than band 10 (designated 10a), which has been reproducibly observed after direct total mitochondrial RNA extraction.

(e) *Physiological nature of the multiple discrete RNA components resolved by agarose/CH_3HgOH slab gel electrophoresis*

A series of experiments was carried out to investigate whether the pattern of poly(A)-containing and poly(A)-lacking RNA species described here reflected a physiological situation. In the experiments detailed above, the labeling conditions had been chosen so as to maximize the incorporation of [^{32}P]orthophosphate into mitochondrial RNA. In particular, low phosphate concentration in the medium (10^{-4} M) and high cell concentration ($1{\cdot}5\times10^6$ cells/ml) were used (see Materials and Methods, section (a)). Since a low phosphate concentration has been previously shown to cause intramitochondrial pool abnormalities (Pica-Mattoccia & Attardi, 1972; Storrie & Attardi, 1972), and since the cell concentration used here during the labeling was such as not to allow exponential growth, the possibility of a perturbation of the normal mitochondrial RNA metabolism had to be considered. Therefore, experiments were carried out using either a tenfold higher phosphate concentration in the medium (10^{-3} M) or a cell concentration (4×10^5 cells/ml) which still allowed exponential growth. The oligo(dT) bound–bound RNA fraction extracted from the Triton X100-insoluble components of cells labeled under the above conditions showed

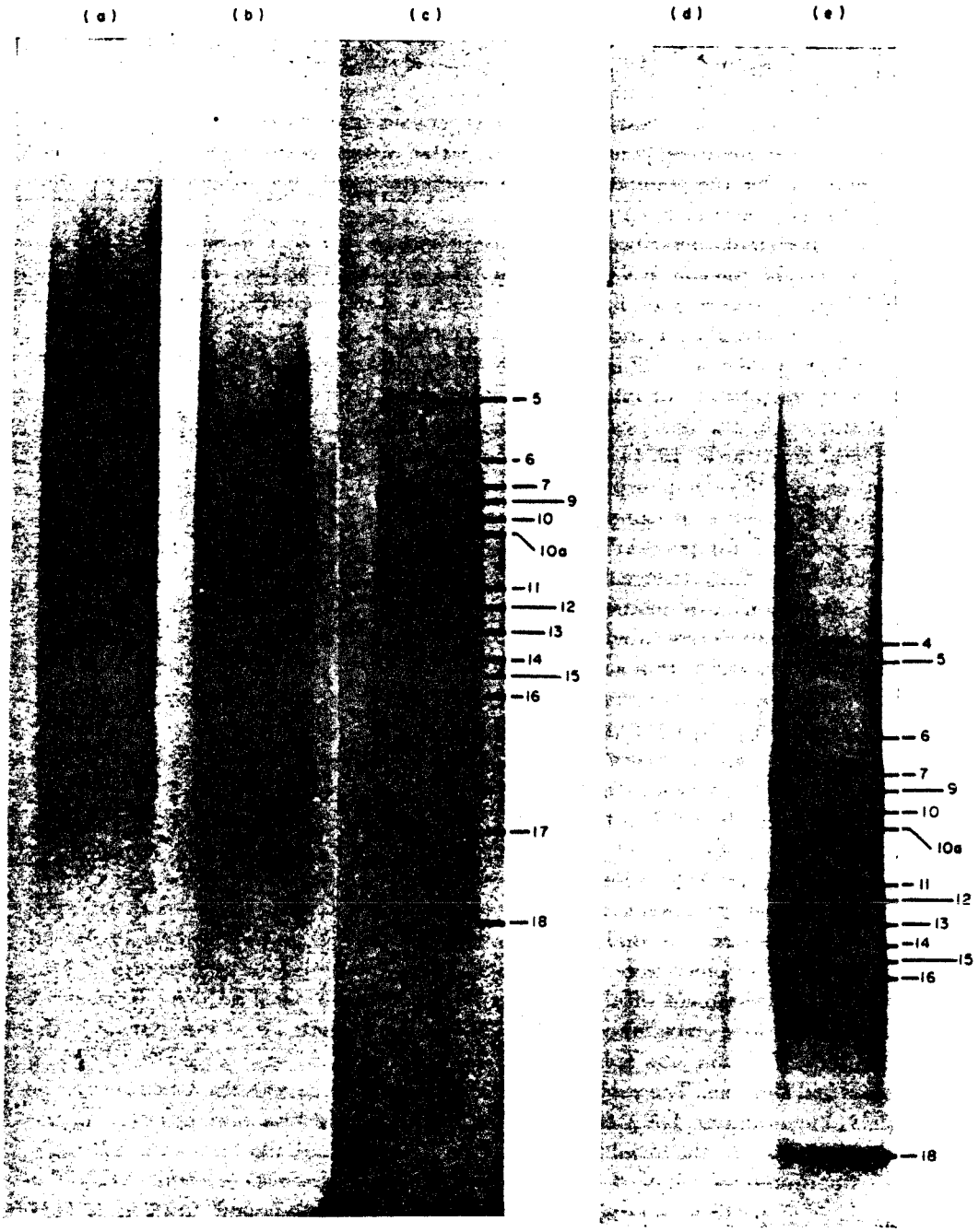

FIG. 5. (a) to (c). Autoradiogram (after 3 h electrophoresis through an agarose (1·2%)/CH_3HgOH slab gel) of the oligo(dT) bound–bound fraction of the RNA extracted from the nuclear pellet (a), or from the 12,000 *g* post-mitochondrial supernatant (b), or from the total mitochondrial fraction (c) of cells labeled as in the experiment of Fig. 1. The samples run in lanes (a) and (b) correspond, in cell equivalents, to $\frac{1}{4}$ of the sample run in lane (c).

(d) and (e). Autoradiogram (after 4 h electrophoresis) of the oligo(dT) bound–bound RNA fraction from the total mitochondrial fraction of cells labeled for 3 h with [^{32}P]orthophosphate in the presence (d) or absence of 1 μg ethidium bromide/ml (e).

all the main discrete components observed under standard labeling conditions; there were, however, in addition, two distinct components migrating faster than component 16 (not shown), which were either absent or present in barely perceptible amounts under the usual conditions. There was also a marked reduction in the relative labeling of the high molecular weight heterogeneous and discrete RNA components migrating slower than component 6. These latter observations suggested a possible interference, under the standard labeling conditions, with the normal rates of processing of mitochondrial RNA species.

In the experiments described above, camptothecin had been used to inhibit high molecular weight nuclear RNA synthesis. The possible effects of this drug on the synthesis and processing of mitochondrial RNA were investigated by comparing the pattern obtained by using this drug with that observed when actinomycin D (0·1 μg/ml) was used to inhibit the synthesis of nuclear rRNA, or with the pattern observed in the absence of inhibitors. Actinomycin D had been used previously in this laboratory in the analysis of mitochondrial poly(A)-containing RNA of HeLa cells (Ojala & Attardi, 1974*a*,*b*). In the presence of actinomycin D, the total incorporation of [^{32}P]orthophosphate into mitochondrial RNA was about 50% of that found in the presence of camptothecin. As shown in Figure 6, the electrophoretic patterns obtained for poly(A)-containing RNA extracted from total mitochondria of cells labeled in the presence of actinomycin D or camptothecin under the standard labeling conditions are qualitatively very similar. However, there are marked differences in the relative labeling of the various discrete components; in addition, the pattern of the RNA from actinomycin D-treated cells exhibits a well-recognizable component migrating faster than component 16 (16a), which is absent in the pattern of the RNA from camptothecin-treated cells labeled under the standard conditions. The electrophoretic patterns of oligo(dT) unbound–unbound RNA from actinomycin D or camptothecin-treated cells are substantially identical (Fig. 6).

The differences observed in the relative proportions of the various electrophoretic components, when the poly(A)-containing RNA fractions from actinomycin D or camptothecin-treated cells were compared, indicated that either one or both of these drugs affected the synthesis and/or processing of mitochondrial RNA in HeLa cells. It was thus important to analyze the pattern of poly(A)-containing mitochondrial RNA under conditions where any possible effect of inhibitors was excluded. This required the development of a procedure for the purification of mitochondrial RNA from contaminating extramitochondrial RNA components. For this purpose, the mitochondrial fraction was treated with RNase and then washed, as described in Materials and Methods. The very small amount of 18 S and 28 S rRNA detectable in the non-poly(A)-containing RNA fraction (Fig. 6) as compared to that expected in the untreated crude mitochondrial fraction (Attardi *et al.*, 1969), illustrates the effectiveness of the procedure followed here in destroying the extramitochondrial RNA. The electrophoretic pattern obtained for the oligo(dT) bound–bound fraction is substantially identical to that obtained for the corresponding fraction from camptothecin-treated cells. This result clearly shows that the great majority, if not all, of the discrete poly(A)-containing RNA components identified here result from normal mitochondrial RNA metabolism. There appears to be, however, a decrease in the relative amount of poly(A)-containing RNA components migrating slower than component 6; by contrast, there is more abundant heterogeneous material in the lower half of the gel in both the bound–bound and the unbound–unbound RNA

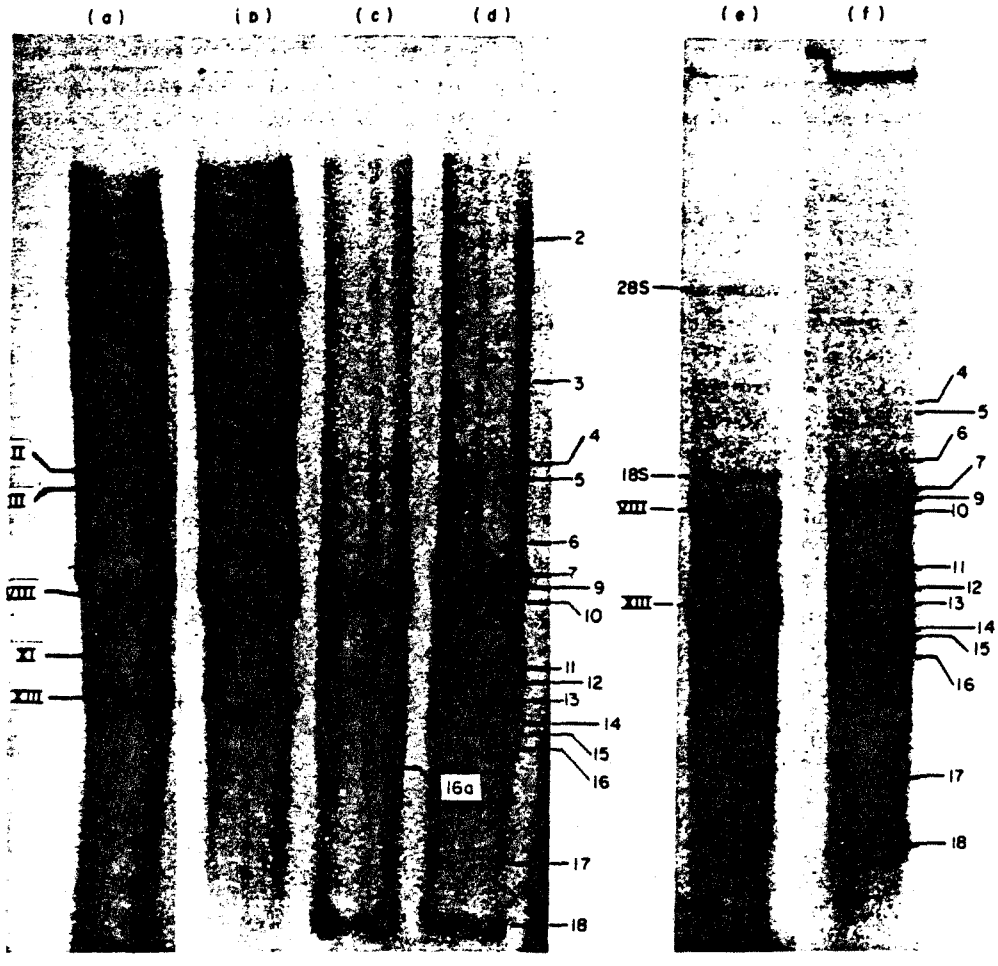

FIG. 6. Autoradiograms, after 4·5 h ((a) to (d)) or 3·5 h ((e) and (f)) electrophoresis through agarose (1·2%)/CH_3HgOH slab gels, of the RNA extracted from the total mitochondrial fraction of cells labeled from 3 h with [^{32}P]orthophosphate in the presence of 0·1 μg actinomycinD/ml (oligo(dT) unbound–unbound (a) and bound–bound (c)), or of 20 μg camptothecin/ml (unbound–unbound (b) and bound–bound (d)), or in the absence of inhibitors (unbound–unbound (e) and bound–bound (f)). The mitochondrial fraction from cells labeled without inhibitors of nuclear RNA synthesis had been treated with RNase and washed, as described in Materials and Methods.

fractions: this heterogeneous material presumably represents RNase digestion products not removed during the washing of mitochondria.

In the electrophoretic runs illustrated in Figure 6, the RNA sample from the RNase-treated mitochondrial fraction of cells labeled in the absence of inhibitors was extracted from about $1{\cdot}3\times10^8$ cells and the autoradiogram exposed for 48 hours; by contrast, the RNA samples from the untreated mitochondrial fraction of cells labeled in the presence of actinomycin D or camptothecin (under otherwise identical conditions) derived from about 2×10^7 cells and the autoradiogram was exposed for 17 hours. Thus, it is clear that the yield of labeled discrete components from RNase-treated mitochondria of cells labeled in the absence of inhibitors is considerably lower

than the yield from untreated mitochondria of cells labeled in the presence of actinomycin D or camptothecin. This lower yield has been reproducibly observed, and may be due to damage, and resultant permeability to RNase, of a substantial fraction of the organelles, and in part probably to losses of the organelles during the washing procedure.

(f) *Size of the discrete RNA components fractionated on agarose/CH_3HgOH slab gels*

In the 1·2% agarose slab gels containing 5 mM-CH_3HgOH routinely used in this work, a linear relationship was found to exist between distance of migration of several standards and the logarithm of their molecular weight in the range between 10^5 and $1{\cdot}5 \times 10^6$. In 0·8% agarose gels with 5 mM-CH_3HgOH, the above-mentioned linear relationship extends up to an M_r of 5×10^6. Figure 7 shows the plot of distance of migration *versus* the logarithm of M_r for the following ^{32}P-labeled markers: *E. coli* 23 S and 16 S rRNAs, HeLa cell 45 S, 32 S, 28 S, 18 S rRNAs, and HeLa cell 16 S, 12 S and 7 S mitochondrial RNAs. The M_r values used for the HeLa cell and *E. coli* rRNA species are those determined by Spohr *et al.* (1976) by electrophoresis in pure formamide at 55°C; for 16 S and 12 S rRNA the values previously estimated by electron microscopic measurements (Robberson *et al.*, 1971), and for 7 S RNA the

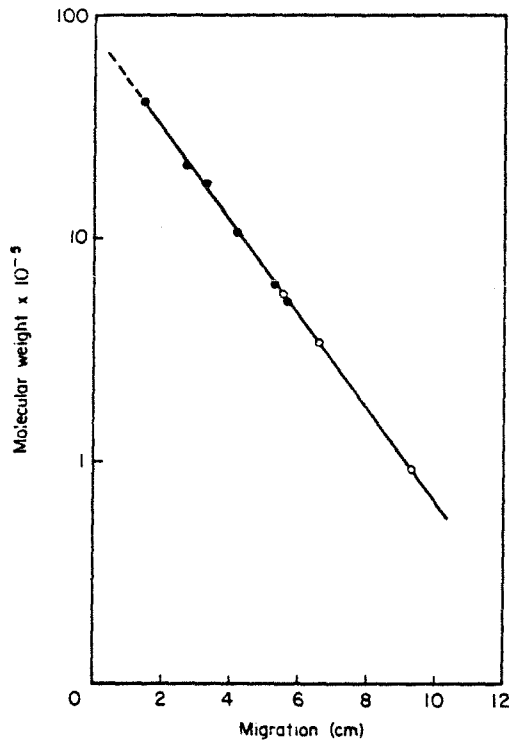

FIG. 7. Plot of the logarithm of molecular weight *versus* migration from the origin in an agarose (0·8%)/CH_3HgOH slab gel for various markers. The markers used were HeLa cell 45 S rRNA ($4{\cdot}1 \times 10^6$), 32 S rRNA ($2{\cdot}1 \times 10^6$), 28 S rRNA ($1{\cdot}75 \times 10^6$), 18 S rRNA ($6{\cdot}05 \times 10^5$) (Spohr *et al.*, 1976); *E. coli* 23 S rRNA ($1{\cdot}05 \times 10^6$) and 16 S rRNA ($5{\cdot}25 \times 10^5$) (Spohr *et al.*, 1976) (●); and HeLa cell mitochondrial 16 S rRNA ($5{\cdot}4 \times 10^5$), 12 S rRNA ($3{\cdot}5 \times 10^5$) (Robberson *et al.*, 1971) and 7 S RNA ($9{\cdot}3 \times 10^4$) (Ojala & Attardi, 1974*b*) (○).

value estimated by sedimentation analysis under denaturing conditions (Ojala & Attardi, 1974*b*) were used. The plot is linear for all these species, which differ widely in G + C content (from 75% for 45 S rRNA (Jeanteur *et al.*, 1968) to 44·6% for 16 S and 12 S rRNA (Attardi & Attardi, 1971)), as expected for fully denatured molecules. Notice that *E. coli* 16 S rRNA migrates slightly faster than HeLa cell mitochondrial 16 S rRNA, in agreement with the above mentioned M_r estimates. Using this standard curve and the migration in 0·8% agarose/CH_3HgOH gels of different poly(A)-containing and non-poly(A)-containing RNA species, we derived for their M_r values those reported in Table 2. The largest discrete component has an M_r around $3{\cdot}4 \times 10^6$, lower than the one expected for a complete transcript ($\sim 5 \times 10^6$). The upper limit of the heterogeneous RNA detectable in the oligo(dT) bound–bound RNA (Fig. 2(a)) and, in greater amount, in the oligo(dT) unbound–unbound RNA (Fig. 2(b)) corresponds to an M_r of about $4{\cdot}5 \times 10^6$. A good correspondence in M_r appears to exist between many of the poly(A)-containing and non-poly(A)-containing RNA components, in particular between components 4 to 12 of the bound–bound fraction and components II to X of the unbound–unbound fraction.

TABLE 2

Molecular weight of discrete RNA components bound and unbound to oligo(dT)-cellulose, estimated from electrophoretic mobility

Oligo(dT)-cellulose bound RNA component	Molecular weight $\times 10^{-5}$	Non-oligo(dT)-cellulose bound RNA component	Molecular weight $\times 10^{-5}$
1	34	I	27
2	28	II	9·0
3	14	III	8·6
4	9·0	IV	6·7
5	8·6	V	6·2
6	6·7	VI	6·0
7	6·2	VII	5·8
8	6·0	VIII (16 S RNA)	5·4‡
9	5·8	IX	4·2
10	5·4	X	4·0
11	4·2	XI	3·9
12	4·0	XII	3·7
13	3·6	XIII (12 S RNA)	3·5‡
14	3·3	XIV	2·7
15	3·2		
16	2·9		
17	1·4		
18(7 S RNA)	0·93†		

Estimates of molecular weights were made on the basis of the standard curve shown in Fig. 7.
† From Ojala & Attardi (1974*b*).
‡ From Robberson *et al.* (1971).

(g) *Sequence complementarity to heavy and light mitochondrial DNA strands of the discrete RNA components fractionated on agarose/CH_3HgOH slab gels*

In earlier work from this laboratory, it was shown that most of the mitochondrial poly(A)-containing RNA from HeLa cells hybridizes with the mtDNA H strand (Ojala & Attardi, 1974*a*): of the discrete components then identified, only one, the

7 S RNA, was found to hybridize with the L strand (Ojala & Attardi, 1974*b*). In the present work, most of the discrete components of the oligo(dT) bound–bound RNA fraction and some of the unbound–unbound RNA fraction have been eluted from a preparative gel, and tested for base sequence complementarity to separated mtDNA strands by RNA–DNA hybridization in DNA excess. Figure 8 shows that the individual poly(A)-containing RNA components, eluted from a preparative gel and rerun on an agarose/CH_3HgOH slab gel, migrate as sharp bands to essentially the same positions as in the first run (in the experiment shown here, a slight distortion in the right half of the gel during drying has resulted in an imperfect alignment of components 14 to 18 with the corresponding bands in the control run). Some of the bands exhibit a slight contamination by material of an adjacent band; more recently, it has been found that this cross-contamination can be almost completely eliminated

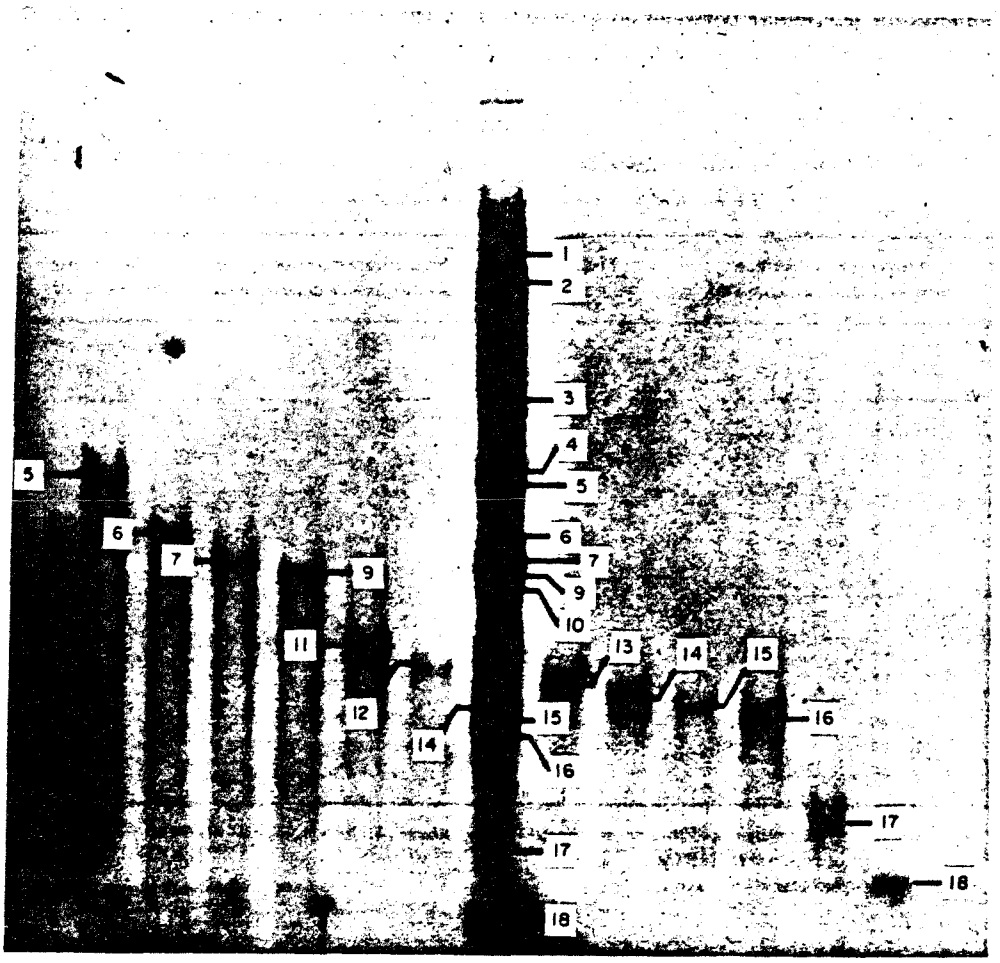

FIG. 8. Autoradiogram, after 4 h electrophoresis through agarose (1·2%)/CH_3HgOH slab gels, of individual oligo(dT) bound–bound RNA components, separated on a preparative gel, electro-eluted and rerun under identical conditions. A sample of the unfractionated preparation was run in parallel as a reference. A slight distortion in the right half of the gel has resulted in a non-perfect alignment of some components with the corresponding bands in the total oligo(dT) bound–bound RNA marker.

by precooling the preparative gel before the cutting. Poly(A)-containing RNA components 5 to 17 from both the Triton X100-insoluble structures and the "polysomal" structures were found to hybridize predominantly to the H strand; in contrast, component 18 (7 S RNA) hybridized mainly to the L strand, as previously reported (Ojala & Attardi, 1974*b*) (Table 3). Among the non-poly(A)-containing RNA components, in addition to the expected 16 S RNA (component VIII) and 12 S rRNA (component XIII), components III and V showed predominant sequence complementarity to the H strand. All the species which hybridized to the H strand showed a significant hybridization to the L strand (20 to 30% for the species extracted from the Triton X100-insoluble structures, 10 to 15% for the species extracted from the "polysomal" structures). As will be shown below, this partial hybridization to the L strand is probably due to heterogeneous material detectable in considerable amount in the electrophoretic patterns of the RNA from the Triton X100-insoluble structures, and in a much smaller amount in the patterns of the RNA from the "polysomal" structures. In agreement with this interpretation, the extent of hybridization with the L strand of the discrete poly(A)-containing RNA components was much reduced if these components were rerun on an agarose/CH_3HgOH gel before being tested (numbers in parentheses in Table 3). Component 18 (7 S RNA) showed a substantial hybridization to the H strand, in confirmation of earlier findings (Ojala & Attardi, 1974*b*); the significance of this phenomenon is not clear.

TABLE 3

Hybridization with separated mitochondrial DNA strands of ^{32}P-labeled RNA components bound and unbound to oligo(dT)-cellulose

Component	Triton X100-insoluble structures (% cts/min hybridized) H strand	L strand	"Polysomal" structures (% cts/min hybridized) H strand	L strand
A. Oligo (dT)-cellulose bound				
5	64	26	77	8
6	55	26	67	8
7	67	21	82	6
9	64	16	75	10
10	74	12	81	7
11	57(75)	21(11)	77	11
12	61	20	78	14
13	61(81)	21(9)		
14	62(66)	20(<1)	77	15
15	62(76)	21(8)	79	11
16	61(73)	19(5)	78	11
17	50(75)	31(21)	69	15
18 (7 S RNA)	34(29)	62(69)	38	59
B. Non-oligo(dT)-cellulose bound				
III	55	29	59	32
V	50	27	58	29
VIII (16 S RNA)	91	3	95	<1
XIII (12 S RNA)	95	<1	96	<1

The RNA components bound–bound and unbound–unbound to oligo(dT)-cellulose were separated on agarose/CH_3HgOH slab gels, and electro-eluted from the gel. The RNA samples (150 to 200 cts/min) were hybridized in solution with an excess of DNA. The numbers in parentheses refer to hybridization data obtained with individual RNA components rerun on gels.

(h) *Nature of the heterogeneous RNA*

The origin of the heterogeneous components present in the electrophoretograms of RNA extracted from the Triton X100-insoluble structures and the "polysomal" structures was investigated by carrying out RNA–DNA hybridization experiments between material eluted from different interband regions of the gel and separated mt DNA strands. The heterogeneous material of the oligo(dT) bound–bound RNA fraction from the Triton X100-insoluble structures (Fig. 2(a)) was found to hybridize with mtDNA to an extent varying in different experiments between 20 and 50% for the RNA in the upper two-thirds of the gel, and between 70 and 90% for the RNA in the lowest third. The non-hybridizable material presumably represents nuclear DNA-coded RNA which has escaped the camptothecin block. The heterogeneous RNA homologous to mtDNA hybridized predominantly to the L strand in the portion of the gel corresponding to a molecular weight range between 7×10^5 and $4{\cdot}5 \times 10^6$, and, by contrast, predominantly to the H strand in the region of lower molecular weight. In order to compare quantitatively the sequence complementarity to the two separated mtDNA strands of the RNA present in the individual bands of the gel and the RNA of the interband regions, the hybridization values obtained were corrected for the fraction of the eluted RNA utilized, and normalized to a unit area of the gel from which the RNA was eluted. As shown in Figure 9, the normalized levels of hybridization of components 5 to 16 with the H strand and of component 18 with

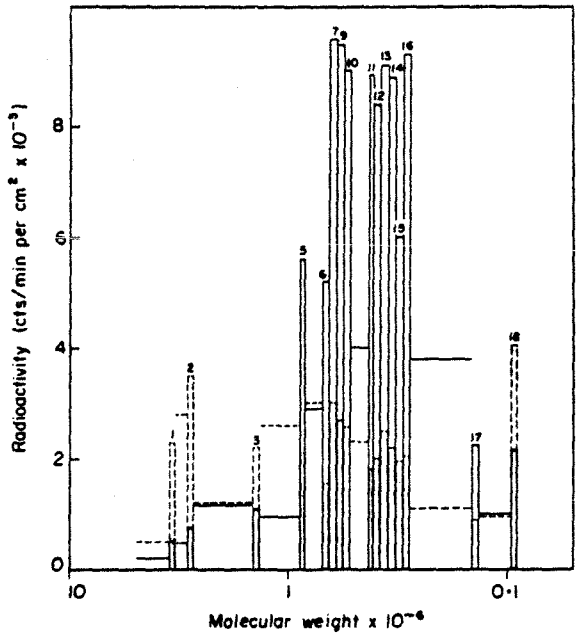

FIG. 9. Hybridization with the mtDNA H and L strands of RNA eluted from different segments of an agarose/CH_3HgOH slab gel of poly(A)-containing RNA. The oligo(dT) bound–bound RNA fraction from the Triton X100-insoluble structures of cells labeled as in the experiment of Fig. 1 was electrophoresed for 3 h; the RNA was eluted from the indicated cuts, collected by ethanol precipitation and centrifugation, and portions (150 to 200 cts/min) were hybridized with H or L mtDNA. The hybridization values for the total eluted samples are normalized to a unit area of the gel. The hybridization levels with the individual discrete RNA components are indicated by bars (numbered), those with interband heterodisperse RNA by horizontal lines. □, ———, hybridization level with the H strand; ▦, – – – –, hybridization level with the L strand.

the L strand are two to five times higher than the hybridization levels with the same strands of the heterogeneous RNA in the closest interband regions. By contrast, the normalized levels of hybridization of components 5 to 17 with the L strand are comparable to the hybridization levels measured for the heterogeneous RNA in the closest interband regions; these results support the idea that the heterogeneous RNA spread throughout the gel is responsible for the partial hybridization of the above mentioned discrete components with the L strand. The values of hybridization with the L strand obtained for the discrete components 1, 2 and 3 (Fig. 9) are not sufficiently high relative to those obtained for the heterogeneous RNA eluted from the closest interband regions that one can accept with confidence such hybridization values as indicative of homology of these components with the L strand.

The heterogeneous RNA from the Triton X100-insoluble structures which was not retained on oligo(dT)-cellulose after two passages was found in general to hybridize with mtDNA to a lower extent than the heterogeneous RNA bound to oligo(dT) (always less than 30% in the upper half of the gel). However, the electrophoretic distribution in this heterogeneous RNA of the molecules complementary to the L or H strand was very similar to that described above.

As previously mentioned, the RNA from the "polysomal" structures contains a much lower amount of high molecular weight heterogeneous components than the RNA from the Triton X100-insoluble structures. Also the contamination by nuclear RNA appears to be considerably less, as judged from the level of hybridization with mtDNA; however, the electrophoretic distribution of the heterogeneous RNA transcribed from the L and H mtDNA strands is similar to that described above for the RNA from the Triton X100-insoluble structures. In fact, all the heterogeneous RNA from the oligo(dT)-bound fraction which migrates in the gel region corresponding to an M_r of more than 10^6, and 50% of the corresponding RNA from the oligo(dT)-unbound fraction, hybridize with mtDNA, and, in particular, exclusively with the L strand.

4. Discussion

The main observation reported here is the unexpectedly large number of discrete components, both poly(A)-containing and non-poly(A)-containing, which are coded for by HeLa cell mtDNA. The reproducibility in the occurrence and relative amounts of these components, even when the RNA is directly extracted from the total mitochondrial fraction, argues against the possibility that these components result from degradation phenomena. Furthermore, the evidence presented above indicates that neither the drugs (camptothecin or actinomycin D) used to block high molecular weight nuclear RNA synthesis, nor the culture conditions existing during the labeling (high cell concentration and low phosphate in the medium) are responsible for the occurrence of these components. However, the use of either camptothecin or actinomycin D apparently has an influence on the relative proportions of the various discrete poly(A)-containing RNA components, as compared to the pattern observed in the absence of inhibitors. This effect is clearly and reproducibly different for the two drugs; furthermore, in the presence of either drug, there is a substantially larger amount of the high molecular weight heterogeneous RNA. Similarly, the use, during the labeling, of a cell concentration which allows exponential growth or of a phosphate concentration in the medium tenfold higher than routinely used has the effect of decreasing the relative labeling of the higher M_r ($>7\times10^5$) RNA components, both

discrete and heterogeneous; moreover, under these conditions, one can see two additional discrete components in the lower M_r range (migrating faster than component 16), which are either absent or present in marginal amount under the usual labeling conditions. The above observations suggest that the processing of mitochondrial RNA is very sensitive to the presence of camptothecin or actinomycin D and to the culture conditions.

The finding that the pattern of poly(A)-containing and non-poly(A)-containing RNA components isolated from RNase-treated mitochondria of cells labeled for three hours with [^{32}P]orthophosphate in the absence of inhibitors of nuclear RNA synthesis is very similar to that observed for the ethidium bromide-sensitive RNA extracted from camptothecin or actinomycin-treated cells has an important implication. It suggests that the great majority, if not all, of the discrete RNA components found in HeLa cell mitochondria are mtDNA coded.

The pattern obtained in the present work, by running through an agarose/CH_3HgOH slab gel the poly(A)-containing RNA extracted from mitochondria of cells labeled in the presence of actinomycin D (Fig. 6(c)), should be compared with the previously published pattern obtained after electrophoresis through cylindrical polyacrylamide/formaldehyde gels of poly(A)-containing RNA from mitochondrial "polysomal" structures labeled under similar conditions (Ojala & Attardi, 1974*b*). Component 1 in the earlier pattern presumably corresponds to unresolved components 7 to 10 of the present work, whereas components 2 to 7 correspond to the present components 11 to 16, and component 8 to the present component 18. The molecular weight estimates obtained in the present work for these components are in good agreement with the previous ones, with the exception of the value for the previous component 1. The absence of higher molecular weight components in the earlier study is presumably due to the fact that only RNA from "polysomal" structures was analyzed.

In the present work, substantially the same electrophoretic components were detected, though with some differences in the relative intensity of the bands, in the RNA from the Triton X100-insoluble structures and in the RNA from the "polysomal" structures (both in the fraction retained and in that not retained on oligo(dT)-cellulose). This suggests that the Triton X100-insoluble material may comprise RNA-containing structures similar or identical to those solubilized by the detergent, in particular, mitochondrial polysomes.

In the RNA from both submitochondrial fractions, many of the discrete RNA components not containing poly(A) (especially those larger than 16 S rRNA) appeared to correspond in molecular weight to poly(A)-containing RNA components. Some of these poly(A)-lacking components were present in small amounts relative to their poly(A)-containing electrophoretic counterparts, while others were present in comparable amounts (e.g., component III *versus* component 5). It seems possible that these poly(A)-lacking components represent precursors of the corresponding poly(A)-containing species, but further experiments are required to establish this point.

The sum of the sizes of the poly(A)-containing RNA components coded for by the H strand ($\sim 6 \times 10^6$) is equivalent to about one and a half times the length of the portion of the H strand which is not occupied by rRNA and tRNA genes ($\sim 3{\cdot}8 \times 10^6$) (Angerer *et al.*, 1976). This suggests that some of the discrete poly(A)-containing RNA components may be precursors to others, or that there may be overlapping transcription of segments of the H strand. The occurrence of overlapping coding sequences in ϕX174 (Linney & Hayashi, 1973; Barrell *et al.*, 1976) and simian virus

40 DNA (Rozenblatt *et al.*, 1976) suggests that overlapping of information may indeed occur in animal cell mtDNA. Furthermore, a comparison of the number and size of the discrete poly(A)-containing RNA species coded for the H strand with the map of the positions of the rRNA and 4 S RNA genes (Angerer *et al.*, 1976) allows some interesting conclusions. For example, the inter-4 S RNA gene spacings on the H strand in the HeLa mtDNA map apparently cannot accommodate all those poly(A)-containing RNA sequences. In particular, there appears to be no DNA stretch as long as the poly(A)-containing RNA component 5. These observations imply that at least some of the H strand-coded poly(A)-containing RNA species must contain one or more tRNA sequences in them. Similar considerations suggest that some of these poly(A)-containing species must contain sequences complementary to tRNA(s) coded for by the L strand (Attardi *et al.*, 1976).

Recently, a detailed physical map of HeLa cell mtDNA has been constructed in our laboratory using *Hpa*II (from *Haemophilus parainfluenzae*) and other restriction enzymes, and aligned with the map of the positions of the rRNA and 4 S RNA genes and the origin of replication (Attardi *et al.*, 1976; Ojala & Attardi, 1977). RNA–DNA hybridization experiments, in progress, between individual restriction fragments and the various RNA species identified here should provide evidence concerning the base sequence relationship among the different poly(A)-containing RNA components, and between these and the poly(A)-lacking species, and should lead to the assignment of their position on the genetic map. We have already been able to determine the map location of the sequences complementary to components 7, 15, 16 and 18 (C. Merkel, D. Ojala & G. Attardi, unpublished observations).

A particular mention should be made of the peculiar behavior exhibited on oligo(dT)-cellulose by the 16 S RNA. It was observed here that a fraction of this RNA species (estimated to be 5 to 10%) was still retained on oligo(dT)-cellulose after a second passage made following a denaturation step. A third cycle of heat denaturation and oligo(dT)-cellulose chromatography removed almost completely this residue of 16 S rRNA from the bound RNA fraction. This behavior was different from that of the 12 S rRNA, which was almost totally eliminated from the bound fraction after two passages. It is not known whether this behavior of the 16 S rRNA reflects the presence of a 3′-terminal poly(A) stretch shorter than the usual mitochondrial poly(A) in a fraction or all of the 16 S rRNA molecules, or of an internal A-rich sequence which is exposed only in a fraction of these molecules or too short to allow effective binding. The retention on oligo(dT)-cellulose of the total large mitochondrial rRNA species from *Drosophila melanogaster* has been recently reported (Spradling *et al.*, 1977). A behavior somewhat similar to that of the 16 S rRNA was exhibited by component 5; about equal amounts of this component were found in the bound and unbound RNA fractions after two cycles of oligo(dT)-cellulose chromatography; after a third cycle, however, the great majority of the bound portion was not retained on oligo(dT)-cellulose.

In addition to the discrete RNA species, a considerable amount of heterogeneous RNA transcribed from mtDNA has been detected in the present work. The great majority of this heterogeneous RNA was associated with the Triton X100-insoluble structures; furthermore, in both these structures and the "polysomal" structures, most of the mtDNA-coded heterogeneous components were found in the RNA fraction not retained on oligo(dT)-cellulose. Labeled nuclear DNA-coded heterogeneous RNA contaminated the mtDNA-coded RNA; this contamination was greater in the RNA

from the Triton X100-insoluble structures than in the RNA from the "polysomal" structures, and, in both RNA samples, more pronounced in the oligo(dT)-unbound than in the bound fraction. In all RNA fractions analyzed, the electrophoretic distribution of the heterogeneous components complementary to the L or H strand was very similar, i.e. predominance of L-strand-coded molecules in the range of M_r values larger than 7×10^5, predominance of H-strand-coded molecules in the lower M_r range. Particularly interesting is the distribution of the L-strand-coded molecules, which extends in the electrophoretogram up to the upper size limit of the detectable heterogeneous RNA, i.e. about $4{\cdot}5\times10^6$. This size is very close to that expected for a continuous transcript of the entire L strand, i.e. 5×10^6. These results strongly suggest that complete transcription of the L strand may occur in the form of long, continuous molecules; a progressive shortening of these primary transcripts could conceivably produce the heterogeneous distribution of molecules hybridizable with the L strand which has been detected in the denaturing gels. Experiments are in progress to test this interpretation. Previous electron microscopic evidence (Aloni & Attardi, 1972) has suggested that at least one of the two strands of HeLa mtDNA is transcribed in the form of a continuous RNA molecule.

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Chapter 3

Isolation and Purification of Mitochondrial RNA Labeled in the Absence of Inhibitory Drugs

INTRODUCTION

In the past, studies using radioactively-labeled mitochondrial RNA have generally relied on the use of drugs to inhibit nuclear labeling. Actinomycin D and Captothecin can be used to obtain mitochondrial RNA which is radiochemically pure; that is to say, the contaminating species are unlabeled or labeled to a much lesser extent than the mitochondrial species of interest. There are two major reasons why this approach is undesirable. First, contamination by nuclear and cytoplasmic RNA does exist; only the radioactive labeling of the contaminants is suppressed or reduced. Second, the drugs used to suppress nuclear labeling thereby create an unusual, non-physiological condition. Although the drug inhibition method is usually acceptable in qualitative studies, it is far less benign with respect to quantitative studies on the metabolism of mitochondrial components. Furthermore, the drugs themselves may have as yet unknown effects on mitochondrial function. Davidson (1976) has discussed at length some of the known effects of Actinomycin D which may include interference with cytoplasmic protein synthesis in addition to its effects in the cell nucleus. Storrie and Attardi (1972) have shown that interference with cytoplasmic protein synthesis has pronounced effects on mitochondrial function, including inhibition of DNA and RNA synthesis.

This chapter describes a method which was developed in order to study RNA metabolism of HeLa mitochondria under conditions which are as close as possible to "normal" cell function. Specifically, HeLa cells grown in spinner cultures can be labeled in the absence of inhibitory

drugs. The method described here is based on the observation (Barnett and Brown, 1967; Attardi et al., 1969) that intramitochondrial RNA is protected from RNase treatment. This suggested that perhaps cytoplasmic and nuclear contamination could be removed by treating intact mitochondria with some RNase. A similar method has been used by Spradling et al. (1977) but their methods of analysis of RNA were rather crude.

An additional problem regarding cell labeling is the problem of the toxicity of the label itself. The next chapter shows that it is possible to label HeLa cells with ^{32}P-orthophosphate in a manner such that the cells maintain exponential growth for 2 to 3 generations.

MATERIALS AND METHODS

Cell Growth and Labeling Conditions

Preparation and growth of cells has been described (Amalric et al., 1978). Some modifications were made in this procedure. Cells were labeled in the absence of inhibitory drugs such as Camptothecin and Actinomycin D at a cell concentration of 1×10^6/ml. Labeling was accomplished by the addition of ^{32}P-orthophosphate to a level of 50 microcuries/ml in the presence of 4×10^{-5} M cold phosphate for 2.5 hours.

Subcellular Fractionation

Cell washing and breakage were carried out as in Amalric et al. (1978) except that no EDTA was included in the buffers used for cell breakage and mitochondrial pelleting, but was instead replaced with 5×10^{-4} M magnesium ion. In some preparations, the RNase treatment of the mitochondrial pellet as described in Amalric et al. (1978) was

used, except that the concentration of RNase A (I.U.B. number 2.7.7.16) was 5 micrograms per milliliter and was supplemented with 50 units/ml of DNase 1 (Worthington, I.U.B. number 3.1.4.5).

In some experiments, mitochondria were treated with micrococcal nuclease (Staphylococcus aureus, I.U.B. number 3.1.4.7) instead of RNase A plus DNase 1. In these experiments, the mitochondrial pellet was suspended in a buffer consisting of 0.25 M sucrose, 0.01 M Tris, pH 8.0, 0.001 M $CaCl_2$. Micrococcal nuclease was added to a concentration of 15 units/ml. The suspension was then transferred to room temperature for 15 minutes, whereupon it was cooled to 0 degrees. EGTA was added to a concentration of 0.002 M in order to destroy the activity of the nuclease. After 5 minutes of EGTA treatment, the suspension was diluted with a large volume of 0.25 M sucrose, 0.01 M Tris, pH 7.4, 0.01 M EDTA and centrifuged at 10 KRPM in an SS-34 rotor. The supernatant containing degraded cytoplasmic material was carefully removed.

Mitochondrial Lysis and RNA Extraction

Mitochondria were lysed by the addition of SDS buffer (0.5% SDS, 0.01 M Tris, pH 7.4, 0.001 EDTA) which had been adjusted to 1% SDS. RNA was then extracted by the pronase-SDS procedure as described in Amalric et al. (1978).

Preparation of Polyadenylated RNA

The procedure as described by Ojala and Attardi (1974a) involving binding and elution of RNA from oligo-deoxythymidylate cellulose was followed with minor modifications. The cellulose used in these preparations was the T3 form (Collaborative Research) rather than T1.

RNA samples were heat denatured in a low salt buffer (0.01 M Tris, pH 7.4, 0.001 M EDTA) at 63 degrees for 4 minutes, then cooled on an ice bath. Binding of polyadenylated RNA was accomplished as before in a buffer containing 0.12 M NaCl, 0.01 M Tris, pH 7.4, 0.001 M EDTA, but elution was with low salt buffer (0.01 M Tris, pH 7.4, 0.001 M EDTA). This step differs from the previous work in the elimination of SDS from the elution buffer. Only one step of binding and elution was used rather than the two steps used in the previous work.

Gel Electrophoresis

Gel analysis was carried out as described by Amalric et al. (1978) using the method of Bailey and Davidson (1976) in which the strong denaturing agent methylmercuric hydroxide is used in agarose gels. Gels were dried onto filter paper as described and subjected to autoradiography using Kodak XR-5 film.

RESULTS

Mitochondrial polyadenylated RNA was prepared from micrococcal nuclease treated, SDS lysed mitochondria labeled with ^{32}P-orthophosphate in vivo as described in the Materials and Methods. Gel electrophoresis was carried out as described in Materials and Methods. The autoradiograph shown in Figure 1 displays the electrophoretic pattern obtained from that preparation in the track on the right. Other tracks show the electrophoretic patterns obtained for mitochondrial RNA prepared from the mitochondrial fraction of Camptothecin-treated cells as described in Amalric et al. (1978).

DISCUSSION

The patterns obtained from Camptothecin-treated cells and from cells labeled in the absence of inhibitory drugs are shown in Figure 1. Each of the tracks containing polyadenylated mitochondrial RNA shows the same pattern of 21 major species. The RNA from micrococcal nuclease treated mitochondria appears to be a little "cleaner" in the sense that the bands of radioactivity stand out from the background with more clarity and contrast. There are no major species present in one preparation and not present in the other.

The fact that micrococcal nuclease treated mitochondria do not contain other bands in addition to the bands labeled in the presence of Actinomycin D suggests that there is no transport of a significant class of messenger RNAs from the cytoplasm into mitochondria. If there were, these mRNAs would be labeled in the absence of Actinomycin and would be protected from micrococcal nuclease digestion once they had entered the mitochondria.

Since the RNA species observed in micrococcal nuclease treated mitochondria appear to be identical to RNA species which have been demonstrated to be of mitochondrial transcriptional origin by DNA-RNA hybridization (Amalric et al., 1978), it can be concluded that this method provides a convenient way of isolating labeled mitochondrial RNA under conditions which are less abusive of cell function than were previously available.

A major advantage of the procedure using micrococcal nuclease is that the activity of the enzyme can be eliminated by the use of suitable

chelating agents as has been done in the present work. The alternate method, using RNase A plus DNase 1, requires extended washing of mitochondria to remove the enzyme activities.

A modification of the micrococcal nuclease treatment of mitochondria in which enzyme treatment is carried out for 30 minutes at 0 degrees rather than for 15 minutes at room temperature has also been used and is described in the Appendix of Chapter 4. No significant loss of purification of mitochondrial polyadenylated RNA was observed after this modification.

Recently, mitochondrial polysomal poly(A)+ RNA has been prepared from micrococcal nuclease treated mitochondria labeled in the absence of inhibitory drugs. The electrophoretic profile of this RNA was essentially indistinguishable from the corresponding polysomal poly(A)+ RNA profile shown in Figure 1, except that the pattern from micrococcal nuclease treated mitochondria had lower levels of heterogeneous background (data not shown).

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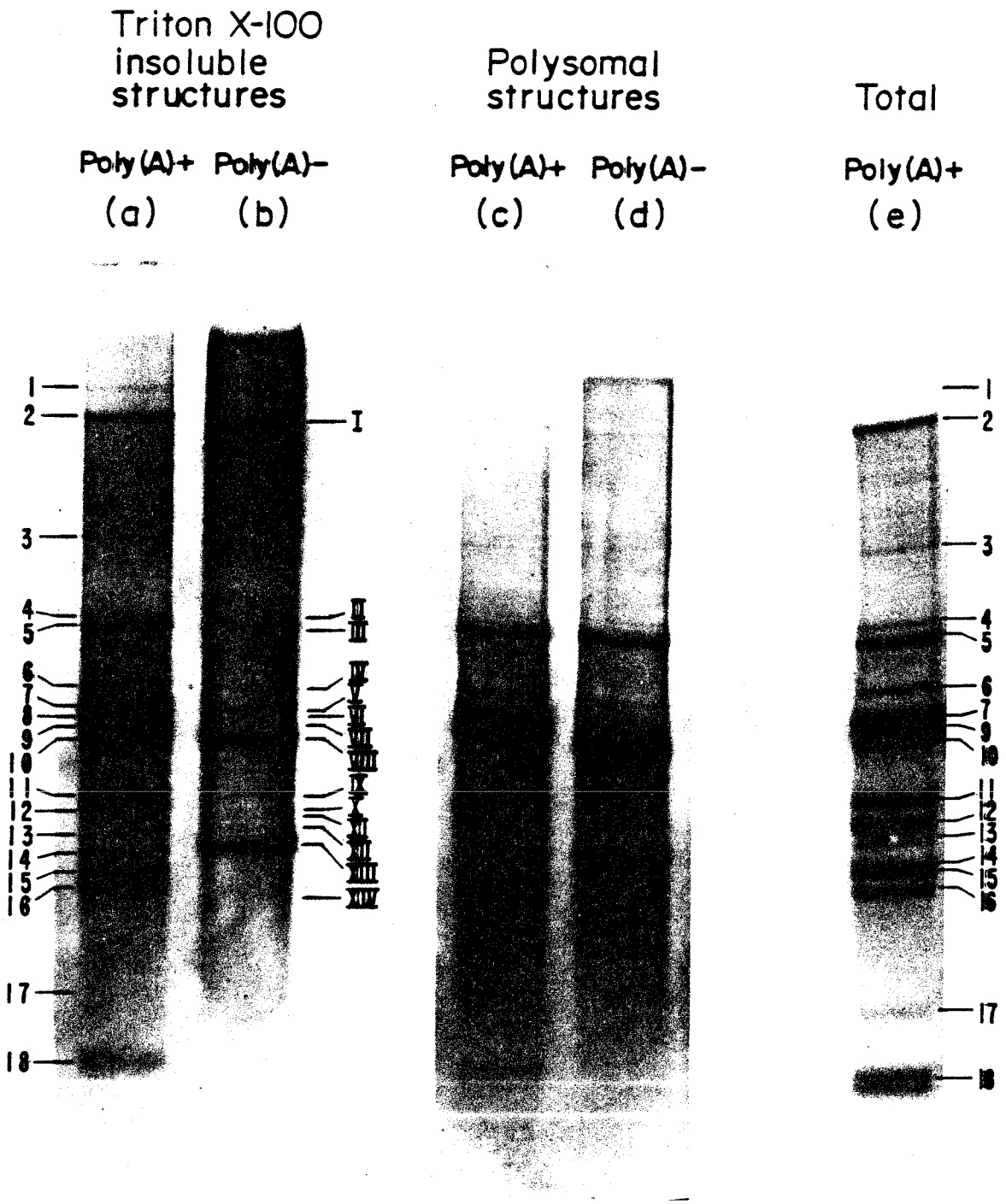
Figure 1

The electrophoretic patterns of ^{32}P-labeled mitochondrial RNA prepared in different ways is shown.

In columns a-d, the mitochondrial RNA from HeLa cells labeled in the presence of the drug Camptothecin is shown. Mitochondria were lysed with the detergent Triton X-100. The lysed material was centrifuged at 12 KRPM (SS-34 rotor) for 25 minutes. Material which was pelleted by that centrifugation is shown in columns a and b. The supernatant was spun through a 15-30% sucrose gradient and the material sedimenting in the polysome region of that gradient is shown in columns c and d.

The RNA whose pattern is shown in column e was purified from mitochondria treated with micrococcal nuclease as described in the text, extracted from cells labeled in the absence of inhibitory drugs.


Triton X-100 insoluble structures
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Poly(A)-
Poly(A)+
Poly(A)-
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Chapter 4

The Metabolic Stability of Individual Mitochondrial RNA Species

INTRODUCTION

The relative stability of eucaryotic cytoplasmic messenger RNA species, with half-lives of hours or days (Perry and Kelley, 1973; Murphy and Attardi, 1973; Galau et al., 1977) is in strong contrast to the relative instability of procaryotic mRNAs, with half-lives on the order of 2 minutes (Watson, 1976). Procaryotic mRNA is largely lacking the distinctive modifications of 3'-polyadenylic acid and 5'-caps to be found in eukaryotic messengers.

The situation with respect to mitochondrial messenger RNA should be of interest for several reasons. Mitochondrial mRNAs do contain 3'-polyadenylic acid, though of a shorter variety than cytoplasmic poly(A), and are translated in polyribosomes which show antibiotic sensitivities rather more like procaryotic polysomes than eucaryotic cytoplasmic polysomes (Perlman et al., 1973). Unlike their cytoplasmic counterparts, mitochondrial mRNAs appear to lack 5'-caps (Grohmann et al., 1978).

This apparent mixture of eucaryote-like and procaryote-like features may stem from an evolutionary origin for mitochondria at or soon after the divergence between procaryotic and eucaryotic forms (Raff and Mahler, 1972) or in contrast, it may stem from a mitochondrial origin as an endosymbiont which has gradually transferred functions to the nucleus (argued, for example, recently by Borst and Grivell, 1978), or perhaps there are other factors of which we are as yet unaware.

Published results of cytoplasmic mRNA half-lives must be considered to be average results which have been weighted in several ways, since there are thousands of different mRNA species in a given cell present

in different prevalence classes (Davidson, 1976).

The mitochondrial system is therefore interesting to consider for another reason, namely, that the products of mitochondrial transcription have been characterized (Amalric et al., 1978) and can be studied both in bulk and individually. The metabolic stabilities of mitochondrial RNA species should be considered both in absolute terms as they relate to the differences between procaryotic and eucaryotic mRNAs and also as a complete, related set of transcription products.

Two recent studies have suggested indirectly that mitochondrial mRNAs may have relatively long half-lives, on the order of hours or days. Lansman and Clayton (1975a and 1975b) largely destroyed the ability of mouse mitochondria to synthesize RNA. Under those conditions, mitochondrial protein synthesis continued essentially undiminished for 48 hours. England et al. (1978) obtained a similar result in enucleated African Green Monkey cells. Hirsch and Penman (1974a) have reported mitochondrial RNA half-lives on the order of 0.5-3 hours using techniques which required the use of two or more inhibitory drugs and which apparently produced bulk aggregated RNA of suspect origin.

For these reasons, it was of interest to measure the metabolic stability of mitochondrial RNA. It appeared that this could be accomplished for the individual RNA species as described in Amalric et al. (1978). It was particularly desirable to avoid drug artifacts, particularly considering the sorry history of RNA half-life measurements using the drug Actinomycin D as an instrument for "pulse-chase" experiments (see Discussion). The techniques involving RNase A and micrococcal

nuclease treatment of intact mitochondria in order to remove contaminating RNA species of cytoplasmic or nuclear origin were developed for this purpose and have been described in Chapter 3 of this manuscript.

There are two practical methods which can be used to measure the half-life of RNA. The first is the "pulse-chase" type of experiment. In this type of measurement, the decay of RNA is typically followed by the loss of radioactive label after the incorporation of new label is blocked in some way. The chase may consist of removing the label from the intracellular pool and the extracellular medium (see Murphy and Attardi, 1973, for example) or may consist of blocking new incorporation of label by the use of some drug. In this work, the drug cordycepin was used as a blocking agent. In all pulse-chase experiments, it is a critical requirement that the "chase" be demonstrated. Residual incorporation of label would theoretically result in an overestimate of the stability of the RNA under examination. Furthermore, the pulse-chase experiment is usually carried out in a manner which may conceivably disrupt cell metabolism. For instance, the blocking agent may have multiple effects, or the physical dilution of the intracellular precursor pools may require extended washing of the cells with much manipulation of the cells by repeated centrifugation or cold treatment (Murphy and Attardi, 1973, for example).

There is another method which is less physiologically disruptive. This method requires that the RNA species of interest and its precursor pools be labeled and purified. If it can be assumed that the rate of synthesis is constant or is some simple function (for example, in

exponentially growing cells, it is assumed that the rate of synthesis is constant for a given number of cells) and if it is also assumed that the RNA species of interest decays with first order kinetics (which can be verified by the pulse-chase experiment), then this equation (Galau et al., 1977) can be written:

$$dR/dt = k_s\ S(t) - k_d\ R$$

This equation relates the amount of radioactive label in RNA (R) to the total rate of incorporation and total rate of loss. Since the decay is assumed to be first order, the total rate of loss is the product of R and some rate constant k_d. Since the total rate of synthesis is constant, the total rate of radioactive incorporation is the product of the rate constant k_s and the specific activity of the precursor pool S(t). Given values for S(t) and R(t) for various times and given an initial condition, the equation can be solved numerically for the values of k_s and k_d. Galau et al. (1977) developed such an approach using a computer program to solve the equation numerically. Once k_d is known, the half-life of the RNA of interest is determined by the equation:

$$t_{\frac{1}{2}} = \text{natural log } (2)/k_d$$

It follows that measurement of RNA incorporation and of the specific activity of the RNA precursor pool over some time course of labeling allows the determination of the half-life of that RNA. This approach was used extensively in this work since it is non-intrusive except for the addition of radioactive label. Extensive controls were done to insure at least that cells continued to grow in mass and number in the normal way under the labeling conditions used for experiments 1 through 3 to be described below.

Since yields may vary for the extraction of RNA from different cultures, RNA values are determined in terms of label/mass, corresponding to the terms used to express precursor specific activity. In the present work, pulse labeling was accomplished by the addition of 5-^{3}H-uridine to cell cultures for different lengths of time. "Specific activity" refers to the quantity of label for some given unit of mass. Since the actual mass of individual mitochondrial RNA species purified from a few grams of HeLa cells is almost vanishingly small, an indirect method was used to determine the mass used for the determination of specific activity. In each of the kinetic experiments to be described, individual cell cultures were "long-term" labeled with ^{32}P-orthophosphate. Each culture received the same amount of ^{32}P label for the same length of time. Any individual RNA species or nucleotide precursor should therefore have been labeled to some particular value of ^{32}P/mass which should not vary from one culture to another. The extent of ^{32}P incorporation in any species should therefore be proportional to the mass in that species. Specific activity values in the experiments to be described are expressed as the ratio ^{3}H-CPM/^{32}P-CPM in which the ^{32}P value represents the mass term.

In some experiments (1, 2, and 3 to be described), ^{32}P labeling was carried out for a total of 24 hours for each culture. In experiments 4 and 5, ^{32}P labeling was not as long (3.5 hours for experiment 4 and 2.5 hours for experiment 5). In the later experiments, it is possible that the value ^{32}P/mass may differ for various RNA species in the same culture. This is due to the fact that the length of ^{32}P labeling does

not constitute several half-lives for all the species studied. The value of ^{32}P/mass should be the same, however, for any particular RNA species for all of the cultures from which it is extracted. Since specific activity is expressed as the ratio of two isotopes, the values for synthesis rates to be calculated from precursor and product specific activities are only relative synthesis rates. This, however, does not affect the determination of half-lives.

In the experiments in which ^{32}P labeling was carried out for 24 hours (1, 2, and 3), the different RNA species probably approached a uniform ^{32}P/mass value for all mitochondrial RNA. In this case, the relative labeling of RNA species with ^{32}P probably reflects closely their relative abundance.

A major difficulty in working with a subcellular organelle such as the mitochondrion is the difficulty in making sure that a soluble pool (such as the nucleoside-triphosphate pool) extracted from that source is not grossly contaminated by material of cytoplasmic or nuclear origin. One way of dealing with this problem is to measure the labeling of cytoplasmic and mitochondrial pools using the best purification techniques available and show that the mitochondrial pool shows different kinetics of labeling. This was accomplished (to be described below) for the CTP pool when labeling was done with ^{3}H-uridine and was also done independently for the ATP pool (described in the chapter on poly-adenylic acid). Another approach is to use some non-soluble fraction of unimpeachable mitochondrial origin from which the precursor pool specific activity can be determined. This was accomplished by using

the nascent RNA from mitochondrial DNA-RNA transcription complexes which can be isolated (Aloni and Attardi, 1972) and have been characterized as to purity (Carré and Attardi, 1978).

Five experiments using the "kinetics of labeling" approach and one pulse-chase experiment are described.

MATERIALS AND METHODS

Cell Growth and Labeling Conditions

Preparation and growth of cells has been described (Amalric et al., 1978). Some modifications were made in this procedure. First, cells were labeled in the absence of inhibitory drugs such as Camptothecin and Actinomycin D. Second, cells in some experiments were maintained at low cell concentrations in order to maintain exponential phase growth throughout the labeling period. Finally, in some experiments, the phosphate concentration of the medium prior to labeling was reduced to 4×10^{-5} M.

Subcellular Fractionation

Cell washing and breakage were carried out as in Amalric et al. (1978) except that no EDTA was included in the buffers used for cell breakage and mitochondrial pelleting, but was instead replaced with 5×10^{-4} M magnesium ion. In some preparations, the RNase treatment of the mitochondrial pellet as described in Amalric et al. (1978) was used, except that the concentration of RNase A (I.U.B. number 2.7.7.16) was 5 micrograms per milliliter and was supplemented with 50 units/ml of DNase 1 (Worthington, I.U.B. number 3.1.4.5).

In some experiments, mitochondria were treated with micrococcal nuclease (Staphylococcus aureus, I.U.B. number 3.1.4.7) instead of RNase A plus DNase 1. In these experiments, the mitochondrial pellet was suspended in a buffer consisting of 0.25 m sucrose, 0.01 M Tris, pH 8.0, 0.001 M $CaCl_2$. Micrococcal nuclease was added to a concentration of 15 units/ml. The suspension was then transferred to room temperature for 15 minutes,

whereupon it was cooled to 0 degrees. EGTA was added to a concentration of 0.002 M in order to destroy the activity of the nuclease. After 5 minutes of EGTA treatment, the suspension was diluted with a large volume of 0.25 M sucrose, 0.01 M Tris, pH 7.4, 0.01 M EDTA and centrifuged at 10 KRPM in an SS-34 rotor. The supernatant containing degraded cytoplasmic material was carefully removed.

Mitochondrial Lysis and RNA Extraction

In some experiments, mitochondria were lysed by the addition of SDS buffer (0.5% SDS, 0.01 M Tris, pH 7.4, 0.001 EDTA, 0.1 M NaCl) which had been adjusted to 1% SDS. RNA was then extracted by the pronase-SDS procedure as described in Amalric et al. (1978). In other experiments, mitochondria were lysed with Triton X-100 as described previously (Ojala and Attardi, 1974; Amalric et al., 1978). The procedure for preparation of polysomes by sucrose gradient sedimentation followed by RNA extraction as described therein was followed in the present experiments.

Preparation of Polyadenylated RNA

The procedure as described by Ojala and Attardi (1974a) involving binding and elution of RNA from oligo-deoxythymidylate cellulose was followed with minor modifications. The cellulose used in these preparations was the T3 form (Collaborative Research) rather than T1. RNA samples were heat denatured in a low salt buffer (0.01 M Tris, pH 7.4, 0.001 M EDTA) at 63 degrees for 4 minutes, then cooled on an ice bath. Binding of polyadenylated RNA was accomplished as before in a buffer containing 0.12 M NaCl, 0.01 M Tris, pH 7.4, 0.001 M EDTA, but elution was with low salt buffer (0.01 M Tris, pH 7.4, 0.001 M EDTA). This step differs

from the previous work in the elimination of SDS from the elution buffer. Only one step of binding and elution was used rather than the two steps used in the previous work.

Gel Electrophoresis

Gel analysis was carried out as described by Amalric et al. (1978) using the method of Bailey and Davidson (1976) in which the strong denaturing agent methylmercuric hydroxide is used in agarose gels. Gels were dried onto filter paper as described and subjected to autoradiography using Kodak XR-5 film. In some cases, the film was exposed at -70 degrees in conjunction with a DuPont "Cronex-lightning plus" image intensification screen.

Elution of Radioactive RNA from Dried Gels

Areas of interest were located on the autoradiographs. The corresponding area of the filter paper containing the dried gel was then excised and placed in low salt buffer (0.01 M Tris, pH 7.4, 0.001 M EDTA) which was heated to 90 degrees for 20 minutes. The soluble material was than mixed with a Xylene based scintillation fluid (Anderson and McClure, 1973) and counted in a Packard scintillation counter. This technique elutes greater than 95% of the radioactivity in the dried gel (data not shown).

In some experiments, individual tracks from dried slab gels were sliced sequentially on a Mickel gel slicer. Areas of the gel containing RNA species of interest were identified by autoradiography, cut from the dried gel, and sliced into 1 millimeter sections. The radioactivity in the individual sections was then eluted by heating in low salt buffer as described above.

Purification and Quantitation of Nucleoside Triphosphates

Isolation of a fraction containing nucleoside triphosphates was carried out using the charcoal binding and elution method described by Humphreys (1973). Separation of nucleoside triphosphates was carried out by the method of Neuhard et al. (1965), procedure 1, and sometimes by the method of Cashel et al. (1969). Elution of the triphosphates was carried out as described in the Neuhard et al. procedure. The eluted material was then mixed with Xylene based scintillation fluid (Anderson and McClure, 1973) and counted in a Packard scintillation counter.

DNA Purification and Strand Separation

The purification of mitochondrial DNA by repeated cesium chloride gradient centrifugation and the purification of separated light (L) and heavy (H) strands by alkaline cesium chloride gradient centrifugation has been described previously (Amalric et al., 1978).

DNA-RNA Hybridization

Hybridization in DNA excess has been described (Amalric et al., 1978).

Preparation of Mitochondrial DNA-RNA Transcription Complexes

The method used for purification of mitochondrial DNA-RNA transcription complexes has been described previously (Aloni and Attardi, 1972). Mitochondria are lysed with SDS buffer and the lysate is run through a 15-30% sucrose-SDS gradient over a cushion consisting of 64% sucrose in SDS buffer. Centrifugation is carried out at 25 KRPM for 5 hours at 20 degrees. The transcription complexes appear as a double peak immediately above the cushion.

Carré and Attardi (1978) have shown that the transcription complexes obtained in this way are largely free of contamination by other mitochondrial material.

Purification of L-strand Hybridizable RNA from Mitochondrial Transcription Complexes

The transcription complex material isolated from sucrose gradients as described above was precipitated by the addition of 2.5 volumes of ethanol. Following centrifugation, deproteinization was accomplished by treatment with pronase in the presence of SDS followed by repeated extractions with phenol:chloroform:isoamyl alcohol (50:50:1) as described previously (Amalric et al., 1978). Following ethanol precipitation and centrifugation, the remaining material was treated with DNase 1 as described (Amalric et al., 1978) except that treatment was carried out with 100 micrograms/ml DNase 1 for 60 minutes at room temperature. The DNased material was then deproteinized as before.

The RNA samples thus obtained were then hybridized to mitochondrial L-strand DNA under conditions of DNA excess as described above. The TCA precipitated DNA-RNA hybrids were eluted from their filter discs by heating in low salt buffer for 15 minutes at 90 degrees.

The DNA-RNA hybrids were subjected to alkali hydrolysis (0.5 N NaOH, 18 hours, 37 degrees).

Alkali hydrolyzable radioactivity was determined following TCA precipitation in the presence of 50 micrograms unlabeled BSA, centrifugation, and neutralization of the supernatant.

RESULTS

Six experiments were performed in order to determine the metabolic stability of mitochondrial RNA. Of these, one experiment was a "pulse-chase" and the others consisted of measuring the kinetics of incorporation of radioactive label into mitochondrial RNA in conjunction with measuring the specific activities of precursor pools.

Figure 1 shows the growth of HeLa cells in spinner cultures in the presence of 5 microcuries/ml ^{32}P-orthophosphate, 1 x 10^{-3} M cold phosphate. Growth as determined by cell concentration (i.e., the number of cells per ml) and by protein concentration is plotted as a function of time both for cells grown in the presence of ^{32}P and for control cultures grown without radioactivity.

In order to grow cells in such a way that they remain in exponential phase, it is necessary to keep them at rather low concentrations, typically 5 x 10^4/ml to 5 x 10^5/ml in HeLa grown in spinner cultures. Under these conditions, it is hard to obtain quantities of RNA and mitochondrial nucleoside triphosphates labeled to an extent adequate for analysis. However, it is possible to obtain large quantities of radioactivity incorporated into cytoplasmic nucleoside triphosphates. Figure 2 shows the kinetics of labeling of mitochondrial and cytoplasmic triphosphate pools as a function of the time of ^{3}H-uridine labeling, normalized to long term ^{32}P label. It can be seen that the UTP specific activities are almost identical for the cytoplasmic and mitochondrial pools. The CTP labeling time course is a little different at early time points, the mitochondrial pool lagging the cytoplasmic pool for perhaps 30-60

minutes but becoming essentially equal in specific activity to the cytoplasmic pool by 1-2 hours. Since the CTP pool specific activity is not very different except at the earliest times of labeling, and since the UTP pools are essentially identical in specific activity, the cytoplasmic nucleoside triphosphate specific activity values were used for the determination of mitochondrial RNA half-lives in experiments 1, 2 and 3. This made it possible to estimate the metabolic stabilities of mitochondrial RNA species in cells growing in exponential phase. This was desirable in order to rule out the effects of increased cell density on RNA stabilities. In other experiments, mitochondrial precursor pools were measured more directly, but these experiments required high cell densities in order to get sufficient radioactive incorporation.

In all of the kinetic experiments, cells were labeled with ^{32}P in order to normalize for differences in yield from time point to time point and in order to identify the mitochondrial RNA species by autoradiography. Figure 3 shows the pattern obtained from an experiment in which cells were grown at low density. HeLa cells were maintained in exponential phase throughout the labeling. Polysomal polyadenylated RNA was isolated as described in Materials and Methods and fractionated by agarose-methylmercury gel electrophoresis. Autoradiography was as described in the Materials and Methods. The typical pattern of mitochondrial RNA is seen for each of the six samples, each sample representing a different time of labeling with ^{3}H-uridine. Since the time and conditions of ^{32}P-labeling were identical for all six cultures, differences in intensity are the result of differences in yield.

This pattern is a little less sharp than other patterns obtained, for example, patterns obtained in chapter 3 of this manuscript. The reason for this is that the small amount of cells recovered after conditions of exponential phase growth results in a small amount of radioactivity recovered in the mitochondrial RNA fraction. In order to obtain exposure of the X-ray film with low levels of radioactivity, this gel was subjected to autoradiography in conjunction with an image intensification screen as described in the Materials and Methods. This increases the sensitivity of autoradiography enormously, but results in some loss of resolution in our hands.

In order to make sure that the RNA bands which were observed by autoradiography corresponded to the pattern of labeling achieved with ^{3}H-uridine, the gel was sliced into 1 millimeter sections after drying as described in the Materials and Methods. This reveals the electrophoretic pattern for both isotopes. The pattern is shown in Figure 4. In Figure 4A, the pattern obtained for all the RNA species of interest after a one hour labeling with ^{3}H-uridine is plotted. In Figure 4B, the patterns obtained for RNA species 11 and 12 is shown for all six times of ^{3}H-uridine labeling. The patterns obtained show clearly that the ^{3}H-uridine and ^{32}P-orthophosphate peaks coincide. This pattern is also useful for another reason. It is possible to see that the material in the regions between peaks is not negligible. This technique of serial slicing makes it possible to obtain the labeling kinetics of the background material and thereby to prove that it does not affect significantly the calculated values found for the half-lives of the

material in the peaks. In one experiment (experiment 2), the background material did affect the calculated half-life values for mitochondrial RNA species. In order to correct this problem, gel background was subtracted from the values for the peaks.

The half-life values for mitochondrial RNA species determined in six separate experiments are listed in Table 1. Experiments 1 through 5 are saturation kinetics experiments. Experiment 6 is the pulse-chase experiment.

The results of the first experiment are briefly summarized in Figure 5. Cells were maintained in exponential phase throughout the period of labeling. Polysomal polyadenylated mitochondrial RNA was obtained from RNase A plus DNase 1 treated mitochondria as described above. The mitochondrial nucleoside triphosphate precursor pool specific activity values were approximated by the cytoplasmic pool specific activity values as discussed above. Since the labeling was carried out for as long as 18 hours, it seems unlikely that the slight differences between cytoplasmic and mitochondrial CTP pools would have much effect on the calculated half-life values. The values for half-lives of mitochondrial RNA species were determined by means of a computer program developed for this purpose by Galau et al. (1977) and kindly supplied by them to us. The calculated values are listed in the first column of Table 1.

The results of the second experiment are briefly summarized in Figure 6. Cells were grown and labeled under the same conditions as in experiment 1. Isolation and RNase treatment of mitochondria was also identical. In this experiment, however, the mitochondrial pellet

purified of contaminating material by RNase treatment was immediately lysed in a buffer consisting of 1% SDS, 0.01 M Tris, pH 7.4, 0.1 M NaCl, 0.001 M EDTA (i.e., "SDS buffer" adjusted to 1% SDS). The purpose of this procedure was to obtain the entire intramitochondrial pool of RNA rather than just the portion sedimenting in the polysome region of a sucrose gradient. RNA obtained by SDS lysis of mitochondria is sometimes referred to in this manuscript as "whole mitochondrial RNA" or the "RNA obtained by SDS lysis of whole mitochondria." The large amount of background material found in the gel electrophoretic pattern of this experiment required that background radioactivity be subtracted from peak radioactivity in order to calculate half-life values. As can be seen in Figure 3, the background radioactivity varies slightly from one region of the gel to another. Separate values for background radioactivity were used for the region from species 7 to 10, the region from species 11 to 16, and for species 18. The calculated half-lives from this experiment are listed in the second column of Table 1.

The results of the third experiment are briefly summarized in Figure 7. Cells were maintained in exponential phase throughout labeling. Mitochondrial polyadenylated polysomal RNA was prepared as in experiment 1. This experiment is essentially a repeat of experiment 1 except that labeling was not carried out for as long a time. Also, RNA yields were somewhat better for this experiment than for experiment 1, resulting in somewhat greater precision in the data for incorporation of label into RNA. The calculated half-lives are listed in the third column of Table 1.

The fourth experiment differed in several ways from the first three. Cells were concentrated to approximately 2 x 10^6/ml. Higher levels of radioactive label were used. The mitochondria were purified of contaminating cytoplasmic and nuclear RNA by treatment with micrococcal nuclease rather than with RNase A. This method gives much higher yields of radioactivity in purified mitochondrial RNA than the methods involving exponential phase cells and RNase A treatment. However, HeLa cells are incapable of sustained growth for more than about one generation in the presence of ^{32}P at this concentration (data not shown). In the experiments carried out using highly concentrated cells to be described here, labeling with ^{32}P was never carried on for more than 3.5 hours.

In experiment 4, it was found that mitochondrial and cytoplasmic nucleoside triphosphate pools are not equal in specific activities under these conditions of growth and labeling. Figure 8A shows the time courses of specific activity for the cytoplasmic and mitochondrial UTP pools obtained in this experiment. Figure 8B shows the time course of incorporated label for some RNA species. Calculations were made using the mitochondrial UTP values to find the mitochondrial RNA half-lives. The results are summarized in the fourth column of Table 1.

The fifth experiment used the novel method referred to in the Introduction. Mitochondrial transcription complexes can be obtained by the method of Aloni and Attardi (1972). The transcription complex RNA is a more immediate precursor to mature mitochondrial RNA than the nucleoside triphosphate pools. The ability to obtain transcription complex RNA provided an opportunity to verify the half-life results

obtained in the other experiments. In order to minimize the chance that some stable or mature RNA would contaminate the transcription complex RNA, only that fraction of the RNA which was hybridizable to mitochondrial L-strand DNA was used in the calculations. This material is known to be extremely unstable (Aloni and Attardi, 1972b; Cantatore and Attardi, 1979) and therefore would represent the specific activity of the precursor pool in an accurate way. Transcription complex RNA was purified, hybridized to mitochondrial L-strand DNA, and precipitated on filters with 5% TCA as described. In order to eliminate the risk that some small DNA fragments from the transcription complexes might contaminate the RNA, alkali hydrolysis was carried out on material eluted from the filters by heating (Jeanteur and Attardi, 1969). Only the radioactivity found to be alkali hydrolyzable was used in the calculations.

Mitochondrial RNA in this experiment was obtained from micrococcal nuclease treated, SDS lysed mitochondrial pellets.

The calculated half-lives obtained in this experiment are listed in the fifth column of Table 1.

The "Pulse-chase" Experiment

Hirsch and Penman have claimed that the drug cordycepin, consisting chemically of 3'-deoxyadenosine, is an inhibitor of mitochondrial transcription (1974b). There are a variety of conflicting and sometimes confusing claims as to the effects and mechanism of action of cordycepin (briefly reviewed in the Discussion). For the purpose of doing a "pulse-chase" type of experiment, the drug would be required to block almost instantaneously incorporation of label into mature mitochondrial RNA

species. The experimental test of whether the drug can be used as the blocking agent in a pulse-chase experiment is whether it blocks the incorporation of label into the molecules of interest.

In order to test this, an experiment was carried out. Cells were pretreated with cordycepin at a final concentration of 50 micrograms/ml for various times. Labeling was then carried out with ^{3}H-uridine for 10 minutes. In one sample, no drug was added but labeling was carried out in the same way as for the other cultures in order to provide a zero-time value. This experiment could thereby show if cordycepin blocks incorporation into mitochondrial RNA, and if so, how long it takes for the block to occur and how effective the block is. Following harvesting of the cultures used in this experiment, the cells of each culture were mixed with equal amounts of ^{32}P-labeled cells in order to correct for differences in yield. Mitochondrial RNA was then obtained from micrococcal nuclease treated, SDS lysed mitochondrial fractions. RNA was then further fractionated by agarose-methylmercuric hydroxide gel electrophoresis. In this way, it was possible to test the effectiveness of cordycepin in blocking incorporation of label into individual RNA species.

The results of this experiment are summarized in Figures 10 and 11. Cordycepin blocks incorporation of radioactive label into most of the mitochondrial RNA species studied within 2.5-5 minutes. The block is close to 100% effective by 15 minutes for most species of RNA. The labeling of some RNA species proved to be resistant to cordycepin inhibition to some extent, in particular the small ribosomal RNA (12S) and the polyadenylated species 18. Species 18 continued to incorporate radio-

activity at an increasing rate for the first 5 minutes after the addition of the drug to cultures and showed a slow decrease in its labeling later. The small ribosomal RNA showed decreasing rates of radioactive incorporation following drug addition, but the inhibition was not strong enough to give a good "chase" for the purpose of determining the half-life of 12S RNA.

The pulse-chase experiment was carried out by prelabeling HeLa cultures in vivo for 2.5 hours with ^{32}P-orthophosphate and "chasing" by the addition of cordycepin to a final concentration of 50 micrograms/ml to block further incorporation of radioactive label into mitochondrial RNA. Prior to cell breakage, equal amounts of cells labeled with ^{3}H-uridine were added to the different cultures in order to correct for differences in yield. The results of this experiment are shown in Figure 10. The decay of individual RNA species as assayed by radioactive label is plotted semilogarithmically for several mitochondrial RNA species. Since there is some scatter in the data, curves were fit by a computer program which determined the best linear least squares fit to the log of the radioactivity values. The values calculated for the mitochondrial RNA half-lives determined in this way are listed in the sixth column of Table 1.

DISCUSSION

The data presented here represent the attempt to measure the metabolic stabilities of the entire set of transcription products of a single genome. RNA half-lives are considered both individually and as elements of a complete system.

Recent studies on the metabolic stability of cytoplasmic messenger RNA in various systems (Greenberg, 1972; Singer and Penman, 1972; Murphy and Attardi, 1973; Galau et al., 1977) show that it is relatively stable. RNA half-lives have been found to be several hours to one or more days, often being roughly equal to the cell generation time. These results are in marked contrast to stability values previously obtained in experiments using Actinomycin D to block transcription, in which the half-lives of messenger RNAs appeared to be several-fold shorter than they actually are (discussed by Perry and Kelley, 1973). This difficulty encountered in the Actinomycin D experiments has been attributed to a secondary action of the drug on translation (Singer and Penman, 1972).

Past experiments on various aspects of RNA synthesis and metabolism of mitochondria have typically used drugs to block nuclear RNA synthesis, in particular, Actinomycin D or Camptothecin. The experiments described here were conducted in the absence of inhibitory drugs (except when cordycepin was used with the intent of obtaining a blockage in incorporation of label into RNA) in order to eliminate the possibility of drug artifacts.

The results show some variability from experiment to experiment. The results of the experiment in which the precursor specific activity data were obtained from mitochondrial DNA-RNA transcription complexes

are the most reliable from the theoretical standpoint because they did not rely on soluble pools of nucleoside-triphosphates. In general, the results of the other experiments are consistent with the results of this experiment.

One possible source of error may be identified in this work. The transcription complex experiment involved purifying the RNA which hybridizes to the light strand of mitochondrial DNA because it is known to be extremely labile and would thus represent the condition of the precursor pools immediately before the time of cell harvesting. The major polyadenylated RNA species which label strongly are transcribed off the heavy strand (i.e., poly(A)+ RNA species 4-17) as demonstrated by Amalric et al. (1978).

Light and heavy strand transcripts may differ in base composition (as may RNA species transcribed from different regions of the same strand). If ^{3}H label in the nucleotide precursor pools were found only in UTP, the RNA species with lower uridylate composition would incorporate less ^{3}H label than RNA species with higher uridylate compositions. The change in labeling would remain proportional, however, for the various RNA species, differing only by some constant factor. The calculated half-life would therefore not be affected by differences in base composition among different RNA species; only the calculated relative rates of synthesis would be affected. In these experiments, however, some label may appear in CTP. Furthermore, the kinetics of the appearance of label in CTP may differ from the kinetics of label appearance in UTP. The actual specific activity for the precursor pool of some RNA species

should in theory be determined according to the base composition of that RNA species, particularly if the different precursors have different kinetics of labeling. For example, if the CTP pool becomes labeled much later than the UTP pool, the effect on RNA labeling would depend on the relative molar ratios of uridine and cytidine in specific RNA species. The use of the L strand hybridizable transcription complex RNA may therefore result in some error in calculated half-lives if the average base composition of the "precursor" RNA differs from the base compositions of the RNA species under investigation.

The same type of error is possible in the interpretation of the other experiments, even though UTP and CTP pool specific activities were measured directly. The values used for pool specific activities were constructed by adding the UTP and CTP specific activities weighted for the relative base composition of an "average" transcript of the mitochondrial H strand. Error is possible if the base composition of some individual RNA species differs significantly from the average base composition.

In spite of the fact that measurements of both precursor specific activity and RNA were made on material obtained in a number of different ways, the values obtained for the half-lives of individual species of RNA do not appear to differ grossly from experiment to experiment. No obvious difference is found for the metabolic stability of polysomal RNA as opposed to RNA extracted from whole mitochondrial lysates. No obvious differences exist in the measured half-lives of RNA obtained from cells growing exponentially as opposed to cells maintained at high cell concentration.

There is one difference which may be significant. The measured half-lives of ribosomal RNA species extracted from polysomes (experiment 1) appear to be somewhat longer than the respective half-lives of ribosomal RNA species obtained from whole mitochondrial lysates (experiment 2). This is not some experimental error of a trivial nature, since the respective half-lives of the polyadenylated species show the opposite relationship in these two experiments.

The Significance of the Results

The values found for the metabolic stabilities of the polyadenylated RNA species range from a minimum of at most 7 minutes (RNA species 1 and 2) to a maximum on the order of 100 minutes (RNA species 14 and 15). The half-lives of the ribosomal RNA species were longer, but not by more than a factor of 2 or 3. There appear to be no stability classes encompassing large fractions of the molecular species but rather a set of individual values.

There is no obvious relationship between the sizes of the molecules and their respective half-lives, as would be expected if RNA degradation were the result of random endonucleolytic events.

Of the polyadenylated RNA species studied, species 2, 4, and 6 appear to be precursors to other species (Ojala et al., manuscript in preparation; Gelfand et al., manuscript in preparation). Species 4 appears to be a precursor to the ribosomal RNA species and species 6 appears to be a precursor to species 9. In each case, the precursor molecule is less stable than the product, but not by a vast difference. The nature of polyadenylated RNA species 2 is discussed elsewhere in

this manuscript (in the chapter on giant L strand transcripts). It may be a precursor to one or more tRNA molecules, or it may have some other function as yet unknown.

RNA Stability and Protein Synthesis

Two sets of experiments should be considered in relation to these results. England et al. (1978) enucleated cultured cells from African Green Monkey. The level of mitochondrial protein synthesis did not drop significantly in the 24 hours following enucleation even though mitochondrial RNA synthesis was strongly inhibited under these conditions. Lansman and Clayton (1975a and 1975b) destroyed most of the transcriptional capacity of the mitochondria of a line of mouse cells by light irradiation of cells whose mitochondrial DNA contained bromodeoxyuridine substituted for thymidine. In this case, mitochondrial protein synthesis continued without significant decline for 48 hours.

It was suggested by the latter authors that mRNA is not rate limiting in mitochondrial protein synthesis based on this finding. It is hard to imagine that there is such a vast excess of mitochondrial mRNA that it can decrease with a half-life of less than two hours for one to two days and still maintain unchanged synthetic ability. This would require a several thousand-fold excess of mRNA over the amount actually required for normal protein synthesis.

There are several possible explanations of this seeming paradox. First, the RNA whose metabolic stability was measured in the study described in this chapter might not be the mRNA used for the synthesis of much of the mitochondrial protein. The evidence presented in Chapters 2 and 3

of this manuscript against the likelihood of import of mRNA into mitochondria of HeLa argue against this possibility. Second, there might be something wrong with the other experiments. The fact that the experiments were carried out by different laboratories using very different methods suggests that this is probably not the case. Last, it is possible that both of the previous works and the results discussed in this manuscript are all basically correct. In that case, the stability of mitochondrial mRNA must have been greater under conditions of enucleation or light-induced destruction of mitochondrial RNA than it is under normal physiological conditions. In other words, the enucleation and light nicking of mitochondrial DNA somehow stabilized mitochondrial mRNAs.

Storrie and Attardi (1972) showed that inhibition of cytoplasmic protein synthesis repressed mitochondrial synthesis of DNA and RNA in HeLa. The enucleation experiments represent an analogous case in which loss of a non-mitochondrial function results in loss of transcription in mitochondria.

It is, therefore, possible that the metabolic turnover of mRNA in HeLa mitochondria is linked to some mitochondrial process such as transcription or DNA synthesis. Another possibility is that the metabolic turnover of mRNA in mitochondria is linked to something which requires both nuclear and mitochondrial gene function.

In some experiments, including experiments 4 and 5 discussed in this chapter, mitochondrial RNA has been obtained after a treatment of the intact mitochondria with micrococcal nuclease, which was carried out at room temperature for 15 minutes. The fact that mitochondrial

RNA survives for 15 minutes at room temperature without noticeable degradation is suggestive that mitochondria may contain very little RNase activity or that the RNase activity which they do contain is highly labile. It is possible to speculate on the possibility that RNase activity in mitochondria depends on some mitochondrial function but there are no data at present in regard to this.

The drug cordycepin (3'-deoxyadenosine) which was used in one of the experiments described here may perturb cellular biochemistry in several ways. Penman et al. (1970) demonstrated that cordycepin inhibits labeling of messenger RNA in HeLa cells while apparently affecting labeling of heterogeneous nuclear RNA much less. It has been argued that the inhibition of labeling of mRNA by cordycepin was due to blockage of polyadenylation of the mRNA precursor in the cell nucleus (Darnell et al., 1971). Rose et al. (1977) have demonstrated that polyadenylation is inhibited in isolated nuclei by cordycepin 5'-triphosphate. Diez and Brawerman (1974) showed that polyadenylic acid sequences attached to large RNA molecules undergo turnover of the adenosine residues at the 3' end. This turnover was found to be essentially insensitive to cordycepin. Klenow and Frederiksen (1964) suggested that cordycepin inhibits the activity of DNA-dependent RNA polymerase.

More recent works suggest that cordycepin may have other effects. Glazer and Peale (1978) found an inhibition of methylation of nuclear RNA in cells treated with cordycepin. Deitch and Sawicki (1979) found that cordycepin-treated cells accumulate in mitotic metaphase. The effect was attributed to inhibition of microtubule formation. Beach

and Ross (1978) have recently obtained results which strongly suggest that cordycepin inhibits the transcription of globin mRNA sequences.

The confused state of the literature and lack of agreement as to the mechanism of action of cordycepin are supportive of the argument that the "chase" in a pulse-chase experiment must be demonstrated directly. In particular, the inhibition of labeling by a putative blocking agent should be demonstrated at the level of specific sequences or species of RNA, as was done in the present work, if there is any question as to sequence specificity of the labeling block.

The somewhat higher half-life values determined by the cordycepin chase experiment may be partly due to an incomplete chase and/or partly due to some secondary action of the drug.

A. Sample Calculation: The Synthesis Rate of Poly(A)+ RNA Species 16

1) In a typical culture, 212 cpm of RNA species 16 were recovered from 0.75 ml of cells (counts corrected for ^{32}P decay between labeling and counting). This value must be converted to mass of RNA, expressed as moles or pg.

2) The molar specific activity of the phosphate pool can be calculated since a small mass of ^{32}P-orthophosphate was added to a large mass of cold phosphate:

$$\frac{4 \text{ mCi}}{(5 \times 10^{-4} \text{moles } l^{-1})(0.8 \text{ l})} = 10^4 \frac{\text{mCi}}{\text{mole}}$$

3) Assuming that moles phosphate = moles nucleotide in RNA,

$$212 \text{ cpm} \cdot \frac{1 \text{ mole}}{10^4 \text{mCi}} \cdot \frac{1 \text{ mCi}}{2.2 \times 10^9 \text{ cpm}} = 9.6 \text{ pmole}$$

$$= 3.17 \times 10^3 \text{ pg}$$

$$= 60 \text{ molecules per cell}$$

This is the experimentally determined steady state level of RNA species 12.

4) Using the calculated K_s value by numerical integration

$$K_s = 0.016 \text{ cpm} - {}^{32}P \cdot \text{min}^{-1}$$

This value must be corrected for ^{32}P recovery, since it was calculated using ^{3}H incorporation values normalized to ^{32}P recovery. Furthermore, the precursor specific activity data were not corrected for the fact that only one phosphate group from a triphosphate ends up in the product molecule. Therefore, the corrected K_s is found by multiplying the calculated value of K_s by these factors:

$$K_s = 0.016 \text{ cpm } {}^{32}P \text{ min}^{-1} \ (212) \ (\tfrac{1}{3})$$

$$K_s = 1.13 \text{ cpm min}^{-1}$$

5) This may be converted to $pg \cdot min^{-1}$ or $pmole \cdot min^{-1}$

$$K_{s_{mole}} = 1.13 \text{ cpm} \cdot \text{min}^{-1} \cdot \frac{1 \text{ mole}}{2.2 \times 10^{13} \text{ cpm}}$$

$$= 5.1 \times 10^{-14} \text{ mole min}^{-1}$$

6) To find the number of RNA molecules synthesized per minute:

$$5.1 \times 10^{-14} \frac{\text{mole nucleotide}}{\text{min}} \cdot \frac{1 \text{ mole RNA}}{880 \text{ mole nucleotide}}$$

$$\cdot 6.2 \times 10^{23} \frac{\text{molecules}}{\text{mole}}$$

$$= 3.59 \times 10^{7} \frac{\text{molecules}}{\text{min}}$$

7) Converting to molecules per minute per cell:

$$\frac{3.59 \times 10^{7} \frac{\text{molecules}}{\text{min}}}{1.1 \times 10^{8} \text{ cells}} = 0.33 \frac{\text{molecules}}{\text{min} \cdot \text{cell}}$$

8) Steady State Level

$$Ss = \frac{K_s}{K_d} = \frac{0.33 \text{ molecules min}^{-1} \text{ cell}^{-1}}{6.3 \times 10^{-3}}$$

$$= 52 \frac{\text{molecules}}{\text{cell}}$$

Comment: If an independent measurement of the steady state level of RNA gives a different result, the calculated synthesis rate may be adjusted proportionately.

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Table 1

The half-lives of individual mitochondrial RNA species are listed as determined in experiments 1-6 described in the text.

		Experiment					
	RNA species	1	2	3	4	5	6
poly(A)+	2						7
	4						39
	5			60*	29	34	87
	6			28*		20	16
	7		41		53	28	112
	9	48	58	74	65	62	116
	11	26	45	30	29	26	56
	12	32	63	36	31	26	51
	13	36*		51*	25*		
	14	60	50	97	93	66 avg	141 avg
	15	50	62	82	66		
	16	57	66	103	81	45	191
	17				35		
	18		53	64	60	20	118
poly(A)-	16S	221	177		127*	53 *	
	12S	297	172		158*	94 *	

*Date not absolutely reliable due to the low level of radioactivity over the background.

Figure 1

The growth of HeLa cells in the presence and absence of ^{32}P is plotted as a function of time. Cells were suspended in Eagle's phosphate medium adjusted to 10^{-3} M cold phosphate and supplemented with 5% dialyzed calf serum. One culture received ^{32}P to a level of 4 microcuries/ml and the other received no ^{32}P.

The increase in cell number is plotted for the culture containing ^{32}P (open circles) and without ^{32}P (filled circles).

The increase in cellular protein is plotted for the culture containing ^{32}P (open triangles) and for the culture without ^{32}P (filled triangles).

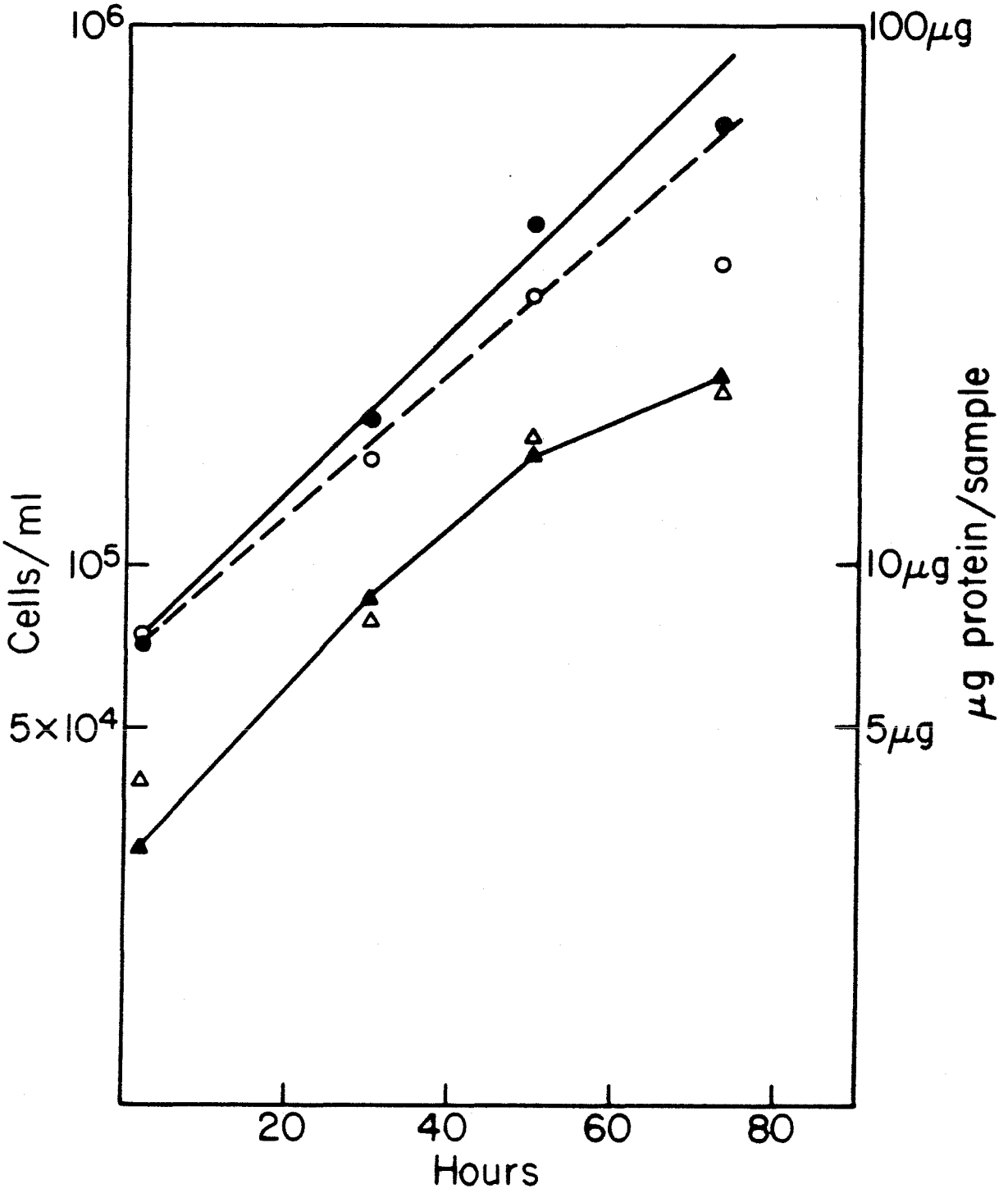

10^6
10^5
5×10^4
Cells/ml
100μg
10μg
5μg
μg protein/sample
20
40
60
80
Hours

Figure 2

Cells were in exponential phase throughout labeling. ^{32}P-orthophosphate was added to a final level of 5 microcuries/ml in the presence of cold phosphate adjusted to a final concentration of 5×10^{-4} M. ^{32}P-labeling was carried out for a total of 24 hours for each culture. At various times after the beginning of ^{32}P-labeling, 5-^{3}H-uridine was added to individual cultures to a final level of 2.5 microcuries/ml.

Mitochondria were purified by banding in a 1-1.7 M sucrose step gradient as described in the Materials and Methods. Nucleoside triphosphates were extracted as described in the Materials and Methods and separated according to the procedure of Neuhard (1965).

Specific activities (^{3}H/^{32}P) of UTP and CTP, both cytoplasmic and mitochondrial, are plotted as a function of ^{3}H-uridine labeling time.

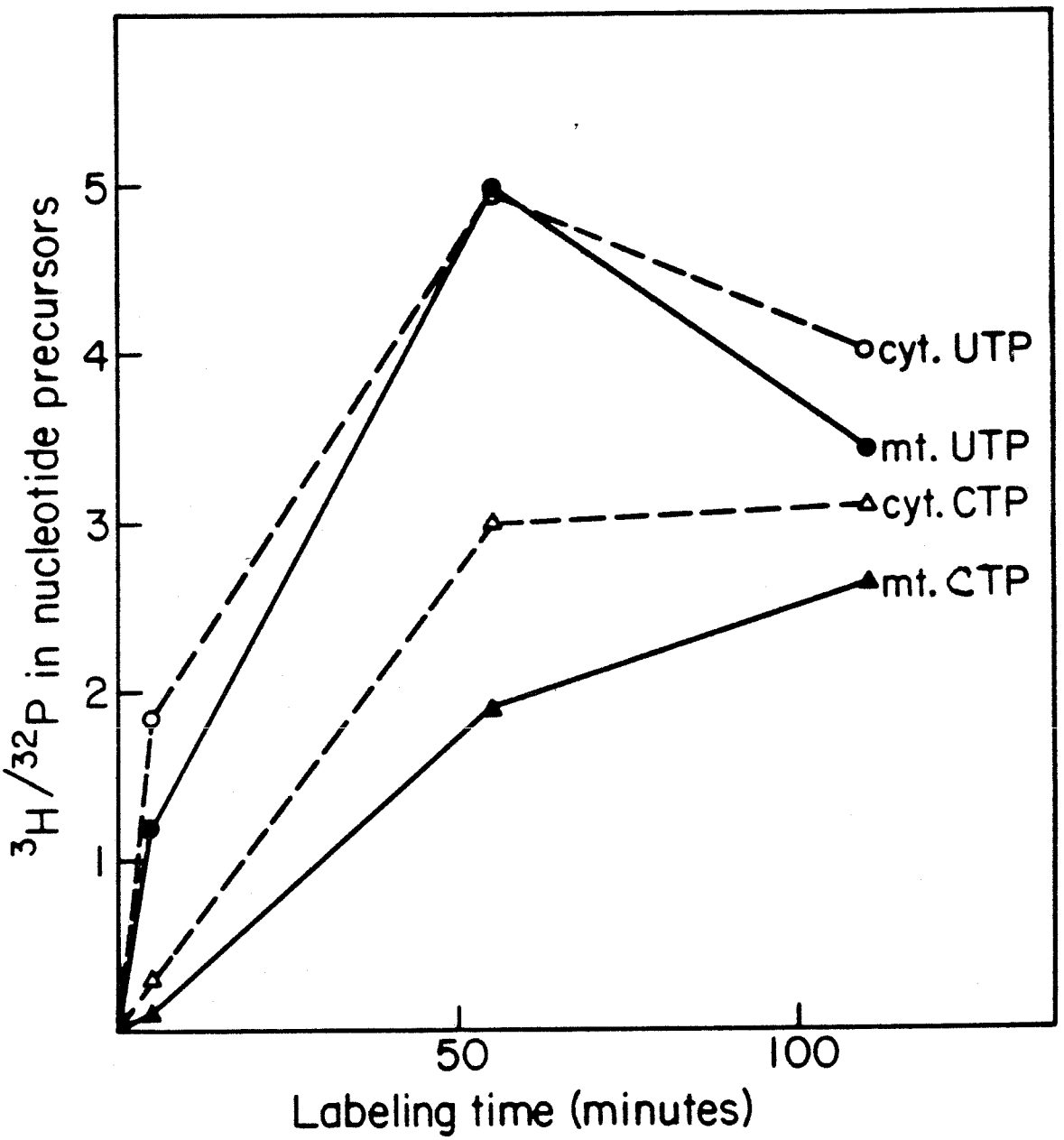

$^{3}H/^{32}P$ in nucleotide precursors
5
4
3
2
1
50
100
Labeling time (minutes)
cyt. UTP
mt. UTP
cyt. CTP
mt. CTP

Figure 3

Cells were in exponential phase throughout labeling. ^{32}P-orthophosphate was added to a final level of 5 microcuries/ml in the presence of cold phosphate adjusted to a final concentration of 5×10^{-4} M. ^{32}P-labeling was carried out for a total of 24 hours for each culture. At various times after the beginning of ^{32}P-labeling, 5-^{3}H-uridine was added to individual cultures to a final level of 2.5 microcuries/ml.

Mitochondrial polysomal polyadenylated RNA was prepared as described in the Materials and Methods from six different cultures. The RNA from the different cultures was analyzed by electrophoresis in parallel tracks of an agarose-methylmercury slab gel as described in Materials and Methods. The dried gel was subjected to autoradiography for two days at -70 degrees C using Kodak XR-5 film in conjunction with a DuPont "Cronex-Lightning Plus" image intensification screen. The image obtained by autoradiography is shown in the figure.

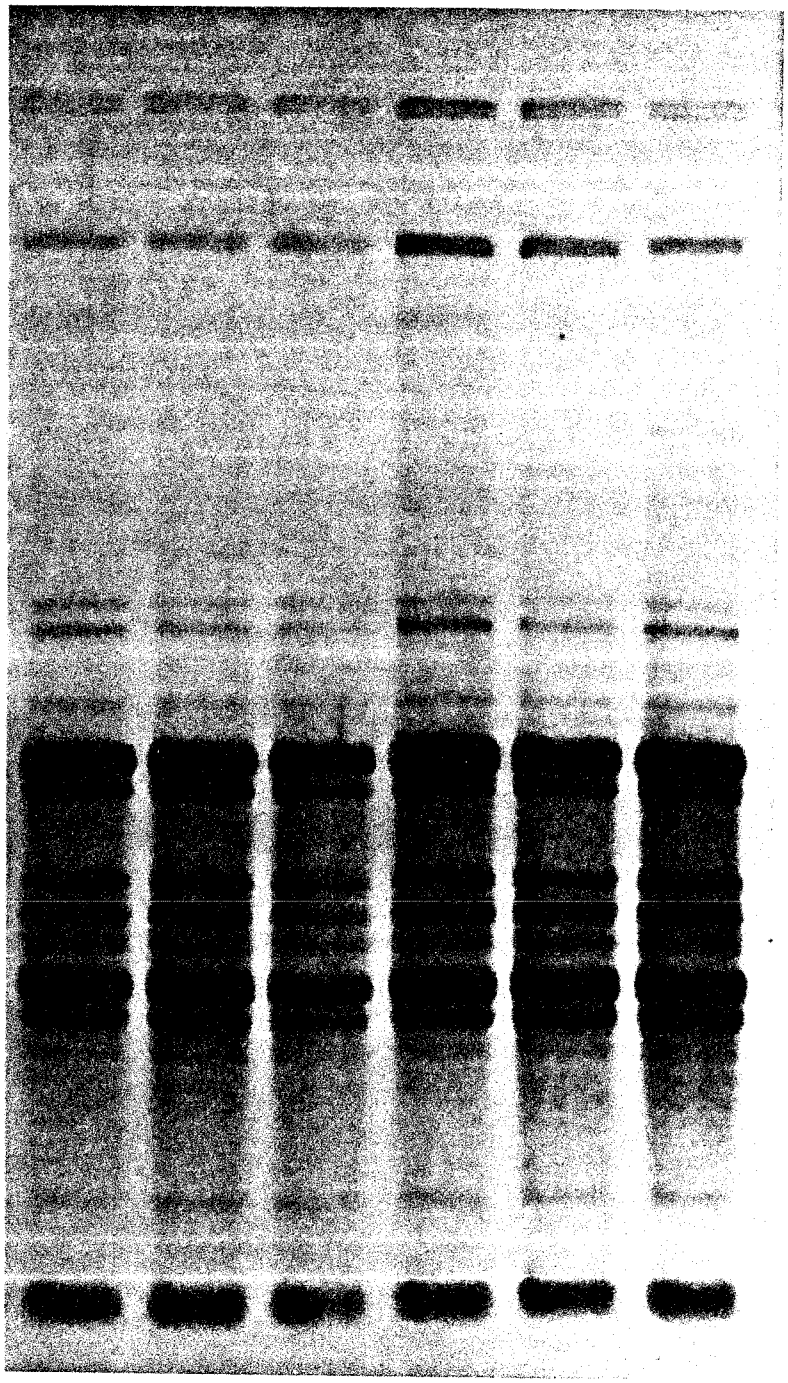


Figure 4

Analysis by serial slicing was carried out on the gel from which the autoradiograph shown in Figure 2 was obtained. The individual tracks of the dried gel were excised and sliced into 1 mm segments and the RNA was eluted from individual slices as described in Materials and Methods. A) The gel track containing RNA from cells labeled for one hour with ^{3}H-uridine was used for this panel. The ^{32}P and ^{3}H counts per minute are plotted as a function of migration in the gel. For the purpose of identification, the positions of the peaks in this plot have been aligned with the positions of mitochondrial poly(A)+ RNA species in the autoradiograph and the peaks have been accordingly marked. B) The labeling patterns obtained for poly(A)+ RNA species 11 and 12 from the six cultures corresponding to different times of exposure of the cells to ^{3}H-uridine.

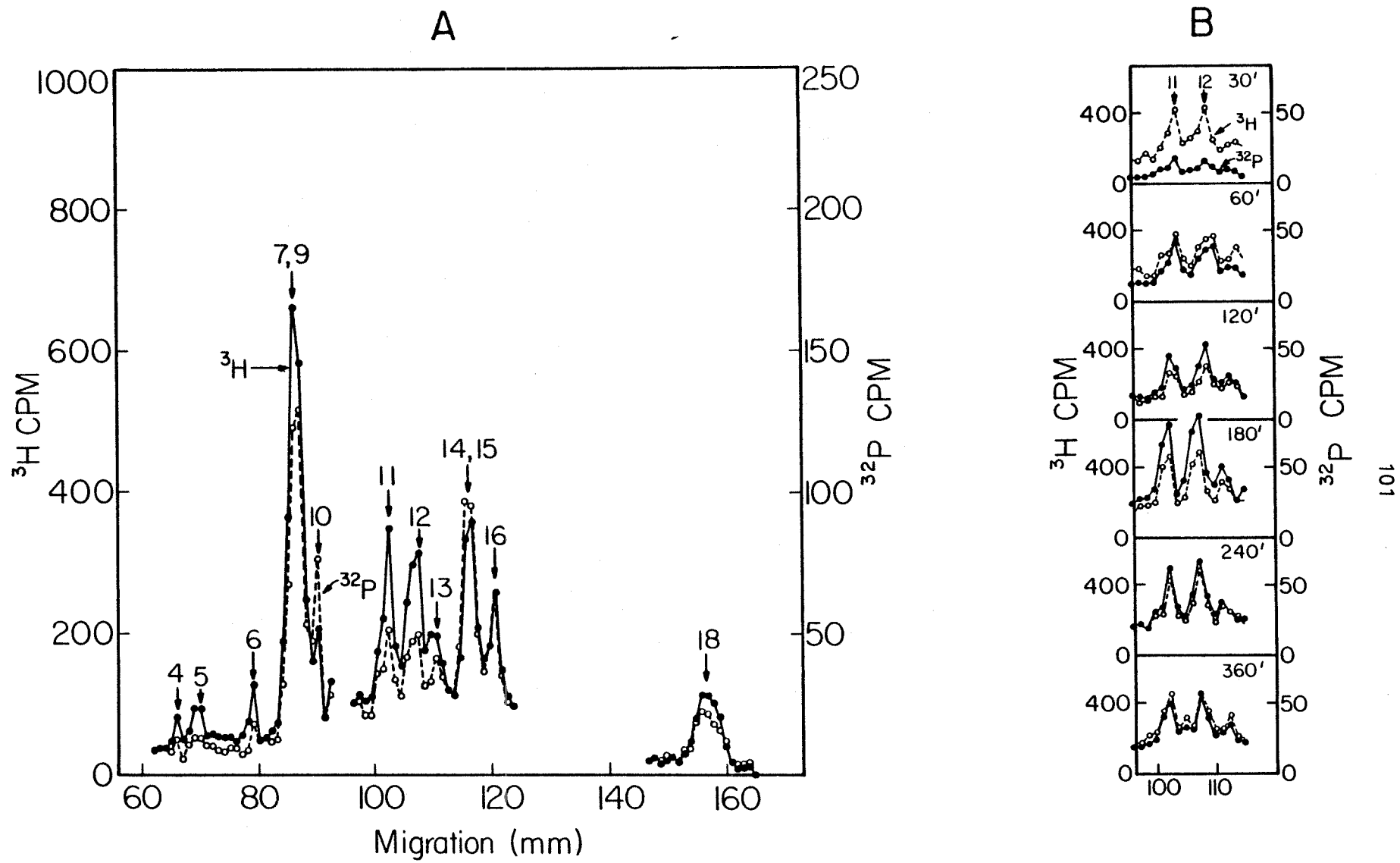

A
B
1000
800
600
400
200
0
250
200
150
100
50
^{3}H CPM
^{32}P CPM
Migration (mm)
60
80
100
120
140
160
4
5
6
7,9
10
11
12
13
14,15
16
18
^{3}H
^{32}P
30'
60'
120'
180'
240'
360'
100
110

Figure 5

Cells were in exponential phase throughout labeling. ^{32}P-orthophosphate was added to a final level of 5 microcuries/ml, in the presence of cold phosphate adjusted to a final concentration of 5 x 10^{-4} M. ^{32}P-labeling was carried out for a total of 24 hours for each culture. At various times after the beginning of ^{32}P-labeling, 5-^{3}H-uridine was added to individual cultures to a final level of 2.5 microcuries/ml.

Polysomal polyadenylated mitochondrial RNA was purified as described in Materials and Methods. RNA was eluted from serially sliced gels as described in Materials and Methods.

A) Cytoplasmic UTP and CTP were extracted as described in Materials and Methods and separated by the procedure of Neuhard et al. (1965). Specific activities (^{3}H/^{32}P) of UTP and CTP are plotted as a function of ^{3}H-uridine labeling time.

B) The specific activity (^{3}H/^{32}P) of mitochondrial polyadenylated RNA species 11 and 16 and the smaller mitochondrial ribosomal RNA are plotted as a function of ^{3}H-uridine labeling time.

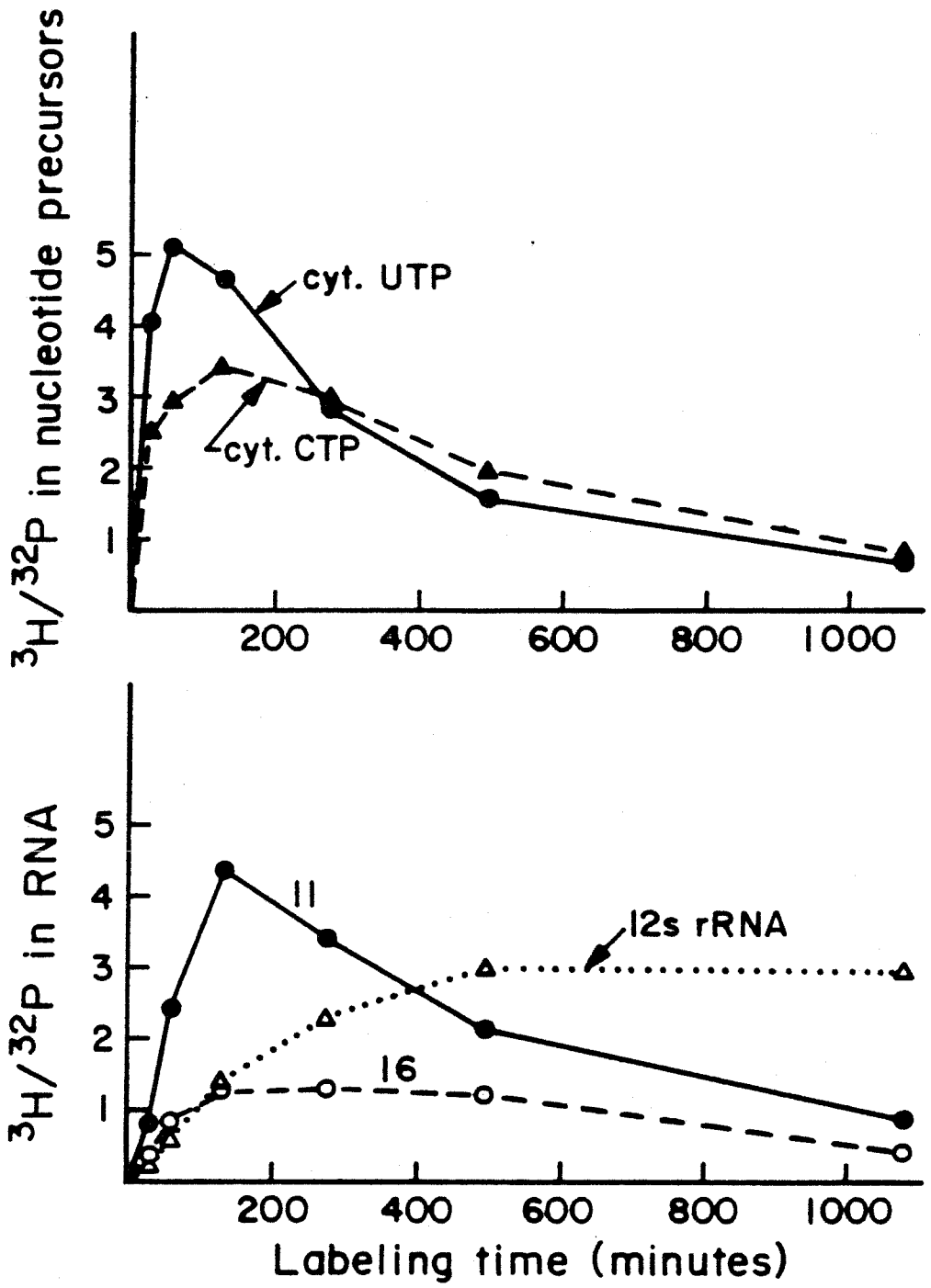

$^3H/^{32}P$ in nucleotide precursors
cyt. UTP
cyt. CTP
1
2
3
4
5
200
400
600
800
1000
$^3H/^{32}P$ in RNA
11
12s rRNA
16
Labeling time (minutes)

Figure 6

Cells were in exponential phase throughout labeling. ^{32}P-orthophosphate was added to a final level of 5 microcuries/ml in the presence of 5 x 10^{-4} M cold phosphate. ^{32}P-labeling was carried out for a total of 24 hours for each culture. At various times after the beginning of ^{32}P-labeling, 5-^{3}H-uridine was added to individual cultures to a final level of 2.5 microcuries/ml.

Mitochondria were treated with RNase A plus DNase 1 and then lysed with SDS buffer as described in Materials and Methods. Cytoplasmic UTP was extracted as described in Materials and Methods and separated according to the procedure of Neuhard et al. (1965). RNA was separated into polyadenylated and non-polyadenylated fractions as described in Materials and Methods.

A) Cytoplasmic UTP specific activity (^{3}H/^{32}P) is plotted as a function of ^{3}H-uridine labeling time.

B) Specific activities (^{3}H/^{32}P) of mitochondrial polyadenylated RNA species 11 and 16 are plotted as a function of ^{3}H-uridine labeling time.

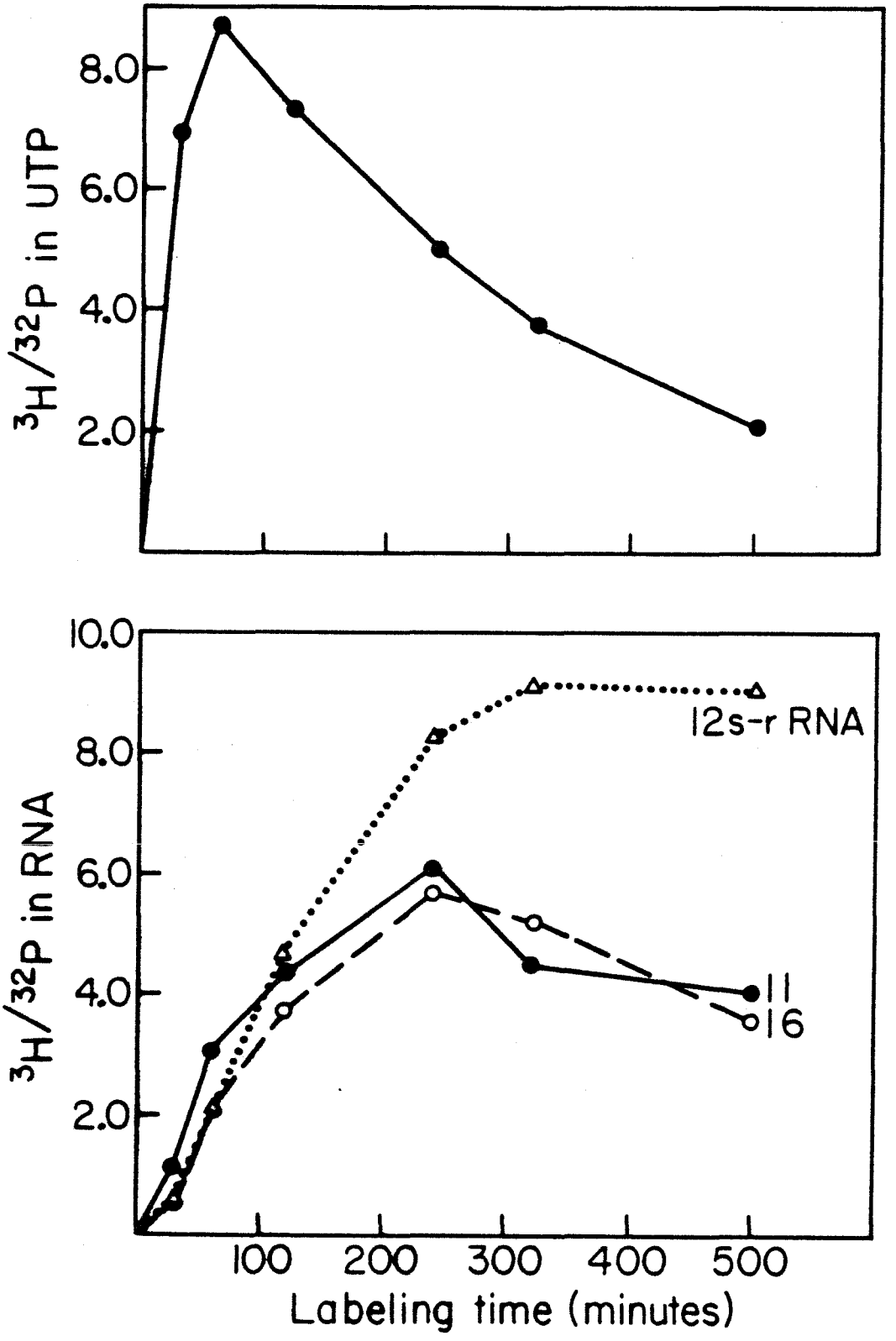

8.0
6.0
4.0
2.0
$^{3}H/^{32}P$ in UTP
10.0
8.0
6.0
4.0
2.0
$^{3}H/^{32}P$ in RNA
12s-r RNA
11
16
100
200
300
400
500
Labeling time (minutes)

Figure 7

Cells were in exponential phase throughout labeling. ^{32}P-orthophosphate was added to a final level of 5 microcuries/ml in the presence of cold phosphate adjusted to a final concentration of 5×10^{-4} M. ^{32}P-labeling was carried out for a total of 24 hours for each culture. At various times after the beginning of ^{32}P-labeling, 5-^{3}H-uridine was added to individual cultures to a final level of 2.5 microcuries/ml.

Mitochondrial polysomal RNA was prepared as described in Materials and Methods. Cytoplasmic UTP and CTP were extracted as described in Materials and Methods and separated according to the procedure of Neuhard et al. (1965).

A) Cytoplasmic UTP and CTP specific activities (^{3}H/^{32}P) are plotted as a function of ^{3}H-uridine labeling time.

B) Specific activities (^{3}H/^{32}P) of polysomal polyadenylated RNA species 11 and 16 are plotted as a function of ^{3}H-uridine labeling time.

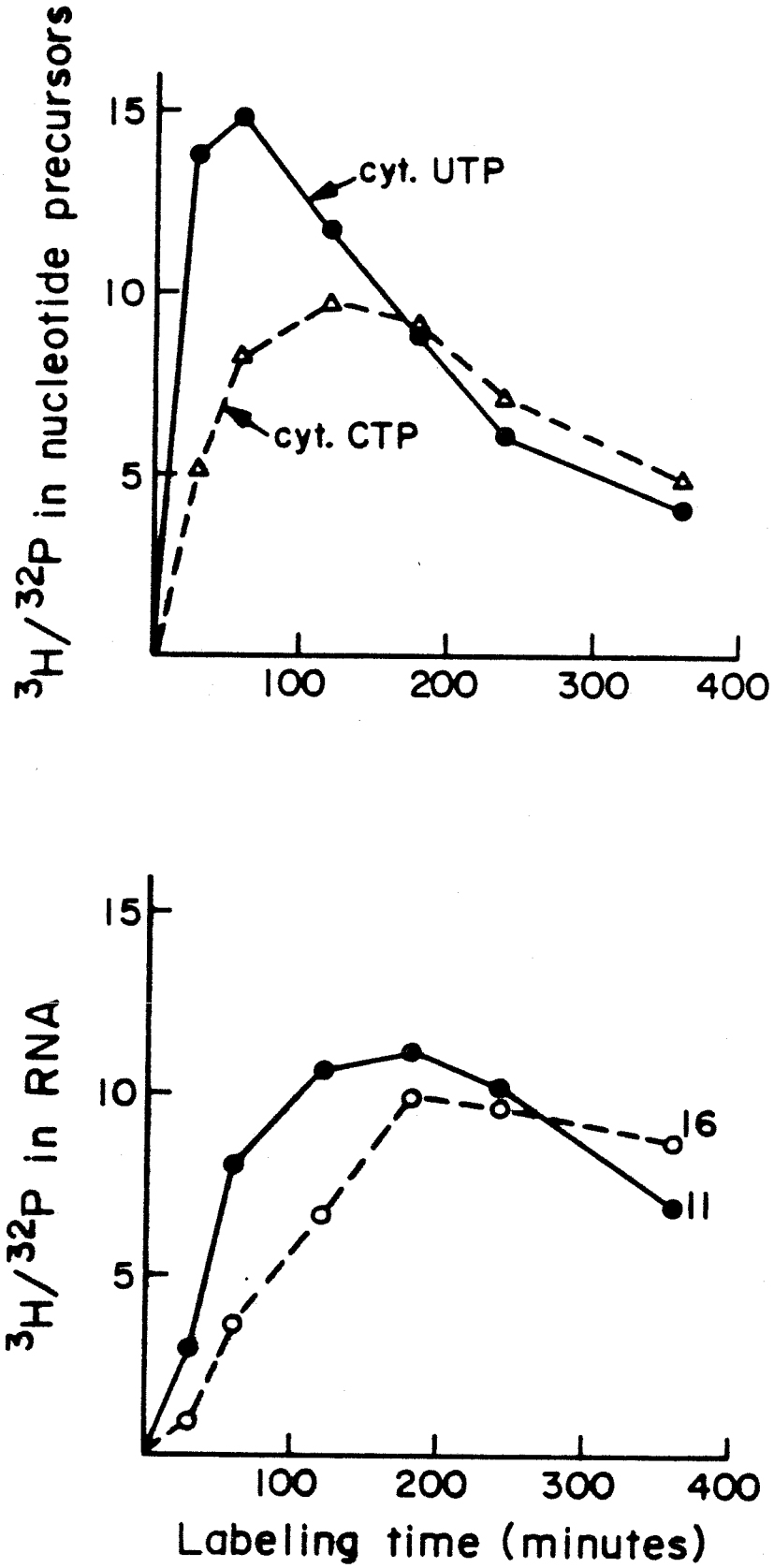

$^3H/^{32}P$ in nucleotide precursors
15
10
5
cyt. UTP
cyt. CTP
100
200
300
400
$^3H/^{32}P$ in RNA
15
10
5
16
11
100
200
300
400
Labeling time (minutes)

Figure 8

Cells were concentrated to a density of approximately 1 x 10^6/ml. All cultures were labeled with ^{32}P-orthophosphate to a final level of 20 microcuries/ml in the presence of 4 x 10^{-5} M cold phosphate for a total of 3.5 hours. 5-^{3}H-uridine was added to individual cultures at various times after the beginning of ^{32}P-labeling to a final level of 20 microcuries/ml. The mitochondrial fraction obtained from each culture was divided into two equal portions. One portion, to be used for the extraction of nucleoside-triphosphates, was treated with RNase A plus DNase 1 according to the procedure described in Materials and Methods. The other portion, to be used for the extraction of "total" mitochondrial RNA, was treated with micrococcal nuclease and lysed with SDS as described in Materials and Methods.

A) Mitochondrial UTP was isolated from sonicated mitochondria isolated as described above. UTP was also prepared from the post-mitochondrial supernatant ("cytoplasm"). UTP was separated by the method of Cashel et al. (1969). Specific activities (^{3}H/^{32}P) are plotted as a function of ^{3}H-uridine labeling time.

B) Mitochondrial RNA was separated into polyadenylated and non-polyadenylated fractions as described in Materials and Methods. Specific activities (^{3}H/^{32}P) of polyadenylated RNA species 11 and 16 and the small mitochondrial ribosomal RNA are plotted as a function of the ^{3}H-uridine labeling time.

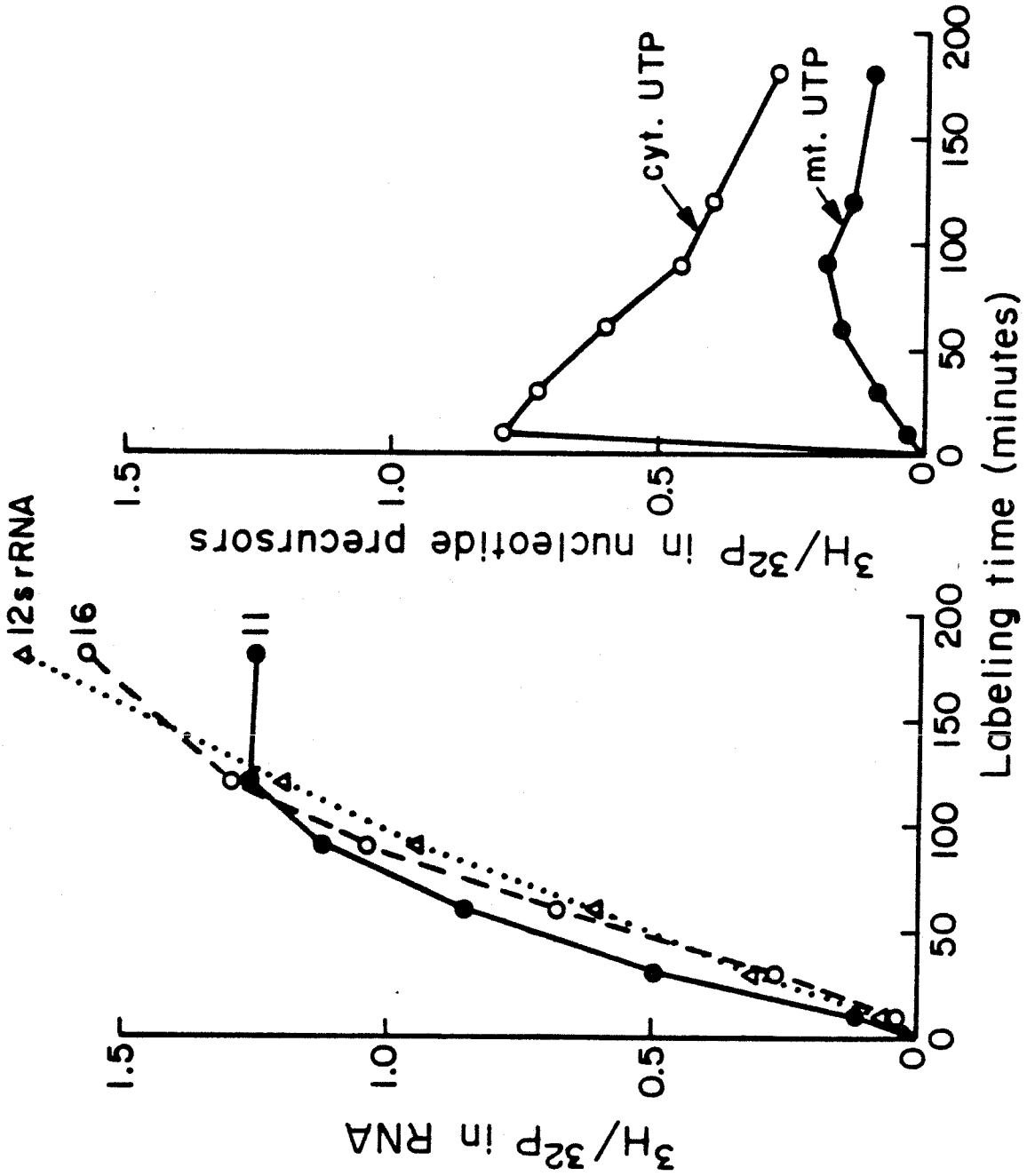

$^3H/^{32}P$ in RNA
1.5
1.0
0.5
0
0
50
100
150
200
Labeling time (minutes)
A 12s rRNA
16ρ
11
4
4
4
8
8
$^3H/^{32}P$ in nucleotide precursors
cyt. UTP
mt. UTP

Figure 9

HeLa cell cultures were labeled with ^{32}P-orthophosphate to a final level of 20 microcuries/ml in the presence of 4 x 10^{-5} M cold phosphate for a total of 3 hours at a cell density of 1 x 10^{6}/ml. 5-^{3}H-uridine was added to individual cultures at various times after the beginning of ^{32}P-labeling to a final level of 20 microcuries/ml. The mitochondrial fraction obtained from each culture was divided into two equal portions. One portion, to be used for the extraction of DNA-RNA transcription complexes, was further purified by banding in a 1-1.7 M sucrose step gradient, as previously described (Ojala and Attardi, 1974b). Purification of RNA hybridizing to L strand DNA was accomplished as described in Materials and Methods. The other portion, to be used for the purification of mitochondrial RNA, was treated with micrococcal nuclease as described in Materials and Methods. Purification of mitochondrial RNA and further fractionation into polyadenylated and non-polyadenylated fractions was accomplished as described in Materials and Methods.

A) The specific activity (^{3}H/^{32}P) of L strand hybridizable RNA from mitochondrial DNA-RNA transcription complexes is plotted as a function of ^{3}H-uridine labeling time.

B) Specific activities (^{3}H/^{32}P) of mitochondrial polyadenylated RNA species 11 and 16 and the mitochondrial small ribosomal RNA are plotted as a function of ^{3}H-uridine labeling time.

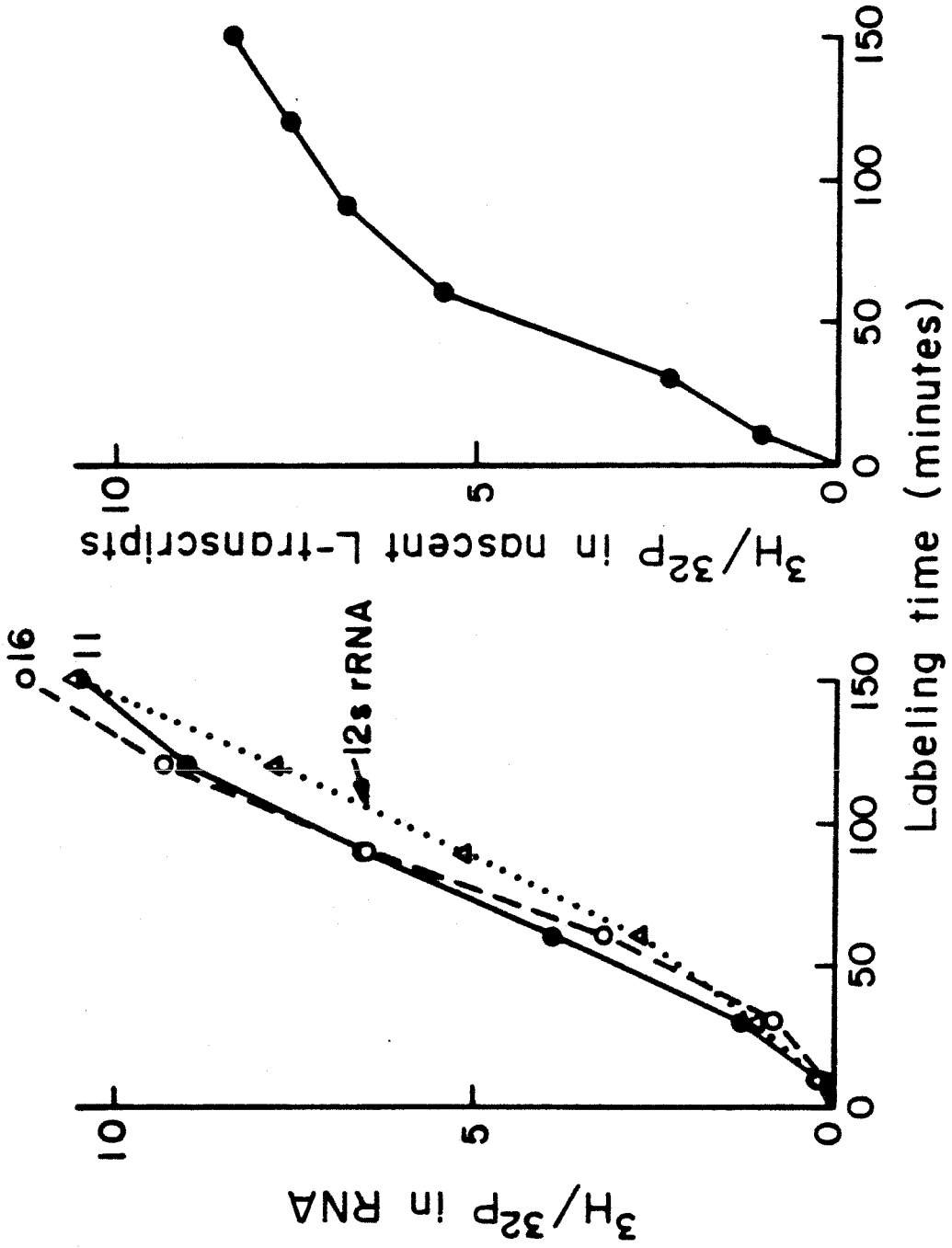

$^3H/^{32}P$ in RNA
$^3H/^{32}P$ in nascent L-transcripts
Labeling time (minutes)
0
5
10
50
100
150
16
11
4
4
4
8
12s rRNA

Figure 10

HeLa cells were suspended in medium adjusted to a concentration of 4×10^{-5} M cold phosphate at a cell concentration of 1.5×10^{6}/ml. Cultures were treated with cordycepin for various times prior to labeling, except one culture received no cordycepin. Following cordycepin treatment, all cultures were labeled with 5-^{3}H-uridine (33 microcuries/ml) for 10 minutes. Following cell harvesting but prior to cell breakage, ^{32}P-labeled cells were added to each culture in equal amounts in order to allow correction for differences in yields.

Mitochondrial RNA was purified from micrococcal nuclease treated, SDS lysed mitochondria as described in Materials and Methods. RNA was separated into polyadenylated and non-polyadenylated fractions by oligodeoxythymidylate cellulose chromatography as described in Materials and Methods.

The figure shows the pattern of labeling for mitochondrial polyadneylated RNA species in the absence of cordycepin (plotted at time zero) and following cordycepin treatment, plotted as a function of the time of cordycepin treatment. All values have been corrected for differences in yield and are expressed as the ratio ^{3}H-CPM/^{32}P-CPM.

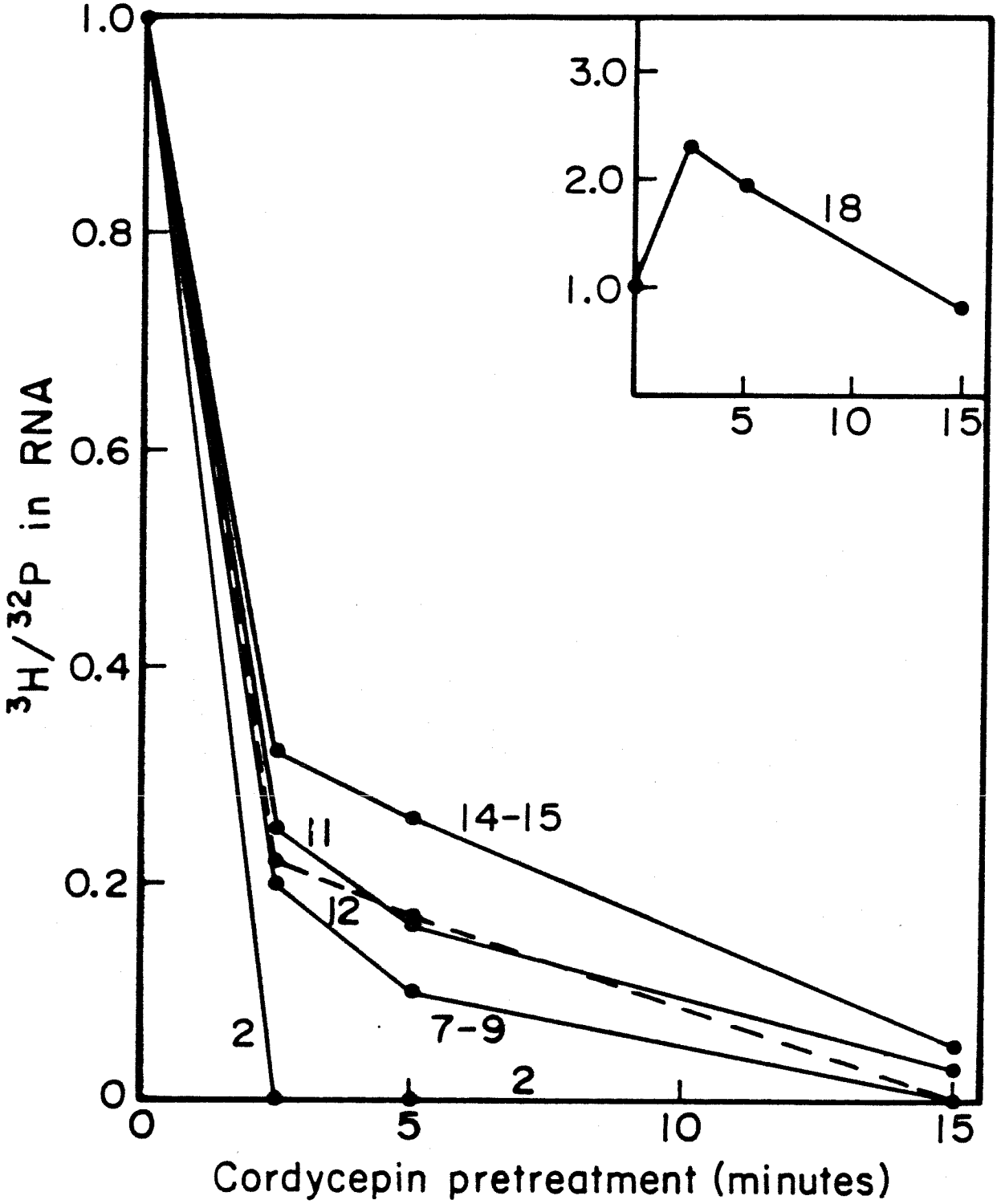

1.0
0.8
0.6
0.4
0.2
0
$^3H/^{32}P$ in RNA
0
5
10
15
Cordycepin pretreatment (minutes)
14-15
11
12
7-9
2
2
3.0
2.0
1.0
18
5
10
15

Figure 11

Mitochondrial ribosomal RNA was labeled and purified in the experiment described in Figure 10 and the text. This figure shows the radioactive labeling of the small rRNA (12S) and the large rRNA (16S) as a function of the time of cordycepin pretreatment, corrected for differences in yield and therefore expressed as the ratio $^{3}H\text{-CPM}/^{32}P\text{-CPM}$.

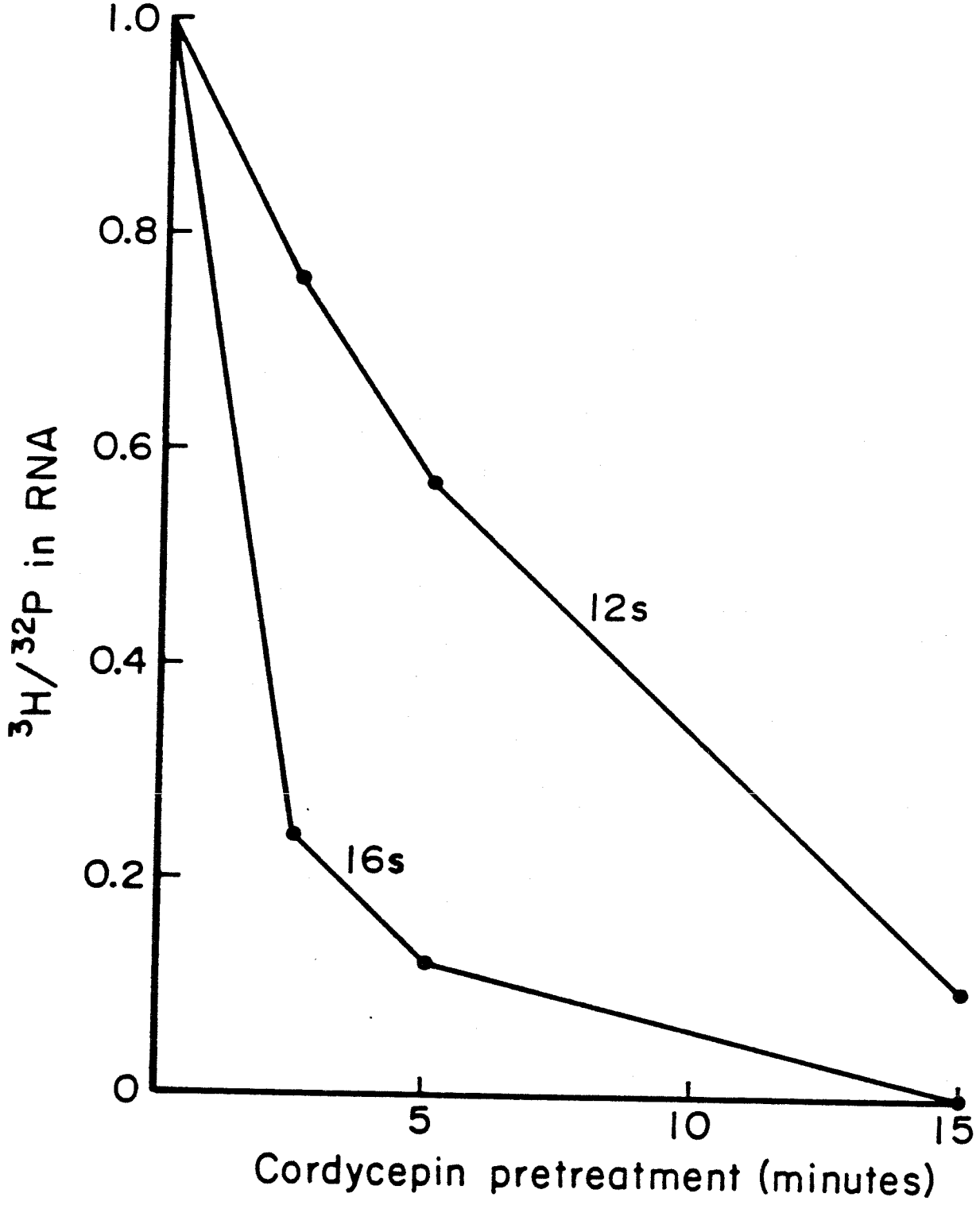

1.0
0.8
0.6
0.4
0.2
0
$^{3}H/^{32}P$ in RNA
12s
16s
5
10
15
Cordycepin pretreatment (minutes)

Figure 12

HeLa cells were suspended at a concentration of 1 x 10^6/ml and labeled with ^{32}P-orthophosphate (30 microcuries/ml) for 2.5 hours in the presence of 4 x 10^{-5} M cold phosphate. Cordycepin was then added to each culture to a final concentration of 50 micrograms/ml except that one culture received no cordycepin and was immediately harvested. The cultures which received cordycepin were harvested after various times.

Prior to cell breakage, ^{3}H-uridine labeled cells were added to each culture in equal amounts in order to allow correction for differences in yields.

RNA was purified from micrococcal nuclease treated, SDS lysed mitochondria as described in Materials and Methods. Separation of polyadenylated RNA from non-polyadenylated RNA, fractionation by agarose-methylmercuric hydroxide gel electrophoresis, and autoradiography were accomplished as described in Materials and Methods.

The electrophoretic pattern of the polyadenylated RNA fraction as determined by autoradiography is shown.

Cordycepin chase after pulse labeling

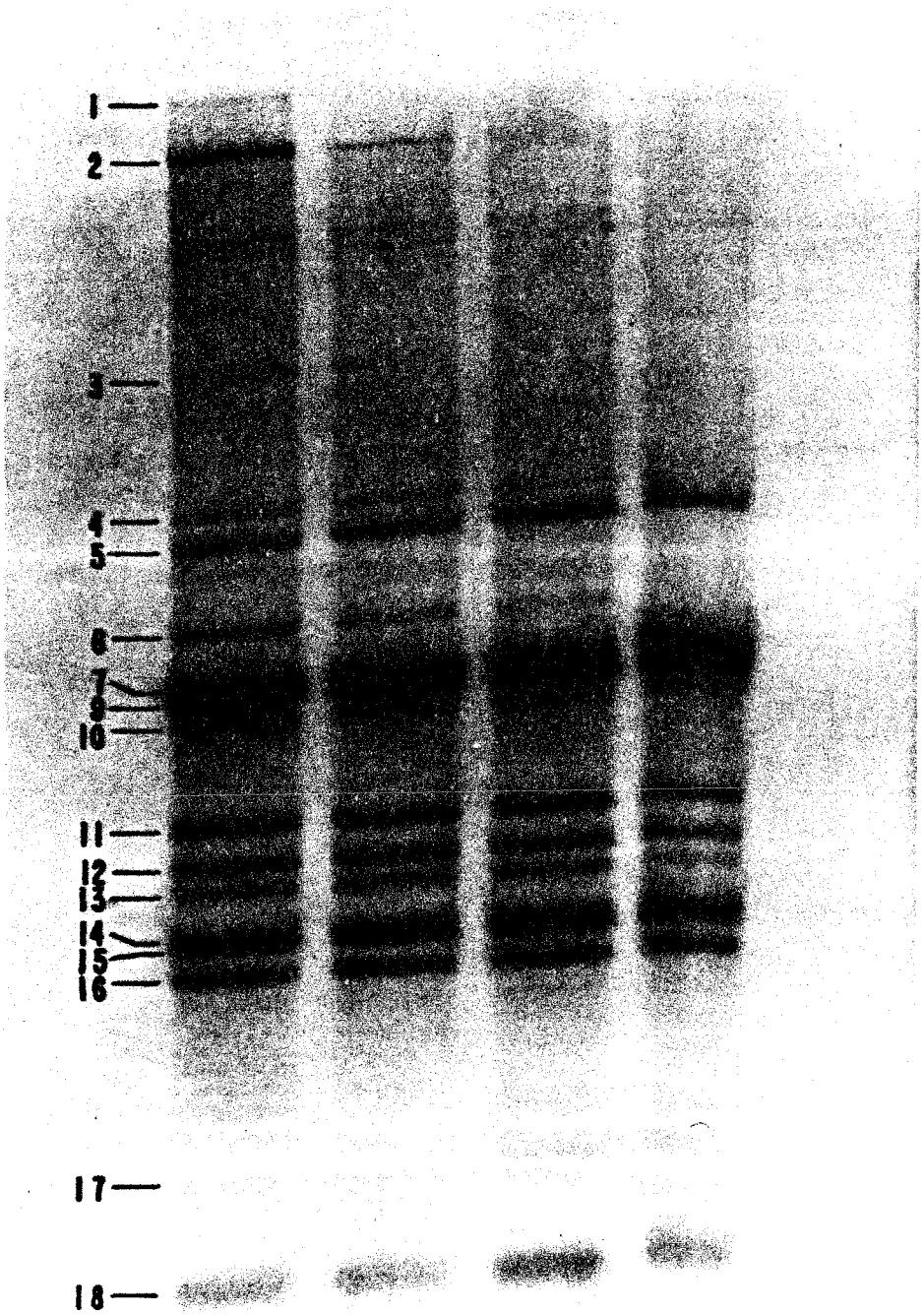
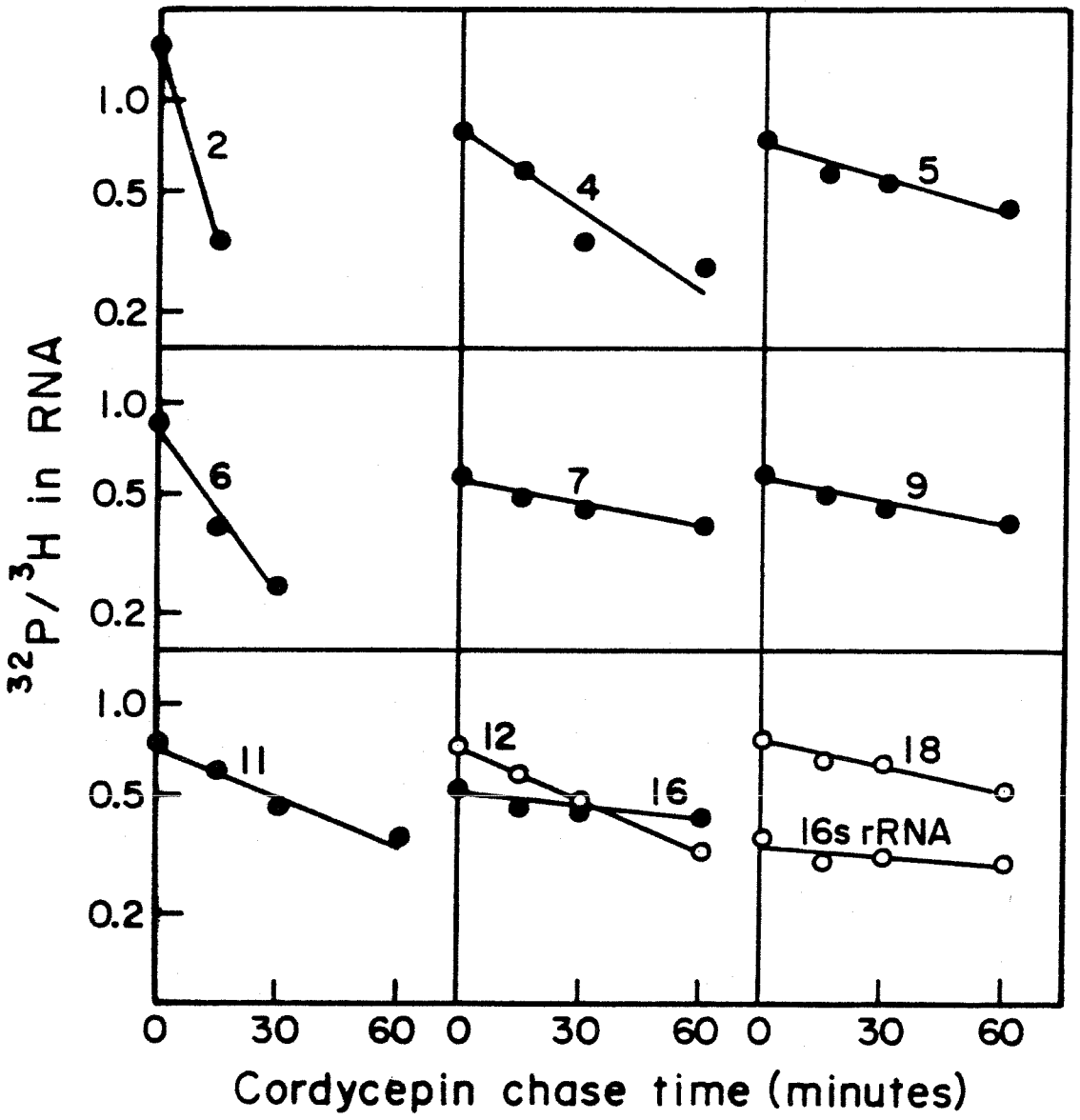


Figure 13

RNA from the pulse-chase experiment described in Figure 12 and the text was analyzed in order to determine the decrease in incorporated radioactivity in individual RNA species as a function of the time of cordycepin chase. RNA bands of interest were located by autoradiography, excised, and the radioactivity was eluted and counted as described in Materials and Methods. All values were corrected for differences in yield and are therefore expressed as the ratio ^{3}H-CPM/^{32}P-CPM. The logarithms of the corrected values of label in individual RNA species are plotted as functions of cordycepin chase time.


1.0
0.5
0.2
2
4
5
6
7
9
11
12
16
18
16s rRNA
$^{32}P/^{3}H$ in RNA
0
30
60
Cordycepin chase time (minutes)

APPENDIX

Purification and Separated Strand Hybridization of Mitochondrial Transcription Complex RNA

Appendix

Purification and Separated Strand Hybridization of Mitochondrial Transcription Complex RNA

Mitochondrial DNA-RNA transcription complexes were isolated and characterized electron microscopically by Aloni and Attardi (1972). The transcription complexes were further analyzed as to their purity and composition by Carré and Attardi (1978) using electron microscopy and biochemical techniques.

The use of mitochondrial DNA-RNA transcription complexes in this work constitutes a novel method for determining the specific activity of the precursor pool of mature RNA species. The RNA obtained by isolation and extensive DNase treatment of mitochondrial transcription complexes was used to represent the pool of direct precursors to mature mitochondrial RNA. The mitochondrial origin of the RNA used in the measurements was demonstrated by its hybridization to mitocohondrial DNA, and only the hybridized RNA was used to determine the precursor pool specific activity.

It was important to show that the transcription complex RNA is uncontaminated by mature mitochondrial RNA. If there were such contamination, the precursor specific activities would be unreliable and therefore the calculated half-lives would also be unreliable. This appendix discusses the purification of mitochondrial DNA and mitochondrial DNA-RNA transcription complexes and describes an experiment to show that the mitochondrial transcription complex RNA is uncontaminated by mature mitochondrial RNA.

In the kinetic experiments, mitochondria to be used for the isolation of transcription complexes were purified of cytoplasmic contamination by banding in a 1-1.7 M sucrose step gradient, as described in Materials and Methods. In the experiment to be described here, the mitochondria were purified of cytoplasmic and nuclear contamination by a modification of the micrococcal nuclease treatment which is described below. The purpose of using the micrococcal nuclease treatment in the purification procedure is to assure that the electrophoretic pattern of transcription complex RNA is not contaminated by RNA of cytoplasmic origin. All other purification procedures were essentially the same in the kinetic experiment and in the experiment to be described here. In particular, the sucrose gradient preparation of transcription complexes used by Aloni and Attardi (1972) was used in both sets of experiments. The sucrose gradient purified mitochondria used in the kinetic experiment may have had slightly more cytoplasmic RNA contamination, but that would not have affected the measured specific activity since this was determined only for RNA hybridizing to mitochondrial DNA.

Mature mitochondrial RNA appears as a set of distinct species when analyzed by agarose methylmercuric hydroxide gel electrophoresis (Amalric et al., 1978). The absence of distinct bands in the electrophoretic pattern of transcription complex RNA would therefore indicate that the transcription complex material is not contaminated by mature RNA. A heterogeneous electrophoretic pattern, showing the existence of radioactive RNA, but not distinct species, can be obtained artifactually as well as from the result of physiological processes. Possible artifacts

in this analysis were therefore considered. In particular, it was necessary to show that the absence of distinct species in the electrophoretic pattern of transcription complex RNA is not the result of degradation during the extraction procedure.

In order to eliminate the problem that observed heterogeneity in size might be due to degradation, a test was available. The extraction of transcription complex RNA is essentially identical to the extraction of total mitochondrial RNA except that there is a sucrose gradient step which is carried out in the presence of SDS. In the experiment to be described, a single culture was labeled and mitochondria extracted. Following the lysis of the mitochondria with SDS buffer, the material was divided into two portions, one of which was immediately subjected to RNA extraction, the other of which was subjected to centrifugation through a sucrose gradient containing 0.5% SDS. Since the transcription complex preparation differs only in the steps following SDS lysis of mitochondria and these steps are carried out in the presence of SDS, the random degradation, if any, suffered by the transcription complex RNA would have to have occurred during the relatively short centrifugation in the presence of SDS. Total mitochondrial RNA which bound to oligodeoxythymidylate cellulose was therefore prepared from the same culture as the transcription complex RNA in order to ascertain if extensive degradation had occurred.

In order to eliminate the danger that any observed heterogeneity in size might be the result of aggregation of small RNA molecules, the method of agarose-methylmercury gel electrophoresis (Bailey and Davidson, 1976) was used.

MATERIALS AND METHODS

Cells were labeled in a medium consisting of Eagle's phosphate medium adjusted to a cold phosphate concentration of 4×10^{-5} M. The cell concentration was approximately 1×10^{6}/ml. Labeling was accomplished by the addition of ^{32}P-orthophosphate to a final level of 50 microcuries/ml. Labeling was carried out for a total of 2.5 hours. At the conclusion of labeling, cells were harvested by pouring the contents of the cell cultures over frozen, crushed NKM buffer (0.13 M NaCl, 0.005 M KCl, 0.0075 M $MgCl_2$). The mitochondrial extraction was carried out according to the procedure of Amalric et al. (1978).

Purification of the mitochondria from cytoplasmic and nuclear RNA contamination was carried out by a modification of the micrococcal nuclease procedure described in Chapter 3 of this manuscript. Micrococcal nuclease was added to mitochondria suspended in 0.25 M sucrose, 0.01 M Tris, pH 7.4, 0.001 M CaCl, as before, but enzyme digestion was carried out at 0 degrees for 30 minutes rather than at room temperature for 15 minutes. All further steps, including EGTA treatment and washing with 0.25 M sucrose, 0.01 M Tris, pH 7.4, 0.01 M EDTA were the same as the earlier method, except that two washing steps were used instead of one.

The mitochondrial pellet was lysed in a buffer consisting of 1% SDS, 0.01 M Tris, pH 7.4, 0.001 M EDTA, 0.1 M NaCl (SDS buffer adjusted to 1% SDS from its previous 0.5% SDS). The lysed mitochondrial material was then divided into equal parts. One part was deproteinized by the SDS-pronase method (described below) in order to prepare total mitochondrial RNA. The other part was mixed with 2 volumes of ethanol and

stored at -20 degrees overnight. The ethanol precipitated material was then used to prepare transcription complexes. Following centrifugation at 10 KRPM for 20 minutes (SS-34 rotor), the pellet was resuspended in SDS buffer adjusted to 2.5% SDS. This material was applied to a sucrose gradient consisting of 15-30% sucrose in SDS buffer over a cushion of 64% sucrose in SDS buffer. Centrifugation was carried out in a Beckman SW-27 rotor for 5 hours at 26,000 RPM at 20 degrees. The gradient was fractionated from the bottom and the transcription complex material was recovered from the region of the gradient at and directly above the cushion. The transcription complex material was then precipitated with 2.5 volumes of ethanol and stored at -20 degrees for later analysis.

The transcription complex material was pelleted by centrifugation for 20 minutes at 20 KRPM (SS-34 rotor). The pellet was resuspended in SDS buffer and deproteinized by the SDS pronase method just as the total mitochondrial RNA sample had been. This method consists of digesting the proteins in the samples by the addition of pronase (self digested) to a final level of 100 micrograms/ml and incubating for one hour at room temperature, followed by several extractions with phenol:chloroform:isoamyl alcohol (50:50:1, v/v). Following the extraction step, all material was saved for later analysis at -20 degrees after the addition of 2.5 volumes of ethanol.

The transcription complex material and the total mitochondrial RNA were then treated with DNase. DNase 1 (Boerhinger, electrophoretically purified, RNase free) was added to samples dissolved in TKM buffer (0.05 M Tris, pH 6.7, 0.025 M KCl, 0.0025 M $MgCl_2$) to a final level of 40 units/ml.

Digestion was carried out at 0 degrees for 30 minutes. Following the digestion step, NaCl was added to 0.1 M, SDS was added to 1%, and pronase was added to 50 micrograms/ml. The pronase digestion was carried out at room temperature for 15 minutes. The material was then extracted with phenol:chloroform:isoamyl alcohol as before except that following the first extraction, SDS buffer was added to the organic phases (equal volume), shaken, separated by centrifugation, and pooled with the original aqueous phase material for the separate preparations.

The total mitochondrial RNA sample was then further fractionated by separation on oligo-deoxythymidylate cellulose as described earlier in this chapter.

Analysis of the fractions produced in this way was by electrophoresis in agarose-methylmercury slab gels and autoradiography as described. The preparation of mitochondria DNA and strand separation by alkaline cesium chloride centrifugation was accomplished as described earlier in this chapter.

RESULTS AND DISCUSSION

Strand Separation of DNA

The separated light and heavy strands of mitochondrial DNA were prepared by alkaline cesium chloride centrifugation. The gradient was fractionated from the bottom and the optical density (260 nm) was determined for each fraction. The optical density pattern is shown in Figure 14. The fractions pooled to be used in hybridization experiments are labeled "Heavy" and "Light."

Characterization of Transcription Complex RNA

Transcription complex RNA and polyadenylated mitochondrial RNA were prepared as described in Materials and Methods from mitochondria treated with micrococcal nuclease by the method described in this appendix.

The transcription complex RNA and total mitochondrial RNA separated into polyadenylated and non-polyadenylated fractions was then analyzed by gel electrophoresis as described in Materials and Methods. The electrophoretic pattern obtained by autoradiography of the dried gel is shown in Figure 15.

The transcription complex RNA obtained in this experiment differs greatly in its electrophoretic pattern from the total mitochondrial RNA. Total mitochondrial RNA consists of strongly labeled discrete bands and some heterogeneous background as can be seen in the polyadenylated RNA track and in the mitochondrial ribosomal RNA species in the non-polyadenylated RNA track. In contrast, the transcription complex RNA is mainly a heterogeneous "smear" of material.

The transcription complex RNA pattern shows a maximum size as determined by gel migration. The slowest migrating (therefore largest) material in the transcription complex is approximately the same size as or perhaps a little larger than the largest size classes seen in the total RNA patterns. Although the resolution of the gel system used is limited for giant transcripts of this order of size, the largest transcripts are at least as large as polyadenylated species 1, identified to be approximately 10,200 nucleotides in length. The largest nascent

transcripts may even be close to the size of the mitochondrial genome, 16,000 nucleotides, but the resolution of the analysis is not sufficient to make this distinction.

There is no obvious contamination of the transcription complex RNA by mature RNA species, with the exception of two faint bands which have the same electrophoretic mobility as the mitochondrial ribosomal 12S and 16S bands. Since there is probably a large precursor molecule for the 12S and 16S rRNA species (Ojala et al., manuscript in preparation), these bands are probably the result of a slight amount of contamination of the transcription complexes by mature mitochondrial rRNA species. The ribosomal RNA is transcribed from the heavy DNA strand (Amalric et al., 1978) and would therefore not hybridize to the L strand in the assay used in the kinetic studies described earlier.

There is no evidence of any appreciable contamination of the transcription complex RNA by mature relatively stable RNA species which hybridize to the L strand. In particular, species 18 (7S RNA) and 4S RNA are not observed in the electrophoretic pattern of transcription complex RNA.

DNA-RNA Hybridization

The purified transcription complex RNA was hybridized to purified L strand mitochondrial DNA as described earlier in this chapter. The method involves incubation of the mixture of labeled RNA and unlabeled DNA at 66 degrees for 48 hours in 2X SSC. Preliminary experiments (data not shown) established that the quantity of DNA (0.5 micrograms in total volume of 1 ml) was far in excess of the amount necessary to achieve the maximum level of hybridization.

Since the transcription complex RNA contains no stable RNA species transcribed from the L strand, the hybridized RNA should faithfully reflect the condition of its precursor pools at or immediately before the time of cell harvesting.

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Aloni, Y., and Attardi, G. (1972) Expression of the mitochondrial genome in HeLa cells. XI. Isolation and characterization of transcription complexes of mitochondrial DNA. J. Mol. Biol. 70, 363-373.

Amalric, F., Merkel, C., Gelfand, R., and Attardi, G. (1978) Fractionation of mitochondrial RNA from HeLa cells by high-resolution electrophoresis under strongly denaturing conditions. J. Mol. Biol. 118, 1-25.

Bailey, J. M., and Davidson, N. (1976) Methylmercury as a reversible denaturing agent for agarose gel electrophoresis. Anal. Biochem. 70, 75-85.

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Figure 14

HeLa mitochondrial DNA was purified by repeated ethidium bromide-cesium chloride centrifugations as described in Materials and Methods. The figure shows the results of an alkaline-cesium chloride strand separation centrifugation (Aloni and Attardi, 1971). Optical density (260 nm) is plotted as a function of gradient fraction.

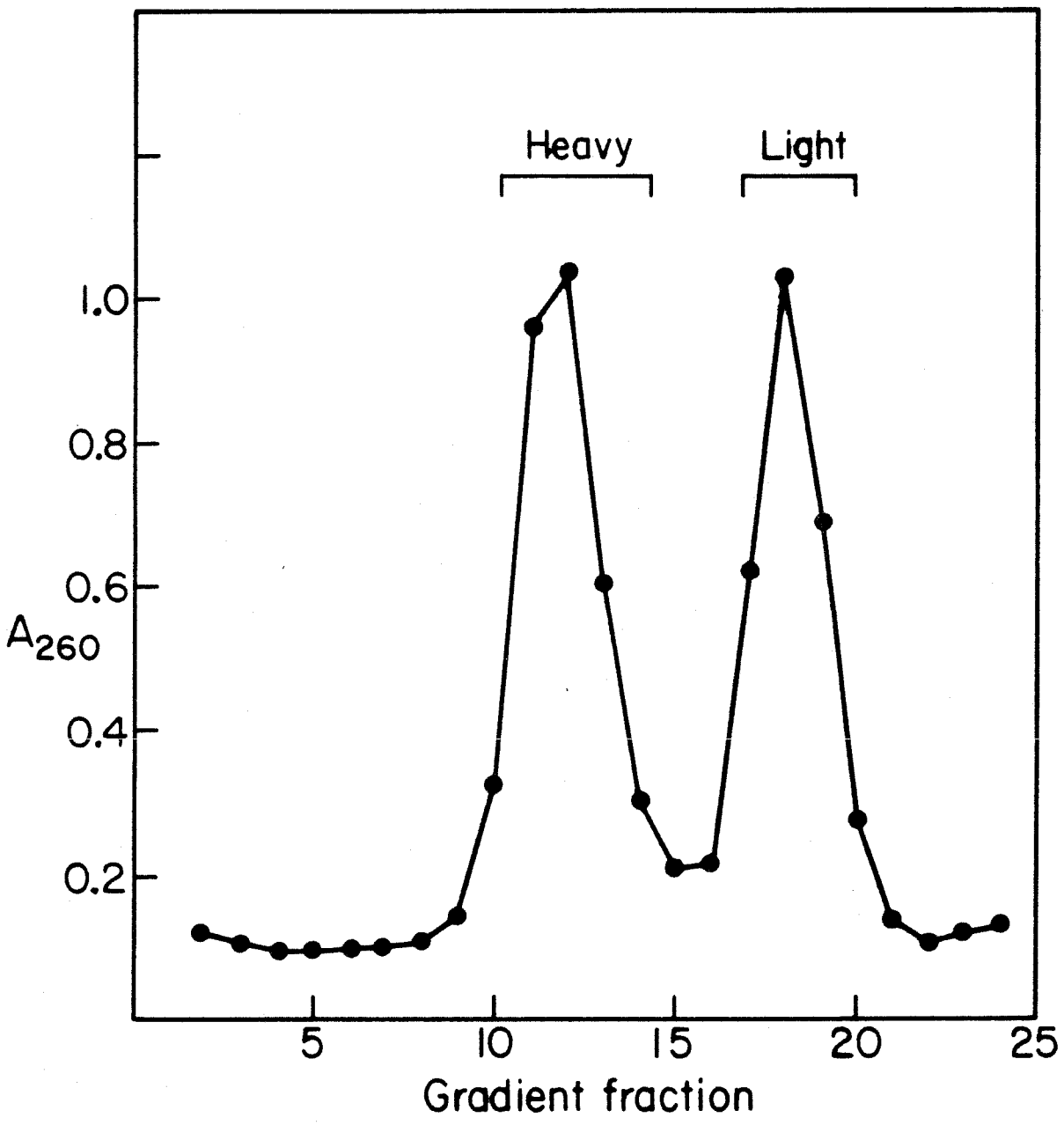
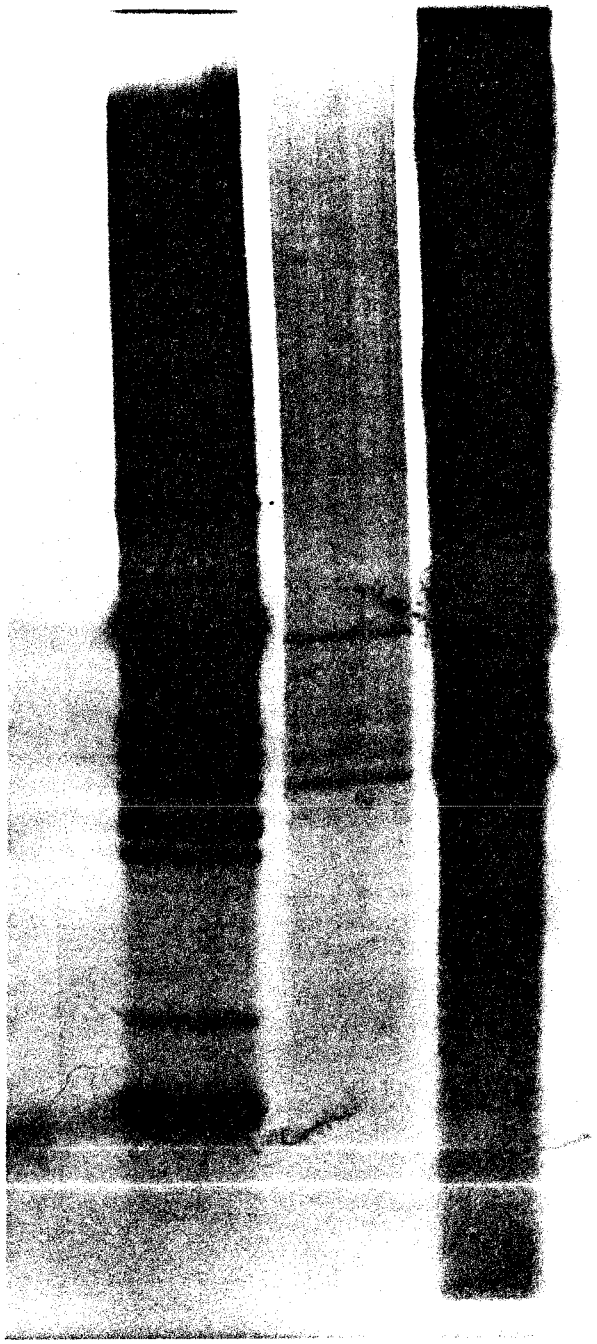

Heavy
Light
1.0
0.8
0.6
A_{260}
0.4
0.2
5
10
15
20
25
Gradient fraction

Figure 15

The electrophoretic patterns of transcription complex RNA, poly(A)+ RNA, and poly(A)- RNA are shown. RNA was prepared and electrophoresed as described in the text. The transcription complex RNA (middle track) was purified from twice the cell volume as the poly(A)+ RNA and ten times the cell volume as the poly(A)- RNA.



Chapter 5

Giant Overlapping Transcripts of Mitochondrial L Strand DNA

INTRODUCTION

The majority of polyadenylated mitochondrial RNA species are transcribed from the heavy ("H") strand of mitochondrial DNA. RNA species 4 through 16 have been demonstrated directly by RNA-DNA hybridization to have H strand origin (Amalric et al., 1978). In this earlier work, the strand specificity of the larger species was not clearly determined. The experiments described in this section concern the large polyadenylated species numbered 1, 2, and 3. These species are demonstrated here to be giant transcripts of the L strand which correspond to a large fraction of the mitochondrial genome map and contain overlapping sequences.

MATERIALS AND METHODS

Figure 1 shows the autoradiogram of ^{32}P-labeled mitochondrial polyadenylated RNA prepared from micrococcal nuclease treated mitochondria, after electrophoresis through an agarose-methylmercuric hydroxide slab gel. RNA species 2 is labeled more intensely than either 1 or 3, but not nearly as intensely as species 11-16. The RNA of interest is eluted from an agarose-methylmercuric hydroxide gel of this type by the following procedure. The wet gel, protected by cellophane wrapping, is subjected to autoradiography at 0-4 degrees for six to twelve hours. The gel and film are aligned by pinhole puncture done prior to developing of the film.

The regions of the gel containing the RNA bands of interest are then excised, crushed with a siliconized glass rod, and incubated in 0.01 M Tris, pH 7.4, 0.001 M EDTA, 0.1% SDS for 10-20 hours at 37 degrees C.

The buffer containing the eluted RNA is centrifuged through glass wool to remove gel fragments. The eluted material is adjusted to 10 mM dithiothreitol (Cleland's Reagent) in order to remove any methylmercuric hydroxide still complexed to the RNA. After 10 minutes at room temperature, the solution is adjusted to 0.2 M NaCl, 25 micrograms of unlabeled yeast tRNA is added as a carrier, and 2.5 volumes of ethanol is added to precipitate the RNA.

By this method, it has been possible to obtain an adequate amount of radioactive species 1, 2, and 3 (500 to 2000 CPM). These RNA species are, as far as we know, pure except for a small amount of heterogeneous material forming a background throughout the upper portion of the gel.

Mitochondrial DNA restriction fragments bound to nitrocellulose filters were prepared according to the Southern procedure (1975). Mitochondrial DNA was digested with the restriction endonucleases HpaII and BamI. It was then run on a 1% agarose gel and transferred to nitrocellulose paper by the Southern technique (1975). Individual membrane filter strips containing the whole range of restriction fragments were cut from large transfer filters and used for the hybridization with individual RNA species eluted from methylmercuric hydroxide agarose gels as described above. RNA was denatured in a low salt buffer (10 mM Tris, pH 7.4, 1 mM EDTA) for 5 minutes at 90 degrees C, then added to the hybridization medium. Reactions were carried out in 2 ml of 2X SSC for 2-3 days at 66 degrees C. At the conclusion of the incubations, the filter strips were washed extensively with hybridization buffer at 66 degrees. The strips were dried and then subjected to autoradiography. The autoradiography was carried out in conjunction with a DuPont

"Cronex-lightning plus" image intensification screen facing the opposite side of the film. The exposure of the film was carried out for 2-21 days at -70 degrees C. These conditions of exposure result in an enormous increase in sensitivity of the film over the traditional exposure without the intensification screen.

"Berk-Sharp" Experiments

In order to determine more accurately the mappings of these RNA species, hybridization reactions were carried out according to the method of Berk and Sharp (1977). In this procedure, RNA is hybridized to DNA. The hybridization mixture is then subjected to S1 nuclease digested in order to hydrolyze non-base paired molecules. The lengths of S1 protected segments are determined by gel electrophoresis. In this case, the RNA consisted of individual mitochondrial poly(A)+ RNA species 1, 2, and 3. The DNA consisted of separated light strands of mitochondrial restriction fragments. The separated strands of restriction fragments were prepared from double stranded restriction fragments by means of heat denaturation followed by separation on a non-denaturing agarose gel run at 4 degrees C. This method presumably takes advantage of differences in the secondary structure of the respective L and H strand segments. Although this method separates the strands, the single strand DNA bands must then be hybridized with known L and H strand mitochondrial DNA in order to determine which is which.

Most of these procedures were done with fragments prepared by HindIII restriction endonuclease. Some HpaII-BamI fragments were also used, in particular Δ 2a and 7.

RESULTS

RNA species 2, purified by the methods described above, was used in separated strand hybrid studies in order to determine its strand specificity. Table 1 summarizes the results of these measurements. There is a slight contamination of the RNA by nuclear DNA coded species. Of the remainder, essentially all hybridizes to the light ("L") strand of mitochondrial DNA. In Amalric et al. (1978), it was not possible to obtain individual RNA bands 1-3 with sufficient purity to make an unambiguous determination of their transcriptional strand specificity. In that study, a measurement of strand specificity on material eluted from the regions of the gel containing these bands suggested that all mitochondrial RNA with the size of species 3 and larger is of L strand origin. The increased yield of band 2 RNA in the present experiments has allowed us to make a determination of its strand specificity directly and unambiguously.

The strand specificity of polyadenylated species 1 and 3 was not determined in this way. It was tentatively assigned to the L strand on the basis of measurements by Amalric et al. (1978) and was confirmed by protection of DNA-RNA hybrids from S1 nuclease in experiments described below.

RNA species 2 was determined by Amalric et al. to be approximately 8500 nucleotides long, whereas species 1 is slightly larger, of approximately 10,200 nucleotides length. Species 3 is approximately half the size of species 2, with a measured length of 4200 nucleotides. Since the mitochondrial genome is slightly larger than 16,000 nucleotide pairs

in size, these species are each a significant fraction of the total mitochondrial genome.

In order to find the mapping locations of these RNA species with respect to the restriction map of the mitochondrial genome, two techniques appeared promising. The first was the "Southern" technique consisting of hybridization of individual RNA species of interest to DNA restriction fragments immobilized on nitrocellulose filters. This technique would provide a rough picture of the map locations of these RNA species, but is limited by the size and gel resolution of the DNA restriction fragments. For example, hybridization of an RNA species to a restriction fragment which is itself several thousand nucleotide pairs in length cannot tell us whether the RNA species hybridizes to all, most, or only one part of that fragment. Also, the Southern technique often results in grossly unequal efficiencies of transfer of the various DNA restriction fragments, at least in our hands, so hybridization of RNA cannot be analyzed quantitatively with respect to different restriction fragments. Nevertheless, this technique appeared to be useful since the large size of the RNA species themselves requires a first approximate positioning in order to use the more exact technique described by Berk and Sharp (1977).

Polyadenylated species 2 and 3 were incubated with Southern blots of restriction enzyme digested mtDNA. The washed and dried blots were then subjected to autoradiography for one to three weeks.

Reproductions of these autoradiograms appear in Figure 2. In order to interpret these photos, it is necessary to consult the restriction

map of the mitochondrial genome (Ojala, 1977) which is reproduced in Figure 3.

The particular restriction map which is of interest here is the HpaII plus BamI digest map. Starting from the origin of replication at 12 o'clock, and going clockwise, the fragments are, in order, 8, 17, 10, Δ2a, Δ2b, 15, 5, 12, 1, 6, 7, and so forth. The numbering scheme refers to the size of the HpaII digest fragments, in which fragment 1 is largest. The restriction endonuclease BamI cuts in only one site, which appears in the HpaII fragment number 2. Fragment 2 is thereby divided by BamI into two new fragments called Δ2a and Δ2b. The BamI digest is used because HpaII fragments 2 and 3, from opposite sides of the genome, do not separate well on gels.

Polyadenylated RNA species 2 hybridizes to fragments 1, Δ2a, 5, 6, and 7, as seen in Figure 2. This corresponds to a region of the map (Figure 3) from 2 o'clock to 7 o'clock, roughly. Within this region, fragments Δ2b, 12, and 15 also occur. Since this technique does not provide good resolution of hybrids to fragments smaller than 9, at least in our hands, the conclusion that species 2 hybridizes to fragments Δ2b, 12, and 15 is only tentative.

Polyadenylated RNA species 1 shows a hybridization pattern similar to that of species 2 (data not shown), though the bands on autoradiographs are faint. This is probably due to our inability to isolate much radioactive label in the form of RNA species 1.

Polyadenylated species 3 hybridizes with some of the fragments showing homology to RNA species 2, in particular fragments 1, Δ2a, and 5. Fragments 6 and 7 do not hybridize to RNA species 3.

The more exact mapping by the Berk-Sharp technique is necessary in order to achieve higher resolution. Before describing these experiments, an experiment should be mentioned which may be of importance in developing a detailed model of the metabolism of these giant L strand transcripts.

Figure 2 shows that there is a non-polyadenylated RNA species which is of nearly identical size to polyadenylated RNA species 2. It must be emphasized that the terms poly(A)+ and poly(A)- refer to oligo-deoxythymidylate cellulose binding and exclusion, respectively. The presence or lack of polyadenosine in these RNA species has not been demonstrated directly, for instance by RNase (A+T1) digestion. The occurrence of poly(A)+ and poly(A)- species of about the same size is most evident for poly(A)+ species 2, 4, and 5 when RNA is prepared under these conditions. The RNA from the non-polyadenylated fraction corresponding in size to species 2 was eluted and hybridized to Southern blots in the same way as poly(A)+ species 2. The autoradiogram from that experiment is shown in Figure 2. Within the limits of this technique, the results are identical. From this it can be concluded that there exists a non-polyadenylated size class of RNA which is very similar or identical to polyadenylated species 2 in its mapping position. This experiment demonstrates the possibility that RNA species 2 exists in polyadenylated and non-polyadenylated forms.

"Berk-Sharp" type Experiments

The restriction enzyme HindIII cuts mtDNA into three fragments (Ojala and Attardi, 1978). Fragment 1 is particularly useful for the

study of these RNA species since it overlaps part of HpaII fragment number 5, all of HpaII fragment number 2, and a large fraction of the genome extending in a counterclockwise direction from fragment 2. It is therefore nicely situated so as to locate the 5' ends of RNA species which extend part of the way into HpaII fragment 2. Interestingly, when the Berk-Sharp procedure was carried out with HindIII fragment 1 on the RNA species 1, 2, and 3, they all gave approximately the same result. All three species were found to extend about 2050 nucleotides into this fragment as discussed below.

Figures 4 and 5 demonstrate the Berk-Sharp experiments carried out for RNA species 1 and 2 (Figure 4) and RNA species 3 (Figure 5). In Figure 4, the HindIII-1 DNA segment protected by hybridization to RNA species 1 is found in column 2. The corresponding HindIII-1 DNA segment protected by hybridization to RNA species 2 (poly(A)+) is found in column 5. Other columns show markers (column 1) and control experiments in which either DNA was omitted (columns 3 and 6) or RNA was omitted (column 4). Figure 5, column 2, shows the corresponding protected segment of HindIII-1 DNA found after hybridization to RNA species 3.

The results obtained by using HindIII fragment 1 are limited in accuracy because the resolution of the gel electrophoretic system is limited for protected segments this large. To the limits of resolution, the segments protected by RNA species 1, 2, and 3 are identical in size.

In order to obtain more accurate mapping of RNA species 2 and 3, additional experiments were carried out using isolated L strand segments

of different restriction fragments. For RNA species 2, the DNA used was obtained from HpaII fragments Δ2a and 7. For RNA species 3, the DNA used was obtained from HindIII fragment 2.

The results obtained using RNA species 2 are shown in Figure 4, columns 7-10. Column 7 shows the protected segment of HpaII-Δ2a to be approximately 390 nucleotides in length. Column 9 shows the corresponding protected segment for HpaII-7 to be approximately 550 nucleotides in length.

Figure 5, column 3 shows the results of hybridization of HindII-2 DNA to RNA species 3. The protected segment is approximately 1230 nucleotides in length.

DISCUSSION

The rough positioning provided by the Southern blot experiments and the more exact positioning provided by the Berk-Sharp experiments allows us to construct a transcription map of the RNA species studied. The positions of RNA species 1, 2, and 3 have been plotted on the restriction map shown in Figure 3.

The position of RNA species 2 is based on highly repeatable Southern blot data and data from Berk-Sharp experiments using an amount of radioactivity in RNA and DNA adequate to obtain unambiguous results. Likewise, the results for RNA species 3 were obtained using an adequate amount of radioactivity.

The conclusion regarding the mapping location of RNA species 1 is less reliable. It has never been possible to obtain as much of this species as of the others and the results may be complicated by the

existence of a heterogeneous background of material in the preparative gels from which this material was obtained (Figure 1). The position assigned to RNA species 1 as shown in Figure 3 is based largely, therefore, on the results of the Berk-Sharp experiments whose results are shown in Figure 4.

Recent findings (Attardi et al., 1979; Cantatore and Attardi, manuscript in preparation) strongly suggest that there is a site for the initiation of L strand transcription near the origin of DNA replication and that transcription occurs "clockwise" from this site with respect to the map shown in Figure 3. There was found to be an abundance of transcription in the region between the origin of DNA replication (12 o'clock on the map) and the 5' terminus of the map locations for RNA species 1, 2, and 3. The common 5' terminus of RNA species 1-3 may therefore be a processing site or, alternatively, there may be a second transcriptional initiation site at or near this 5' terminus.

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TABLE 1

Poly(A) + RNA species 2 was hybridized to purified light (L) and heavy (H) strands of mitochondrial DNA and to human nuclear DNA as described in Materials and Methods

DNA	Fraction Hybridized
L	0.816
H	0.019
nuclear	0.165

Considering only the non-nuclear hybridization, the values are 0.977 (L) and 0.023 (H).

Figure 1

The figure shows the autoradiograph of a typical preparative (i.e., undried) agarose-methylmercuric hydroxide slab gel with tracks containing mitochondrial poly(A)+ RNA (right) and mitochondrial poly(A)- RNA (left). Cells were labeled with ^{32}P-orthophosphate (50 microcuries/ml) in the presence of 4×10^{-5} M cold phosphate for 2.5 hours in the absence of inhibitory drugs. Mitochondria were treated with micrococcal nuclease as described in Chapter 3 in order to eliminate contaminating RNA of cytoplasmic or nuclear origin. RNA was extracted from mitochondria and electrophoresed as described in Materials and Methods.

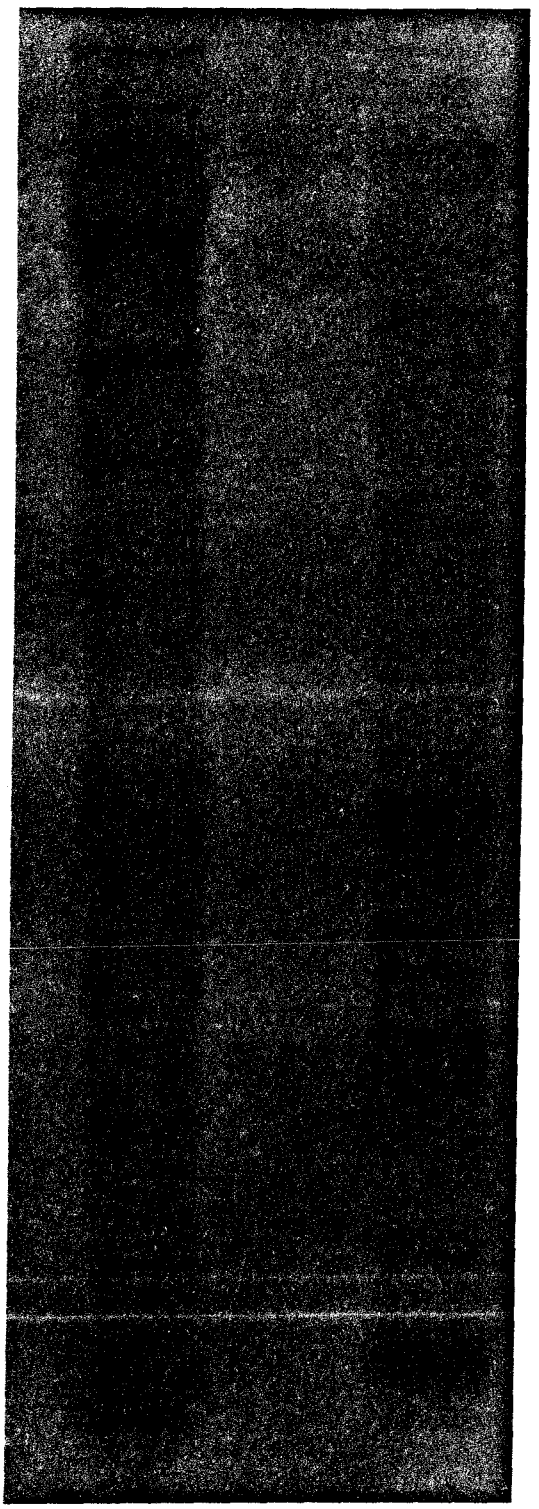


Figure 2

The results of Southern blot hybridizations as described in the text are shown. The marker column (M) shows the typical pattern of DNA fragments obtained by electrophoresis of mitochondrial DNA which has been digested with restriction enzymes Hpa II and Bam I. The adjacent columns show the results when RNA species 2, labeled with ^{32}P, is hybridized with nitrocellulose filters containing these DNA fragments. In one experiment (second from right column), small nitrocellulose blots containing DNA restriction fragments were individually hybridized to RNA species 2. These small blots were then arranged vertically for autoradiography.

The column on the right shows the Southern blot hybridization pattern for RNA species 3.

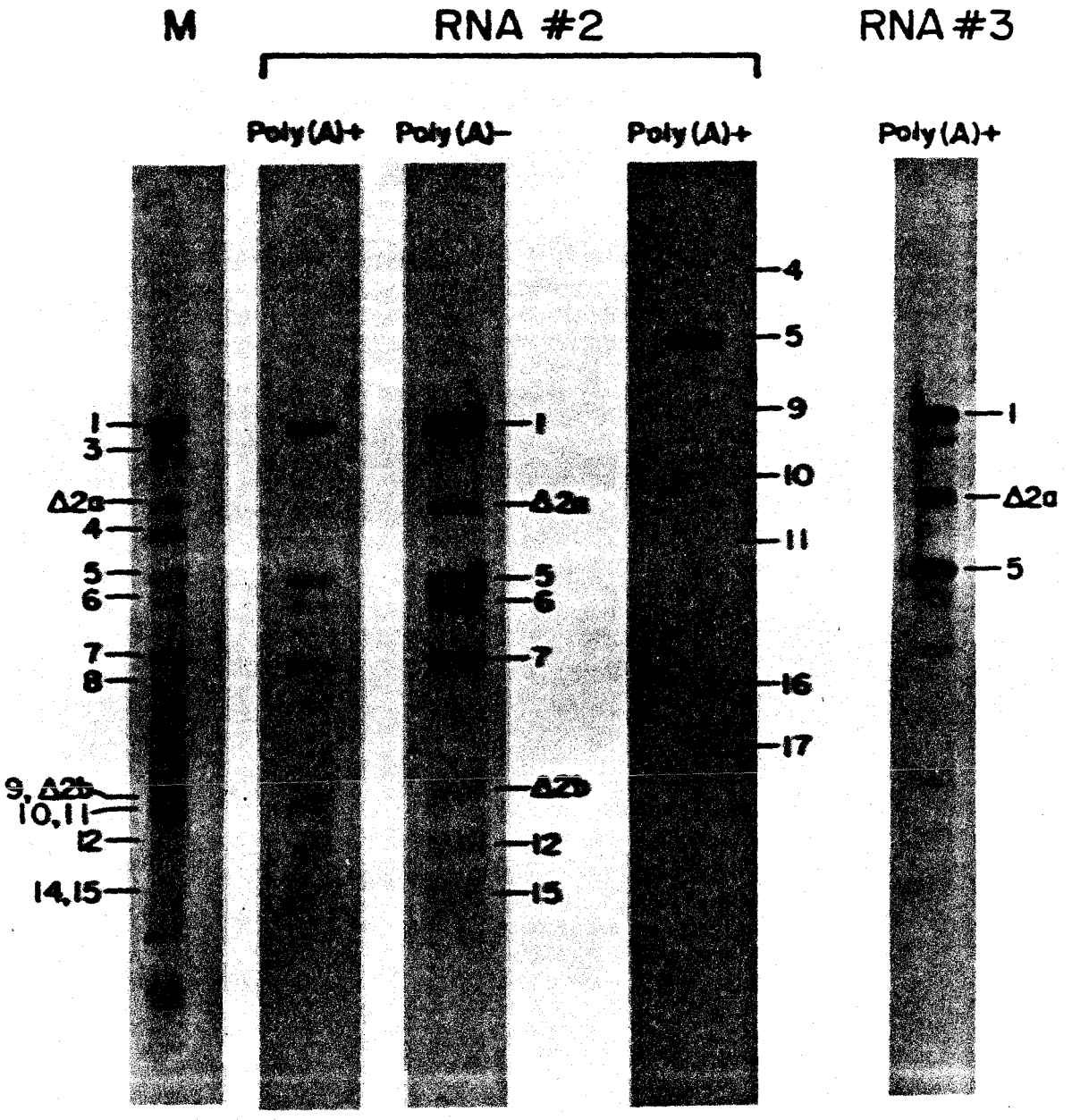

M
RNA #2
RNA #3
Poly (A)+
Poly (A)−
Poly (A)+
Poly (A)+
1
3
Δ2a
4
5
6
7
8
9, Δ2b
10, 11
12
14, 15
1
Δ2a
5
6
7
Δ2b
12
15
4
5
9
10
11
16
17
1
Δ2a
5

Figure 3

The restriction map of HeLa mitochondrial DNA is taken from Ojala and Attardi (1977). The cutting sites of restriction enzymes Hpa II, Eco RI, Hind III, and Bam I are plotted.

The positions of RNA species 1, 2, and 3 as determined by hybridization studies (see Discussion) are plotted as arcs outside of the restriction map.

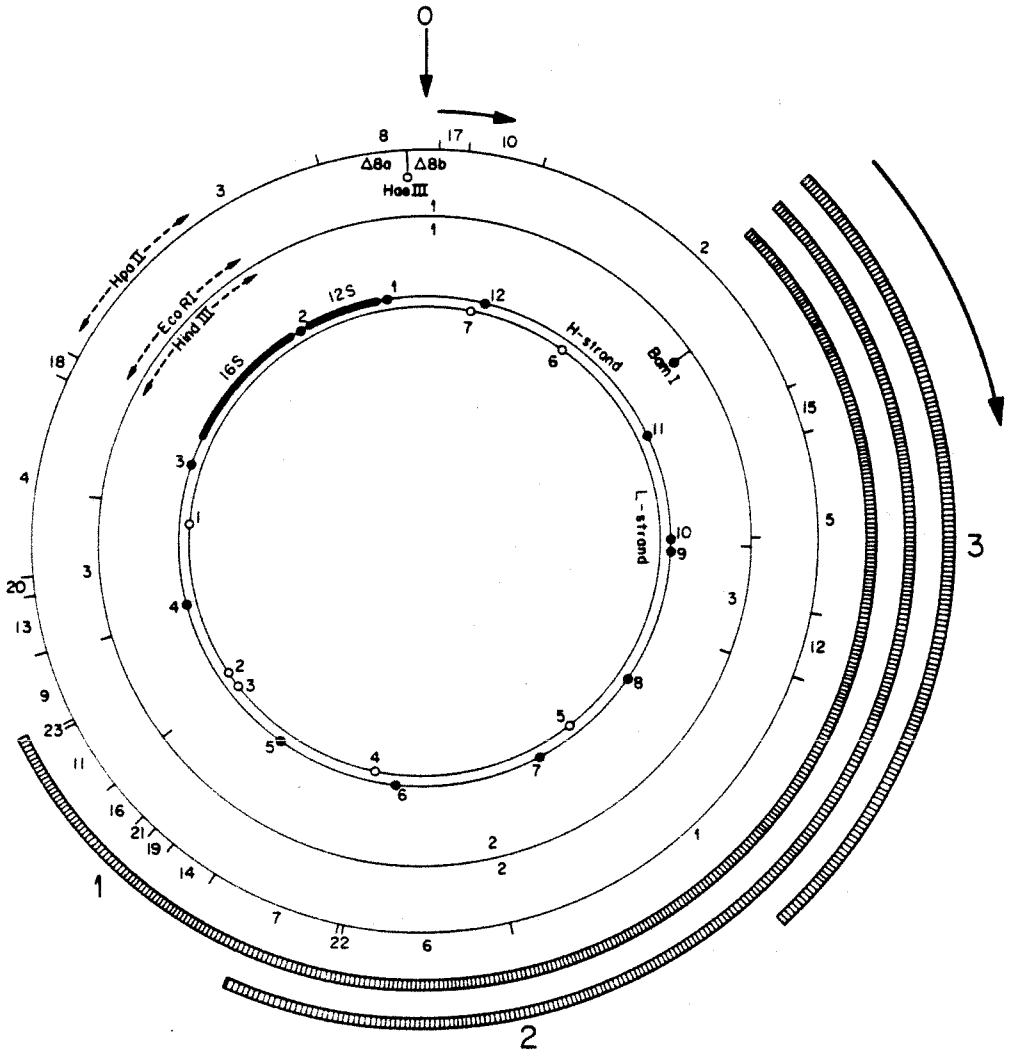

0
Δ8a
Δ8b
HaeIII
HpaII
EcoRI
HindIII
12S
16S
H-strand
L-strand
BamI
1
2
3

Figure 4

The results of Berk-Sharp studies with RNA species 1 and 2 are shown. RNA species were hybridized to isolated L strand DNA obtained from individual restriction fragments. Following S1 nuclease treatment to digest unhybridized molecules, the material was electrophoresed through a non-denaturing gel.

Column 1: Hpa II + Bam I digested mitochondrial DNA: markers for 2-6

2: RNA species 1 plus Hind III fragment 1

3: Same as 2, except no DNA

4: Same as 2, except no RNA

5: RNA species 2, Hind III fragment 1

6: Same as 5, except no DNA

7: RNA species 2, Hpa II fragment 2a

8: Same as 7, except no DNA

9: RNA species 2, Hpa II fragment 7

10: Same as 9, except no DNA

11: Hpa II = Bam I digested mitochondrial DNA: markers for 7-10

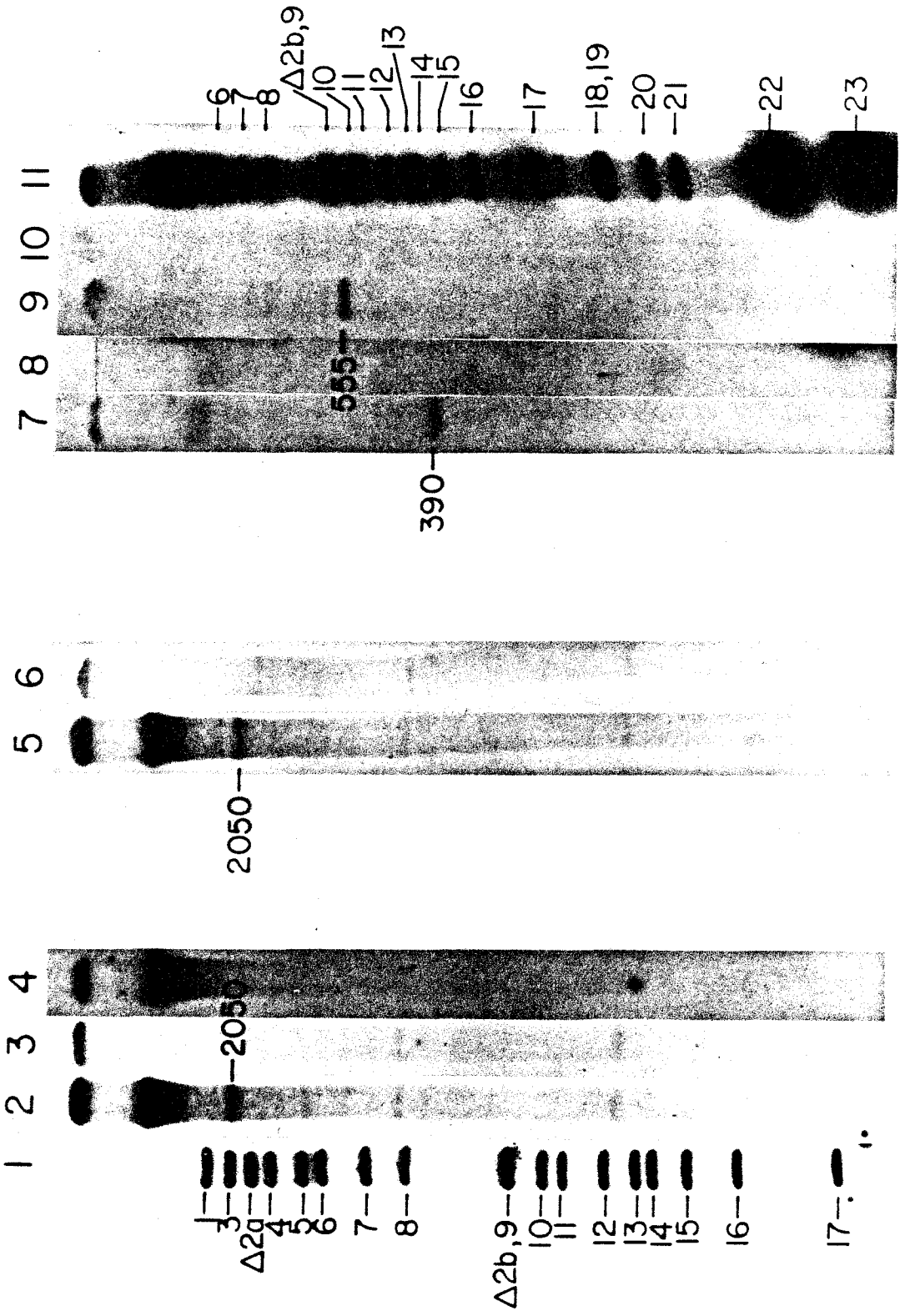

1 2 3 4
2050
1—
3—
Δ2a—
4—
5—
6—
7—
8—
Δ2b,9—
10—
11—
12—
13—
14—
15—
16—
17—
5 6
2050—
7 8 9 10 11
555—
390—
6—
7—
8—
Δ2b,9
10—
11—
12—
13—
14—
15—
16—
17—
18,19
20—
21—
22—
23—

Figure 5

Berk-Sharp hybridization studies were carried out as described for Figure 4 and in the text. RNA species 3 was hybridized to purified L strand DNA obtained from individual restriction fragments.

Column 1: Hpa II + Bam I digested mitochondrial DNA

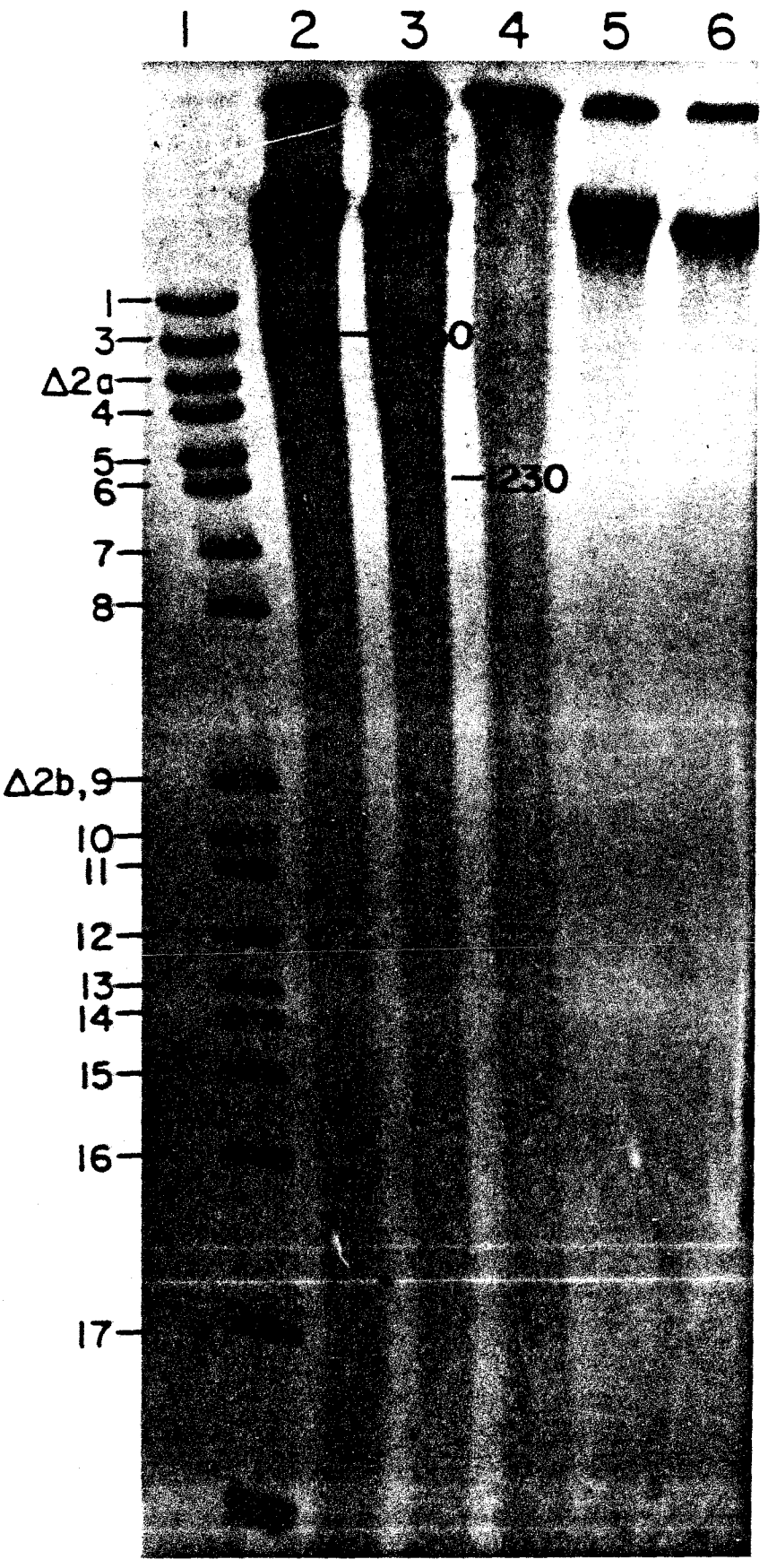
2: RNA species 3, Hind III fragment 1

3: RNA species 3, Hind III fragment 2

4: RNA species 3 alone

5: Hind III fragment 1 alone

6: Hind III fragment 2 alone


1
2
3
4
5
6
1
3
Δ2a
4
5
6
7
8
Δ2b,9
10
11
12
13
14
15
16
17
0
230

Chapter 6

Investigation of "Free" Polyadenylic Acid of HeLa Mitochondria: Its Possible Relation to Polyadenylic Acid Covalently Linked to Large RNA Molecules

INTRODUCTION

Most eucaryotic messenger RNA molecules contain a poly(A) tail at the 3' end (Brawerman, 1974). Some exceptions exist (Sonenshein et al., 1976; Milcarek et al., 1974), and particularly histone mRNAs (Adesnik and Darnell, 1972; Greenberg and Perry, 1972). The metabolism of poly(A) in mammalian cell nuclei and cytoplasm is complex. The initial synthesis of poly(A) occurs in the nucleus (Darnell et al., 1971) but there is turnover of adenylate residues at the 3' ends of poly(A) segments in both nucleus and cytoplasm (Diez and Brawerman, 1974; Sawicki et al., 1977).

The existence of a short 3'-poly(A) in a small fraction of E. coli mRNAs has been reported (Nakazato et al., 1975).

The question of the function of poly(A) remains controversial. Revel and Groner (1978) have reviewed evidence which suggests that poly(A) helps to maintain mRNA in an undegraded state. Most of the experiments reported involved the translation of mRNAs in vitro in foreign translation systems and must therefore be interpreted with caution.

Mitochondrial RNA of HeLa has been shown to contain poly(A) (Ojala and Attardi, 1974a; Perlman et al., 1973). The polyadenylated RNA species include presumptive messenger RNAs, isolated from mitochondrial polyribosomes (Ojala and Attardi, 1974a; Amalric et al., 1978). Mitochondrial poly(A) is shorter than nuclear or cytoplasmic poly(A) in HeLa, being 50-60 nucleotides in length (Ojala and Attardi, 1974b; Perlman et al., 1973).

HeLa mitochondria contain poly(A) both "bound", that is, covalently linked to relatively larger RNA molecules, and "free", consisting of

poly(A) molecules containing no (or at most a few) non-adenylate residues (Ojala and Attardi, 1974b).

The function of "free" poly(A) is unknown. The previous study (Ojala and Attardi, 1974b) could not rule out the possibility that "free" poly(A) is derived from the decay of poly(A)+ RNA (the term "poly(A)+ RNA" is used here to mean an RNA molecule containing both poly(A) and non-poly(A) segments) either in vivo or as the result of extraction procedures. It was found that the labeling of "free" poly(A) is insensitive to ethidium bromide present at levels sufficient to block mitochondrial transcription almost completely. This result shows that "free" poly(A) labeling does not require mitochondrial transcription, but the interpretation may be complicated by pathological effects of the drug.

The present study was designed to rule out the possibility that "free" poly(A) is derived from poly(A)+ RNA. If it were, the poly(A) segments bound to large RNA molecules should incorporate radioactive label prior to the incorporation of label into "free" poly(A) as in other precursor-product systems. The studies to be described below show that this is not the case.

In the previous study (Ojala and Attardi, 1974b), Actinomycin D was routinely used prior to in vivo labeling of cell cultures in order to block incorporation of radioactive label into cytoplasmic RNA. In the present study, the existence of "free" poly(A) is demonstrated in mitochondria of HeLa labeled in the absence of inhibitory drugs in order to rule out the possibility that "free" poly(A) is produced as an artifact of Actinomycin D treatment.

MATERIALS AND METHODS

Growth of HeLa cells in spinner culture, labeling with 2,8-^{3}H-adenosine in the presence of 0.1 microgram/ml Actinomycin D, cell breakage, and purification of mitochondria were carried out according to the methods described previously (Ojala and Attardi, 1974b). Fractionation of RNA by oligo-deoxythymidylate cellulose chromatography and analysis of formaldehyde-denatured RNA by electrophoresis through cylindrical polyacrylamide gels containing 3% formaldehyde were also as described (Ojala and Attardi, 1974a). Fractionation of cylindrical polyacrylamide gels has been described previously (Maizel, 1966; Lederman and Attardi, 1973) and involves forcing the gel through a metal screen into a stream of aqueous buffer. The fragmented gel particles produced in this way are collected directly into scintillation vials allowing elution of RNA from the gels and scintillation counting of the resulting eluted radioactivity in a quantitative way. In the experiments to be described, quantitation of RNA eluted from cylindrial polyacrylamide gels was only attempted for small (approximately 60 nucleotides; Perlman *et al.*, 1973) RNA of the size of mitochondrial polyadenylic acid, in order to avoid any potential problems resulting from inefficient elution of large RNA species from acrylamide gels.

In some experiments, cells were labeled *in vivo* in the absence of inhibitory drugs such as Actinomycin D. In those experiments, mitochondria were purified of contaminating RNA of cytoplasmic and nuclear origin by the RNase A treatment described in Chapter 3.

In order to purify RNA for further analysis, slab gel electrophoresis

was used in some cases. The gel consisted of 15% polyacrylamide, 0.5% bisacrylamide, 5 M urea, in a buffer consisting of 0.05 M Tris-borate, pH 8.3, 0.001 M EDTA. RNA was dissolved in 5 M urea and heated to 90 degrees for one minute before being applied to the gel. Following electrophoresis, RNA purified in this way was located by autoradiography. Alignment of the gel and the film was accomplished by pinhole puncture and RNA bands of interest were excised. Elution of RNA from the gel was accomplished by soaking crushed gel fragments in a buffer consisting of 0.5 M sodium acetate, 0.001 M EDTA, 0.1% SDS for 6 to 12 hours.

In one experiment, double labeled ATP was prepared and purified by charcoal binding technique (Humphreys, 1973) and thin layer chromatography (Cashell et al., 1969).

Base composition analysis of alkali hydrolyzed RNA was accomplished by ion exchange chromatography with Dowex 1-X8 resin as previously described (Attardi et al., 1966).

RESULTS

"Free" Poly(A) from Mitochondria Directly Lysed with SDS

The "free" poly(A) which was detected in the previous work (Ojala and Attardi, 1974b) was obtained by a procedure in which mitochondria were lysed in the detergent Triton X-100, following which the lysate was centrifuged through a sucrose gradient. The "free" poly(A) was most prevalent in the region of the gradient containing the slowest sedimenting structures.

Precautions were taken in that work in an attempt to minimize the effects of endogenous RNase activity including the use of RNase inhibitors such as polyvinyl sulfate and rat liver supernatant (alleged to have anti-RNase activity; Blobel and Potter, 1966). Nevertheless, it seemed possible that "free" poly(A) was generated by some RNase activity during the rather lengthy extraction procedure. An experiment was devised to test this possibility within the limits of the techniques available. If "free" poly(A) were absent from the RNA of mitochondria lysed with SDS immediately following mitochondrial isolation and pelleting, this would be evidence that "free" poly(A) is created by degradation of larger molecules during extraction and purification. If "free" poly(A) is present in extracts of SDS lysed mitochondria, then it cannot have been created entirely by RNase activity following mitochondrial lysis.

In this experiment, cells were suspended at a concentration of 1×10^6/ml in Eagle's phosphate medium supplemented with 5% dialyzed calf serum. Actinomycin D (0.1 micrograms/ml) was added 30 minutes prior to labeling. Labeling was accomplished by the addition of 2,8-^{3}H-adenosine (3.75 microcuries/ml) for 60 minutes.

Mitochondria were isolated as described in Materials and Methods, including treatment with 0.03 M EDTA prior to the final mitochondrial pelleting step. The mitochondria were lysed in SDS buffer (0.5% SDS, 0.01 M Tris, pH 7.4, 0.001 M EDTA, 0.1 M NaCl) and run through a 15-30% sucrose-SDS gradient formed over a 64% sucrose cushion. Centrifugation was carried out for 24 hours at 25 KRPM, 20 degrees, in an SW-27 rotor.

The gradient was fractionated from the bottom and a small sample of each fraction was counted directly by mixing with a Xylene-based

scintillation fluid (Anderson and McClure, 1973). The pattern of radioactivity determined in this way is shown in Figure 1. The regions of the gradient labeled A, B, and C in Figure 1 correspond to the bottom, middle, and top portion of the gradient, respectively. The fractions comprising the three gradient cuts were pooled for further analysis.

RNA was extracted from the gradient cuts by the SDS-pronase method as described previously (Amalric et al., 1978) except that cut C was extracted directly with phenol:chloroform:isoamyl alcohol without first being subjected to pronase digestion.

The purification of polyadenylated RNA and extraction of polyadenylic acid by digestion with RNase A plus T1 RNase has been described previously (Ojala and Attardi, 1974b).

A sample of the poly(A)+ RNA of each of the gradient cuts A, B, and C was divided into equal halves. One was subjected to RNase digestion in order to obtain polyadenylic acid, while the other was not.

The resulting samples of poly(A)+ RNA and poly(A) were denatured by formaldehyde treatment and analyzed by electrophoresis through cylindrical polyacrylamide gels containing formaldehyde as described in Materials and Methods. The electrophoretic patterns of ^{3}H label determined in this way are shown in Figure 2. The peaks corresponding to "free" polyadenylic acid as demonstrated by Ojala and Attardi (1974b) are labeled. The poly(A) obtained by RNase treatment of poly(A)+ RNA is shown in the right column of Figure 2. In each case, the poly(A) electrophoretic pattern in the right column corresponds to the pattern of non-RNased material to its immediate left.

This experiment demonstrates the isolation of "free" poly(A) from SDS lysates of HeLa mitochondria. As can be seen in the lowest panels of Figure 2, the top portion of the sucrose gradient contains poly(A)+ RNA (only the poly(A)+ fraction was analyzed in this way) which is largely "free" poly(A) based on its size and the fact that the peak appears almost identical in both the RNased and non-RNased samples. "Free" poly(A) is therefore not generated as an artifact of prolonged centrifugation following mitochondrial lysis unless the same type of artifactual production of "free" poly(A) occurs in the presence of SDS, an unlikely possibility. In this experiment, as in the previous work (Ojala and Attardi, 1974b), the "free" poly(A) is prevalent in the region of the gradient containing the slowest sedimenting structures.

Base Composition of "Free" Poly(A)

The demonstration of the existence of a mitochondrial poly(A) polymerase (Jacob et al., 1974) suggests that the synthesis of poly(A) may require some primer sequence. "Free" poly(A) may conceivably be synthesized starting with a non-poly(A) oligonucleotide primer.

In an attempt to discover whether "free" poly(A) contains non-adenylate residues, "free" poly(A) was subjected to base composition analysis. The "free" poly(A) used for this analysis was first purified in a way which promised better resolution than sucrose gradients can offer. Mitochondrial poly(A)+ RNA, labeled in vivo with ^{32}P was separated by polyacrylamide-urea slab gel electrophoresis. Several RNA species of about the expected size for "free" poly(A) (i.e., somewhat smaller than 4S RNA) were chosen for further analysis to be described below. Each

was compared with known "free" poly(A) in a mixing experiment and subjected to base composition analysis.

HeLa cells were suspended at a concentration of 2 x 10^6/ml and labeled for 2 hours with 50 microcuries/ml ^{32}P-orthophosphate in the presence of 4 x 10^{-5} M cold phosphate.

Mitochondria were purified of contaminating RNA of cytoplasmic and nuclear origin by treatment with RNase A plus DNase 1 as described in Materials and Methods.

Poly(A)-containing RNA was purified and then electrophoresed through a 15% acrylamide, 0.5% bis, 5 M urea slab gel as described in Materials and Methods. The pattern obtained by autoradiography is shown in Figure 3. Four bands, labeled A, B, C, and D, were chosen for further analysis. After elution of the RNA from the gel, base compositions were determined as described in Materials and Methods and the results are summarized in Table 1. All of the bands tested contain non-adenylate residues. A, B, and C are each about 70% adenylic acid. D is 89% adenylic acid.

The eluted ^{32}P-labeled RNA bands were analyzed with respect to the question of which, if any, corresponds to the "free" poly(A) found previously by cylindrical gel analysis (Ojala and Attardi, 1974b; also experiments described in this chapter). Individual ^{32}P-labeled bands were mixed with ^{3}H-adenosine labeled mitochondrial poly(A)+ RNA. The various mixtures were electrophoresed through cylindrical polyacrylamide gels in the presence of formaldehyde as described in Materials and Methods. The electrophoretic patterns obtained in this way are shown in Figure 4.

In addition, the lowest panel of Figure 4 shows the pattern obtained when ^{32}P-labeled poly(A)+ RNA, RNased to obtain polyadenylic acid, is mixed with ^{3}H-adenosine-labeled poly(A)+ RNA. It can be seen that RNA band D corresponds most closely to "free" poly(A).

The material shown as band "D" in Figure 4 electrophoreses in a region which corresponds to the middle of the ^{3}H-labeled "free" poly(A). The ^{32}P-labeled material was eluted from the middle of a broad band detected in the acrylamide-urea slab gel. Subsequently, material from the upper and lower regions of "band D" was eluted from the slab gel, mixed with ^{3}H-poly(A)+ RNA, and analyzed by cylindrical gel electrophoresis. The material coelectrophoresed with the leading and trailing edges of the ^{3}H-labeled "free" poly(A) peak (data not shown).

The results of the mixing experiments show that "free" poly(A) can be detected in mitochondrial lysates labeled with ^{32}P in the absence of inhibitory drugs. Band D of the acrylamide gel corresponds to the "free" poly(A) detected previously by cylindrical gels. Furthermore, band D consists mainly of adenylate residues with perhaps 11% non-adenylate residues, corresponding to an average of 5 or 6 per full length molecule.

Labeling Kinetics of "Free" and Bound Poly(A)

In order to test whether the labeling of bound poly(A) precedes the labeling of "free" poly(A), the kinetics of labeling of these species was investigated. If bound poly(A) were found to label earlier than "free" poly(A), this would be consistent with the idea that bound poly(A) is a precursor to "free" poly(A). If this were found to be untrue, it would demonstrate that "free" poly(A) is not derived from bound poly(A).

"Free" poly(A) is defined by its electrophoretic mobility in polyacrylamide gels as demonstrated in Ojala and Attardi (1974b) and in Figure 2. "Bound" poly(A) is defined as polyadenylic acid which electrophoreses with molecules the size of mitochondrial poly(A) (i.e., about the same as "free" poly(A)) after it has been released from larger molecules by treatment with RNase A plus T1 RNase (Ojala and Attardi, 1974b). Experimentally, the label incorporated into bound poly(A) may be determined by first finding the extent of labeling of "free" poly(A) and total poly(A) (determined by RNase digestion of total polyadenylated RNA). "Bound" poly(A) labeling is the difference between "free" and total poly(A) labeling.

Cells were suspended at 5 x 10^5/ml and treated with 0.1 microgram/ml Actinomycin D 30 minutes prior to labeling. Labeling was accomplished by the addition to each culture of 2,8-^{3}H-adenosine to 2.5 microcuries/ml. Individual cultures were labeled for various times ranging from 5 to 120 minutes.

Mitochondria were isolated according to the method of Ojala and Attardi (1974b), including a short treatment with 0.03 M EDTA prior to the final mitochondrial pelleting. Mitochondrial pellets were lysed in SDS buffer and polyadenylated RNA was purified as described in Materials and Methods.

A sample of the polyadenylated RNA from each culture was divided into two equal halves, one of which was treated with RNase A plus T1 RNase to obtain purified "total" poly(A), the other of which was not. The total RNA and total poly(A) sample from each culture were analyzed

by cylindrical gel electrophoresis in the presence of formaldehyde as described in Materials and Methods. A typical electrophoretic pattern of total poly(A)+ RNA obtained from SDS lysed mitochondria is shown in Figure 5, representing the culture labeled with ^{3}H-adenosine for 60 minutes. The "free" poly(A) is present as a peak migrating in the region from fractions 40 to 50. The "free" poly(A) and total poly(A) electrophoretic patterns obtained from each culture are plotted in Figure 6 (only the regions of the gel corresponding to these classes of RNA are shown).

The incorporation of radioactive label into each peak representing "free" or total poly(A) was calculated by summing the radioactivity of the gel fractions and subtracting background radioactivity (as can be seen, the background is always relatively low compared to the radioactivity in the peaks).

A rough idea of the time course of labeling of "free" and total poly(A) may be obtained by plotting the total radioactivity found in each peak as a function of labeling time. Figure 7 shows such a plot in which poly(A) labeling of the different cell cultures was corrected for differences in yield according to the optical density (260 nm) of the total mitochondrial RNA.

The relative labeling of "free" and "bound" poly(A) is depicted in Figures 8 and 9. Figure 8 shows the ratio of radioactive incorporation into "free" and total poly(A) as a function of labeling time. Figure 9 shows the ratio of "free" and bound poly(A) as a function of labeling time.

It can be seen that at the earliest time of labeling (5 minutes), most of the poly(A) labeling occurs in "free" poly(A). The fraction of label in "free" poly(A) decreases as labeling time increases. By the end of the longest labeling time (120 minutes), most of the incorporation is in the bound fraction.

In order to determine what fraction of ^{3}H label went into poly(A) in the polyadenylated RNA fraction, the resistance of each poly(A)+ RNA sample to digestion by RNases A plus T1 was measured. A sample of the mitochondrial poly(A)+ RNA from each culture was subjected to RNase digestion, the trichloroacetic acid precipitable radioactivity being determined before and after RNase digestion. Figure 10 shows the RNase resistance plotted as a function of labeling time. A continuous decrease in RNase resistance over the time course of labeling is found. After a 5 minute labeling, over 60% of the label in poly(A)+ RNA is in poly(A).

In the kinetic experiment just described, the drug Actinomycin D was used to suppress nuclear labeling of RNA. In order to show that the results obtained are not artifacts of the drug treatment, an attempt was made to repeat the results obtained in the kinetic experiment without using inhibitory drugs. The method described in Amalric et al. (1978) and in Chapter 3, using RNase A plus DNase 1 on intact mitochondria to remove contaminants of nuclear and cytoplasmic origin was used.

Cells were suspended at a concentration of 5×10^{5}/ml and labeled for various times with 2,8-^{3}H-adenosine (6.25 microcuries/ml for shortest labeling time, 4 microcuries/ml for other cultures). Prior to cell

breakage, ^{32}P-labeled cells were added in equal amounts to each culture.

The poly(A)+ RNA was purified as described in Materials and Methods from mitochondria purified of nuclear and cytoplasmic contaminants by treatment with RNase A plus DNase 1. As in the previous kinetic experiment, samples of the poly(A)+ RNA of each culture were divided into equal portions, one of which was subjected to digestion with RNases A plus T1, the other of which was not. The determination of "free" and total poly(A) labeling was accomplished by summing the radioactivity values of the respective peaks in acrylamide gels, as before.

The electrophoretic patterns of the poly(A)+ RNA and purified poly(A) of one culture are shown in Figure 11. The 5 minute ^{3}H label profile shows a pronounced "free" poly(A) component and a slight amount of label in other regions. The 60 minute ^{32}P profile shows the existence of large RNA species and a comparatively small amount of label in "free" poly(A).

Figure 12 shows the electrophoretic patterns obtained for "free" poly(A) for all three cultures labeled for 2, 5, and 20 minutes, respectively with ^{3}H-adenosine. A pronounced "free" poly(A) region is seen after all labeling times, from 2 to 20 minutes.

Figure 13 shows the ratio of "free" poly(A) to total poly(A), both determined from the electrophoretic profiles as described for the previous kinetic experiment. As in the other kinetic experiment, labeling of "free" poly(A) precedes the labeling of bound poly(A). In this experiment, ^{3}H-labeling was carried out for as short a period as 2 minutes. "Free" poly(A) contained the majority of label in poly(A) after this

labeling period. In a manner similar to the other kinetic experiment, the "free" poly(A) labeling was a decreasing fraction of the total poly(A) labeling as labeling time increased.

This experiment shows that the labeling kinetics produced in the other experiment are not dependent upon the presence of Actinomycin D, since this experiment was carried out without the use of inhibitory drugs.

Labeling Kinetics of "Free" Poly(A)

The kinetics of labeling of "free" poly(A) were measured in an experiment in which the kinetics of labeling of the ATP pool were also determined. This might reveal whether "free" poly(A) is turned over rapidly or slowly, or if there is more than one stability class.

Cells were suspended at a concentration of 1×10^6/ml. "Long-term" labeling was accomplished by the addition to each culture of ^{32}P-orthophosphate to a level of 15 microcuries/ml in the presence of 10^{-4} M cold phosphate. ^{32}P-labeling was carried out for 2.5 hours for each culture. Short-term ^{3}H-labeling was carried out by the addition of 2-^{3}H-adenosine (10 microcuries/ml) at various times after the beginning of ^{32}P labeling and for intervals ranging from 5 to 20 minutes.

Following cell harvesting and breakage, mitochondria were treated with RNase A plus DNase 1 to remove contaminating RNA of nuclear and cytoplasmic origin. A sample of the mitochondria from each culture was saved in order to determine the specific activity of ATP, defined as the ratio of ^{3}H/^{32}P in this precursor. Another sample of the mitochondria from each culture was used to prepare poly(A)+ RNA by SDS lysis and the procedure described in Materials and Methods. ATP was isolated

by charcoal adsorption (Humphreys, 1973) and purified by ion exchange thin layer chromatography (Cashel et al., 1969).

The ATP specific activity is plotted in Figure 14 as a function of ^{3}H-adenosine labeling time. The mitochondrial ATP pool is rapidly labeled to a maximum value by about 10 minutes under these conditions and maintains a nearly constant value for another 10 minutes.

The ^{3}H radioactivity in "free" poly(A) was determined from the patterns obtained by electrophoresis through acrylamide-formaldehyde gels as described in Materials and Methods.

The "free" poly(A) ^{3}H radioactivity values were corrected for differences in yield as determined by ^{32}P radioactivity in the non-polyadenylated mitochondrial RNA fraction.

The labeling of "free" poly(A) is plotted in Figure 15 as a function of ^{3}H-adenosine labeling time. The label in "free" poly(A) rises rapidly for the first 10 minutes, corresponding to a rapid rise in the precursor specific activity. The maximum rate of incorporation of label (observed between 5 and 10 minutes) is not maintained during the final 10 minutes of labeling, even though the precursor specific activity does not decrease. This suggests a fairly rapid turnover of "free" poly(A) (i.e., half-life less than 10 minutes) but the data are not sufficient to allow an accurate calculation of the half-life.

DISCUSSION

The existence of "free" polyadenylic acid in HeLa mitochondria was demonstrated by Ojala and Attardi (1974b). That work did not rule out the possibility that "free" poly(A) is produced by degradation of

poly(A) covalently linked to larger RNA either in vivo or as the result of the extraction procedure. One suggestive result of the earlier work was that "free" poly(A) labeling is totally insensitive to ethidium bromide present at a level high enough to inhibit mitochondrial transcription. This implied that "free" poly(A) synthesis does not require mitochondrial transcription but does not prove that "free" poly(A) can't derive from some process involving labeling and release of poly(A) covalently linked to larger RNA molecules. The present study suggests strongly that "free" poly(A) is not derived from poly(A) covalently linked to larger RNA molecules and is not the product of some artifact of Actinomycin D treatment.

If "free" poly(A) were derived from "bound" poly(A), then bound poly(A) should incorporate radioactive label before "free" poly(A) does. The results of kinetic experiments in the present work show that the opposite is the case. "Free" poly(A) was found to incorporate label before bound poly(A) both in the presence and absence of Actinomycin D.

Another possibility which is ruled out is that there might be a small pool of bound poly(A) which is rapidly labeled and converted to "free" poly(A). All the evidence in the present study from labeling for one to two hours with either ^{3}H-adenosine or ^{32}P suggests that the pool of bound poly(A) is much larger than the pool of "free" poly(A).

The most likely explanation for the data shown in this study is that "free" poly(A) is maintained as a relatively small pool of molecules which are rapidly synthesized and turned over.

The turnover of "free" poly(A) might occur by decay or it might occur by the conversion of "free" poly(A) to bound poly(A).

In one experiment described here, mitochondria were lysed with SDS and the lysate was spun through a sucrose SDS gradient. A large fraction of the oligo-deoxythymidylate binding RNA from the slowest sedimenting region of the gradient is indistinguishable from the "free" poly(A) found by other methods of preparation. Though not proof, this result is consistent with the idea that "free" poly(A) exists in soluble form rather than attached to large, faster sedimenting structures.

The results of this work lead to the question as to what function, if any, "free" polyadenylic acid has in mitochondrial metabolism. It might serve no function as such, except as a storage form of adenosine. Abraham and Jacob (1978) have suggested that one function of cellular poly(A) is to serve as a storage form of adenine nucleotides. The relatively small amounts of "free" poly(A) compared to other poly(A) in the cell suggests that it is probably not important as a storage form of adenosine since it is a miniscule fraction of cellular poly(A).

An alternative possiblity is that "free" poly(A) is a precursor to some or all bound poly(A). The data presented here do not prove this but are consistent with it. It is also possible that "free" poly(A) is produced by a poly(A)-polymerase of HeLa mitochondria in the absence of any critical biological function for this substance, but only as a result of the inefficiency of the system.

The possibility that "free" poly(A) is converted to bound poly(A) might have seemed outlandish in the recent past. Recent discoveries of RNA ligase activity, for example in the processing of tRNA molecules (Peebles et al., 1978), lends credibility to this hypothesis. The ligation

of poly(A) to a larger RNA molecule would differ from the processing of tRNA molecules because the poly(A) and the RNA molecule to which it becomes bound are not originally transcribed as parts of a larger precursor molecule.

The rigorous demonstration that "free" poly(A) is a precursor to bound poly(A) would depend on technical capabilities which do not now exist. A pulse-chase experiment might be possible if it were possible to block nearly immediately the labeling of "free" poly(A). The drug cordycepin (3'-deoxyadenosine) was tested as a potential blocking agent. Some inhibition of labeling of "free" poly(A) occurred, but it did not appear to be a strong enough inhibition to allow a pulse-chase experiment (data not shown). Rose and Jacob (1976) have recently found that several agents inhibit the mitochondrial poly(A) polymerase to various extents. Cordycepin was one of the agents tested in this study and was found to partially inhibit enzyme function.

It is conceivable that a "free-poly(A) ligase" exists in HeLa mitochondria. If this is the case, it may be possible to demonstrate ligation of "free" poly(A) to large precursor molecules in vitro. This would be strongly suggestive that "free" poly(A) is a precursor to bound poly(A) in vivo.

It is not yet clear how poly(A) metabolism of mitochondria compares with poly(A) metabolism in nucleus and cytoplasm. It is known that poly(A) covalently linked to large RNA molecules undergoes replacement of its 3'-terminal residues both in nucleus and cytoplasm (Diez and Brawerman, 1974). It is not known whether a similar situation exists in mitochondria.

The possibility that poly(A) is originally "preformed" before addition to large RNA molecules as a complete unit has not been seriously considered with respect to nuclear RNA metabolism. A recent report (Sawicki et al., 1977) contains data which may bear on this question. It was found that the labeled poly(A) segment of nuclear heterogeneous RNA is full-sized even when the labeling time is as short as one minute. The authors interpreted the lack of smaller poly(A) segments to mean that poly(A) segments are synthesized in less than one minute. In contrast to this interpretation, sequential digestion by a 3'-exonuclease showed that an average of about 30 nucleotides at the 3' terminus were labeled in one minute. The authors concluded that two processes were occurring - synthesis of a few new, full-sized poly(A) segments and 3' end turnover of previously synthesized, full-sized poly(A).

These data appear to be subject to another interpretation, however: Poly(A) might be preformed and added to HnRNA as full-sized segments.

Nothing in this or previous work demonstrates rigorously that "free" poly(A) is synthesized inside mitochondria. The most powerful tool available for other kinds of RNA, namely, hybridization to purified mitochondrial DNA, is obviously not useful for this purpose. The existence of a mitochondrial poly(A)-polymerase (Jacob et al., 1974) is suggestive but not conclusive. For example, mitochondrial poly(A) might be originally synthesized elsewhere and be subjected to 3'-end turnover within mitochondria. However, no evidence of any RNA import into mitochondria exists.

The poly(A) polymerases previously reported (Jacob et al., 1974; Edmonds and Winters, 1976) require some sort of primer, though the actual

primer specificity remains unclear. Small oligonucleotides may function as primers as well as larger RNA molecules or poly(A).

The base composition of "free" poly(A) was determined in the present work. Only 89% of the "free" poly(A) label was found in adenylate residues. The 11% of label found in non-adenylate residues may be the result of small (5 or 6 nucleotides) non-adenylate primer sequences covalently linked to poly(A) or the "free" poly(A) may have been contaminated by other RNA molecules.

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Table 1

The base compositions of RNA species A-D were determined as described in the text.

	Base Composition (%)			
RNA species	A	U	G	C
A	70	14	4	11
B	72	14	5	9
C	71	15	14	0
D	89	4	3	5

Figure 1

HeLa cells were suspended in 400 ml of Eagle's phosphate medium supplemented with 5% dialyzed calf serum at a concentration of 1 x 10^6 cells/ml. Thirty minutes prior to labeling, Actinomycin D was added to a concentration of 0.1 micrograms/ml. Labeling was accomplished by the addition of 2,8-^{3}H-adenosine to a level of 3.75 microcuries/ml. Labeling was carried out for 1 hour.

Mitochondria were prepared and lysed in SDS buffer as described in Materials and Methods. Lysed mitochondrial material was centrifuged through a 15-30% sucrose gradient containing SDS buffer at 25 KRPM for 24 hours at 20 degrees. The gradient was fractionated from the bottom and a sample of each fraction was counted with an aqueous scintillation counting system as described in Materials and Methods. The radioactive pattern of the fractionated gradient is plotted in the figure.

Fractions from the regions of the gradient labeled A, B, and C were pooled for further analysis.

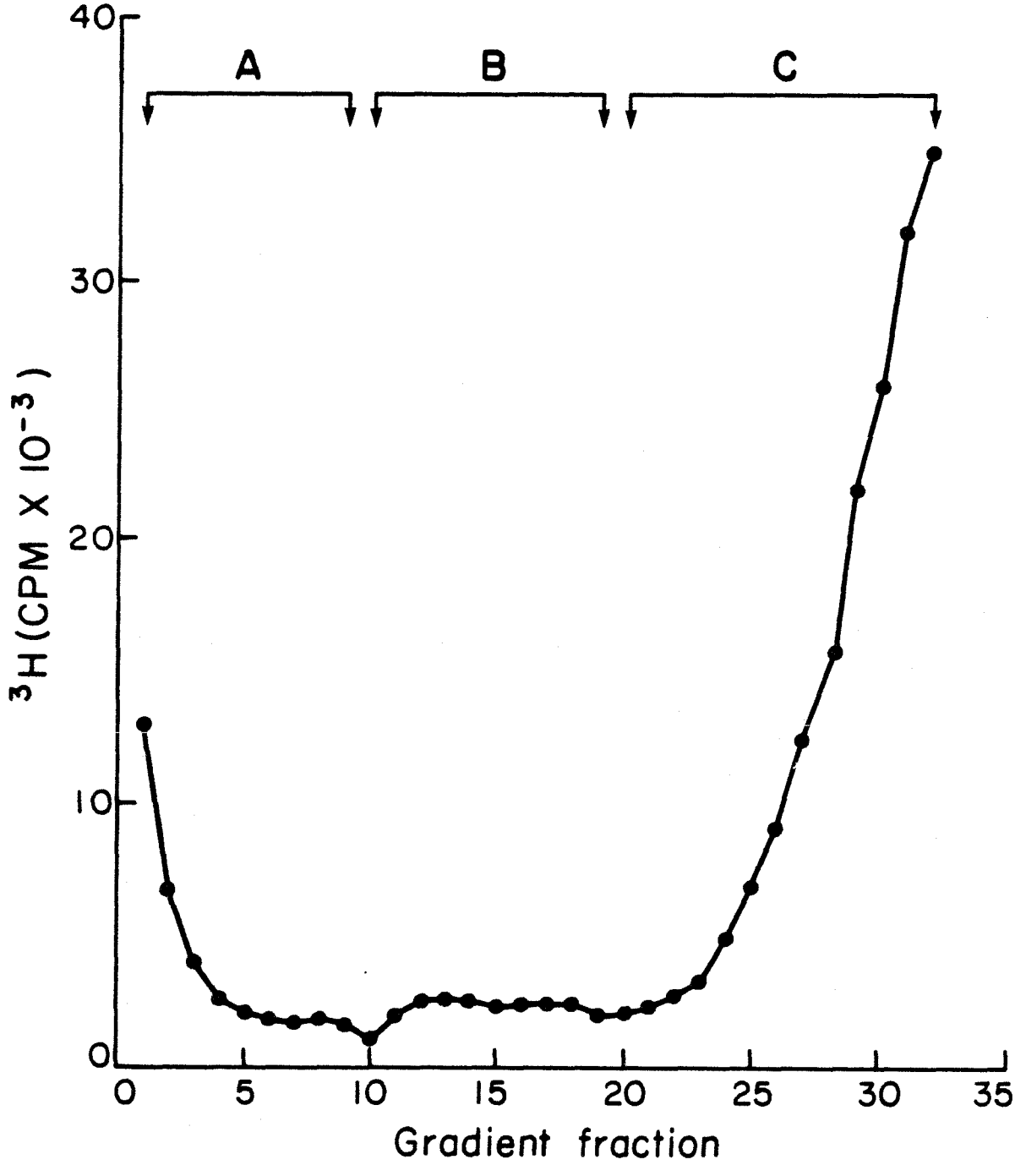

A
B
C
40
30
20
10
0
^{3}H (CPM X 10^{-3})
0
5
10
15
20
25
30
35
Gradient fraction

Figure 2

Pooled material from the sucrose-SDS gradient of Figure 1 was analyzed. RNA was extracted from the pooled fractions and further fractionated by oligo-deoxythymidylate cellulose chromatography as described in Materials and Methods.

The polyadenylated RNA purified from each region of the gradient was then divided into equal portions. One portion was treated with RNase A plus T1 RNase as described in Materials and Methods. The other was not. The various samples of RNA, both RNase treated and untreated, were analyzed by electrophoresis through 4% acrylamide, 0.2% bis, 3% formaldehyde cylindrical gels which were mechanically fractionated as described in Materials and Methods. The electrophoretic pattern of ^{3}H label obtained from each sample is plotted. From top to bottom, the rows display the patterns obtained from the bottom of the sucrose gradient (A), the middle of the gradient (B), and the top of the gradient (C).

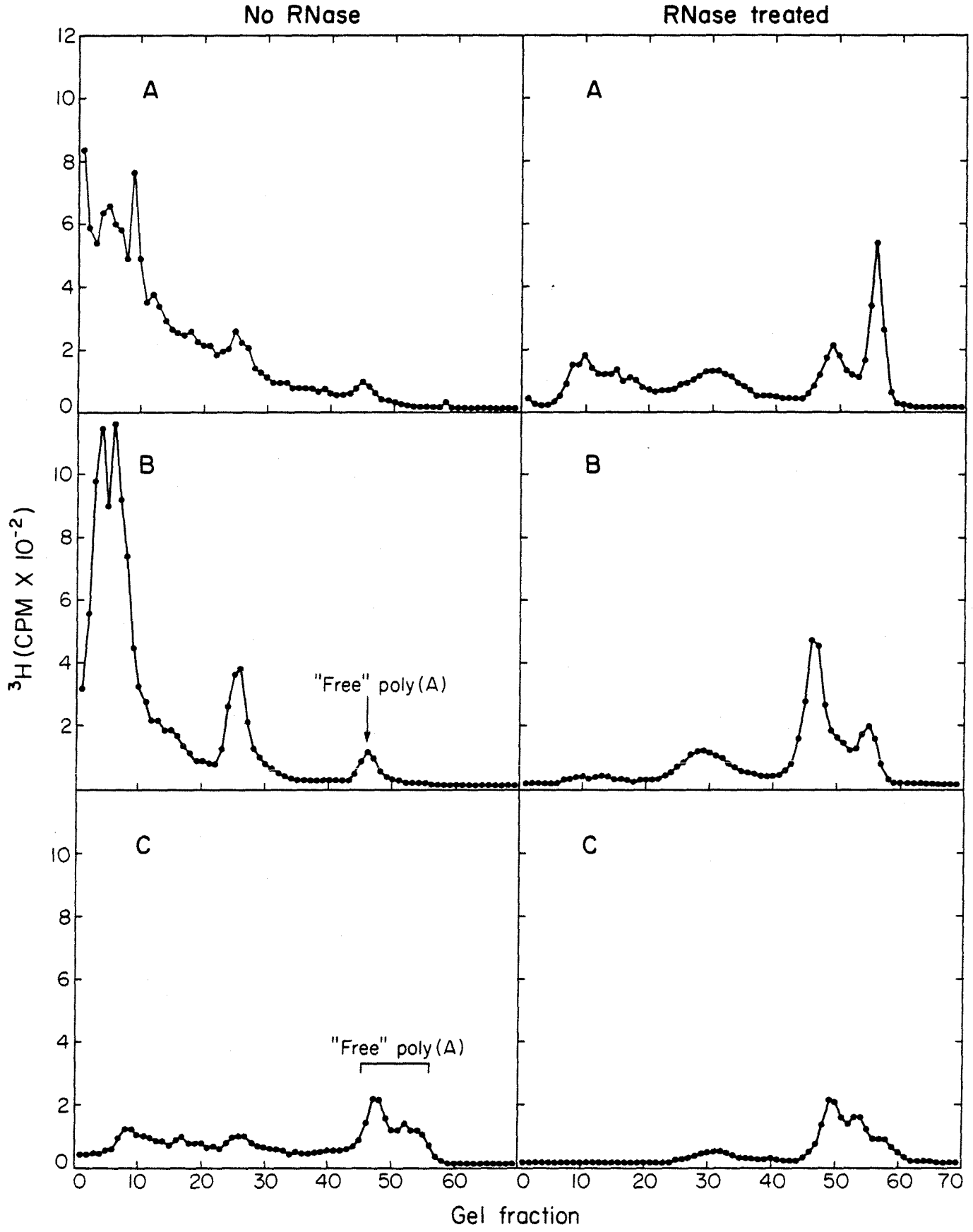

No RNase
RNase treated
A
A
B
B
C
C
"Free" poly(A)
"Free" poly(A)
^{3}H (CPM X 10^{-2})
Gel fraction
0
2
4
6
8
10
12
0
10
20
30
40
50
60
70

Figure 3

^{32}P-labeled mitochondrial poly(A)+ RNA and poly(A)- RNA were prepared from RNase treated mitochondria as described in the text. RNA was fractionated by electrophoresis through a denaturing polyacrylamide slab gel (15% acrylamide, 0.5% bis, 5 M urea). Autoradiography was performed on the gel and radioactive RNA bands of interest were excised.

The figure shows the electrophoretic patterns of poly(A)+ RNA and poly(A)- RNA determined by autoradiography.

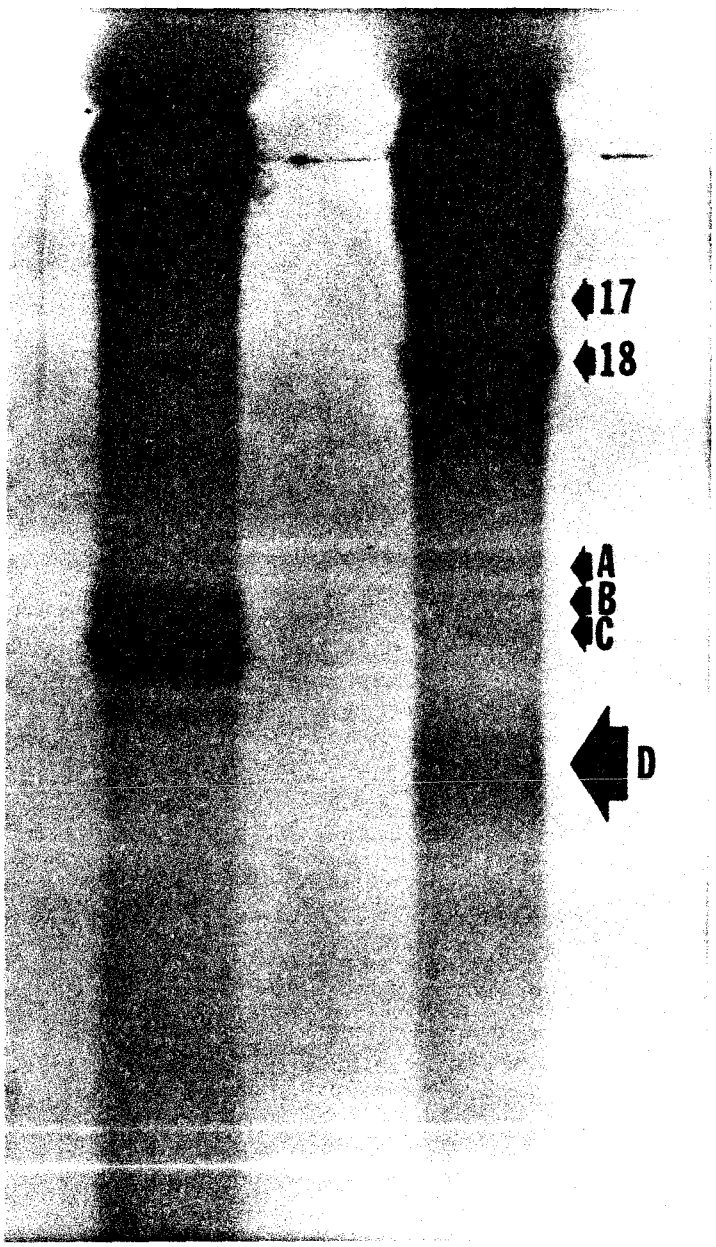

17
18
A
B
C
D

Figure 4

^{32}P-labeled RNA fractions excised from the gel described in Figure 3 and the text were analyzed. Each fraction (A, B, C, and D) was mixed with ^{3}H-labeled mitochondrial poly(A)+ RNA and electrophoresed through a cylindrical polyacrylamide gel (4% acrylamide, 0.2% bis) containing 3% formaldehyde. The ^{3}H-labeled poly(A)+ RNA patterns (open circles) contain distinct "free" poly(A) peaks in the region of fractions 48-58. The ^{32}P-labeled RNA fractions recovered from the slab gel (filled circles) are seen to have specific mobilities; band "D" (fourth from top panel) coelectrophoreses with the middle of the ^{3}H "free" poly(A).

The lowest panel contains ^{32}P-labeled mitochondrial poly(A) produced by RNase digestion of ^{32}P-labeled poly(A)+ RNA mixed with ^{3}H-labeled poly(A)+ RNA.

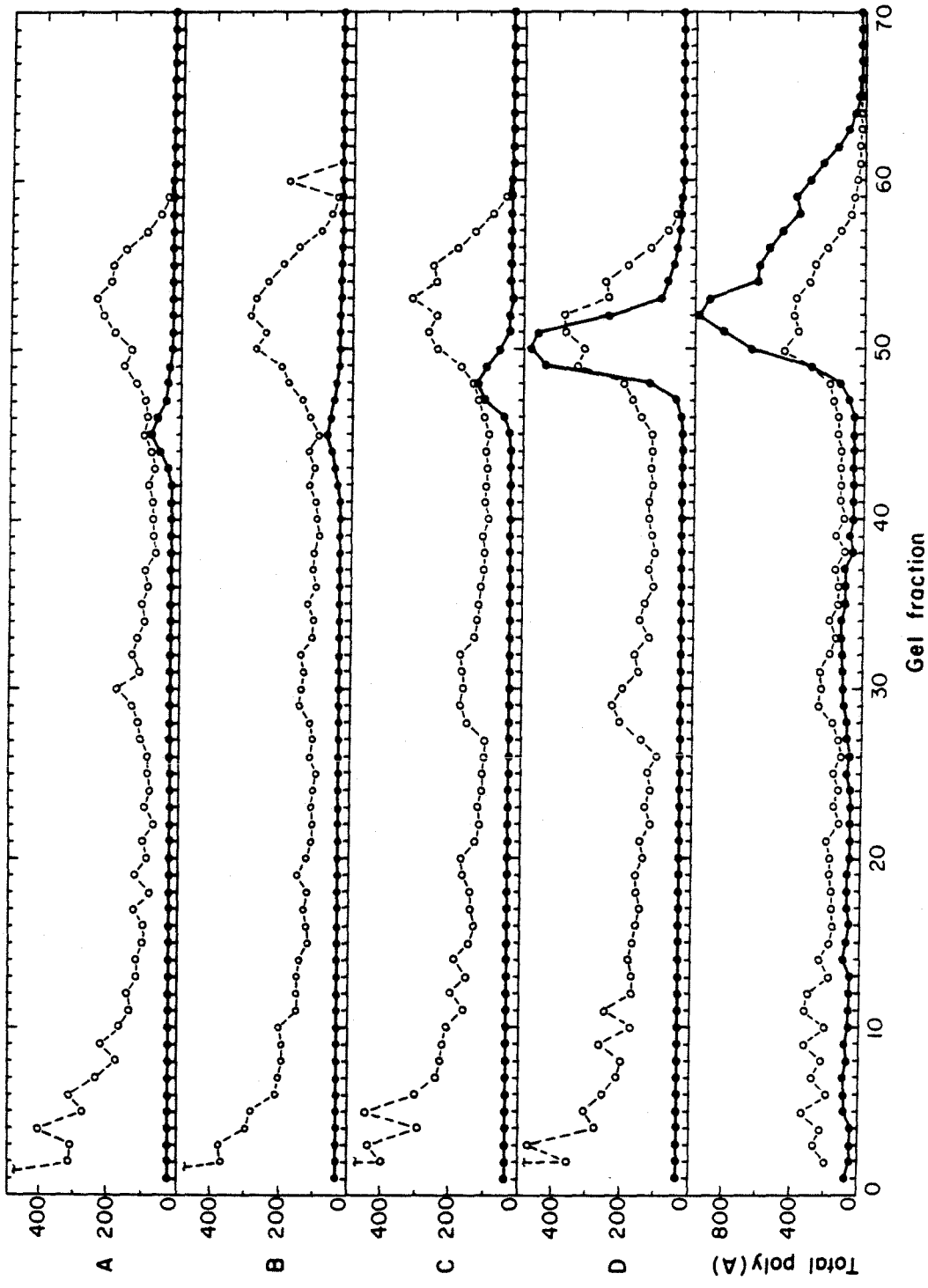

A
B
C
D
Total poly(A)
400
200
0
800
0
10
20
30
40
50
60
70
Gel fraction

Figure 5

Mitochondrial polyadenylated RNA labeled with 2,8-^{3}H-adenosine for 60 minutes in the presence of 0.1 microgram/ml Actinomycin D was prepared as described in the text.

RNA was analyzed by electrophoresis through a cylindrical acrylamide gel containing 3% formaldehyde as described in Materials and Methods. The electrophoretic pattern of ^{3}H label obtained by fractionation of the gel as described in Materials and Methods is plotted.

Upper: No RNase treatment.

Lower: Treated with RNase.

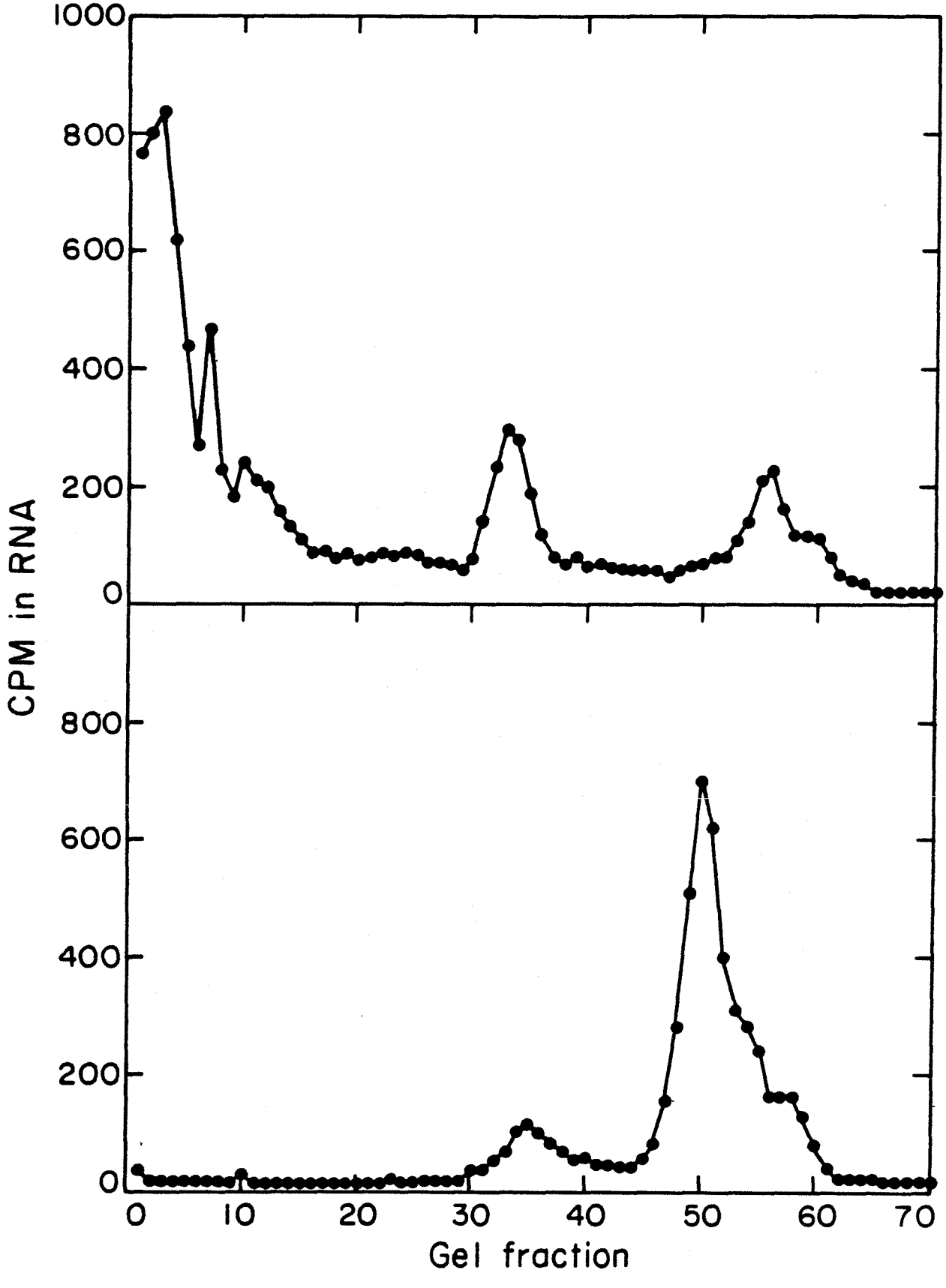

1000
800
600
400
200
0
800
600
400
200
0
CPM in RNA
0
10
20
30
40
50
60
70
Gel fraction

Figure 6

HeLa cells were labeled in vivo for various times with 2,8-^{3}H-adenosine in the presence of Actinomycin D. Mitochondrial polyadenylated RNA was prepared from these cells as described in Materials and Methods.

The poly(A)+ RNA obtained from each culture was then divided into two equal portions, one of which was treated with RNase A plus T1 RNase as described in Materials and Methods, while the other remained untreated.

The RNA obtained from each culture was analyzed by electrophoresis through a 4% acrylamide, 0.2% bis cylindrical gel containing 3% formaldehyde as described in Materials and Methods. The electrophoretic pattern obtained by fractionation of each gel as described in Materials and Methods is plotted. Only the region of each gel containing material the size of mitochondrial poly(A) is shown.

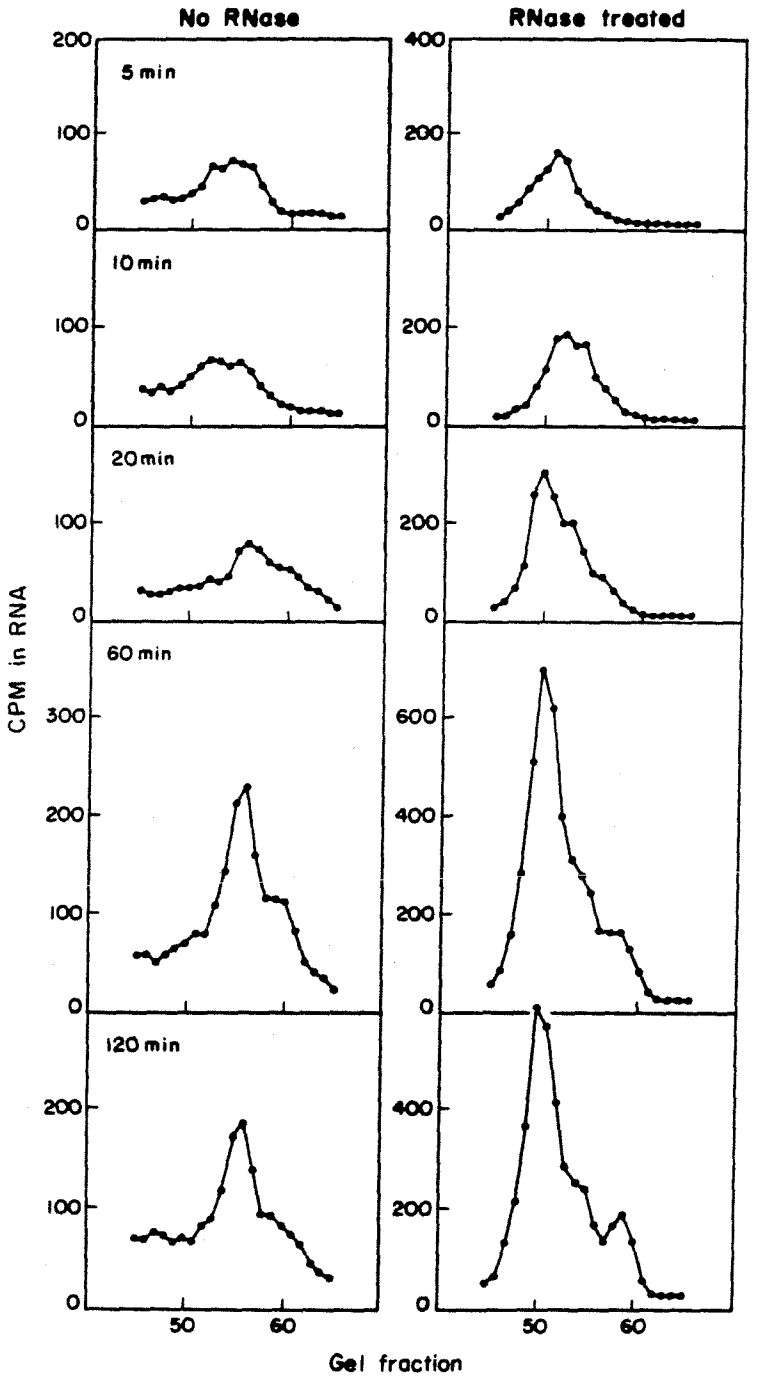

No RNase
RNase treated
5 min
10 min
20 min
60 min
120 min
CPM in RNA
Gel fraction
200
100
0
400
300
50
60

Figure 7

"Free" and total poly(A) labeling were determined as described in the text. The incorporation of radioactive label in each poly(A) fraction is plotted as a function of labeling time. Each value has been corrected for differences in yield as determined by the optical density (260 nm) of mitochondrial RNA.

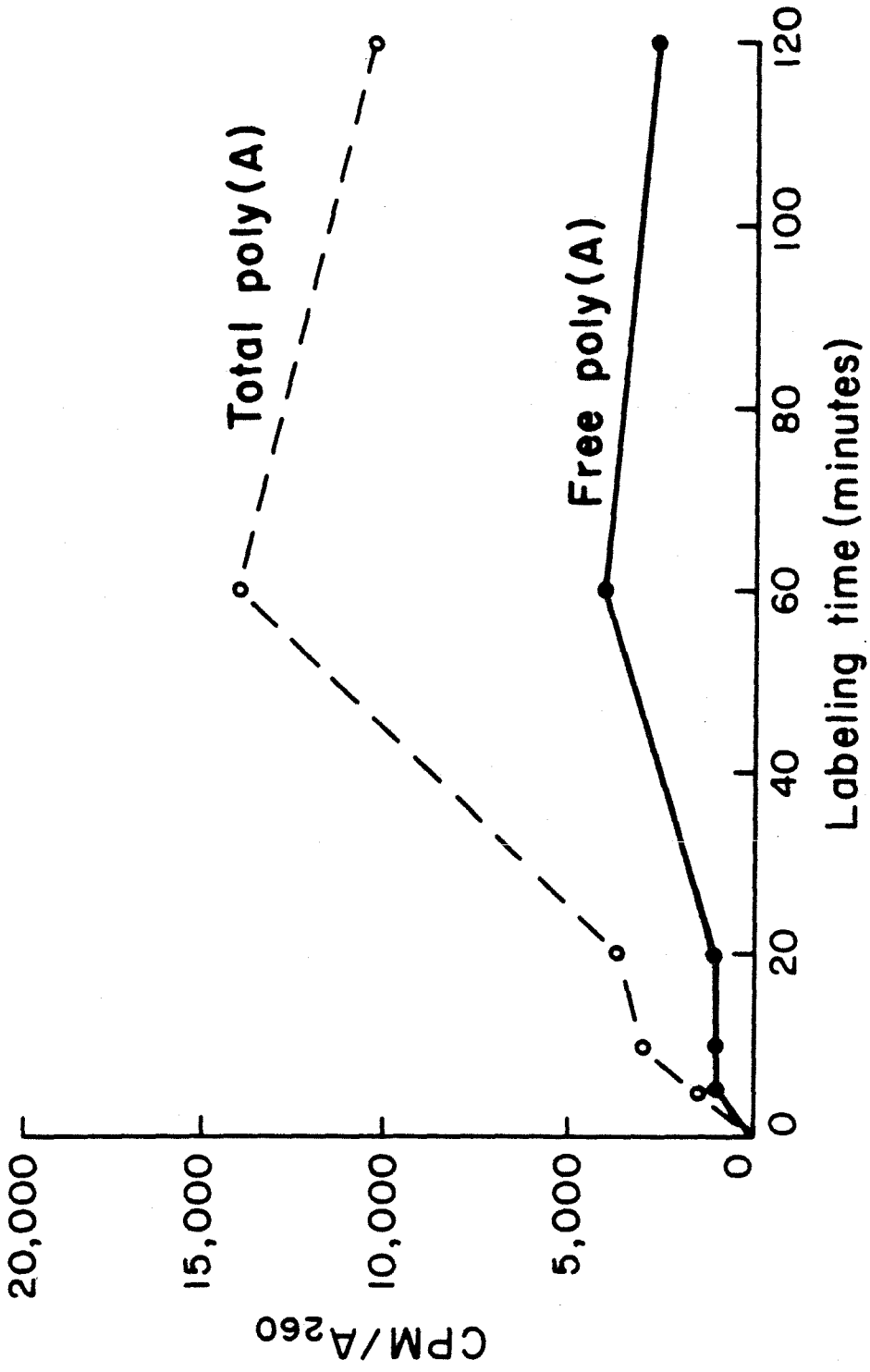

Total poly(A)
Free poly(A)
CPM/A260
20,000
15,000
10,000
5,000
0
0
20
40
60
80
100
120
Labeling time (minutes)

Figure 8

The incorporation of radioactive label into the "free" and total poly(A) was determined for each culture as described in the text. The ratio of "free" to total poly(A) labeling is plotted as a function of the time of labeling.

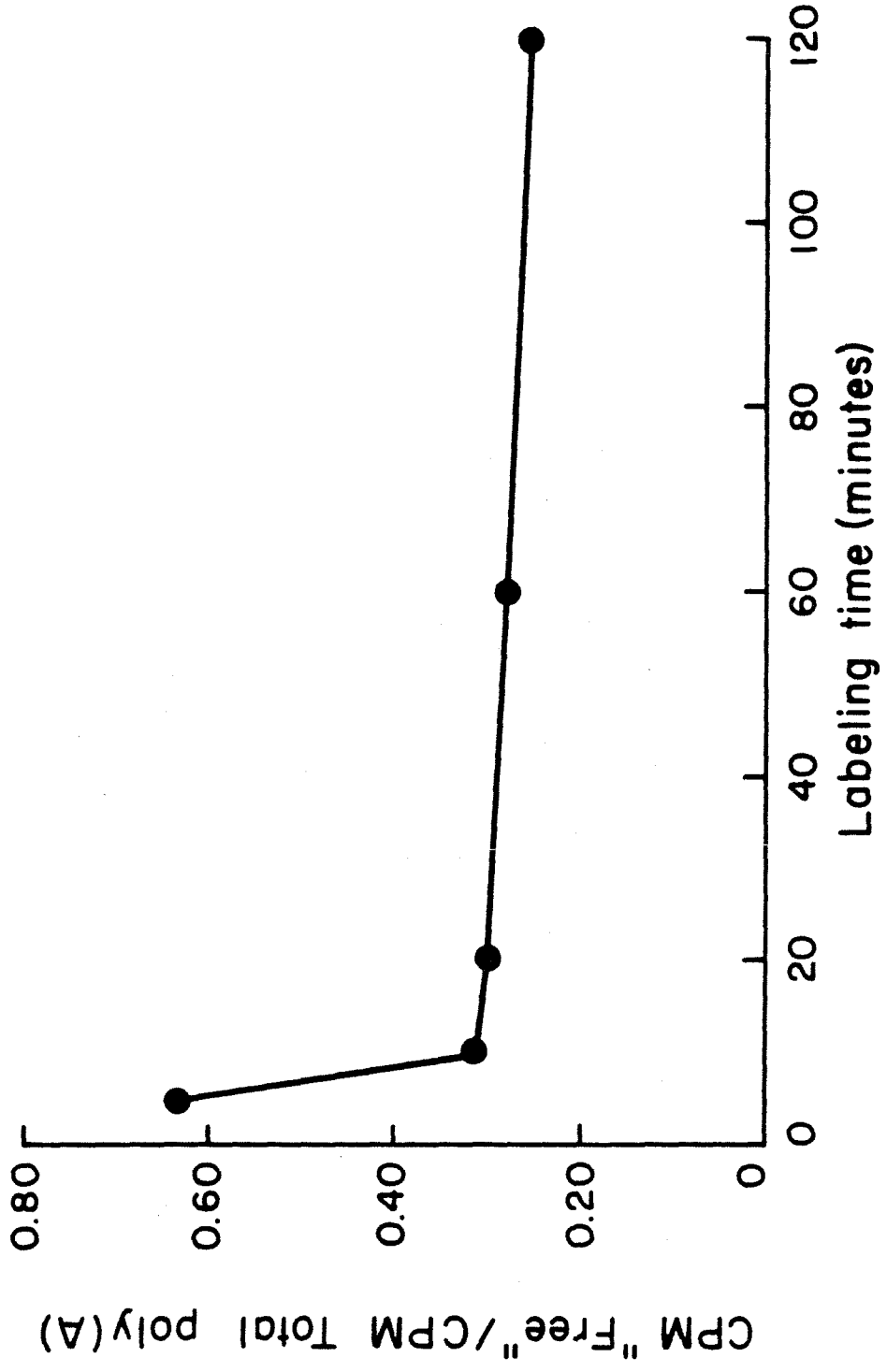

CPM "Free"/CPM Total poly(A)
0.80
0.60
0.40
0.20
0
0
20
40
60
80
100
120
Labeling time (minutes)

Figure 9

The incorporation of radioactive label into "free" and bound poly(A) was determined for each culture as described in the text. The ratio of labeling in "free" and bound poly(A) is plotted as a function of labeling time.

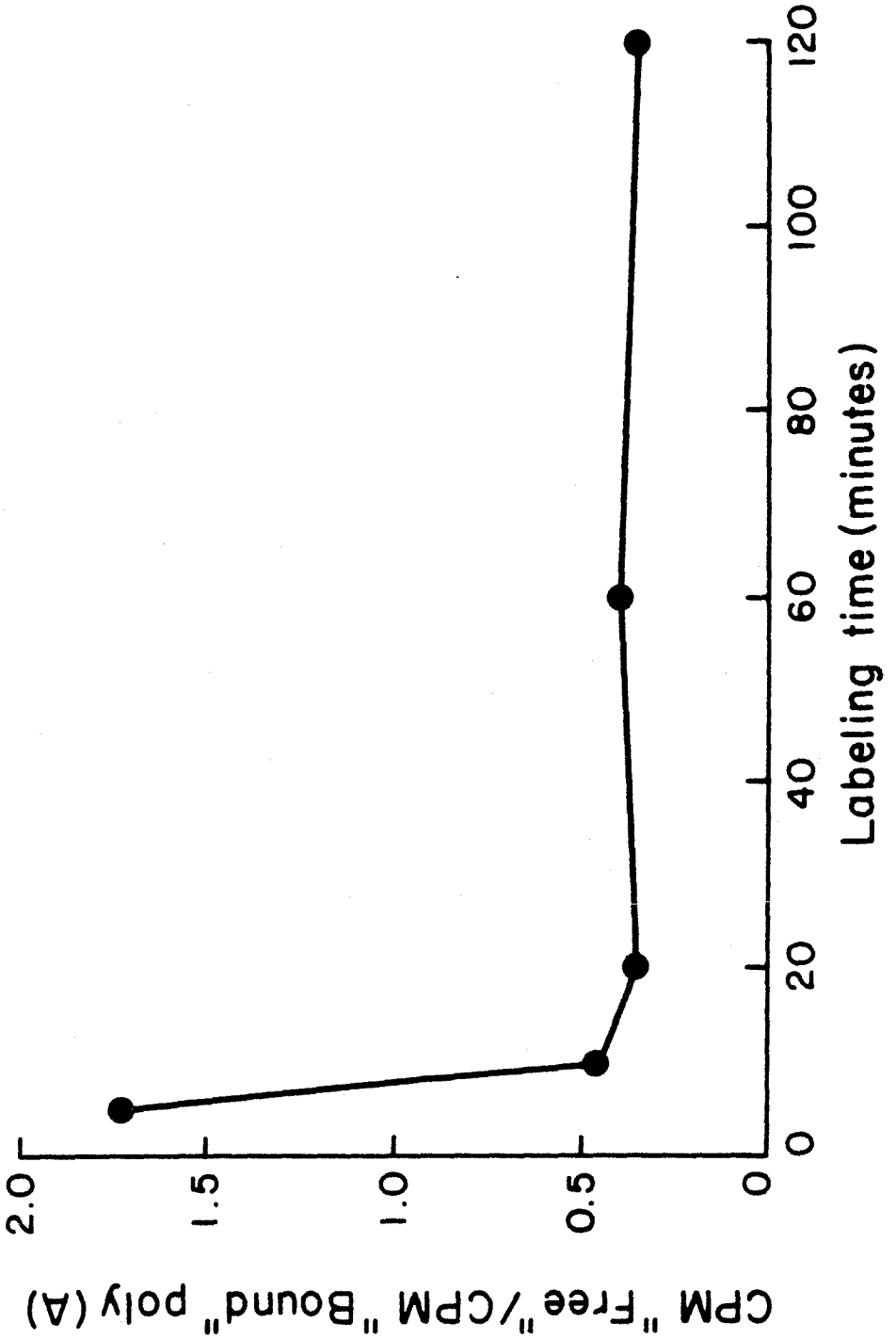

CPM "Free"/CPM "Bound" poly (A)
2.0
1.5
1.0
0.5
0
0
20
40
60
80
100
120
Labeling time (minutes)

Figure 10

A sample of the mitochondrial poly(A)+ RNA from each culture was tested for resistance to digestion by RNase A plus T1 RNase as described in the text. The RNase resistance was calculated as the fraction of TCA precipitable radioactivity which resisted enzyme digestion. RNase resistance is plotted as a function of labeling time.

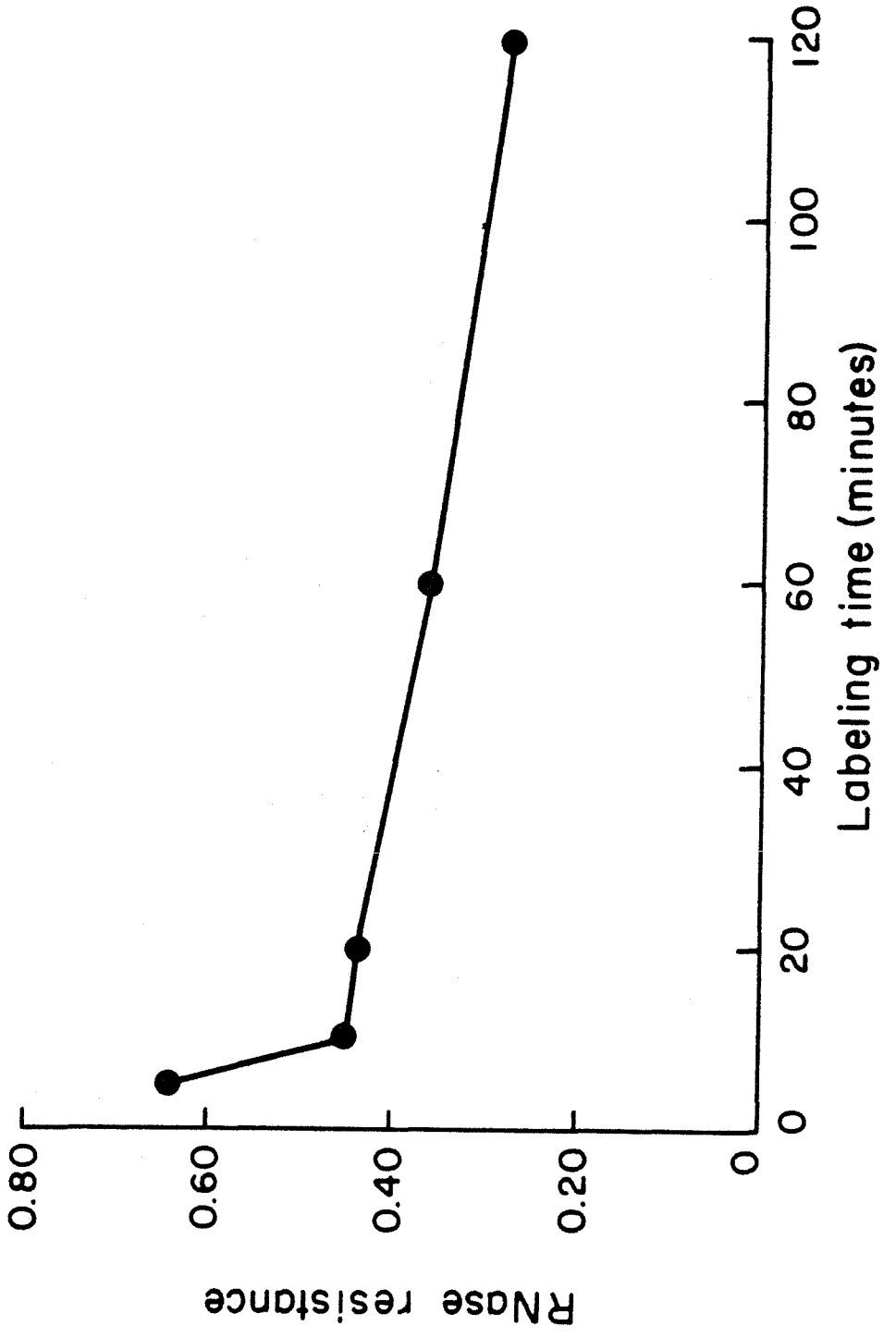

RNase resistance
0.80
0.60
0.40
0.20
0
0
20
40
60
80
100
120
Labeling time (minutes)

Figure 11

Cells were labeled with 2,8-^{3}H-adenosine for 5 minutes in the absence of inhibitory drugs as described in the text. Prior to cell breakage, ^{32}P-labeled cells (1 hour labeling) were mixed with the ^{3}H-labeled cells. Mitochondria were treated with RNase A plus DNase 1 to eliminate contaminating RNA of cytoplasmic or nuclear origin. Poly(A)+ RNA was purified from SDS lysed mitochondria and analyzed by electrophoresis through a cylindrical polyacrylamide gel containing formaldehyde as described in Materials and Methods.

The figure shows the electrophoretic pattern obtained from that gel. The short-term ^{3}H-labeled RNA (filled circles) shows significant labeling in the "free" poly(A) region. The long-term ^{32}P-labeled RNA (open circles) shows a distribution containing a small amount of material the size of "free" poly(A) and much more material of larger sizes.

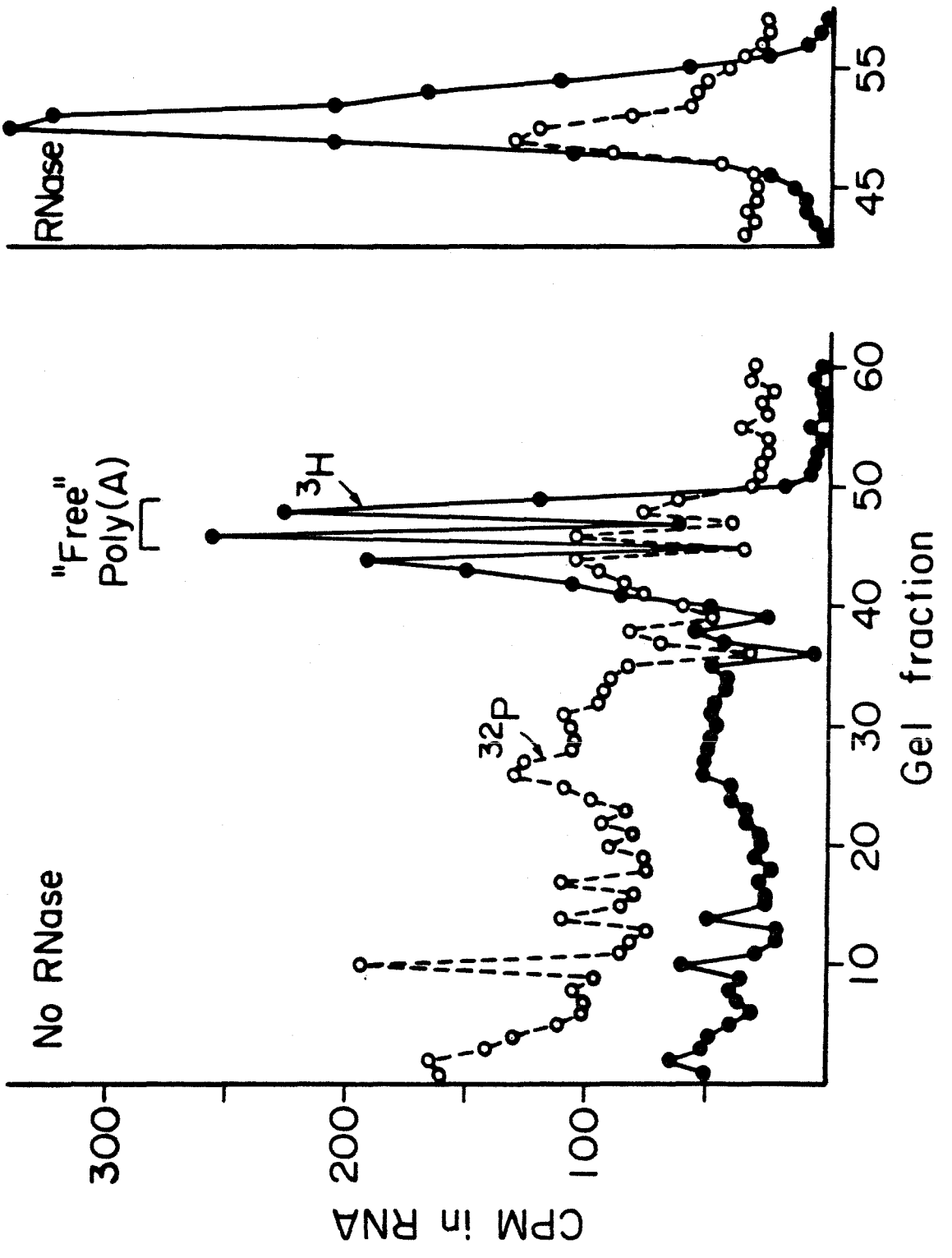

No RNase
RNase
"Free" Poly(A)
^{3}H
^{32}P
CPM in RNA
300
200
100
10
20
30
40
50
60
45
55
Gel fraction

Figure 12

Cultures were labeled for various times with ^{3}H-adenosine in the absence of inhibitory drugs. The poly(A)+ RNA was extracted from the mitochondrial fraction of each culture as described in Materials and Mehods.

Prior to cell breakage, ^{32}P-labeled cells were added to each culture.

Analysis of the RNA was by electrophoresis through cylindrical polyacrylamide-formaldehyde gels as described in Materials and Methods. The electrophoretic pattern obtained from the region of the gel containing "free" poly(A) is plotted for each gel.

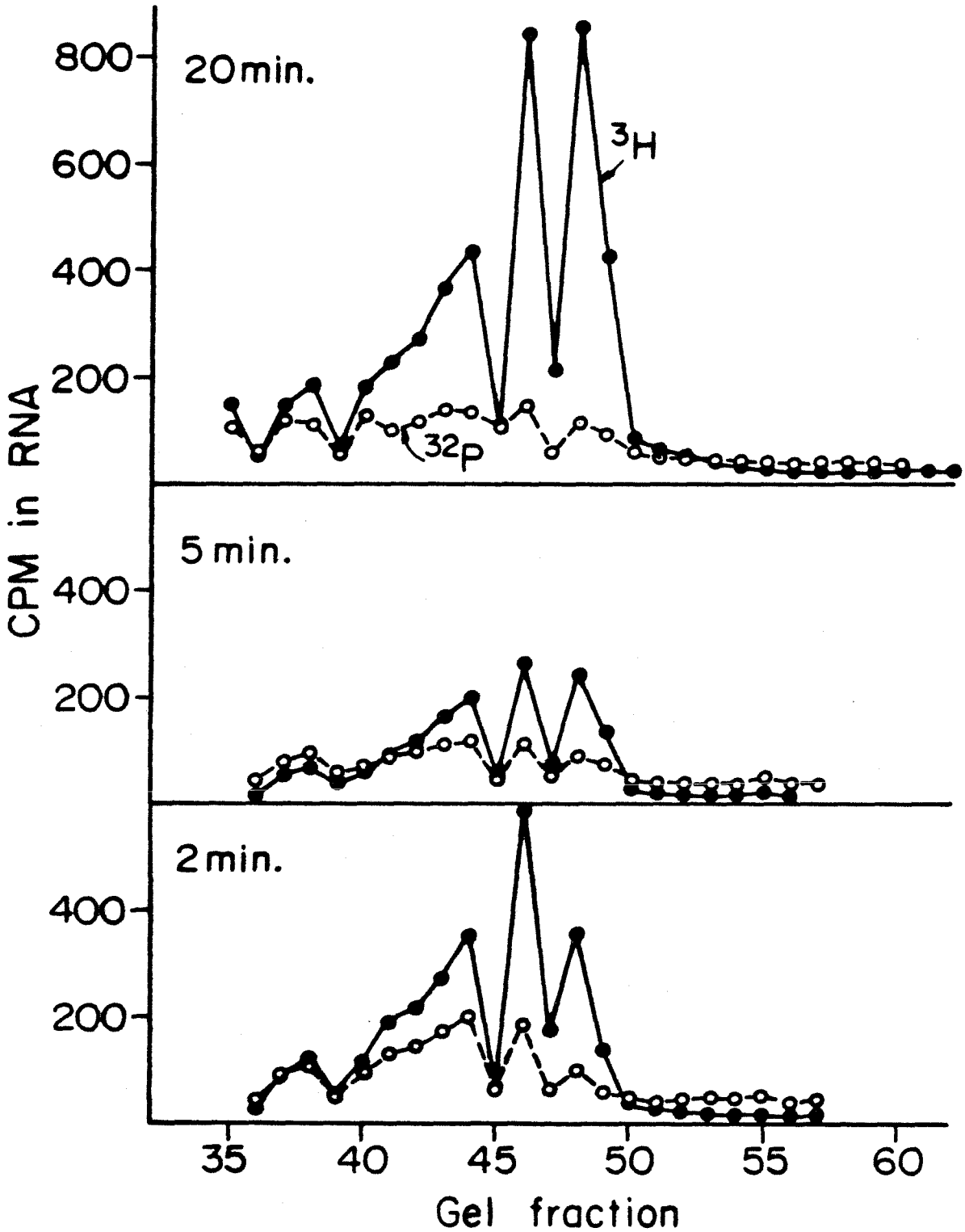

20 min.
5 min.
2 min.
^{3}H
^{32}P
CPM in RNA
800
600
400
200
400
200
400
200
35
40
45
50
55
60
Gel fraction

Figure 13

The ratio of label incorporated in "free" and total poly(A) was determined as described in the text for the mitochondrial poly(A)+ RNA from the experiment described in Figures 11 and 12 and the text.

The ratio of label in "free" and total poly(A) is plotted as a function of labeling time.

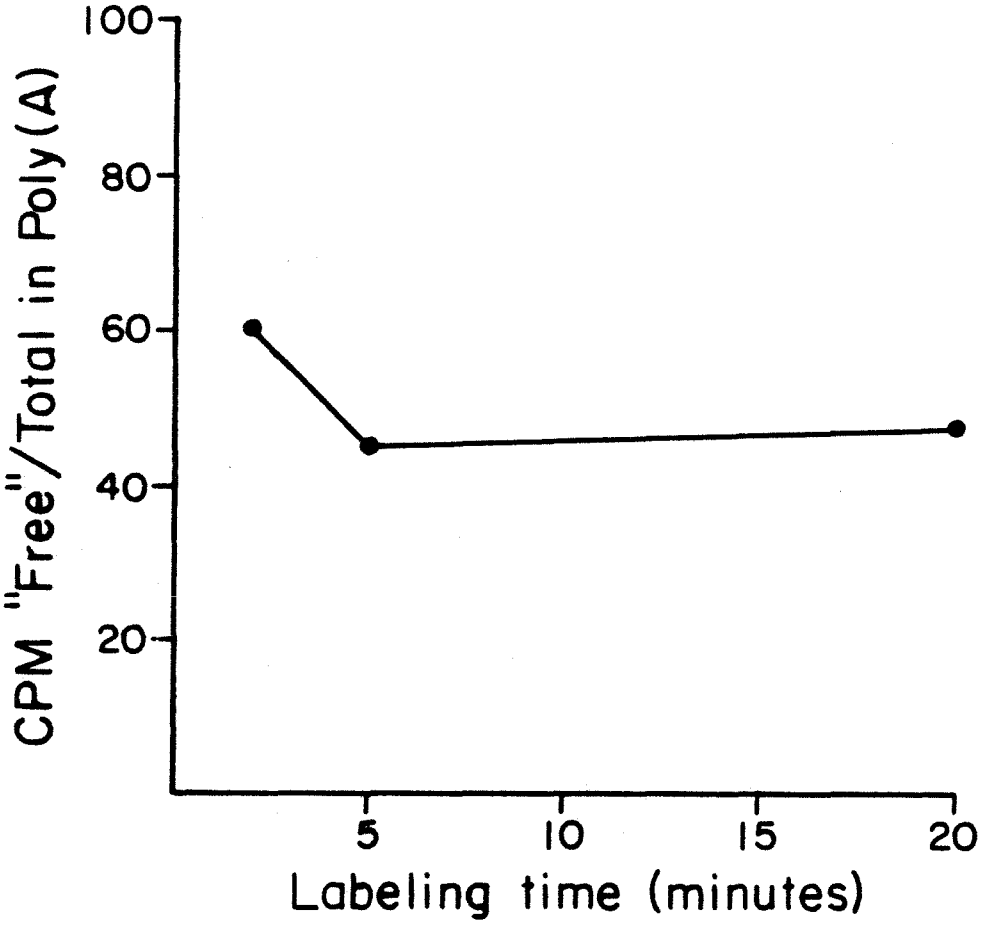

100
80
60
40
20
CPM "Free"/Total in Poly(A)
5
10
15
20
Labeling time (minutes)

Figure 14

Cell cultures were long-term labeled with ^{32}P-orthophosphate as described in the text. At various times after the addition of ^{32}P, pulse labeling was accomplished by the addition of 2,8-^{3}H-adenosine. The specific activities of the mitochondrial and cytoplasmic (i.e., post-mitochondrial supernatant) ATP pools were determined as described in Materials and Methods.

The ATP specific activity, expressed as the ratio $^{3}H/^{32}P$, is plotted as a function of ^{3}H labeling time for the ATP extracted from the mitochondrial and cytoplasmic compartments.

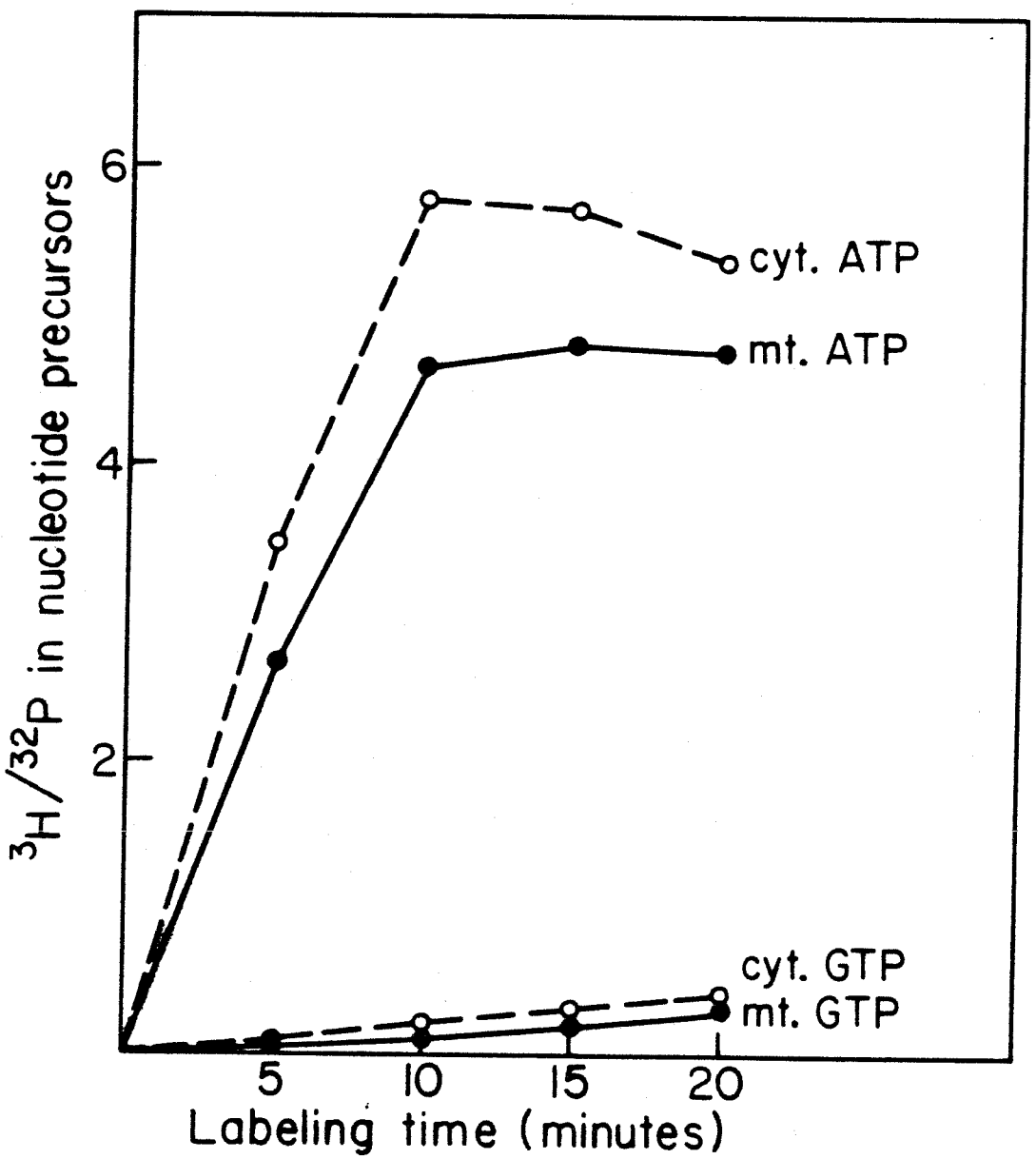
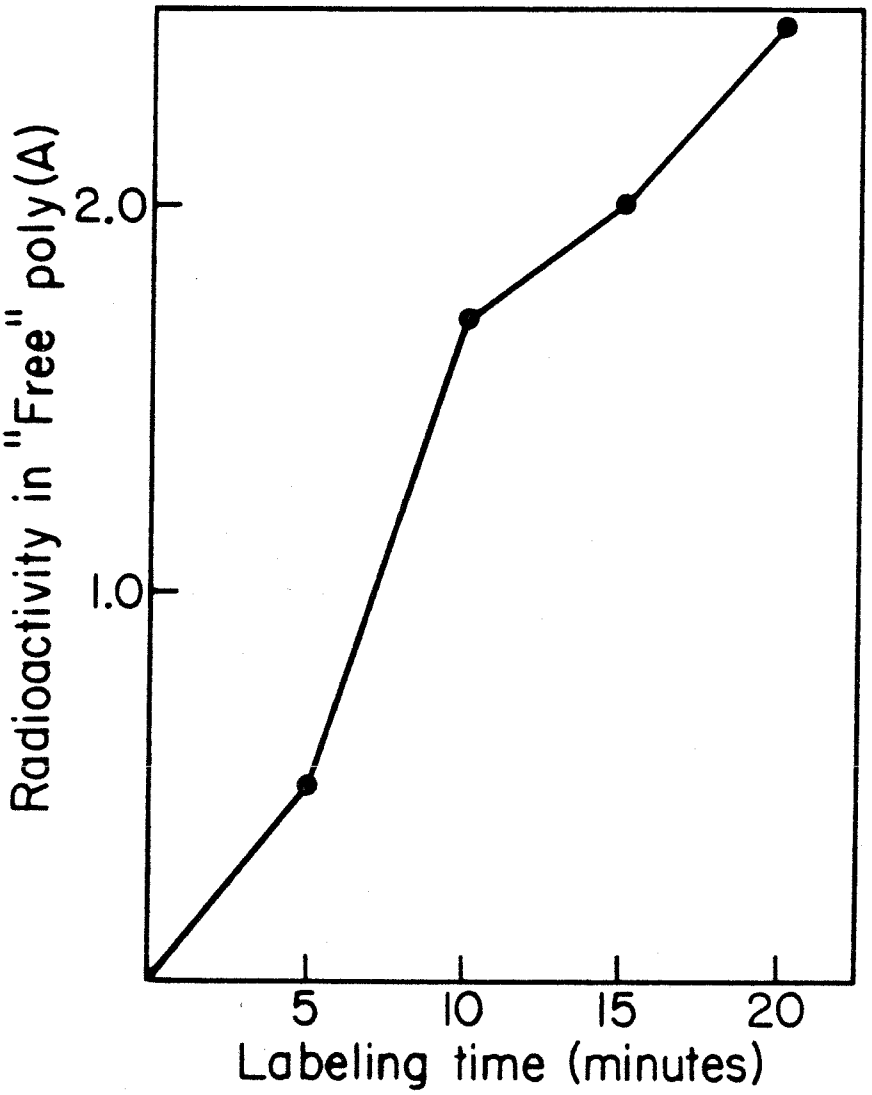

$^{3}H/^{32}P$ in nucleotide precursors
6
4
2
cyt. ATP
mt. ATP
cyt. GTP
mt. GTP
5
10
15
20
Labeling time (minutes)

Figure 15

The incorporation of ^{3}H label in "free" poly(A) was determined as described in the text for the mitochondrial RNA from the cell cultures described in Figure 14.

In order to correct for differences in yield, the values of ^{3}H label in "free" poly(A) have been divided by the ^{32}P radioactivity in mitochondrial poly(A)- RNA from the corresponding cultures.

The incorporation of radioactivity into "free" poly(A), corrected for differences in yield, is plotted as a function of ^{3}H labeling time.


Radioactivity in "Free" poly(A)
2.0
1.0
5
10
15
20
Labeling time (minutes)