The Control of RNA Synthesis in Isolated HeLa Cell Mitochondria

Thesis by

George L. Gaines, III

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1986

(Submitted January 1986)

Acknowledgements

I am indebted to many people for their help during my graduate career at Caltech. Those mentioned below deserve special thanks:

Giuseppe Attardi as my advisor and mentor,

Jane Houldsworth and Jan Hoh for friendship and help in writing this thesis,

Jeff Masters and Stuart Kim for friendship, support, and advice,

Ram S. Puranam for help in deciphering respiration,

Mike King and Cesaré Rossi for helping with experiments and making clones,

Arger Drew for growing approximately 1/5 of my weight in HeLa cells,

Graphic Arts and Renee Thorf for helping me get this thesis together and typed,

The Weigle Memorial Fund for financial support.

Abstract

The transcription of DNA and processing of RNA in mitochondria was investigated using isolated HeLa cell mitochondria. The intact organelles transcribe their DNA and process their RNA both qualitatively and quantitatively similar to the *in vivo* situation. Changing the conditions for the transcription reactions allowed the identification of a processing pathway for the small ribosomal RNA species, and an RNA species from the region surrounding the origin of light-strand replication with novel electrophoretic properties. Removal of the mitochondria from the cellular environment simplified the investigations of the nuclear-cytoplasmic influences upon the mitochondrial transcription. Differential sensitivities of all three transcription events, synthesizing the rRNAs, mRNAs, and light-strand RNAs, was shown to exist to a small molecular weight factor(s) present in the cytoplasm, and the availability of energetic substrates. These sensitivities indicate a link between cytoplasmic and respiratory/oxidative phosphorylative control of mitochondrial transcription.

TABLE CONTENTS

INTRODUCTION

> Large numbers of students in the modern era regard metabolic pathways as a solved problem. Science, however, has a way of providing employment to its loyal servants, so the fields of enzyme regulation and metabolic pathways, far from being dead, may be entering one of their most exciting phases (1).

Interest in the fields of enzyme regulation and metabolic pathways has been rekindled partly as a result of the discovery of the potential molecular and genetic relationships between development, homeostasis and oncogenesis, and also because of the newly acquired ability to further investigate previously studied subjects using the powerful techniques of molecular biology. To understand the control of cellular development in normal or abnormal (oncogenic) situations, it is necessary to characterize the basic metabolic processes functioning in cells. Intracellular metabolism is achieved through the interactions of enzymes of multiple levels. We have begun to understand, at a molecular level, how some individual enzymes function (for example, 2-4). Groups of enzymes can be linked together *visa vie* reactants and products to form the primary metabolic pathways (for example, 5, 6). Furthermore, these pathways interact to form complex networks that, in eukaryotic cells are partly compartmentalized within organelles (for review, 7, 8). Finally, one investigating higher eukaryotic metabolism may have to consider the interactions not only within cells, but among cells (for review, 9). Coordination of the metabolic processes needed for homeostasis and development is achieved through regulation at every level. The disruption of this coordination through a change in a regulatory process may result in abnormal development or oncogenesis (10, 11).

The techniques developed during the past ten years resulting from the fusion of genetics and biochemistry have considerably added to the tools now available to help delineate metabolic pathways and investigate their coordination. In addition, regulatory mechanisms known to operate in prokaryotic systems serve as models

for the more complicated control schemes present in eukaryotic cells. This foundation of knowledge coupled with the application of molecular biological techniques was used in this thesis to gain further insights into the control of metabolic processes within human cells. In particular, this work has focused on molecular events occurring within mitochondria—the organelles intimately involved in the energetic pathways of cells.

The first definitive observations of mitochondria were reported in 1890 by Richard Altman (12). He noted that "bioblasts" varied widely in number per cell and morphology between different tissues within the same organism. Many researchers have confirmed these observations and also identified differences in mitochondrial morphology between species. Further work has shown that heterogeneity among mitochondria occurs not only morphologically but also genetically and metabolically (for review, 13). Classically, these heterogeneous organelles have been studied for their most important function—as the site of aerobic respiration in cells (for review, 14).

The initial link between the mitochondria and the site of energy production from aerobic oxidation of lipids and carbohydrates was made in 1949 by Kennedy and Lehninger (15). They showed that the citric acid cycle (or tricarboxylic acid cycle) and the β oxidation of fatty acids were localized in the mitochondrion. These studies culminated the classical work done by Engelhardt, Belitzer, Ochoa, and Kalckar, who identified the process of oxidative phosphorylation which allows the conservation of energy derived from biological oxidation (for review, 16). Further work by Lehninger, Green, Hatefi, Chance, Racker, Mitchell, and others has elucidated the pathways and mechanisms of electron transport and oxidative phosphorylation occurring in these organelles (for review, 16-19). However, the control of these energy transforming pathways and their interactions with other cellular processes is just beginning to be determined. Clearly, the better

understanding of the genetic and metabolic interactions within the cell will help further define these control mechanisms.

Mitochondria are unique in that they are the only organelles in animal cells other than nuclei which contain genetic material (20). However, mitochondrial genomes differ greatly among species. These genomes in metazoan cells are covalently closed circular DNA molecules of about 15,000 to 20,000 base pairs (21). Protozoan mitochondrial DNAs are more heterogeneous in structure; *Paramecium* mitochondria contain linear DNA molecules, *Plasmodium* have circular mitochondrial DNA genomes, and the kinetoplasts of *kinetoplastidae* contain catenated molecules of mini-circular DNAs or maxi-circles of roughly 20,000 to 40,000 base pairs (22). Yeast and other fungi have circular mitochondrial genomes which range in size from roughly 20,000 base pairs to about 80,000 base pairs, depending upon the species (22, 23). Higher plants have the largest mitochondrial genomes, ranging up to 200,000 base pairs (24). The differences in sizes of genomes appears to be due to the presence or absence of repeated sequences, introns and A-T rich spacers (22). The larger genomes of the fungal mitochondria contain introns which in some cases code for an enzyme (maturase) utilized in the splicing of some of the mitochondrial mRNAs (25, 26). In contrast, the genomes of metazoan mitochondria are amazingly compact (27-30).

The heterogeneity of mitochondrial genomes includes their genetic organization. The relative position of genes within the genome common to all mitochondria varies from species to species. Even though mitochondrial DNA of *Saccharomyces cerevisae* and human cells contain many of the same genes, no relationship in the sequence of genes is observed (22, 23, 29). Furthermore, despite their size and structural similarities, sea urchin, *Drosophila*, and human mitochondrial genomes have differing genetic arrangements (29, 31-33). In

addition, certain species contain genes in their mitochondrial DNA which appear in the nuclear DNA of other species. The best documented example of "Promiscuous" mitochondrial DNA is that of the dicyclohexylcarbodiimide (DCCD)-binding subunit of the ATPase complex (ATPase subunit 9) (34). This gene is found in the yeast mitochondrial but not the nuclear genome and vice versa in human cells (35). Interestingly, *Neurospora* mitochondrial DNA contains a pseudogene for ATPase subunit 9, whereas the functional gene is encoded in the nucleus (36).

The heterogeneity of mitochondrial genomes among species is interesting from an evolutionary standpoint. Lower and higher eukaryotic mitochondria may have evolved from a common bacterial ancestor, as suggested by the endosymbiotic theory (22). However, the environmental pressures experienced by different species has resulted in radically varying mitochondrial genomes. These pressures selected for very compact mitochondrial genomes in metazoans. This may have resulted because of a slight replicative advantage enjoyed by a smaller genome, especially during the amplification of mitochondrial DNA during oocyte development (37). In contrast, in lower eukaryotes, selection might have occurred for mitochondria with a more plastic genome and gene expression system. Hence, these genomes evolved with introns, spacers and possibly more transcriptional control sequences (7, 23). In essence, the evolution of the many mitochondrial genomes possibly represents a balance between the need for responsiveness to environmental pressures through the plasticity of genomic organization and expression versus the ease of replication due to loss of extra nucleotides.

The similarity of mitochondrial genomes within a species (27, 39, 40), and near homogeneity within an organism (38, 41), does not exclude differences in the morphology and function of the organelles. For example, mitochondria of rat liver parenchymal cells and spermatozoa appear very different (42). In addition,

differences in the numbers and shapes of mitochondria can occur during the development of a specific tissue or cell. These changes are accompanied by alterations in transcription rates and respiratory capacities of the mitochondria (43). This is exemplified physiologically by the observation that neonatal mammals have a marked tolerance to anoxia compared with adults (44, 45). Interestingly, this tolerance correlates periodically with the developing brain's low oxidative activity (46). During growth, the increase in oxidative capacity of the brain is correlated biochemically with a three- to ten-fold increase in the respiratory enzyme content per mitochondrion (47). This is accompanied by an increase in the surface area of the inner membrane, rather than an enlargement of each mitochondrion (47, 48). The acquisition of maximum oxidative capacity in the nerve cells in the rat brain is signalled by the expression of an antigenic mitochondrial surface protein—MIT-23 (49). Hence, it is evident that mitochondria can change morphologically and functionally during the differentiation of their "host" cell.

Finally, abnormal differentiation resulting in oncogenesis can also cause changes in mitochondria. For example, mitochondria from tumors differ from those of normal tissues in their membranous lipid and protein content (10, 50-52), the type of substrate they oxidize during respiration (50), the magnitude of their acceptor control ratio (State III versus State IV respiration) (10, 50, 53), their capacity to accumulate Ca^{+2} (10, 53), and their rate of protein synthesis and turnover (10, 50, 51). Since the changes in mitochondrial morphology and function seen during normal or abnormal development are not the consequence of differences in the mitochondrial genomes (7, 10), this heterogeneity must result from nuclear and cytoplasmic interactions with the mitochondria.

Every aspect of mitochondrial biogenesis and therefore its many metabolic roles in the cell, depend in part or totally upon nuclear functions. The formation

of the electron transport and oxidative phosphorylation activities require not only the replicational, transcriptional and translational functions within the mitochondria, but also the expression and import of proteins involved in energy transforming processes. In fact, the only proteins which are encoded by the mammalian mitochondrial genome are ones involved directly in the electron transport and ATPase activities (27, 54). These protein components, the six subunits of the NADH dehydrogenase complex (complex 1) (54), the ATPase subunits 6 and 8 (55), the cytochrome oxidase subunits 1, 2, and 3 (56, 57), and cytochrome b (27), interact with their respective nuclear genome-encoded subunits to form complexes 1, 3, and 4 of the respiratory chain and the ATPase (7). It has been estimated that an additional 300-400 mitochondrial proteins are encoded in the nucleus (7). Hence, the coordination of both the mitochondrial and nuclear gene expression systems is necessary for the biogenesis of the mitochondria and production of the electron transport and ATPase activities. In addition, the differential expression of the 300-400 nuclearly encoded mitochondrial proteins allows for the morphological and functional heterogeneity seen among mitochondria in the same organism.

Another form of nuclear/cytoplasmic-mitochondrial interactions is evident at a metabolic level and is regulated by the energetic demands of the cell. The cytoplasmic glycolytic and mitochondrial respiratory pathways are intimately coordinated, a phenomenon which was reported over 120 years ago by Louis Pasteur (for review, 58). He observed that per gram of glucose, more yeast cells are formed in the presence of air than in its absence. More importantly, he also noted that the glucose was utilized more slowly in the presence of air. The mechanisms which control this switch between aerobic respiration and anaerobic fermentation, now called the Pasteur effect, are not well understood even today. It is apparent, though, that the nucleus, cytoplasm and mitochondria play crucial

roles in balancing the activities of these two energy transforming processes (59).

Regulation of many of the metabolic pathways within mitochondria is highly dependent upon the energetic state of the organelle (60-70). Clearly, any intra-mitochondrial process which requires ATP will be controlled in part by the extent of oxidative phosphorylation occurring within the organelle. The regulation of these processes will depend upon the requirements (K_m) of the various enzymes for ATP and ADP/phosphate and the intra-mitochondrial fluctuations in the concentrations of these nucleotides. In addition, enzymes may also be directly affected by the magnitude of the inner membrane's electrochemical gradient, or other phenomenon which reflect the level of respiration (63, 64, 69, 70). Several mechanisms have been proposed to explain the observations that some enzymatic processes within the mitochondria are controlled by changes in the ATP concentration and by the electron transport and oxidative phosphorylative activities. These mechanisms include conformational changes in the proteins (68, 69), thiol-disulfide exchanges within enzyme complexes (67) and the phosphorylation-dephosphorylation of enzymes (65, 71). All of these mechanisms presumably cause changes which affect the relative K_ms of the substrates exhibited by the enzymes. Also, if ATP or ADP also is one of the substrates, several energetic regulatory mechanism can affect a single enzyme or complex (66, 68). Further work will most likely delineate other molecular mechanisms for energetic control of enzymes and increase the understanding of those already mentioned.

From the above discussion, it is evident that control of metabolic pathways within mitochondria is mediated at several levels: genetic (role of mitochondrial and nuclear genomes in the biogenesis and maintenance of a functional mitochondrion), metabolic (interactions of enzymes, substrates and products between the cytoplasm and mitochondria), and molecular (direct or indirect

effects on enzymes by the energetic environment of the mitochondrion itself). Each of these levels is in turn regulated in part by the other two and the cellular environment. Furthermore, each level may contribute to the heterogeneity previously described. At present, very little is known about the details of the coordination of regulation within in each level and between levels. Genetic approaches using yeast systems have begun to elucidate some of the interactions at each level (72). Once the interactions are defined, then control mechanisms can be studied. Unfortunately, genetic avenues are as yet unavailable for the researchers interested in mammalian mitochondrial systems. Other approaches have been taken to investigate interactions at all three levels. The discussion will be limited to approaches used for the study of the control of mammalian mitochondrial gene expression.

The first approach taken was to study the interactions between cytoplasmic protein synthesis and mitochondrial gene expression using the different sensitivities of the two translation systems to various drugs (73, 74). Secondly, the temporal aspects of mitochondria replication, transcription, and translation with that of the cell cycle has been studied in synchronized cells (73, 75). These two sets of studies has shown that both the cytoplasmic protein synthesis and the cycles of cell growth and division are important in the maintenance and control of mitochondrial transcription and replication. In contrast, the amount of translation in mitochondria remains constant during the cell cycle and furthermore, continues for several hours after the inhibition of cytoplasmic protein synthesis is initiated. The third approach utilized the techniques of enucleation by treatment of cells with cytochalasin B and subsequent centrifugation to remove the nuclear but not cytoplasmic influences upon the mitochondria (77). Interestingly, transcription rapidly declined without nuclear input, whereas translation continued at a stable rate for greater than 24 hours. Taken together, these observations suggest two

different regulatory systems exist for gene expression in mitochondria. The amount of replication and transcription is influenced by labile cytoplasmic factors (some of which may be the polymerases) which fluctuate with the cell cycle. The proteins and factors involved in translation on the other hand, are either very stable, or in large excess.

The work presented here was carried out using a different approach (78, 79), one which separated the mitochondria from both nuclear and cytoplasmic influences by physical means, and thereby allowed the reconstitution of several of the interactions mentioned above. This separation was achieved by mechanically lysing HeLa cells and isolating the intact mitochondria by centrifugation and washings. Simultaneously, the cytoplasmic fraction was retained for the reconstitution experiments. The mitochondria could then be assayed for transcriptional capacity and processing fidelity by analyzing the incorporation of radioactive nucleotides into their RNA using gel electrophoretic techniques. These isolated mitochondria were shown to be competent in transcription and processing of RNA, comparable to that found during the *in vivo* situation. It was then possible to study both the effects of changes in their energetic or metabolic environments and the above-mentioned influences of cytoplasmic factors upon the processes involved in synthesis and maturation of RNA. The molecular analysis of these effects was possible because of the preexisting information about the HeLa cell mitochondrial DNA and its *in vivo* transcription products.

The human mitochondrial genome has been sequenced (80) and the mitochondrial RNAs have been precisely mapped (81-87). A diagram of the mitochondrial genome and the map positions of the corresponding RNAs is shown in Fig. 1. The extremely compact organization is evident, illustrated by almost a complete absence of non-coding stretches, with the exception of the D-loop region. The main coding strand, or heavy (H) strand, contains the majority of

tRNA genes, both rRNA genes, and all but one of the protein coding genes. These genes are not separated by more than several nucleotides (80-82, 86), and in some cases, polyadenylation of the mRNA species contributes to the formation of termination codons for subsequent translation (80). Furthermore, two cases exist in which reading frames on the H strand overlap. Four proteins are synthesized from the two mRNAs (RNAs 14 and 7) corresponding to the overlapped genes (encoding two ATPase proteins and two NADH dehydrogenase proteins, respectively) (54, 88). The light (L) strand contains the genes for eight tRNAs; one significant open reading frame that probably represents a protein coding gene (88) and a small RNA purported to be involved in the initiation of mitochondrial DNA replication (89) and the control of translation (85).

The transcription of the HeLa cell mitochondrial genome and processing of the resulting RNAs has been studied extensively *in vivo*. Both strands of the circular genome are almost completely transcribed (90-92). Three major initiation sites have been mapped; two for the transcription of H strand and one for the L strand (Fig. 2) (83, 93). The transcripts are processed cotranscriptionally to give discrete mRNAs, rRNAs, and tRNAs (94). The rates of synthesis and degradation of most of these RNAs has been described using double labeling (^{3}H and ^{32}P) techniques (95). The combination of the information about the compact nature of the mitochondrial genome with the kinetic data of the RNA synthesis has allowed the formation of models for either the synthesis or the processing of these RNAs. The tRNA punctuation model for mitochondrial RNA processing suggests that the tRNAs act as processing sites and signals for the maturation of the RNA species (96-100). The overlapping transcription units model explains the observation that the amounts of *in vivo* synthesis of the 12S and 16S rRNAs is much higher than that of the mRNAs encoded in the H strand (101). The use of the *in vitro* transcription system described here has been used to study both of these events.

The tRNA punctuation model suggests the existence of an enzyme(s) capable of either recognizing all 22 tRNA sequences or some common element among tRNAs, or alternatively, non-enzymatic, self-catalytic processing by those tRNAs could mediate the cleavage of the nascent RNA into the respective species. The absence of mutants in mammalian mitochondrial systems has hindered the experimental confirmation of this model. However, evidence for the requirement of correct tRNA structure for the processing has been shown by Gaines and Attardi using an isolated organelle transcription system (102; Chapter 1, this thesis). This work demonstrated that the 12S rRNA could be initially synthesized as a longer molecule (12S*) with a 5' extension containing the phenylalanine tRNA and ~20 nucleotide leader. This precursor molecule (12S*) could be subsequently processed to give the mature 12S rRNA. The inhibition of processing occurred in the presence of ethidium bromide and proflavin, drugs that are known to intercalate into tRNA structures (102-104). Further evidence for the necessity of a correct tRNA structure for processing of the mitochondrial RNAs was provided by the identification and characterization of RNA 9L (Chapter 3, this thesis), a relatively stable leader RNA for the cytochrome oxidase I mRNA. This molecule is transcribed from the H strand corresponding to the anti-tRNAs or complements of four L-strand tRNAs (Tyr, Cys, Asn, and Ala) and also contains the origin of L-strand replication (Fig. 1). Computer analysis of the sequence using the Nussinov-Jacobson (105) program has suggested that the most thermodynamically stable conformation of RNA 9L is not a series of four tRNA cloverleaf structures, but rather a series of long stems and loops. This conformation inhibits the processing of internal anti-tRNA sequences, most likely because they do not form cloverleaf structures. The processing at either the 5' or 3' end of the molecule appears to occur at or very near sites recognized as cloverleaf structures. It is also interesting to note that the anti-tRNA structure is recognized in other regions of transcription, as seen in the processing of RNAs 5 and 11 (Fig. 1).

As mentioned earlier, it was unknown if the processing was by a self-catalyzed event or an enzymic reaction, like the RNase-P reaction found in *E. coli* (106-108). Evidence in support of an RNase-P like enzyme mediated event has come from work also done in this laboratory. Claus Doersen has purified a mitochondrially associated enzyme, distinct from the cytoplasmic RNase-P like activity, which will process a bacterial tRNA precursor to the correct tRNA (109). However, as yet the purification of a mitochondrial enzymatic activity capable of processing an exogenously added mitochondrial tRNA-containing transcript has not been documented.

The combination of two lines of evidence led to the suggestion that two overlapping transcription units existed for the H strand. Surprisingly, Gelfand and Attardi (95) showed that the relative rates of transcription for the rRNAs and mRNAs were roughly 10-50 to 1. Since Montoya and coworkers showed that the only sites of initiation for transcription on the H strand existed 5' to the 12S rRNA gene, overlapping transcription units were postulated to exist. In addition, two RNA species were identified which are transcribed from the region containing the rRNA genes (Fig. 1, 2) (101). RNA b4, a polyadenylated RNA, has kinetic properties of synthesis similar to all of the H-strand mRNAs. RNA u4a, in contrast, is not polyadenylated and has synthesis rates similar to the 12S and 16S rRNAs. These findings, in combination with the data about initiation sites and synthesis rates, clearly strengthened the argument that the rRNAs and mRNAs were synthesized in different transcriptional events. Furthermore, it was suggested that the control of the termination or attenuation at the 3' end of 16S was mediated by the site of initiation (101). According to this idea the synthesis of rRNAs occurs by the initiation of transcription at the upstream I_{HR} (Fig. 2) site and termination of transcription at the 3' end of 16S. In contrast, the synthesis of H-strand mRNAs occurs by the initiation of transcription at the

downstream I_{HT} (Fig. 2) site and transcription continues around the great majority of the genome (93, 101).

The recent observations that these two transcription units were indeed separable (102, 110) has allowed the direct test of their control. The first evidence which indicated that the synthesis of rRNA could be separated from that of the mRNA was shown using the drug actinomycin D in an isolated mitochondrial transcription system (102). This work showed that the labeling of the rRNAs was more sensitive to this drug than was the labeling of H-strand mRNA species. Considerably more evidence presented in this work (Chapters 4 and 5, this thesis) suggests that the two transcriptional units are separable under physiological conditions and this separation may be involved in a crucial control mechanism for mitochondrial transcription. Finally, a small cytoplasmic factor(s) has been identified that may have the properties expected to control mitochondrial transcription in a cell-cycle dependent manner (73, Chapter 5, this thesis).

Two lines of evidence have pointed to the interactions between the nucleo/cytoplasmic influences as being crucial to the control of metabolic pathways sequestered within the mitochondrion. The first line is based on the observations that mitochondria are heterogeneous. One aspect of heterogeneity results from the differing mitochondrial genomes. Evolutionarily, this may have resulted from different selective pressures experience by ancestral cells. Any evolutionary track taken by the mitochondrial genome must have been accompanied by a corresponding compatible pathway taken by the nucleus. Hence, through time, nuclear influences have been critical to mitochondrial genome heterogeneity. The other aspect of mitochondrial heterogeneity depends upon the developmental pathway taken by the cell, as observed by morphological and functional differences of the organelles. These interactions occur through space and most likely depend upon the response of the nuclei and cytoplasm to the cells' environment.

The second line of evidence that nuclei and cytoplasmic influences are required comes from the realization that the great majority of the molecules (proteins, lipids, nucleotides, etc.) are made in the cytoplasm. Therefore, control of mitochondrial biogenesis and function occurs because of the expression of genes in the nucleus. Furthermore, experimentation has shown that intimate control of several mitochondrial functions depends upon factors present in the cytoplasm. Clear examples of this dependence are the Pasteur effect on respiratory activity and the cell-cycle control of mitochondrial transcription and replication. The work presented here describes the development of a system designed to investigate these nuclear/cytoplasmic-mitochondrial interactions and its initial uses in the study of the controls of transcription and RNA processing in mitochondria.

References

1. Koshland, D. E., Jr. 1984. Control of enzyme activity and metabolic pathways. *Trends in Biochemistry. Sci.* **9,** 155-159.
2. Stryer, L. 1975. Biochemistry. W. H. Freeman and Co., San Francisco, pp. 46-68 and 153-176.
3. Pabo, C. O., and Sauer, R. T. 1984. Protein-DNA recognition. *Ann. Rev. Biochem.* **53,** 293-321.
4. Chothia, C. 1984. Principles that determine the structure of proteins. *Ann. Rev. Biochem.* **53,** 537-572.
5. Henry, S. A., Klig, L. S., and Loewy, B. S. 1984. The genetic regulation and coordination of biosynthetic pathways in yeast: amino acid and phospholipid synthesis. *Ann. Rev. Genet.* **18,** 207-231.
6. Gottesman, S. 1984. Bacterial regulation: Global regulatory networks. *Ann. Rev. Genet.* **18,** 415-441.
7. Tzagoloff, A. 1982. Mitochondria. Plenum Press, New York and London.
8. Thorpe, N. O. 1984. Cell Biology. John Wiley and Sons, Inc., New York.
9. Williams, R. H. (ed.). 1974. Textbook of Endocrinology. W. B. Saunders, Philadelphia.
10. Pedersen, P. L. 1978. Tumor mitochondria and the bioenergetics of cancer cells. *Prog. in Exp. Tumor Res.* **22,** 190-274.
11. Varmus, H. E. 1984. The molecular genetics of cellular oncogenes. *Ann. Rev. Genet.* **18,** 553-612.
12. Tzagoloff, A. 1982. Mitochondria, pp. 23-24.
13. Tzagoloff, A. 1982. Mitochondria, pp. 15-40 and 267-320.
14. Lehninger, A. L. 1971. Bioenergetics, 2nd edition. W. A. Benjamin, Inc., Menlo Park, California.

15. Kennedy, E. P., and Lehninger, A. L. 1949. Oxidation of fatty acids and tricarboxylic acid cycle intermediates by isolated rat liver mitochondria. *J. Biol. Chem.* **179,** 957-972.

16. Slater, E. C. 1966. Oxidative Phorphorylation. **In** Comprehensive Biochemistry. M. Florkin and E. H. Stotz (eds.), Vol. 14. Elsevier Publishing Company, Amsterdam, London, and New York, pp. 328-350.

17. Kalckar, H. M. 1975. Origins of the concept of oxidative phosphorylation: retrospectives and perspectives. **In** Reflections on Biochemistry. A. Kornberg, B. L. Horecker, L. Cornudella, and J. Oro (eds.). Pergamon Press, Oxford and New York, pp. 27-32.

18. Slater, E. C. 1975. Bioenergetics: Past, present and future. **In** Reflections on Biochemistry. A. Kornberg, B. L. Horecker, L. Cornudella, and J. Oro (eds.). Pergamon Press, Oxford and New York, pp. 45-55.

19. Lehninger, A. L. 1975. Biochemistry, 2nd edition. Worth Publishers, Inc., New York, pp. 443-541.

20. Tzagaloff, A. 1982. Mitochondria, pp. 267-322.

21. Gillham, N. W. 1978. Organelle Heredity. Raven Press, New York.

22. Gray, M. W., and Doolittle, W. F. 1982. Has the Endosymbiont Hypothesis been proven? *Microbiol. Rev.* **46,** 1-42.

23. Borst, P., and Grivell, L. A. 1978. The mitochondrial genome of yeast. *Cell* **15,** 705-723.

24. Leaver, C. J., and Gray, M. W. 1982. Mitochondrial genome organization and expression in higher plants. *Ann. Rev. Plant Physiol.* **33,** 373-402.

25. Nargang, F. E., Bell, J. B., Stohl, L. L., and Lambowitz, A. M. 1984. The DNA sequence and genetic organization of a *Neurospora* mitochondria plasmid suggest a relationship to introns and mobile elements. *Cell* **38,** 441-453.

26. Waring, R. B., Davies, R. W., Lee, S., Grisi, E., Berks, M. M., and Scazzocchio, C. 1981. The mosaic organization of the apocytochrome b gene of *Aspergellus nidulans* revealed by DNA sequencing. *Cell* **27,** 4-11.

27. Attardi, G. 1985. Animal mitochondrial DNA: an extreme example of genetic economy. *Int. Rev. Cytol.* **93,** 93-145.

28. Borst, P., and Grivell, L. A. 1981. Small is beautiful—portrait of a mitochondrial genome. *Nature* **290,** 443-444.

29. Attardi, G. 1981. Organization and expression of the mammalian mitochondrial genome: a lesson in economy. *Trends in Biochemical Sciences* **6,** 86-89 and 100-103.

30. Bibb, M. J., van Etten, R. A., Wright, C. T., Walberg, M. W., and Clayton, D. A. 1981. Sequence and gene organization of mouse mitochondrial DNA. *Cell* **26,** 167-180.

31. Clary, D. O., and Wolstenholme, D. R. 1983. Nucleotide sequence of a segment of *Drosophila* mitochondrial DNA that contains the genes for cytochrome c oxidase subunits II and III and ATPase subunit 6. *Nucl. Acids Res.* **11,** 4211-4227.

32. Clary, D. O., and Wolstenholme, D. R. 1983. Genes for cytochrome c oxidase subunit 1, URF2, and three tRNAs in *Drosophila* mitochondria DNA. *Nucl. Acids Res.* **11,** 6859-6872.

33. Roberts, J. W., Grula, J. W., Posakony, J. W., Hudspeth, R., Davidson, E. H., and Britten, R. J. 1983. Comparison of sea urchin and human mt DNA: Evolutionary arrangements. *Proc. Natl. Acad. Sci. USA* **80,** 4614-4618.

34. Ellis, J. 1982. Promiscuous DNA-chloroplast genes inside plant mitochondria. *Nature* **299,** 678-679.

35. Attardi, G., Chomyn, A., and Mariottini, P. 1984. Genetic control of chloroplast and mitochondrial H^+-ATPases. In H^+-ATPase (ATP synthetase): Structure, Function, Biogenesis of the F_oF_1 Complex of Coupling Membranes. S. Papa, K. Altendorf, L. Ernster, and L. Packer (eds.). Adriatica Editrice, Bari, Italy, pp. 25-40.

36. van den Boogaart, P., Samallo, J., and Agsteribbe, E. 1982. Similar genes for a mitochondrial ATPase subunit in the nuclear and mitochondrial genomes of *Neurospora crassa*. *Nature* **298**, 187-189.

37. Chapman, R. W., Stephens, J. C., Lansman, R. A., and Avise, J. C. 1982. Models of mitochondrial DNA transmission genetics and evolution in higher eucaryotes. *Genet. Res.* **40**, 41-57.

38. Laipis, P. J., Hauswirth, W. W., O'Brien, T. W., and Micheals, G. S. 1980. A physical map of bovine mitochondrial DNA from a single animal. *Biochem. Biophys. Acta* **565**, 22-32.

39. Brown, W. M. 1980. Polymorphism in mitochondrial DNA of humans as revealed by restriction endonuclease analysis. *Proc. Natl. Acad. Sci. USA* **77**, 3605-3609.

40. Aquadro, C. F., and Greenberg, B. O. 1983. Human mitochondrial DNA variation and evolution: analysis of nucleotide sequences from seven individuals. *Genetics* **103**, 287-312.

41. Hauswirth, W. W., Van De Walle, M. J., Laipis, P. J., and Olive, P. D. 1984. Heterogeneous mitochondrial DNA D-loop sequences in bovine tissue. *Cell* **37**, 1001-1007.

42. Thorpe, N. O. 1984. Cell Biology, pp. 388-399.

43. England, J. M., and Attardi, G. 1976. Analysis of RNA synthesized by an isolated rat brain synaptosomal fraction. *J. Neurochem.* **16**, 617-626.

44. Mayor, F. 1975. Brain damage and perinatal biochemistry. **In** Reflections on Biochemistry. A. Kornberg, B. L. Horeckev, L. Cornudella, and J. Oro (eds.). Pergamon Press, Oxford and New York, pp. 365-375.

45. Benson, R. C. 1984. Current Obstetric and Gynecologic Diagnosis and Treatment, 5th edition. Lange Medical Publications, Los Altos, California, p. 828.

46. Wilkin, G. P., Reijnierse, G. L. A., Johnson, A. L., and Balazs, R. 1979. Subcellular fractionation of rat cerebellum: separation of synaposomal populations and heterogeneity of mitochondria. *Brain Res.* **164,** 153-163.

47. Gregson, N. A., and Williams, P. L. 1969. A comparative study of brain and liver mitochondria from new-born and adult rats. *J. Neurochem.* **16,** 617-626.

48. Wilbur, D. O., and Patel, M. S. 1974. Development of mitochondrial pyruvate metabolism in rat brain. *J. Neurochem.* **22,** 709-715.

49. Hawkes, R., Niday, E., and Matus, A. 1982. MIT-23: A mitochondrial marker for terminal neuronal differentiation defined by a monoclonal antibody. *Cell* **28,** 253-258.

50. Burk, D., Woods, K. M., and Hunter, J. 1967. On the significance of glycolysis for cancer growth with special reference to morris rat hepatomas. *J. Nat. Cancer Inst.* **38,** 839-863.

51. Hochman, J. H., Schindler, M., Lee, J. G., and Ferguson-Miller, S. 1982. Lateral mobility of cytochrome c on intact mitochondrial membranes as determined by fluorescence redistribution after photobleaching. *Proc. Natl. Acad. Sci. USA* **79,** 6866-6870.

52. Woldegiorgis, G., and Shrago, E. 1985. Adenine nucleotide translocase activity and sensitivity to inhibitions in hepatomas: Comparison of the ADP/ATP carrier in mitochondria and in a purified reconstituted liposome system. *J. Biol. Chem.* **260,** 7585-7590.

53. Johnson, L. V., Summerhayes, I. C., and Chen, L. B. 1982. Decreased uptake and retention of Rhodamine 123 by mitochondria in feline sarcoma virus—transformed mink cells. *Cell* **28,** 7-14.

54. Chomyn, A., Mariottini, P., Cleeter, M. W. J., Ragan, C. I., Matsuno-Yagi, A., Hatefi, Y., Doolittle, R. F., and Attardi, G. 1985. Six unidentified reading frames of human mitochondrial DNA encode components of the respiratory-chain NADH dehydrogenase. *Nature* **314,** 592-597.

55. Chomyn, A., Mariottini, P., Gonzalez-Cadavid, N., Attardi, G., Strong, D. D., Trovato, D., Riley, M., and Doolittle, R. F. 1983. Identification of the polypeptides encoded in the ATPase 6 gene and in the unassigned reading frames 1 and 3 of human mtDNA. *Proc. Natl. Acad. Sci. USA* **80,** 4435-4439.

56. Hare, J. F., Ching, E., and Attardi, G. 1980. Isolation, subunit composition, and site of synthesis of human cytochrome c oxidase. *Biochemistry* **19,** 2023-2030.

57. Ching, E., and Attardi, G. 1982. High resolution electrophoretic fractionation and partial characterization of the mitochondrial translation products from HeLa cells. *Biochemistry* **21,** 3188-3195.

58. Sols, A. 1975. The Pasteur effect in the allosteric era. **In** Reflections on Biochemistry. A. Kornberg, B. L. Horecker, L. Cornudella, and J. Oro (eds.). Pergamon Press, Oxford and New York, pp. 199-206.

59. Racker, E. 1980. From Pasteur to Mitchell: a hundred years of bioenergetics. *Fed. Proc.* **39,** 210-215.

60. Tornheim, K. 1985. Activation of muscle phosphofructokinase by fructose 2,6-bisphosphate and fructose 1,6-bisphosphate is differently affected by other regulatory metabolites. *J. Biol. Chem.* **260,** 7985-7989.

61. Gasser, S. M., Daum, G., and Schatz, G. 1982. Import of proteins into mitochondria: Energy-dependent uptake of precursors by isolated mitochondria. *J. Biol. Chem.* **257**, 13034-13041.

62. Reid, G. A., and Schatz, G. 1982. Import of proteins into mitochondria: Yeast cells grown in the presence of carbonyl cyanide m-chlorophenyl-hydrazone accumulate massive amounts of some mitochondrial precursor polypeptides. *J. Biol. Chem.* **257**, 13056-13061.

63. Schatz, G., and Butow, R. A. 1983. How are protein imported into mitochondria? *Cell* **32**, 316-318.

64. Hay, R., Böhni, P., and Gasser, S. 1984. How mitochondria import proteins. *Biochem. Biophys. Acta* **779**, 65-87.

65. Reed, L. J. 1981. Regulation of mammalian pyruvate dehydrogenase complex by a phosphorylation-dephosphorylation cycle. *Current Topics in Cellular Regulation.* **18**, 95-106.

66. Desautels, M., and Goldberg, A. L. 1982. Liver mitochondria contain an ATP-dependent vanadate-sensitive pathway for the degradation of proteins. *Proc. Natl. Acad. Sci. USA* **79**, 1869-1873.

67. Pettit, F. H., Humphreys, J., and Reed, L. J. 1982. Regulation of pyruvate dehydrogenase kinase activity by a protein thiol-disulfide exchange. *Proc. Natl. Acad. Sci. USA* **79**, 3945-3948.

68. Hammes, G. G. 1982. Unifying concept for the coupling between ion pumping and ATP hydrolysis or synthesis. *Proc. Natl. Acad. Sci. USA* **79**, 6881-6884.

69. Hatefi, Y., Yagi, T., Phelps, D. C., Wong, S.-Y., Vik, S. B., and Galante, Y. M. 1982. Substrate binding affinity changes in mitochondrial energy linked reactions. *Proc. Natl. Acad. Sci. USA* **79**, 1756-1760.

70. Yagi, T. Matsumo-Yagi, A., Vik, S. B., and Hatefi, Y. 1984. Modulation of the kinetics and the steady-state level of intermediates of mitochondrial coupled reactions by inhibitors and uncouplers. *Biochem.* **23,** 1029-1036.

71. Walsh, K., and Koshland, D. E., J. 1985. Branch point control by the phosphorylation state of isocitrate dehydrogenase: A quantitaive examination of the fluxes during a regulatory transition. *J. Biol. Chem.* **260,** 8430-8437.

72. Yaffe, M., and Schatz, G. 1984. The future of mitochondrial research. *Trends in Biochemical Sciences* **9,** 179-181.

73. Attardi, G., Costantino, P., England, J., Lynch, D., Murphy, W., Ojala, D., Posakony, J., and Storrie, B. 1975. The bioenergetics of mitochondria in HeLa cells: A molecular and cellular study. **In** Genetics and Biogenesis of Mitochondria and Chloroplasts. C. W. Birky, P. Perlman, and T. J. Byers (eds.). Ohio State University Press, Columbus, Ohio pp.3-65.

74. Storrie, B., and Attardi, G. 1972. Expression of the mitochondrial genome in HeLa cells. XIII. Effect of selective inhibition of cytoplasmic or mitochondrial protein synthesis on mitochondrial nucleic acid synthesis. *J. Mol. Biol.* **71,** 177-199.

75. Pica-Mattoccia, L., and Attardi, G. 1971. Expression of the mitochondrial genome in HeLa cells. V. Transcription of mitochondrial DNA in relationship to the cell cycle. *J. Mol. Biol.* **57,** 615-621.

76. England, J. M., and Attardi, G. 1974. Expression of the mitochondrial genome in HeLa cells. XXI. Mitochondrial protein synthesis during the cell cycle. *J. Mol. Biol.* **85,** 433-444.

77. England, J., Costantino, P., and Attardi, G. A. 1978. Mitochondria RNA and protein synthesis in enucleated African green monkey cells. *J. Mol. Biol.* **119,** 455-462.

78. Novitski, C. E. 1979. Ph.D. Thesis, California Institute of Technology, Pasadena, California.

79. Gadaleta, M. N., Giuffrida, A. M., Renis, M., Serra, I., Deldrete, G., Geremia, E., and Saccone, C. 1979. Macromolecular synthesis in mitochondria isolated from different regions of developing rat brain. *Neurochem. Res.* **4**, 25-35.

80. Anderson, S., Bankier, A. T., Barrell, B. G., deBruijn, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J. H., Staden, R., and Young, I. G. 1981. Sequence and organization of the human mitochondrial genome. *Nature* **290**, 457-465.

81. Ojala, D., Montoya, J., and Attardi, G. 1980. The putative mRNA for subunit II of human cytochrome c oxidase starts directly at the translation initiator codon. *Nature* **287**, 79-82.

82. Ojala, D., Merkel, C., Gelfand, R., and Attardi, G. 1980. The tRNA genes punctuate the reading of genetic information in human mitochondrial DNA. *Cell* **22**, 39-403.

83. Cantatore, P., and Attardi, G. 1980. Mapping of nascent light and heavy strand transcripts on the physical map of HeLa cell mitochondrial DNA. *Nucl. Acids Res.* **8**, 2605-2625.

84. Ojala, D., and Attardi, G. 1980. Fine mapping of the ribosomal RNA genes of HeLa cell mitochondrial DNA. *J. Mol. Biol.* **138**, 411-420.

85. Ojala, D., Crews, S., Montoya, J., Gelfand, R., and Attardi, G. 1981. A small polyadenylated RNA (7S RNA), containing a putative ribosome attachment site maps near the origin of mitochondrial DNA replication. *J. Mol. Biol.* **150**, 303-314.

86. Montoya, J., Ojala, D., and Attardi, G. 1981. Distinctive features of the 5' terminal sequences of the human mitochondrial mRNAs. *Nature* **290**, 465-470.

87. Dubin, D. T., Montoya, J., Timko, K. D., and Attardi, G. 1982. Sequence analysis and precise mapping of the 3' ends of HeLa cell mitochondrial ribosomal RNAs. *J. Mol. Biol.* **157**, 1-19.

88. Chomyn, A. *et al.*, 1983. Identification of the polypeptides. pp. 4435-4439.

89. Chang, D. D., and Clayton, D. A. 1985. Priming of the human mitochondrial DNA replication occurs at the light-strand promoter. *Proc. Natl. Acad. Sci. USA* **82**, 351-355.

90. Aloni, Y., and Attardi, G. 1971. Expression of the mitochondrial genome in HeLa cells. II. Evidence for complete transcription of the mitochondrial DNA. *J. Mol. Biol.* **55**, 251-270.

91. Aloni, Y., and Attardi, G. 1971. Symetric *in vivo* transcription of mitochondrial DNA in HeLa cells. *Proc. Natl. Acad. Sci. USA* **68**, 1957-1961.

92. Murphy, W. I., Attardi, B., Tu, C., and Attardi, G. 1975. Evidence for complete symmetrical transcription *in vivo* of mitochondrial DNA in HeLa cells. *J. Mol. Biol.* **99**, 809-814.

93. Montoya, J., Christianson, T., Levens, D., Rabinowitz, M. and Attardi, G. 1982. Identification of the initiation sites for heavy strand and light strand transcription in human mitochondrial DNA. *Proc. Natl. Acad. Sci. USA* **79**, 7195-7199.

94. Attardi, G. 1984. RNA synthesis and processing in mitochondria. **In** Processing of RNA. D. Apirion, (ed.). CRC Press, Inc., Boca Raton, Florida, pp. 228-290.

95. Gelfand, R., and Attardi, G. 1981. Synthesis and turnover of mitochondrial ribonucleic acid in HeLa cells: The mature ribosomal and messenger ribonucleic acid species are metabolically unstable. *Mol. Cell. Biol.* **1**, 497-511.

96. Ojala, D., Montoya, J., and Attardi, G. 1981. The tRNA punctuation model of RNA processing in human mitochondria. *Nature* **290**, 470-474.

97. Crews, S., and Attardi, G. 1980. The sequence of the small ribosomal RNA gene and the phenylalanine tRNA are joined end to end in the human mitochondrial DNA. *Cell* **19**, 775-784.

98. Eperon, I. C., Anderson, S., and Hierlich, D. P. 1980. Distinctive sequence of human mitochondrial ribosomal RNA genes. *Nature* **286**, 460-467.

99. Battey, J., and Clayton, D. A. 1980. The transcription map of human mitochondrial DNA implicates transfer RNA excision as a major processing event. *J. Biol. Chem.* **255**, 11599-11606.

100. Van Etten, R. A., Walberg, M. W., and Clayton, D. A. 1980. Precise localization and nucleotide sequence of the two mouse mitochondrial rRNA genes and three immediately adjacent novel tRNA genes. *Cell* **22**, 157-170.

101. Montoya, J., Gaines, G. L., and Attardi, G. 1983. The pattern of transcription of the human mitochondrial rRNA genes reveals two overlapping transcription units. *Cell* **34**, 151-159.

102. Gaines, G. and Attardi, G. 1984. Intercalating drugs and low tempertures inhibit synthesis and processing of ribosomal RNA in isolated human mitochondria. *J. Mol. Biol.* **172**, 451-466.

103. Bittman, R. 1969. Studies of the binding of ethidium bromide to transfer ribonucleic acid: absorption, fluorescence, ultracentrifugatin and kinetic investigations. *J. Mol. Biol.* **46**, 251-268.

104. Liebman, M., Rubin, J., and Sundaralingam, M. 1977. Nonintercalative binding of ethidium bromide to nucleic acids: Crystal structure of an ethidium - tRNA molecular complex. *Proc. Natl. Acad. Sci. USA* **74**, 4821-4825.

105. Nussinov, R., and Jacobson, A. 1980. Fast algorithm for predicting the secondary structure of single-stranded RNA. *Proc. Natl. Acad. Sci. USA* **77**, 6309-6313.

106. Wells, B. D., and Cantor, C. R. 1977. A strong ethidium binding site in the acceptor stem of most or all transfer RNAs. *Nucl. Acids Res.* **4**, 1667-1680.

107. Gurevitz, M. Jain, S. K., and Apirion, D. 1983. Identification of a precursor molecule for the RNA moiety of the processing enzyme RNase P. *Proc. Natl. Acad. Sci. USA* **80**, 4450-4454.

108. Bothwell, A. L. M., Stark, B. C., and Altman, S. 1976. Ribonuclease P substrate specificity: Cleavage of a bacteriophage ϕ80-induced RNA. *Proc. Natl. Acad. Sci. USA* **73**, 1912-1916.

109. Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N., and Altman, S. 1983. The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme. *Cell* **35**, 849-857.

110. Doersen, C.-J., Guerrier-Takada, C., Altman, S., and Attardi, G. 1985. Characterization of an RNase P activity from HeLa cell mitochondria. *J. Biol. Chem.* **260**, 5942-5949.

111. Gaines, G., and Attardi, G. 1984. Highly efficient RNA-synthesizing system that uses isolated human mitochondria: New initiation events and *in vivo*-like processing patterns. *Mol. Cell. Biol.* **4**, 1605-1617.

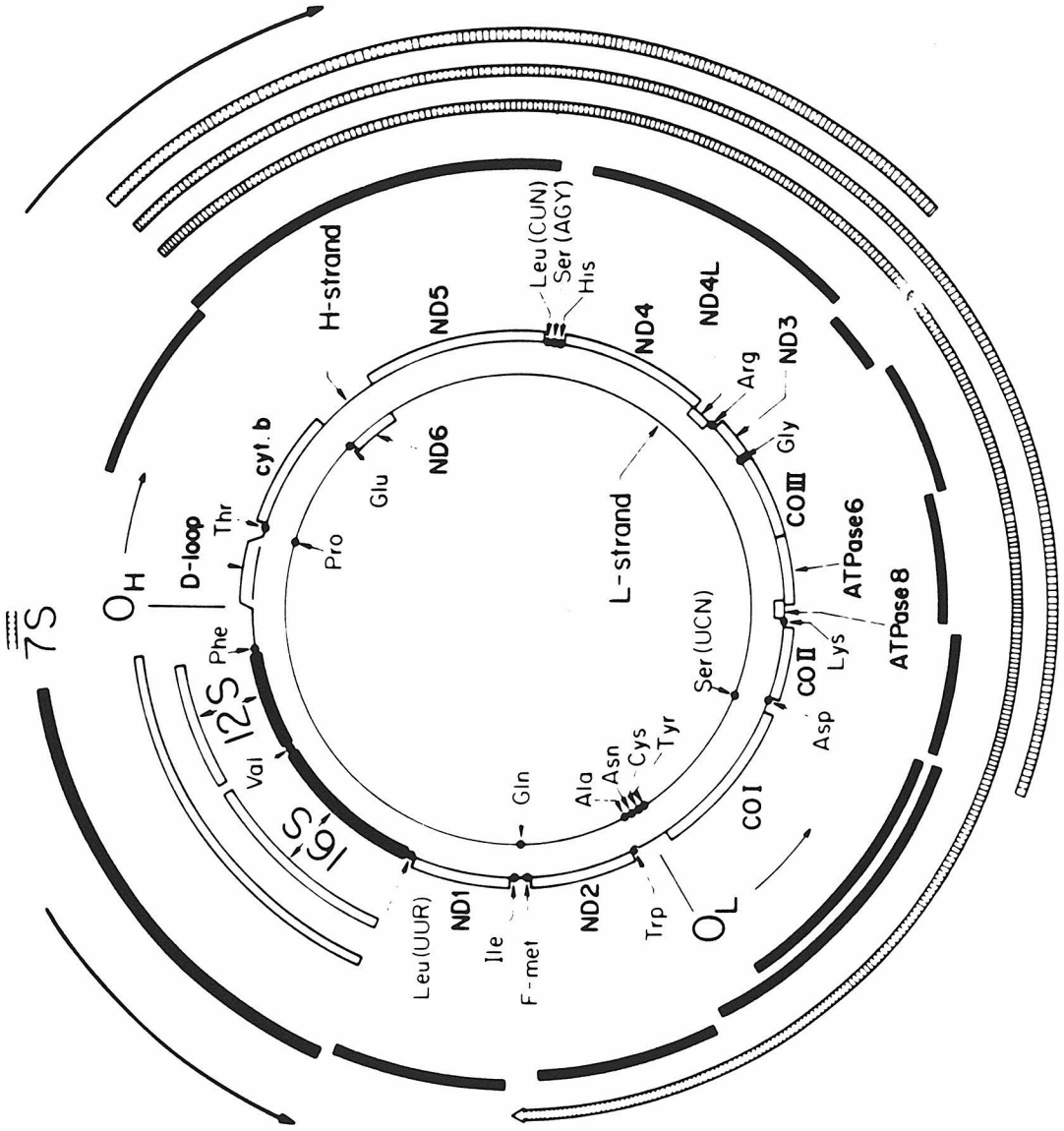

7S
O_H
D-loop
Thr
cyt. b
Phe
12S
Val
16S
H-strand
ND5
Leu(CUN)
Ser(AGY)
His
ND4
ND4L
Arg
ND3
Gly
Glu
ND6
Pro
L-strand
COIII
ATPase6
ATPase8
Lys
COII
Asp
Ser(UCN)
Gln
Ala
Asn
Cys
Tyr
COI
Leu(UUR)
ND1
Ile
F-met
ND2
Trp
O_L

Fig. 1. Diagram of the circular HeLa cell mitochondrial genome and map of the corresponding transcripts (27). The two inner circles show the positions of the rRNA genes, mRNA genes (and reading frames) and tRNA genes. The mapping positions of the oligo(dT)-cellulose bound and nonbound H-strand transcripts are indicated by solid andopen bars, respectively, and the L-strand oligo(dT)-cellulose bound transcripts by hatched bars. The leftward and rightward arrows indicate the direction of H- and L-strand transcription. The origins of replication are marked O_H and O_L. Modified from (27).

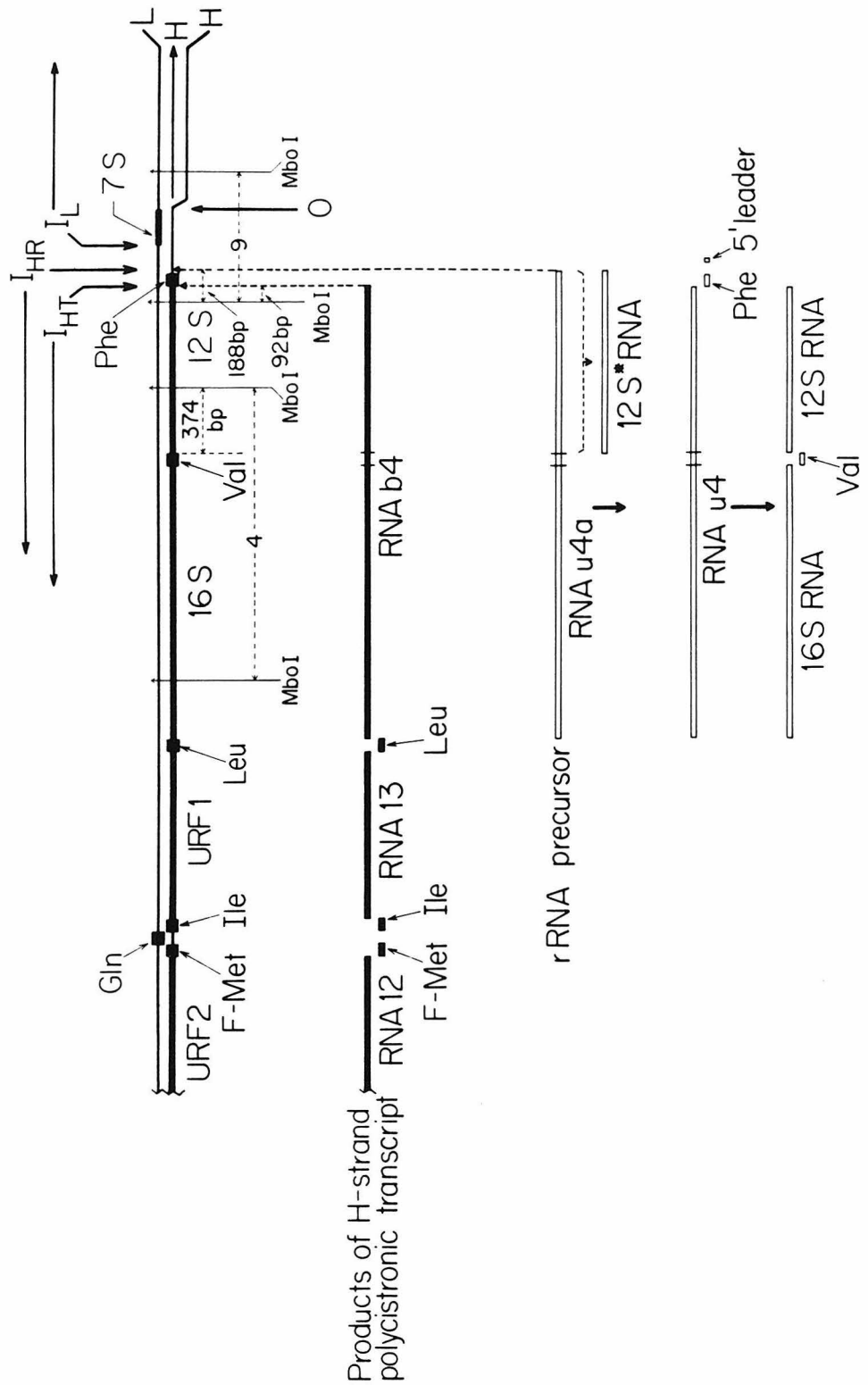

L
H
H
I_{HR}
I_L
I_{HT}
7 S
Phe
12 S
188bp
92bp
MboI
MboI
MboI
MboI
O
9
374 bp
Val
16 S
4
RNA b4
Leu
URF1
Leu
Gln
Ile
F-Met
URF2
RNA 13
Ile
F-Met
RNA 12
Products of H-strand polycistronic transcript
rRNA precursor
RNA u4a
12 S*RNA
RNA u4
Phe
5' leader
16S RNA
12S RNA
Val

Fig. 2. Diagram of a portion of the HeLa cell mitochondrial genome and transcriptional map surrounding the H-strand origin of replication. Shown are the pathways of maturation for the rRNAs and the H-strand mRNAs. Taken from Chapter 1 (92).

CHAPTER 1

Intercalating drugs and low temperatures inhibit synthesis and processing of ribosomal RNA in isolated human mitochondria

J. Mol. Biol. (1984) **172**, 451–466

Intercalating Drugs and Low Temperatures Inhibit Synthesis and Processing of Ribosomal RNA in Isolated Human Mitochondria

GEORGE GAINES AND GIUSEPPE ATTARDI

Division of Biology, California Institute of Technology Pasadena, Calif. 91125, U.S.A.

(Received 12 September 1983)

Mitochondrial DNA transcription in isolated HeLa cell mitochondria faithfully reproduces the *in vivo* process. In this system, actinomycin D, proflavine and ethidium bromide preferentially inhibit the formation of ribosomal RNA over that of messenger RNA, strongly supporting independent controls of the two overlapping transcription units involved in their synthesis. The processing step removing the $tRNA^{Phe}$ sequence at the 5′ end of the ribosomal RNA precursor is uniquely sensitive to low temperature, proflavine and ethidium bromide.

1. Introduction

Analysis of the mapping and kinetic properties of oligo(dT)-cellulose-bound and -unbound transcripts of the ribosomal DNA region of human mitochondrial DNA has revealed the occurrence of two distinct transcriptional events in this region (Attardi *et al.*, 1982; Montoya *et al.*, 1982,1983). These events are correlated with the two initiation sites for heavy-strand transcription identified recently (Montoya *et al.*, 1982). In particular, transcription starting at I_{HR}, ~25 bp† upstream from the $tRNA^{Phe}$ gene, terminates at or near the 3′ end of the 16 S rRNA gene, and is responsible for the synthesis of the bulk of rRNA (Fig. 1). The oligo(dT)-cellulose-unbound RNA species u4A and u4 represent, respectively, the primary transcript of this pathway and an intermediate lacking the $tRNA^{Phe}$ and the 5′ leader. Transcription starting at I_{HT}, very near the 5′ end of the 12 S rRNA gene, results in the synthesis of a polycistronic molecule corresponding to almost the entire H-strand (Fig. 1). This transcript is destined to be processed to the oligo(dT)-cellulose-bound RNA b4 spanning the rDNA region and to the downstream encoded messenger RNA and transfer RNA species.

The mechanisms involved in controlling transcriptional initiation at either site (I_{HR} and I_{HT}), potential termination at the 3′ end of the 16 S rRNA gene and maturation of the two rRNA species remain obscure. With the aim of

† Abbreviations used: bp, base-pairs; H-strand, heavy strand; L-strand, light strand; mtDNA, mitochondrial DNA; nt, nucleotides.

0022-2836/84/040451-16 $03.00/0

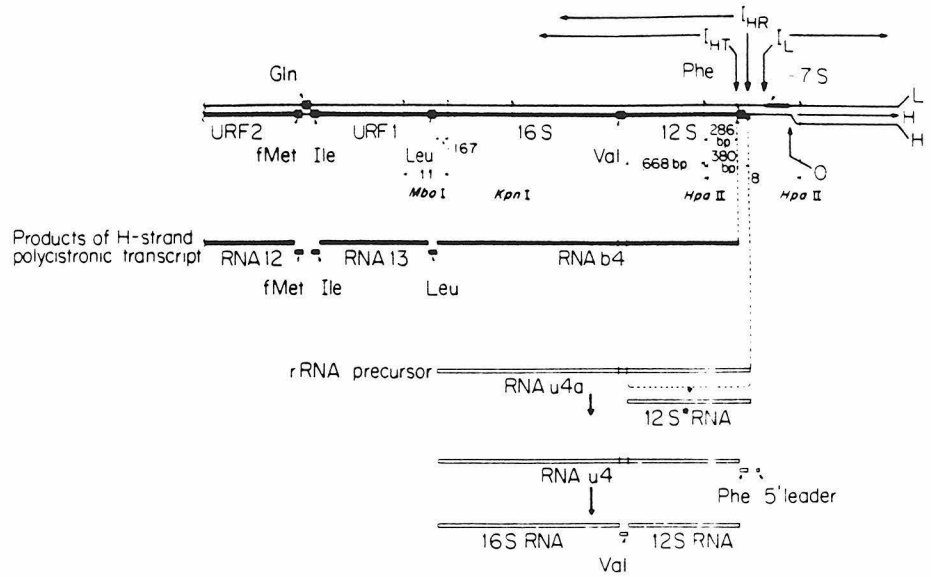


FIG. 1. Portion of the HeLa cell mtDNA genetic and transcription maps illustrating the rRNA gene region and the adjacent regions (Ojala & Attardi, 1980; Crews & Attardi, 1980; Anderson *et al.*, 1981; Ojala *et al.*, 1980). In the upper portion of the diagram, the leftward and rightward heavy arrows indicate the direction of H- and L-strand transcription, respectively, and the downward heavy arrows, the initiation sites for H-strand (I_{HT} and I_{HR}) and L-strand transcription (I_L) (Montoya *et al.*, 1982); the upward heavy arrow indicates the location of the origin of H-strand synthesis (O) (Crews *et al.*, 1979). The lower portion of the diagram shows the precise mapping positions of the large rDNA transcripts identified in previous work (Montoya *et al.*, 1983; Amalric *et al.*, 1978). The *Hpa*II, *Kpn*I-*Hap*II and *Mbo*I fragments utilized in the S_1 mapping experiments are shown. URF1 and −2, unidentified reading frames 1 and 2 (Anderson *et al.*, 1981).

investigating these phenomena further, we have developed an *in vitro* system for mtDNA transcription utilizing isolated HeLa cell mitochondria. Synthesis and processing of rRNA in isolated yeast mitochondria has been described (Boerner *et al.*, 1981; Groot *et al.*, 1981; Newman & Martin, 1982). In the present work, experiments with isolated HeLa cell mitochondria exposed to intercalating drugs or low temperatures have confirmed the existence of independent controls of rRNA and mRNA syntheses, and identified a critical 5′-end processing site in the rRNA precursor, suggesting a hierarchy of steps in the maturation of the rRNAs.

2. Materials and Methods

(a) In vitro *labeling experiments*

The conditions used were a modification of those described by Novitski (1979). In particular, intact mitochondria isolated from about 1 g of HeLa cells were incubated in 1 ml of 0·035 M-Tris · HCl (pH 7·4), 0·075 M-KCl, 0·0075 M-$MgCl_2$, 10% glycerol, 1 mg bovine serum albumin/ml, 0·001 M-ATP, and 100 μCi of [α-^{32}P]UTP ml (NEN $\geq$ 400 Ci/mmol UTP) at 30°C for 90 min. After incubation, the mitochondria were washed at 4°C with 0·25 M-sucrose, 0·01 M-Tris · HCl (pH 7·4), 0·00015 M-$MgCl_2$, and total mitochondrial nucleic acids were extracted and fractionated as described below.

(b) *Extraction, fractionation and analysis of mitochondrial RNA*

Isolation of total nucleic acids from HeLa cell mitochondria by the micrococcal nuclease procedure, oligo(dT)-cellulose chromatography, electrophoresis through 1·4% (w/v) agarose–CH_3HgOH slab gels and isolation of the individual RNA species were carried out as described previously (Ojala *et al.*, 1980; Montoya *et al.*, 1981).

(c) *Isolation and strand separation of restriction fragments*

The human mtDNA *Hpa*II fragment 8 and the *Kpn*I-*Hpa*II fragment shown in Fig. 1 were isolated from the plasmid pBHK2, which contains the middle-sized one of the 3 *Kpn*I fragments of human mtDNA (Greenberg *et al.*, 1983). The *Mbo*I fragment 11 was isolated by polyacrylamide gel fractionation of *Mbo*I-restricted mtDNA. Separation of the strands of *Hpa*II-8 was carried out as previously described (Cantatore & Attardi, 1980).

(d) S_1 *nuclease protection analysis*

Hybridization of separated strands of restriction fragments or whole fragments with *in vitro* labeled RNA species was carried out under conditions of high formamide concentration favoring RNA/DNA hybridization over DNA/DNA reassociation (Casey & Davidson, 1977), as previously described (Ojala *et al.*, 1980). The S_1 nuclease (Sigma) was used at 100 to 500 units in a 0·2 ml vol. for 30 min at 45°C. Electrophoretic analysis of the protected DNA segments was performed under native conditions in 5% polyacrylamide slab gels in Tris/borate/Mg^{2+} buffer (Maniatis *et al.*, 1975).

3. Results

(a) *mtDNA transcription in isolated mitochondria*

Isolated HeLa cell mitochondria, when incubated in the presence of [α-^{32}P]UTP in an appropriate buffer, support transcription of their DNA in a way closely resembling the *in vivo* process. Figure 2 shows a comparison between *in vitro*-labeled mitochondrial RNA species and *in vivo* transcription products. In both patterns, one recognizes the characteristic set of transcripts described previously (Montoya *et al.*, 1983; Amalric *et al.*, 1978). However, polyadenylation appears to be less extensive *in vitro*, as shown by the comparison of the oligo(dT)-cellulose-bound and -unbound fractions in the *in vitro* (lanes 2 and 3) and the *in vivo* labeled RNA (lanes 4 and 5). Furthermore, there are some quantitative differences in the relative labeling of some of the species *in vivo* and *in vitro*. Two RNA species corresponding in mobility to the rRNA precursors identified *in vivo*, u4a and u4 (Montoya *et al.*, 1983), appear to be very prominent in the pattern of *in vitro* synthesized oligo(dT)-cellulose-unbound RNA (lane 2). Furthermore, the band corresponding to polyadenylated RNA 12 is very pronounced, mostly due to the presence of an abundant oligo(dT)-cellulose-unbound component(s) (Fig. 2, lanes 1 and 2). In order to verify the identity of the putative RNAs, u4a and u4, and of the species comigrating with RNA 12, their 5′ and 3′ ends were mapped by S_1 protection experiments.

After hybridization with unlabeled *Hpa*II-8 H-strands (Fig. 1), *in vitro* labeled RNA u4 protected a fragment of ~286 nt in length, while RNA u4a protected a main fragment of ~380 nt in length (Fig. 3(a)). These results were identical to those previously obtained with the corresponding *in vivo* synthesized RNAs, thus

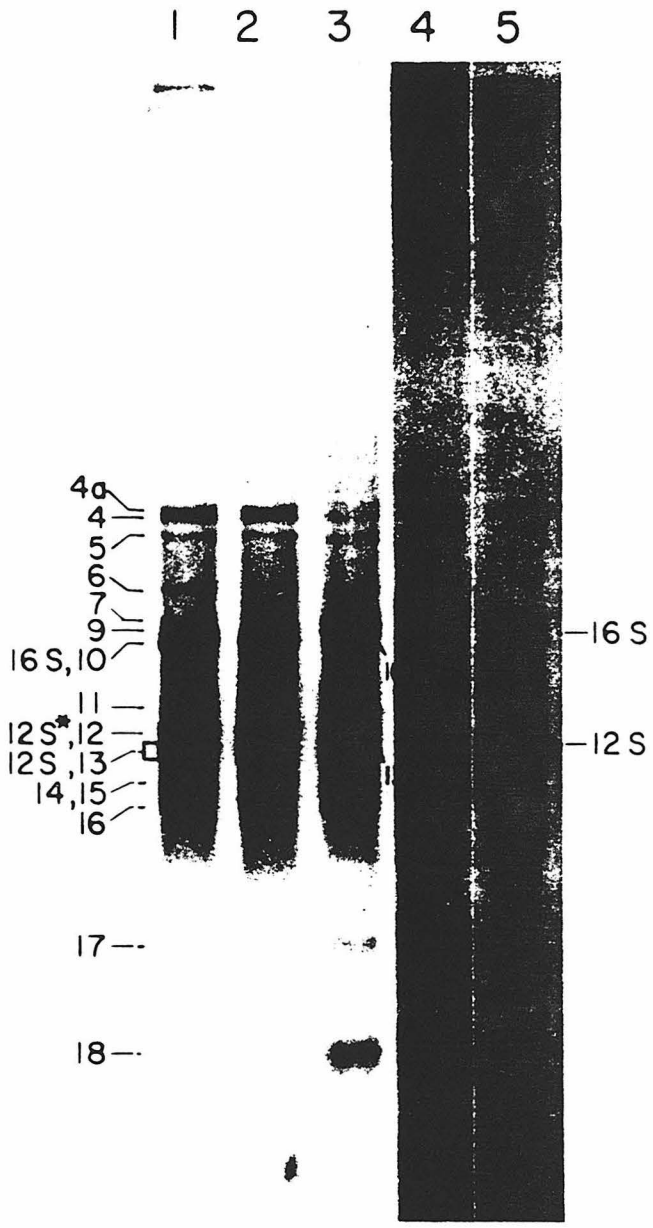


FIG. 2. Comparison of HeLa cell mtDNA transcription in isolated organelles (lanes 1 to 3) and *in vivo* (lanes 4, 5). *In vitro* labeled mitochondrial RNA was fractionated on an oligo(dT)-cellulose column, and the total RNA and the bound and unbound fractions were separated electrophoretically on a 1·4% agarose/CH_3HgOH slab gel. The pattern of *in vitro* labeled total mitochondrial RNA is shown in lane 1. Lane 2 shows the pattern of the oligo(dT)-cellulose-unbound RNA fraction, and lane 3 that of the bound fraction. Tenfold more mitochondrial RNA was applied to lane 3 than to lanes 1 or 2. *In vivo* labeled mitochondrial RNA was prepared and separated into oligo(dT)-cellulose-bound (lane 4) and unbound (lane 5) fractions, as previously described (Ojala *et al.*, 1980). The high molecular weight species in the unbound fraction of *in vivo* labeled material are DNA, as shown by their sensitivity to DNase (J. Montoya, unpublished observations).

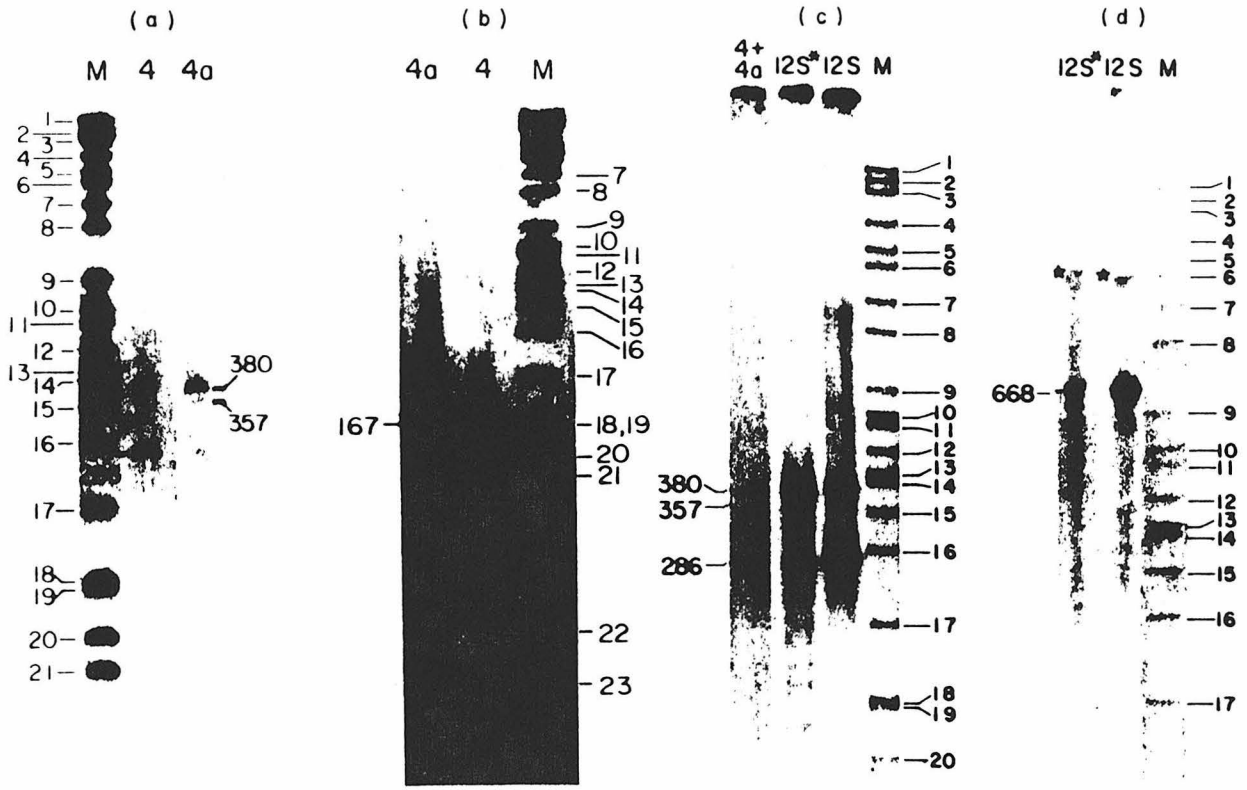


FIG. 3. Nuclease S_1 mapping of the 5′ ends and 3′ ends of rDNA transcripts. (a) Samples of RNAs u4 (lane 2) and u4a (lane 3) from mitochondria labeled *in vitro* with [α-^{32}P]UTP were eluted from slices of an agarose/CH_3HgOH gel, and hybridized with unlabeled *Hpa*II-8H strands. After digestion with S_1 nuclease, the protected segments were analyzed by electrophoresis through a 5% polyacrylamide slab gel in Tris/borate/Mg^{2+} buffer. (b) Samples of *in vitro* labeled RNAs u4a (lane 1) and u4 (lane 2), were hybridized with unlabeled *Mbo*I fragment 11; after S_1 digestion the protected fragments were analyzed as in (a). (c) Samples of *in vitro* labeled RNAs 12 S, 12 S* and u4 + u4a were hybridized with *Hpa*II-8H strands, and subsequently treated as in (a). (d) Samples of *in vitro* labeled RNAs 12 S and 12 S* were hybridized with the *Kpn*I-*Hpa*II fragment (Fig. 1), and subsequently treated as in (a). The stars indicate a minor S_1-resistant band, probably representing RNA/RNA hybrids between a small amount of RNA 11 contaminating the 12 and 12 S* RNA samples and complementary RNA (Ojala *et al.*, 1980). M, 3′-end-labeled mtDNA *Hpa*II digest marker.

placing the 5′ end of *in vitro* synthesized RNA u4 at or near the 5′ terminus of the 12 S rRNA gene and the 5′ end of the *in vitro* synthesized RNA u4a at about 25 nt upstream from the 5′ terminus of the $tRNA^{Phe}$ gene (Fig. 1). A minor protected fragment of ~353 nt in length observed in the S_1 protection pattern obtained with *in vitro* labeled RNA u4a (Fig. 3(a)) is probably derived from a minor RNA species that has its 5′ end very close to the 5′ terminus of the $tRNA^{Phe}$ gene.

After hybridization with unlabeled *Mbo*I fragment 11, a protected fragment of ~167 nt was obtained with both *in vitro* labeled RNA u4a and RNA u4 (Fig. 3(b)). These results indicated that, as the equivalent *in vivo* synthesized RNA species, the two *in vitro* labeled species have identical 3′ ends, corresponding exactly to the 3′ termini of the 16 S rRNA gene (Dubin *et al.*, 1982).

It seemed possible that the abundant component migrating with polyadenylated RNA 12 represented incompletely processed 12 S RNA, still carrying one or both adjacent tRNA sequences. The 5′ and 3′ ends of the 12 S RNA and of the RNA from the prominent band 12 were analyzed by S_1 mapping experiments. Although there was a certain degree of cross-contamination between the two RNAs, the two samples gave clearly distinct patterns. As shown in Figure 3(c), hybridization of *in vitro* labeled RNA from band 12 with unlabeled *Hap*II-8 H-strands yielded a main protected fragment of ~380 nt. This result was consistent with the presence in that band of a major component (termed here 12 S*) differing at its 5′ end from 12 S RNA by the presence of the $tRNA^{Phe}$ sequence and the 5′ leader. A minor protected fragment of ~357 nt obtained with the band 12 RNA sample presumably corresponds to a 12 S RNA processing intermediate carrying the $tRNA^{Phe}$ sequence, but not the 25 nt leader, at its 5′ end. As expected, mature 12 S RNA yielded a main protected fragment of ~286 nt. The S_1 protection pattern obtained in a control experiment using a mixture of RNAs u4a and u4 showed the three protected fragments of approximately 380, 357 and 286 nt described previously (Montoya *et al.*, 1983). At the 3′ end, the 12 S and 12 S* RNA appeared to coincide, since they both protected an ~668 nt long fragment (Fig. 3(d)).

The *in vitro* results described above indicated that the processing of the rRNA species was considerably slower in isolated organelles, with the resulting accumulation of RNAs u4a and u4 and the appearance of processing intermediates, like the 12 S* RNA and the precursors lacking the 5′ leader, a phenomenon that had not been previously observed *in vivo*. These features of the *in vitro* system were exploited in this work to obtain an insight into the pathway of rRNA synthesis and processing.

In the analysis described below, because of the large excess of the 12 S RNA and 12 S* RNA over the amount of the comigrating RNA 12 and RNA 13, respectively (conservatively estimated from densitometric measurements to be more than fivefold; Fig. 2), and of 16 S RNA over the amount of RNA 10 (more than 20-fold), the intensities of the corresponding bands in the electrophoretic patterns of unfractionated mitochondrial RNA were interpreted to reflect primarily the behavior of the rRNA species. For the quantitative analysis of the mRNA under different conditions, the densitometric data for the RNA species 7, 9 and 6 (the latter being the precursor of RNA 9; Ojala *et al.*, 1980) were routinely

used; however, the RNA species 11, 14+15, and 16 showed similar quantitative behavior.

(b) *Effects of intercalating drugs*

Four drugs that are known to intercalate into DNA (Gale *et al.*, 1981) were tested, i.e. actinomycin D, proflavine, ellipticine and ethidium bromide, using the *in vitro* transcription system described above. All four drugs had an overall inhibitory effect upon mtDNA transcription; however, dramatic differences in the response to each drug of rRNA and mRNA synthesis, and of rRNA processing, were observed.

In isolated HeLa cell mitochondria, mitochondrial RNA synthesis was found to be fairly sensitive to actinomycin D (Fig. 4). At drug concentrations $\geq 1{\cdot}3\ \mu$M, more than 95% of organelle RNA synthesis was inhibited. All RNA species were affected, although the synthesis of the rRNA components showed a higher sensitivity (Fig. 4(a)). Particularly noticeable was the effect of the drug on the labeling of 16 S RNA, which was almost totally abolished (being reduced to $<5\%$) at a concentration of actinomycin D of $0{\cdot}8\ \mu$M; this concentration still allowed labeling of 12 S and 12 S* RNAs and that of the mRNAs (as exemplified in Fig. 4(b) by RNA species 7, 9 and 6; the latter being the precursor of RNA 9; Ojala *et al.*, 1980) to about 20% of the control values. The decline in the labeling of 16 S RNA, was accompanied by an increase in the relative labeling of a component (RNA 16a) migrating slightly faster than RNA 16. This component corresponds exactly in migration to an RNA species previously observed in the pattern of mitochondrial RNA labeled *in vivo* in the presence of actinomycin D (Montoya *et al.*, 1983; Amalric *et al.*, 1978) and shown by RNA sequencing to correspond to the proximal one-third of 16 S RNA (J. Montoya, unpublished observations). Densitometric tracings indicate that labeling of RNA 16a could account for a major portion of the decrease in relative labeling of 16 S RNA (not shown). Also to be noted in Figure 4(a) is the relative increase in the labeling of RNA 18 (7 S RNA) (Ojala *et al.*, 1981) and, to a smaller extent, of RNA 17 (Ojala *et al.*, 1980).

Proflavine, an acridine derivative, and ellipticine, a derivative of 6-*H*-pyrido(4,3-*b*)carbazole, two intercalating drugs of similar molecular size, shape, charge and helix-unwinding angle (Gale *et al.*, 1981), showed strikingly different effects on mitochondrial RNA synthesis and processing in isolated organelles (Fig. 5). Proflavine at a concentration of $20{\cdot}4\ \mu$M caused a marked reduction (by 75 to 90%) in the labeling of the rRNA species (12 S, 12 S* and 16 S) with only a moderate inhibition ($\sim 40\%$) of mRNA labeling (Fig. 5(b) and (d)); at higher concentrations, mRNA labeling was also inhibited, although clearly much less than the labeling of the rRNA species. Furthermore, the decrease in the labeling of 12 S* RNA with increasing drug concentrations was much less pronounced than that of the 12 S and 16 S RNA components. As a result, the ratio of labeling of the 12 S* and 12 S bands (which should reflect mainly the relative proportions of the 12 S* and 12 S RNA species) increased by more than a factor of 4 in going from a drug concentration of 4 μM to one of $101{\cdot}5\ \mu$M

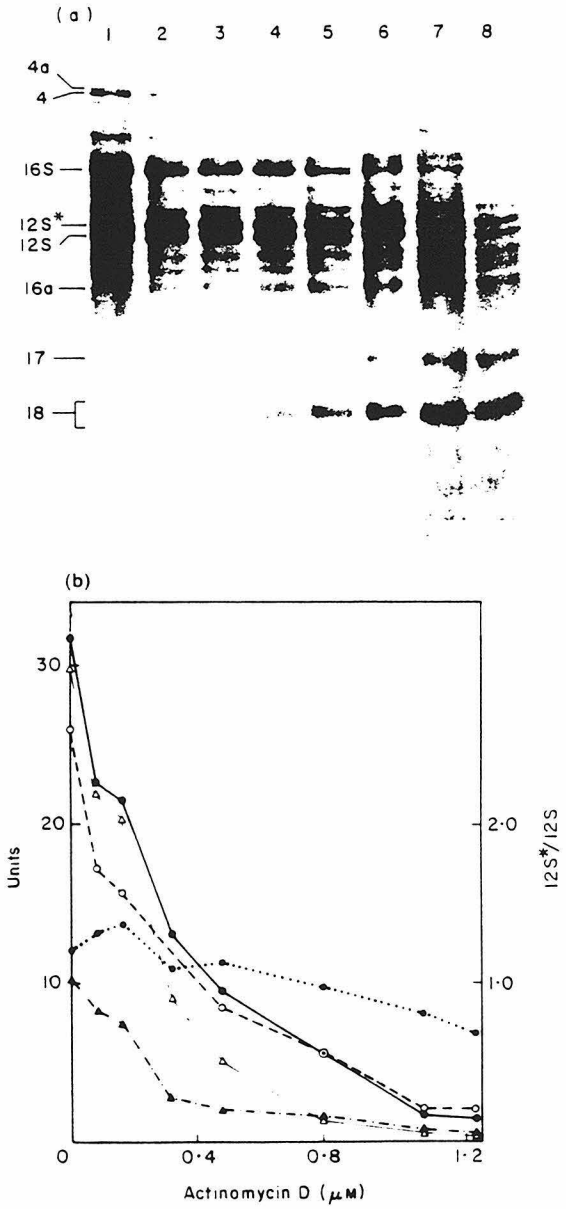


FIG. 4. Effect of actinomycin D on mtDNA transcription in isolated organelles. (a) *In vitro* labeling of mitochondrial RNA was carried out as described in Materials and Methods, with the exception that each incubation mixture contained mitochondria from about 0·5 g of HeLa cells and that the incubation time was 30 min. Varying amounts of actinomycin D were added just before incubation, and mitochondrial RNA was isolated as described. Lanes 1 to 8: no drug, 0·16, 0·32, 0·48, 0·64, 0·8, 1·1 and 1·27 μM-actinomycin D, respectively. In order to obtain fairly uniform exposures, the relative amounts of material electrophoresed were 1, 2, 3, 5, 5, 5, 5 and 5 in lanes 1 to 8, respectively. (b) The autoradiogram was scanned with a Joyce–Loebl double-beam densitometer and the peaks were analyzed with a digitizer. —●—●—, 12 S*; – – ○ – – ○ – –, 12 S; –△–△–, 16 S; ···●···●···, 12S*/12 S; ·–·–▲·–·–▲·–·–, 6+7+9 S.

(Fig. 5(d)). Similarly, the labeling of RNA u4a relative to that of RNA u4 increased in the presence of increasing concentrations of the drug. These observations suggested an interference by proflavine in the processing step involved in the removal of the $tRNA^{Phe}$ or $tRNA^{Phe}+5'$ leader at the 5′ end of the

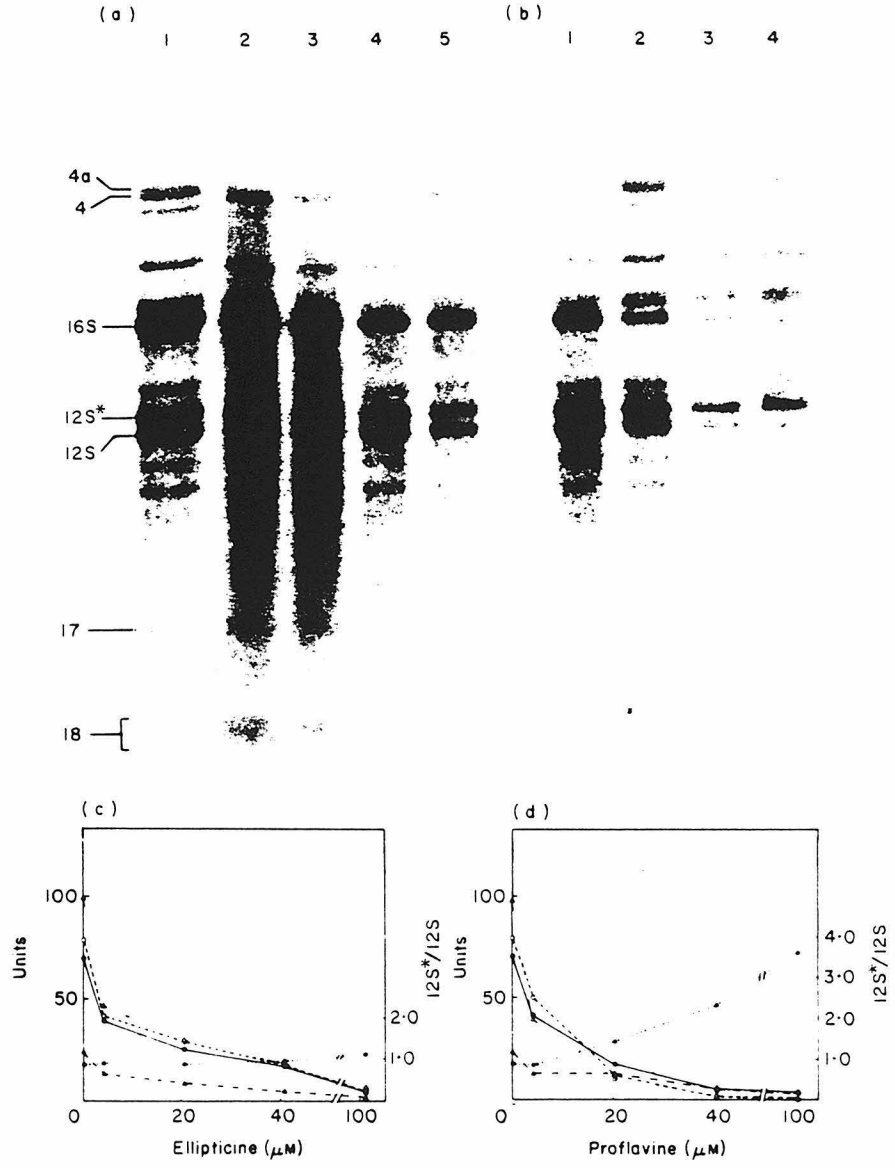


FIG. 5. Effects of ellipticine and proflavine on mtDNA transcription in isolated organelles. (a) and (b) *In vitro* labeling of mitochondrial RNA as in Fig. 4(a). (a) Lanes 1 to 5: no drug, 4·1, 20·3, 40·6 and 101·5 μM-ellipticine, respectively; (b) lanes 1 to 4: 4·1, 20·4, 40·7 and 101·8 μM-proflavine, respectively. The relative amounts of material electrophoresed were 1, 2, 2, 2 and 5 in (a), lanes 1 to 5, respectively, and 2, 5, 10 and 20 in (b), lanes 1 to 4, respectively. (c) and (d) Quantitation of the autoradiograms as in Fig. 4(b). Symbols as in Fig. 4.

rRNA precursors. The nature of the high molecular weight component appearing at the higher proflavine concentrations is unknown.

Ellipticine, in contrast to proflavine, caused parallel inhibition of labeling of the rRNA and mRNA species over the whole range of drug concentrations tested (4 to 101 μM) (Fig. 5(a) and (c)). Furthermore, the 12 S, 12 S* and 16 S RNA components were affected in a quantitatively similar way.

In striking contrast to the effects of actinomycin D, no increase in the relative labeling of RNA 18 (7 S RNA) and RNA 17 with increasing drug concentrations was observed with either proflavine or ellipticine. Furthermore, no evidence of a component migrating as RNA 16a, increasing in relative labeling with drug concentration, was detected.

The effects of ethidium bromide (Gale *et al.*, 1981) (at concentrations about one-tenth of those used for proflavine) on mitochondrial RNA synthesis and processing in isolated organelles were very similar to those of the latter drug (Fig. 6). Thus, in the presence of ethidium bromide, the labeling of the rRNA species was preferentially inhibited relative to that of the mRNAs: at the higher concentrations of the drug ($\geq 2\,\mu$M), the decrease in the labeling of 12 S* RNA was much less drastic than that of 12 S and 16 S RNA, and similarly, the band of RNA u4a became relatively more pronounced than the band of RNA u4. (Although the bands u4a and u4 were not well resolved, the predominant band in each lane was identifiable from its migration relative to that of RNA 5.) Also, no increase in the relative labeling of RNAs 18 and 17 was observed with ethidium bromide.

(c) *A cold-sensitive step in rRNA processing*

The experiments described in the previous sections were all carried out at 30°C. In order to obtain further insight into the various steps involved in mitochondrial rRNA synthesis and processing, the effects of the temperature of incubation on these events were investigated.

Raising the incubation temperature from 30°C to 37°C increased the level of overall mitochondrial RNA labeling by ~70%, while decreasing the temperature to 26°C or 21°C reduced the labeling by ~50% and 75%, respectively (Fig. 7(c)). However, there were marked differences in the relative labeling of the various rRNA components and their precursors at different temperatures. At 37°C, the labeling of 12 S* RNA was decreased relative to that of 12 S RNA, whereas the opposite effect occurred at 26°C, and more markedly, at 21°C (Fig. 7(a) and (c)). Furthermore, at the lower temperatures, the labeling of RNA u4a was more pronounced than that of RNA u4; there was also a decrease in the labeling of 16 S RNA, especially noticeable at 21°C. These results suggested that the step involved in the removal of the $tRNA^{Phe}$ or $tRNA^{Phe}$ + leader from the rRNA precursors was strongly inhibited at low temperatures. Also, the formation of 16 S RNA appeared to be strongly affected at suboptimal temperatures, possibly due to a premature termination of the transcripts. Finally, at 21°C, and to a lesser extent at 26°C, new bands appeared in the region between RNAs 16 and 17, indicating abnormal synthesis and/or processing events.

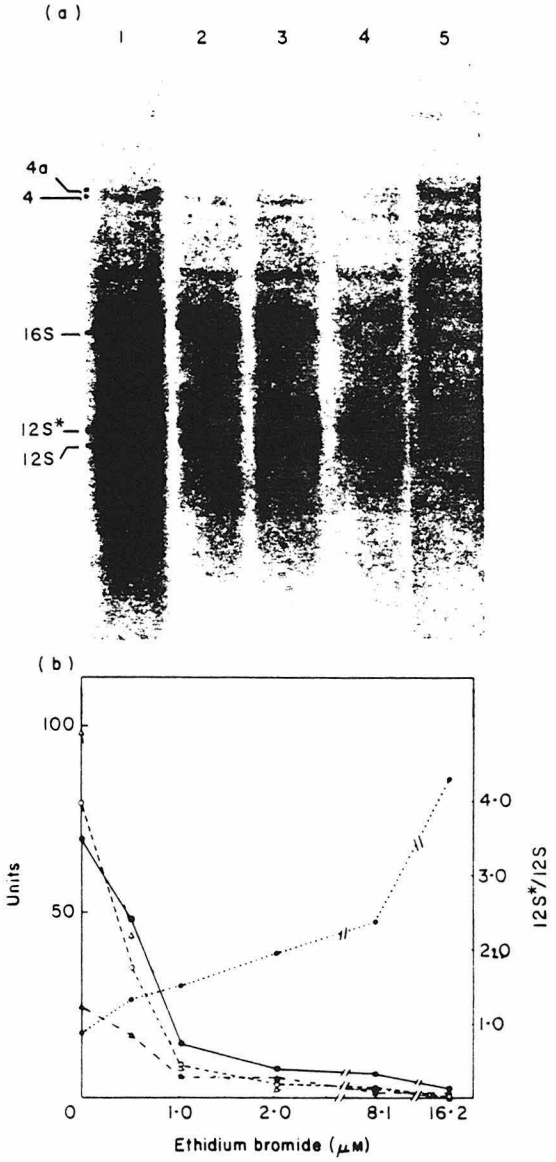


FIG. 6. Effect of ethidium bromide on mtDNA transcription in isolated organelles. (a) *In vitro* labeling of mitochondrial RNA as in Fig. 4(a). Lanes 1 to 5: no drug, 0·5, 1·0, 4·1 and 16·2 μM-ethidium bromide, respectively. The relative amounts of material electrophoresed were 1, 1, 1, 5 and 10 in lanes 1 to 5, respectively. (b) Quantitation of the autoradiogram as in Fig. 4(b). Symbols as in Fig. 4.

(d) *Temperature shift–chase experiment*

In order to obtain direct evidence that the effect of low temperature and intercalating drugs on the relative labeling of the 12 S* and 12 S RNA species was at the level of the 5′-end processing of 12 S* RNA, a temperature shift–chase experiment was carried out. To discriminate between RNA synthesis and

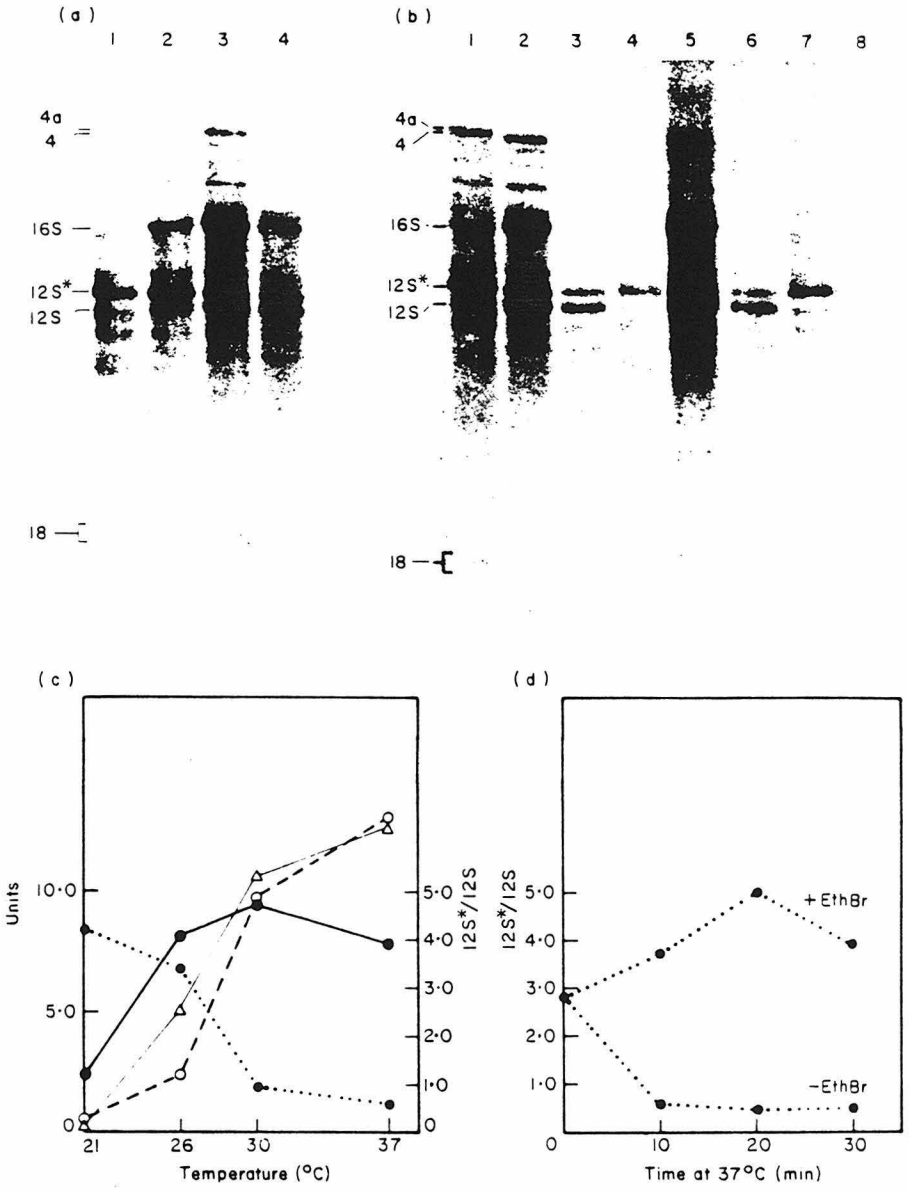


FIG. 7. (a) Effects of temperature upon mtDNA transcription in isolated organelles. *In vitro* labeling of mitochondrial RNA as in Fig. 4(a), except as specified below. Lanes 1 to 4: mitochondria incubated at 21°C, 26°C, 30°C and 37°C, respectively. The relative amounts of material electrophoresed were 10, 5, 2·5, and 1 respectively, in lanes 1 to 4. (b) A temperature shift–chase experiment was carried out as follows. Samples of mitochondria from about 0·5 g HeLa cells each were incubated at 21°C. After 30 min, drugs were added to the appropriate samples, and all samples were incubated for an additional 2 min. The samples, with the exception of that analyzed in lane 1, were then immediately transferred to 37°C and further incubated for 10 min (lanes 2 to 4), 20 min (samples not shown) or 30 min (lanes 5 to 7), with or without addition of drugs as indicated below. Lane 1: mitochondria incubated at 21°C for 30 min. Lanes 2 and 5: mitochondria incubated at 37°C without addition of drugs after the initial 30 min at 21°C. Lanes 3 and 6: mitochondria incubated at 37°C in the presence of 5·1 μM-actinomycin D after the initial 30 min at 21°C. Lanes 4 and 7: mitochondria incubated at 37°C in the presence of 5·1 μM-actinomycin D and 16·2 μM-ethidium bromide (EthBr). Lane 8: mitochondria incubated at 21°C for 30 min without [α-^{32}P]UTP, then treated with actinomycin D (5·1 μM) and, after 2 min, incubated for 30 min at 37°C in the presence of [α-^{32}P]UTP. Lanes 2 and 5 contain one-half the amount of RNA of the other lanes. (c) and (d) Quantitation of the effects of temperature and temperature shift–chase as in Fig. 4(b). Symbols as in Fig. 4.

processing events, advantage was taken of the observation reported above that actinomycin D inhibits mtDNA transcription without greatly affecting the relative proportions of 12 S* and 12 S RNAs. Mitochondria were incubated in the presence of [α-^{32}P]UTP for 30 minutes and then shifted to 37°C with or without addition of 5·1 μM-actinomycin D. As shown in Figure 7(b) (lane 8), actinomycin D effectively blocked further incorporation of [α-^{32}P]UTP. At the time of the temperature shift the 12 S* RNA band was very prominent and there was little or no labeled 12 S RNA (Fig. 7(b), lane 1). After 10 minutes at 37°C in the presence of actinomycin D, the 12 S* band had markedly decreased in intensity, while the 12 S band had correspondingly increased to become the prominent band (Fig. 7(b), lane 3, and (d)). The effect was more pronounced after a 20-minute or 30-minute chase (Fig. 7(b), lane 6). At the same time, there was an almost complete disappearance of labeled RNA u4a, with only faint u4a and u4 bands being visible. As shown in Figure 7(b) (lanes 4 and 7) and Figure 7(d), addition of ethidium bromide to 16·2 μM at the time of the temperature shift, together with actinomycin D, blocked completely the processing of 12 S* RNA to 12 S RNA and, partially, that of RNA u4a to RNA u4. These results indicated that the intercalating drug acted, in a similar way to the low temperature, by preventing the removal of the 5′-terminal tRNA^{Phe} from the rRNA precursors. In the absence of actinomycin D, this processing event presumably also occurred, although it was masked by the continuation of RNA synthesis at an appreciably higher rate after the temperature shift (Fig. 7(b), lanes 2 and 5).

4. Discussion

The mtDNA transcription system utilizing isolated organelles that is described here faithfully reproduces the *in vivo* process. Such a system lends itself to experimental manipulation without the constraints of the nucleocytoplasmic environment, and should prove invaluable for the study of the energetic and metabolic requirements of mtDNA transcription, of the action of mitochondrially targeted drugs and of nucleocytoplasmic–mitochondrial interactions.

(a) *Mode of action of intercalating drugs*

The different effects of the intercalating drugs tested here on mtDNA transcription are presumably related to their different modes of action. There is good evidence from other systems (Gale *et al.*, 1981; Richardson *et al.*, 1966) that actinomycin D acts primarily by slowing down the chain-elongation reaction, and that it binds selectively at G·C base-pairs. The results obtained here with this drug are consistent with this mechanism of action and base-specificity. The lower sensitivity to this drug of the labeling of 12 S (+12 S*) RNA and 7 S RNA, two RNA species that are close to the initiation sites of the corresponding transcription units (Montoya *et al.*, 1983), would be predicted on the basis of a predominant action of actinomycin D at the level of chain elongation. The small size of 7 S RNA and its relatively low G+C content (38%) (Ojala *et al.*, 1981) probably also contributed to the lower sensitivity of its labeling to the drug.

Premature termination may account for the more marked inhibition by actinomycin D of the labeling of 16 S RNA. Accumulation of RNA 16a in the presence of this drug is likely to result from the occurrence of a preferential premature termination site at about one-third of the distance from the 5′ end of the 16 S RNA gene. There is, on the contrary, no obvious explanation for the lower sensitivity to actinomycin D of the labeling of RNA 17; it is possible that this phenomenon reflects a stabilization of this RNA species under the experimental conditions used.

There is good evidence that proflavine and ethidium bromide act preferentially at the level of initiation of transcription by *Escherichia coli* RNA polymerase, without any clear indication of base specificity (Gale *et al.*, 1981; Richardson *et al.*, 1966; Richardson, 1973). The effects of the two drugs on mtDNA transcription are consistent with this mode of action; however, the more-marked effects on 16 S than on 12 S gene transcription suggest that they could act at the level of elongation as well, favoring pausing of the polymerase or premature termination or read-through at the normal termination point. Ellipticine, on the contrary, did not show a more pronounced inhibitory effect on 16 S RNA labeling, suggesting that its block of transcription may be exclusively at the level of initiation.

(b) *Dissociation of the two H-strand transcription events*

The differential sensitivity of mitochondrial rRNA and mRNA syntheses to actinomycin D, proflavine and ethidium bromide point to some critical differences between the two overlapping transcription units involved in the synthesis of the two RNA classes. The difference in behavior of the two transcription units cannot simply be due to a different degree of inhibition by the intercalating drugs of the two initiation reactions. In fact, this mechanism would not account for the premature termination of transcription within the 16 S rRNA gene induced by actinomycin D, which is observed in the absence of a corresponding drug effect on the transcription of the downstream regions of mtDNA. Rather, the existence of two different RNA polymerases and/or specific secondary structures of the rRNA precursor and their possible interaction with other molecules, such as ribosomal proteins, may be involved.

(c) *Pathway of rRNA processing*

The temperature-shift and intercalating-drug (proflavine and ethidium bromide) experiments reported here have shown that the processing of the 5′ ends of both 12 S* and u4a can be dissociated from RNA synthesis. One can exclude the possibility that this effect is due to a block of mitochondrial protein synthesis, since it is not seen during chloramphenicol inhibition of mitochondrial translation (unpublished observations). Although one cannot rule out a direct action of the low temperature or intercalating drugs on the processing enzyme or an effect due to membrane perturbations, it is tempting to speculate that a change in secondary or tertiary structure of the rRNA precursor, affecting the recognition or activity

of the processing enzyme, underlies both the effect of temperature and that of intercalating drugs. An effect of intercalating drugs on RNA secondary structure has been previously postulated. Proflavine and ethidium bromide have been shown to inhibit the processing of nuclear rRNA precursors (Snyder *et al.*, 1971; Yannarell *et al.*, 1977). Proflavine has also been shown to block the processing of cellular heterogeneous nuclear RNA (Yannarell *et al.*, 1977) and of simian virus 40 (SV40) nuclear RNA precursors (Chiu *et al.*, 1980), and to prevent transcription termination on *Tetrahymena* rDNA (Westergaard *et al.*, 1979) and SV40 DNA (Hay *et al.*, 1982). Strong binding of ethidium bromide to tRNA has also been described (Bittman, 1969; Wells & Cantor, 1977; Liebman *et al.*, 1977). In view of the suggested role of the tRNA sequences as recognition signals in the processing of the H-strand transcripts (Attardi *et al.*, 1982; Ojala *et al.*, 1980), it is quite possible that binding of intercalating drugs to the $tRNA^{Phe}$ is involved in the block of the 5′-end processing of the rRNA precursors. Whatever the mechanism may be for the inhibition of this 5′-end processing, it is clear that there is a strong specificity of the intercalating drug involved, since neither actinomycin D nor ellipticine has this effect. Furthermore, it appears that this processing site is uniquely sensitive to low temperature, proflavine and ethidium bromide, among the many sites at which tRNA processing occurs in the mtDNA transcripts (Attardi *et al.*, 1982; Ojala *et al.*, 1980). This unique sensitivity may reflect very strong structural constraints, in terms of secondary structure and/or protein binding, for processing at this site.

The observations reported here on the effects of intercalating drugs and low temperature on *in vitro* mtDNA transcription and RNA processing confirm the conclusion, previously reached on the basis of kinetic arguments (Montoya *et al.*, 1983), that processing of the rRNA precursor at the $tRNA^{Val}$ site is an early event, and that formation of 12 S RNA does not require completion of the 16 S RNA, which probably occurs in most cases in nascent molecules still attached to the template. According to this model, formation of the u4a and u4 rRNA precursors is not a prerequisite for mature rRNA synthesis, and these species may be normally involved in the formation of only a minor portion of the rRNAs.

(d) *Implications for other* in vitro *transcription and RNA processing systems*

The differential response to intercalating drugs and abnormal temperatures, which has been observed here for the various mtDNA transcription and RNA processing events occurring in isolated HeLa cell mitochondria suggests that such agents could be useful in the dissection of other *in vitro* systems; in particular, nuclear DNA transcription and RNA processing. Although intercalating drugs have previously been used *in vivo* to study their effects on transcription or RNA processing, very rarely have they been utilized under the more controllable *in vitro* conditions. In a well-characterized system, the use of either judiciously graduated concentrations of intercalating drugs with different modes of action, or abnormal temperatures, may provide valuable information for the recognition of multiple promoters, for the ordering of processing events, and for the identification of rate-limiting steps and very short-lived intermediates.

These investigations were supported by National Institutes of Health grants GM-11726 and T32 GM-07616. We thank Mike Van Dyke and Peter Dervan for a gift of ellipticine and valuable discussions. We also thank Ms Arger Drew for excellent technical assistance.

REFERENCES

Amalric, F., Merkel, C., Gelfand, R. & Attardi, G. (1978). *J. Mol. Biol.* **118**, 1–25.

Anderson, S., Bankier, A. T., Barrell, B. G., de Bruijn, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. E., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J. H., Staden, R. & Young, I. G. (1981). *Nature (London)*, **290**, 457–465.

Attardi, G., Cantatore, P., Chomyn, A., Crews, S., Gelfand, R., Merkel, C., Montoya, J. & Attardi, G. (1982). In *Mitochondrial Genes* (Slonimski, P., Borst, P. & Attardi, G., eds), pp. 51–71. Cold Spring Harbor Laboratory, New York.

Bittman, R. (1969). *J. Mol. Biol.* **46**, 251–268.

Boerner, P., Mason, T. L. & Fox, T. P. (1981). *Nucl. Acids Res.* **9**, 6379–6390.

Cantatore, P. & Attardi, G. (1980). *Nucl. Acids Res.* **8**, 2605–2625.

Casey, J. & Davidson, N. (1977). *Nucl. Acids Res.* **4**, 1539–1552.

Chiu, N. H., Bruszewski, W. B. & Salzman, N. P. (1980). *Nucl. Acids Res.* **8**, 153–168.

Crews, S. & Attardi, G. (1980). *Cell*, **19**, 775–784.

Crews, S., Ojala, D., Posakony, J., Nishiguchi, J. & Attardi, G. (1979). *Nature (London)*, **277**, 192–198.

Dubin, D. T., Montoya, J., Timko, K. D. & Attardi, G. (1982). *J. Mol. Biol.* **157**, 1–19.

Gale, E. F., Cundliffe, E., Reynolds, P. E., Richmond, M. H. & Waring, M. J. (1981). In *The Molecular Basis of Antibiotic Action*, pp. 258–401, Wiley & Sons, New York.

Greenberg, B. D., Newbold, J. E. & Sugino, A. (1983). *Gene*, **21**, 33–49.

Groot, G. S. P., Van Harten-Loosbroeck, N., Van Ommen, G.-J. B. & Pijst, H. L. A. (1981). *Nucl. Acids Res.* **9**, 6369–6378.

Hay, N., Skolnik-David, H. & Aloni, Y. (1982). *Cell*, **29**, 183–193.

Liebman, M., Rubin, J. & Sundaralingam, M. (1977). *Proc. Nat. Acad. Sci., U.S.A.* **74**, 4821–4825.

Maniatis, T., Jeffreys, A. & van de Sande, H. (1975). *Biochemistry*, **14**, 3787–3794.

Montoya, J., Ojala, D. & Attardi, G. (1981). *Nature (London)*, **290**, 465–470.

Montoya, J., Christianson, T., Levens, D., Rabinowitz, M. & Attardi, G. (1982). *Proc. Nat. Acad. Sci., U.S.A.* **79**, 7195–7199.

Montoya, J., Gaines, G. L. & Attardi, G. (1983). *Cell*, **34**, 151–159.

Newman, D. & Martin, N. (1982). *Plasmid*, **7**, 66–76.

Novitski, C. E. (1979). Ph.D. thesis, California Institute of Technology, Pasadena, California.

Ojala, D. & Attardi, G. (1980). *J. Mol. Biol.* **138**, 411–420.

Ojala, D., Merkel, C., Gelfand, R. & Attardi, G. (1980). *Cell*, **22**, 393–403.

Ojala, D., Crews, S., Montoya, J., Gelfand, R. & Attardi, G. (1981). *J. Mol. Biol.* **150**, 303–314.

Richardson, J. P. (1973). *J. Mol. Biol.* **78**, 703–714.

Richardson, J. P., Slayer, H. S. & Hall, C. E. (1966). *J. Mol. Biol.* **21**, 83–114.

Snyder, A. L., Kann, H. E. Jr & Kohn, K. W. (1971). *J. Mol. Biol.* **58**, 555–565.

Wells, B. D. & Cantor, C. R. (1977). *Nucl. Acids Res.* **4**, 1667–1680.

Westergaard, O., Gocke, E., Nielsen, O. F. & Leer, J. C. (1979). *Nucl. Acids Res.* **6**, 2391–2402.

Yannarell, A., Niemann, M., Schumm, D. E. & Webb, T. E. (1977). *Nucl. Acids Res.* **4**, 503–511.

Edited by P. Chambon

CHAPTER 2

Highly efficient RNA-synthesizing system that uses isolated human mitochondria: new initiation events and *in vivo*-like processing patterns

MOLECULAR AND CELLULAR BIOLOGY, Aug. 1984, p. 1605–1617
0270-7306/84/081605-13$02.00/0

Vol. 4, No. 8

Highly Efficient RNA-Synthesizing System That Uses Isolated Human Mitochondria: New Initiation Events and In Vivo-Like Processing Patterns

GEORGE GAINES AND GIUSEPPE ATTARDI*

Division of Biology, California Institute of Technology, Pasadena, California 91125

Received 21 March 1984/Accepted 14 May 1984

A highly efficient RNA-synthesizing system with isolated HeLa cell mitochondria has been developed and characterized regarding its requirements and its products. In this system, transcription is initiated and the transcripts are processed in a way which closely reproduces the in vivo patterns. Total RNA labeling in isolated mitochondria proceeds at a constant rate for about 30 min at 37°C; the estimated rate of synthesis is at least 10 to 15% of the in vivo rate. Polyadenylation of the mRNAs is less extensive in this system than in vivo. Furthermore, compared with the in vivo situation, rRNA synthesis in vitro is less efficient than mRNA synthesis. This is apparently due to a decreased rate of transcription initiation at the rRNA promoter and probably a tendency also for premature termination of the nascent rRNA chains. The 5′-end processing of rRNA also appears to be slowed down, and it is very sensitive to the incubation conditions, in contrast to mRNA processing. It is suggested that the lower efficiency and the lability of rRNA synthesis and processing in isolated mitochondria may be due to cessation of import from the cytoplasm of ribosomal proteins that play a crucial role in these processes. The formation of the light-strand-coded RNA 18 (7S RNA) is affected by high pH or high ATP concentration differently from the overall light-strand transcription. The dissociation of the two processes may have important implications for the mechanism of formation and the functional role of this unusual RNA species. The high efficiency, initiation capacity, and processing fidelity of the in vitro RNA-synthesizing system described here make it a valuable tool for the analysis of the role of nucleocytoplasmic-mitochondrial interactions in organelle gene expression.

Recent work from this laboratory has shown that the heavy (H) strand of HeLa cell mitochondrial DNA (mtDNA) is the site of two overlapping transcription events (4a, 12, 19, 20). A considerable amount of evidence suggests that one of these events is responsible for the synthesis of the bulk of rRNA, whereas the other results in the synthesis of a polycistronic molecule which corresponds to almost the entire H strand and is destined to be processed to yield the mRNAs and most of the tRNAs encoded in the H strand (Fig. 1). The initiation of the two H-strand transcription events and that of light (L)-strand transcription occur in close proximity to each other in the region of mtDNA upstream of the origin of H-strand synthesis (10). This clustering of the three putative promoters may underlie the integrated control of expression of the corresponding transcription units, whereas their proximity to the origin of H-strand synthesis may reflect the existence of a link between transcription events and mtDNA replication. To investigate further, under conditions allowing easy experimental manipulations, the molecular mechanisms, controls, and interrelationships of the mtDNA transcription and RNA processing events which occur in HeLa cell mitochondria, we developed a system for studying RNA synthesis in isolated organelles. In this system, mtDNA transcription and RNA processing proceed in a way closely resembling, qualitatively and quantitatively, the in vivo processes. By appropriately varying the conditions of incubation, we have been able to affect differentially various DNA transcription and RNA processing phenomena occurring in mitochondria and thus to obtain information concerning the mechanisms involved and the interrelationship between the individual events.

* Corresponding author.

MATERIALS AND METHODS

Cell growth and mitochondria isolation. HeLa cells were grown in suspension in modified Eagle medium supplemented with 5% calf serum, with a continuous flow of 5% CO_2 in air being flushed over the medium to keep the pH constant (15). Exponentially growing cells were harvested, washed two times with 1 mM Tris-hydrochloride (pH 7.0) (25°C)–0.13 M NaCl–5 mM KCl–7.5 mM $MgCl_2$, and broken in one-half of the packed cell volume of 3.5 mM Tris-hydrochloride (pH 7.8)–2 mM NaCl–0.5 mM $MgCl_2$ by using a Thomas homogenizer with a motor-driven Teflon pestle. The homogenate was immediately mixed with 1/9 of the cell volume of 0.35 M Tris-hydrochloride (pH 7.8)–0.2 M NaCl–50 mM $MgCl_2$ and spun for 3 min at 900 × *g* to pellet unbroken cells and nuclei. The supernatant was recentrifuged under the same conditions. The final supernatant was portioned into Eppendorf tubes and spun at full speed (12,700 × *g*) for 1 min in an Eppendorf microfuge. The mitochondrial pellets were washed one time with the appropriate incubation buffer and pelleted. All manipulations up to this point were carried out at 4°C.

In vitro labeling and isolation of mitochondrial nucleic acids. Samples of the mitochondrial fraction, each derived from ~0.5 g of HeLa cells (~0.7 mg of mitochondrial protein), were resuspended in 0.5 ml of the appropriate incubation buffer in Eppendorf tubes. The standard medium contained 10% glycerol, 35 mM Tris-hydrochloride (pH 7.8), 20 mM NaCl, 5 mM $MgCl_2$, 1 mM ATP, 1 mg of bovine serum albumin (BSA) per ml, and 5 to 50 μCi of [α-^{32}P]UTP (400 to 600 Ci/mmol). Unless otherwise specified, incubation was at 37°C for 30 min. This system differs from the RNA-synthesizing system with isolated HeLa cell mitochondria previously developed in this laboratory (C. E. Novitski, Ph.D. thesis, California Institute of Technology, Pasadena,

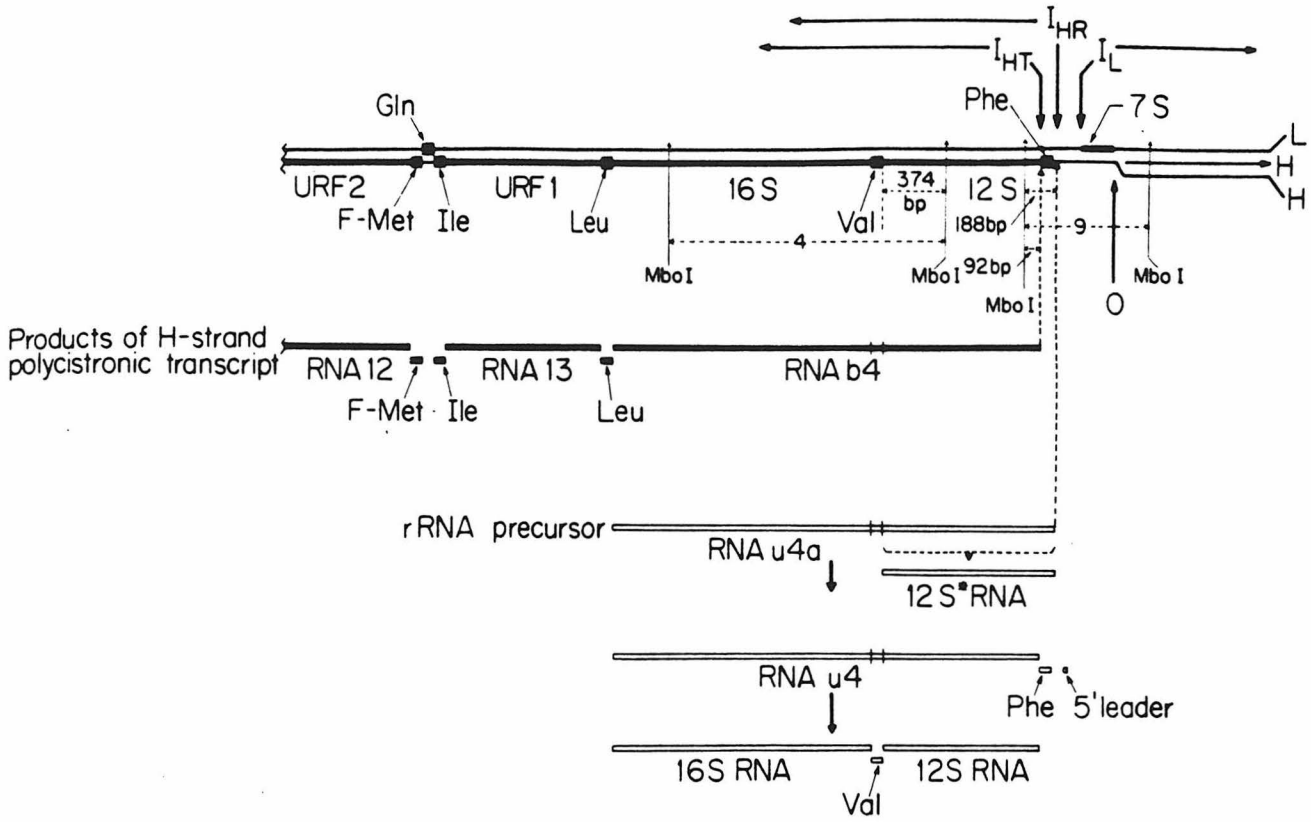


FIG. 1. Portion of the HeLa cell mtDNA genetic and transcription maps illustrating the rRNA gene region and the adjacent regions (2, 9, 24, 26). The downward arrows in the upper portion of the diagrams indicate the initiation sites for H-strand (I_{HT} and I_{HR}) and L-strand transcription (I_L) (19), and the upward arrow indicates the position of the origin of H-strand synthesis (O) (10). The leftward and rightward arrows indicate the direction of H- and L-strand transcription, respectively. In the lower portion of the diagram, the precise mapping positions of the rDNA transcripts and mRNAs identified in previous work (12, 20, 26) are shown. The *Mbo*I fragments utilized in the S1 mapping experiments are also shown.

1979) in the lower concentrations of monovalent cation and Mg^{2+}, the higher pH, and the higher temperature of incubation.

After the incubation, the mitochondrial samples were pelleted at 12,700 × *g* for 1 min and washed two times with 10% glycerol–10 mM Tris-hydrochloride (pH 6.8)–0.15 mM $MgCl_2$ at 4°C. The pellets were suspended in 1 ml of 10% glycerol–10 mM Tris-hydrochloride (pH 8.0)–1 mM $CaCl_2$, incubated with 200 U of micrococcal nuclease each at room temperature for 20 min, and then washed two times with 10% glycerol–10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA at 4°C. The mitochondrial samples were then lysed with 0.35 ml of 0.5% sodium dodecyl sulfate–10 mM Tris-hydrochloride (pH 7.4)–0.15 M NaCl–1 mM EDTA (sodium dodecyl sulfate buffer) and incubated with 100 μg of pronase for 15 min at 30°C. Phenol extraction, fractionation by oligodeoxythymidylate [oligo(dT)]-cellulose chromatography, and electrophoretic analysis in CH_3HgOH-agarose gels of the mitochondrial nucleic acids were carried out as previously described (21, 24, 26). A portion of each extracted nucleic acid sample was acid precipitated (1 N HCl, 50 mM sodium pyrophosphate) and collected on a GF/C membrane for determination of total incorporated radioactivity. Quantitation of the autoradiograms of the CH_3HgOH-agarose gels was carried out by using a Joyce-Loebl double-beam densitometer and analyzing the peaks with a digitizer.

S1 protection analysis. Hybridization of in vitro-labeled RNA species with M13-cloned single-stranded *Mbo*I fragment 4 or 9 of human mtDNA (M. King, unpublished data) was carried out under high-formamide conditions favoring RNA-DNA hybridization over DNA-DNA reassociation (8). Briefly, samples of labeled RNA were mixed with the appropriate unlabeled M13-cloned DNA fragment, lyophilized, resuspended in 20 μl of 80% formamide–20 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] buffer (pH 6.4)–0.375 M NaCl–0.5 mM EDTA, heated to 68°C for 10 min, and incubated at 49°C for 6 to 8 h. S1 digestion buffer (0.25 M NaCl, 40 mM sodium acetate [pH 4.5], 3 mM $ZnCl_2$, 10 μg of denatured salmon sperm DNA per ml) was added to 200 μl, and the samples were then digested with 165 U of S1 nuclease (Sigma Chemical Co.) at 41°C for 30 min. The reaction was stopped by addition of 200 μl of sodium dodecyl sulfate buffer, followed by two ethanol precipitations. The S1-resistant products were analyzed by electrophoresis through an 8% polyacrylamide–8 M urea gel, followed by autoradiography. To quantitate the radioactivity associated with the S1-resistant fragments, appropriate bands were cut from the dried gels and counted in a scintillation counter by using a toluene-based scintillation fluid. Similarly sized slices cut immediately above and below the bands in question were subjected to the same analysis to provide an estimate of the background radioactivity.

Strand homology analysis of in vitro mtDNA transcripts. In

vitro-labeled RNA and separated strands of total mtDNA were hybridized, and the hybrids were analyzed as previously described (7).

RESULTS

Electrophoretic patterns of RNA labeled in isolated mitochondria. The oligo(dT)-cellulose-bound and unbound fractions of the in vitro-synthesized mitochondrial RNA exhibited electrophoretic patterns in CH_3HgOH-agarose slab gels similar to those observed for the in vivo-synthesized RNA (Fig. 2). Almost all the in vivo-synthesized oligo(dT)-cellulose-bound RNA species previously identified (1, 20) (Fig. 2, lane 3) are recognizable in the pattern of the in vitro-synthesized oligo(dT)-cellulose-bound RNA (lane 2). (The three large L-strand-coded RNA species 1, 2, and 3 were visible in some experiments after very long exposure of the autoradiogram.) The major oligo(dT)-cellulose-unbound RNA species produced in vivo, i.e., the large (16S) and small (12S) rRNA and the tRNAs (lane 4), are also present in the in vitro pattern (lane 1). Hence, mtDNA is transcribed and RNA is processed in isolated mitochondria in a way qualitatively similar to that for intact cells. However, there are some quantitative differences between the mtDNA transcription and RNA processing events in the two experimental situations, as discussed below.

Compared with the in vivo pattern, the most important difference is that the rate of rRNA synthesis in isolated organelles is considerably reduced relative to the rate of mRNA synthesis. This decrease is especially pronounced for 16S RNA. From densitometric measurements it was estimated that the labeling of the small rRNA is only 2 to 10 times that of the individual mRNA species (for example, mRNAs 9, 14, 15, and 16), instead of 20 to 40 times, as it is in vivo (4). Furthermore, the observed ratio of labeling of the large to the small rRNA species is, under optimal conditions, 0.1 to 0.5 in different experiments, compared with ~1.6, which is expected for equimolar amounts of the labeled species. The RNA species designated as 12S* in the pattern of in vitro-synthesized RNA, which comigrates with mRNA 12 on CH_3HgOH-agarose gels (Fig. 2, lane 1), has been identified as a precursor of 12S rRNA, carrying at its 5′ end the $tRNA^{Phe}$ sequence and the 5′ leader (12), indicating that the 5′-end processing of the rRNAs is somewhat slowed under these conditions.

Polyadenylation of the mRNAs appears to be less efficient in isolated organelles. Under the standard incubation conditions specified above, the proportion of the total in vitro-labeled RNA which was retained on oligo(dT)-cellulose varied considerably in different experiments (between 1 and 10%) for unknown reasons. Although the highest proportion of oligo(dT)-cellulose-bound RNA observed in the present work (~10%; Fig. 2) is within the range of retention values observed for mitochondrial RNA labeled in vivo during a 15-min [5-^{3}H]uridine pulse (10 to 15%; A. Chomyn, unpublished data), this is mainly due to the much lower efficiency of rRNA synthesis in isolated mitochondria, which results in a marked decrease in the amount of oligo(dT)-cellulose-unbound RNA. The mRNAs synthesized in vitro are indeed less extensively polyadenylated than the in vivo-synthesized species. In fact, the large majority of every mRNA labeled in isolated organelles (>80%) did not bind to oligo(dT) cellulose (Fig. 2), whereas the bulk of the individual in vivo-synthesized mRNAs bound. As a result, oligo(dT)-cellulose chromatography of in vitro-labeled RNA (Fig. 2, lanes 1 and 2) did not allow the near-complete separation of rRNA and mRNA species that is obtained with in vivo-labeled material

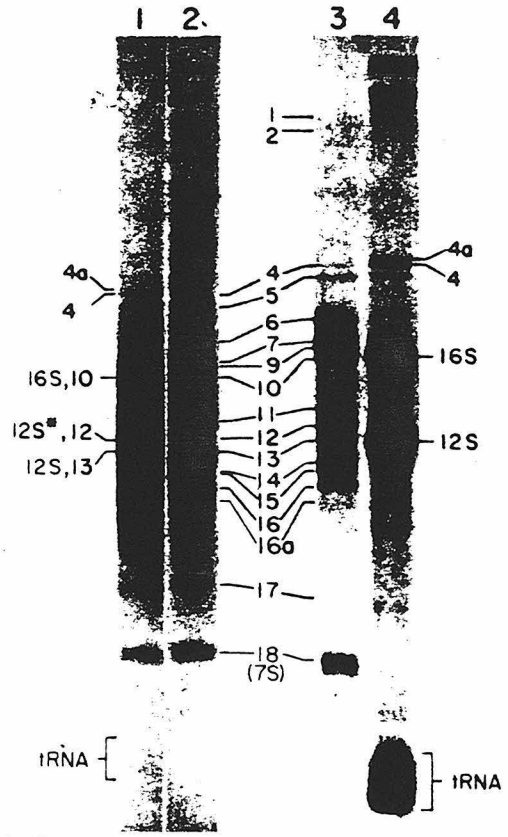


FIG. 2. Comparison of HeLa cell mtDNA transcription in isolated organelles (lanes 1 and 2) and in vivo (lanes 3 and 4). Shown are electrophoretic patterns in agarose-CH_3HgOH gels of total RNA (lane 1) and oligo(dT)-cellulose-bound RNA (lane 2) from mitochondria labeled in vitro with [α-^{32}P]UTP for 30 min at 37°C and of oligo(dT)-cellulose-bound (lane 3) and unbound mitochondrial RNA (lane 4) labeled in vivo with $^{32}P_i$ for 4 h in the presence of 0.1 μg of actinomycin D per ml. The sample run in lane 2 was derived from five times the amount of mitochondria as the sample in lane 1; the samples run in lanes 3 and 4 were derived from equivalent amounts of cells.

(Fig. 2, lanes 3 and 4) (1, 20). In the present work, a rough estimate of the contribution of mRNAs 12 and 13, respectively, to the overall labeling of the bands formed by (12S* plus 12) RNA and (12S plus 13) RNA was obtained from the labeling of other mRNAs; in fact, almost all mRNAs are known to have very similar steady-state amounts and kinetic properties (13) and have been found to be labeled to a similar extent in vitro under a variety of experimental conditions (12; present work). Furthermore, because of the usually large excess of 16S RNA over the amount of its polyadenylated equivalent, RNA 10 (20, 26) (Fig. 2, lanes 1 and 2), the intensity of the band (16S plus 10) has been interpreted to primarily reflect, under standard conditions, the behavior of the mature oligoadenylated large rRNA species (11). The observation that the rRNA species 16S, 12S*, and 12S exhibit a coordinate quantitative behavior under different conditions (see below) supports the validity of the criteria followed here.

The three intensely labeled, slowly moving bands, which are DNase sensitive (data not shown), observed in the pattern of the in vivo-synthesized oligo(dT)-cellulose-

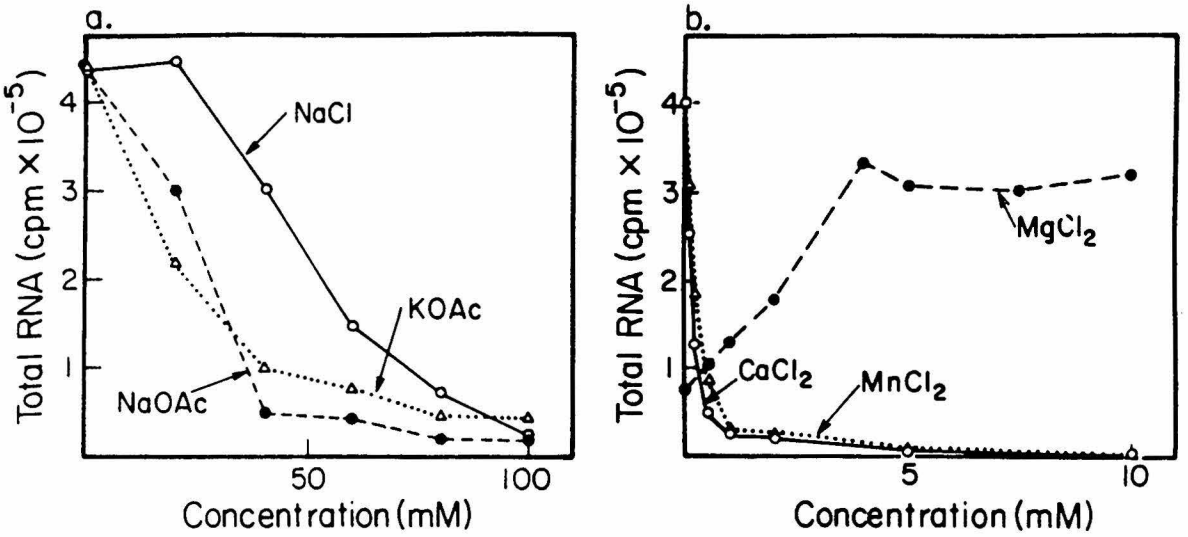


FIG. 3. Effects of salts of monovalent and divalent metals on overall mitochondrial RNA labeling in isolated organelles. The data plotted represent the radioactivity incorporated into total RNA after incubation of mitochondria from 0.5 g of HeLa cells in the presence of 8 μCi of [α-^{32}P]UTP. The standard incubation conditions described in the text were used, except that in the experiments shown in (a) various concentrations of NaCl, NaOAc, or KOAc, as the salt of monovalent metal were used, and in (b) various concentrations of $MgCl_2$, $CaCl_2$, or $MnCl_2$, as the salt of divalent metal, were used.

unbound mitochondrial nucleic acids (Fig. 2, lane 4) but not in that of the in vitro products (lane 1), represent different forms of mtDNA. In the experiment shown in Fig. 2, the in vivo labeling was done in the presence of a low concentration of actinomycin D to prevent the labeling of high-molecular-weight cytoplasmic RNAs. The in vitro-labeled RNA shows no trace of cytoplasmic rRNA, indicating the absence of any contamination of the mitochondrial fraction used here by active nucleolar transcription complexes. In the typical in vitro patterns, particularly in the patterns obtained under nonstandard conditions (see below), one can see some minor unidentified bands, which may represent prematurely terminated or incompletely processed H-strand transcripts or L-strand transcripts; their nature is presently being investigated.

Effects of incubation conditions on RNA labeling in isolated mitochondria. The patterns shown in Fig. 2 were obtained with RNA synthesized in vitro under optimal conditions for mitochondrial RNA labeling, as specified above. Figure 3a shows the effects of various concentrations of Na and K salts on the level of [^{32}P]UMP incorporation into RNA in vitro. NaCl and KCl affect the incorporation of label into RNA in similar ways. Thus, addition of either salt to 20 mM had either no effect or a slight stimulatory effect on the incorporation of the precursor, whereas higher concentrations progressively reduced the labeling, causing almost complete inhibition at 100 mM. NaOAc and KOAc had a more marked inhibitory effect on RNA labeling than NaCl and KCl at comparable concentrations, with a 30 to 50% reduction at 20 mM and an 80 to 90% reduction at 40 mM (Fig. 3a). Thus, the anions contributed to the inhibitory effects in the experiments described above. Both the chloride and the acetate salts of Na and K affect similarly the labeling of the various RNA species (see Fig. 4). All further experiments were carried out at 20 mM NaCl.

The effects of various concentrations of $CaCl_2$, $MnCl_2$, and $MgCl_2$ on in vitro labeling of mitochondrial RNA are shown in Fig. 3b. Increasing concentrations of $CaCl_2$ and $MnCl_2$ had progressively inhibitory effects on [^{32}P]UMP incorporation, with both salts suppressing the RNA labeling by more than 90% at 1 mM and by more than 98% at 10 mM. In contrast to $CaCl_2$ and $MnCl_2$, increasing concentrations of $MgCl_2$ stimulated progressively the in vitro incorporation of [^{32}P]UMP; this reached a maximum (corresponding to a 400% increase) at 4 mM $MgCl_2$ and remained constant at this level up to 10 mM. In contrast to Na and K salts, $CaCl_2$ and $MnCl_2$ had differential effects on the labeling of the various RNA species (Fig. 4). In particular, the labeling of the rRNA species (16S, 12S*, and 12S) was more affected than that of the mRNA species by the addition of increasing concentrations of $MnCl_2$ and $CaCl_2$. Furthermore, there was a progressive increase in the relative amount of the rRNA precursor u4a and of 12S* rRNA (12, 20) (Fig. 1), due to inhibition of the 5′-end processing step which removes the $tRNA^{Phe}$ and the 5′ leader.

Figure 5b shows the effect of varying the pH of the incubation buffer on in vitro labeling of mitochondrial RNA. A maximum [^{32}P]UMP incorporation into total RNA was observed at about pH 7.5 (at 37°C); a very similar pH dependence curve was found for the labeling of the oligo(dT)-cellulose-bound RNA fraction. Figure 5a shows the electrophoretic patterns of total mitochondrial RNA labeled in vitro at different pHs. There were considerable changes in the RNA patterns with pH. For example, above pH 7.5 (Fig. 5a, lanes 3 and 4), there was a decrease in the relative labeling of the higher-molecular-weight RNA species (species 4, 4a, 5, 6, 7, and 9), an increase in the background of heterogeneous RNA, and the appearance of new bands slower moving than RNA 17. These changes are possibly related and may reflect a tendency to degradation or premature termination of the transcripts. The most striking finding, however, was the dramatic increase with pH (between 6.9 and 7.9) in the relative labeling of RNA 18 (7S RNA) (25) (Fig. 5a and c). Furthermore, there was a progressive decrease with pH in the labeling of RNA 12S* relative to that of 12S RNA (Fig. 5a), suggestive of more effective 5′-end processing of the 12S rRNA precursor. Also, there was a progressive decrease in the labeling of 16S RNA and a

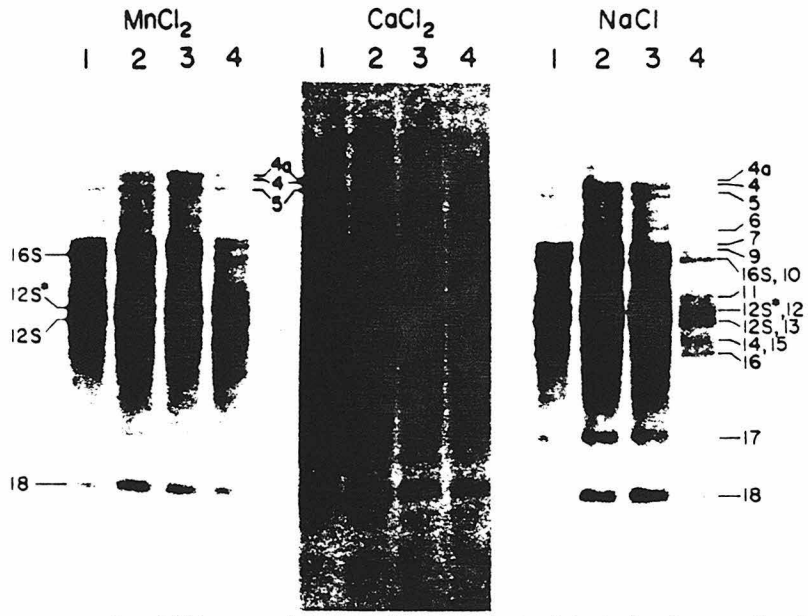


FIG. 4. Electrophoretic patterns of mtDNA transcription products synthesized in isolated organelles in the presence of various concentrations of $MnCl_2$, $CaCl_2$, or NaCl. Conditions were as specified in the legend for Fig. 3. In the experiments shown in the left ($MnCl_2$) and middle ($CaCl_2$) panels, the concentrations of the salt tested were 0, 100, 200, and 500 μM, respectively, in lanes 1 through 4. In the experiment shown in the right panel (NaCl), the salt concentrations were 0, 20, 60, and 100 mM, respectively, in lanes 1 through 4. The relative amounts of RNA electrophoresed were 1, 2, 4, and 8, respectively, in lanes 1 through 4 of the left panel, and 1, 1, 3, and 6, respectively, in lanes 1 through 4 of the middle panel; equal amounts of material were run in the four lanes of the right panel.

corresponding increase in the labeling of a component (appearing as a fairly broad band) which migrates slightly faster than mRNA 16. This component corresponds in migration to a previously described polyadenylated species (RNA 16a; reference 1) (see also Fig. 2), which represents prematurely terminated 16S RNA (12) (see below). Hybridization experiments with RNA labeled at different pHs and an excess of H strands or L strands showed that, in contrast to the behavior of 7S RNA, the proportion of labeled L-strand transcripts in the total RNA remained relatively constant over the pH range of 7.1 to 7.9 (at 37°C) (Fig. 5c).

Figure 6a shows the effects on RNA labeling of varying the CTP, GTP, UTP, and ATP concentrations over the range of 0 to 5 mM. Clearly different effects were produced by the four nucleoside triphosphates (NTPs). Increasing concentrations of both CTP and GTP progressively reduced to similar extents the incorporation of label into RNA. A more marked depression of [^{32}P]UMP incorporation was produced by the addition of increasing amounts of UTP above 0.01 mM, whereas the reduction of labeling by this nucleotide was negligible at lower concentrations (see Fig. 6a insert). To discriminate within the UTP effect between inhibition of incorporation of the labeled precursor and the consequence of dilution of the specific activity of [α-^{32}P]UTP, a parallel experiment was carried out by using [α-^{32}P]CTP as a labeled precursor and various amounts of unlabeled UTP. The inhibition curve thus obtained was very similar to that observed for the inhibition of [^{32}P]UMP incorporation by GTP (data not shown). Therefore, the three NTPs GTP, CTP, and UTP appeared to have very similar inhibitory effects on RNA labeling. In the case of UTP, the major portion of the reduction of [^{32}P]UMP incorporation produced by unlabeled UTP was due to the decrease in specific activity of the labeled precursor (Fig. 6a). However, this reduction was much less than expected from the dilution factor corresponding to each UTP concentration. This suggested that the intramitochondrial UTP pool was large compared with the amount taken in and, furthermore, that there was an increased uptake of exogenous UTP with increasing UTP concentrations, due to the more favorable concentration differential (see below). Gel analysis of the RNA products showed no differential effects of the addition of CTP, GTP, and UTP on the labeling of the various RNA species (data not shown).

In contrast to the effect of the NTPs mentioned above, addition of increasing concentrations of ATP stimulated RNA labeling in isolated mitochondria, with a maximum at 1 to 2 mM (Fig. 6a); at higher concentrations, the stimulatory effect decreased and disappeared at 5 mM. A control experiment with [α-^{32}P]ATP showed that at least 50% of the 1 mM ATP originally present in the incubation medium could still be recovered from the medium as ATP after 30 min of incubation of the organelles at 37°C. A differential effect of ATP addition on [^{32}P]UMP incorporation into total RNA and into oligo(dT)-cellulose-bound RNA was observed. The relative labeling of the oligo(dT)-cellulose-bound RNA increased at high ATP concentrations (>1 mM) (Fig. 7b). Gel analysis of the oligo(dT)-cellulose-bound RNA species labeled in the presence of different ATP concentrations (Fig. 7a) revealed a parallel behavior of the labeling of the various RNA species; however, at the highest concentration (5 mM), there was a dramatic drop in the labeling of 7S RNA (RNA 18) (Fig. 7a and c) and an increase in the relative labeling of 16S RNA, with the concomitant disappearance of the putative 16a RNA band. Hybridization experiments with RNA labeled in the presence of different ATP concentrations and an excess of H strands or L strands showed that the amount of labeled L-strand transcripts varied roughly in proportion to the total

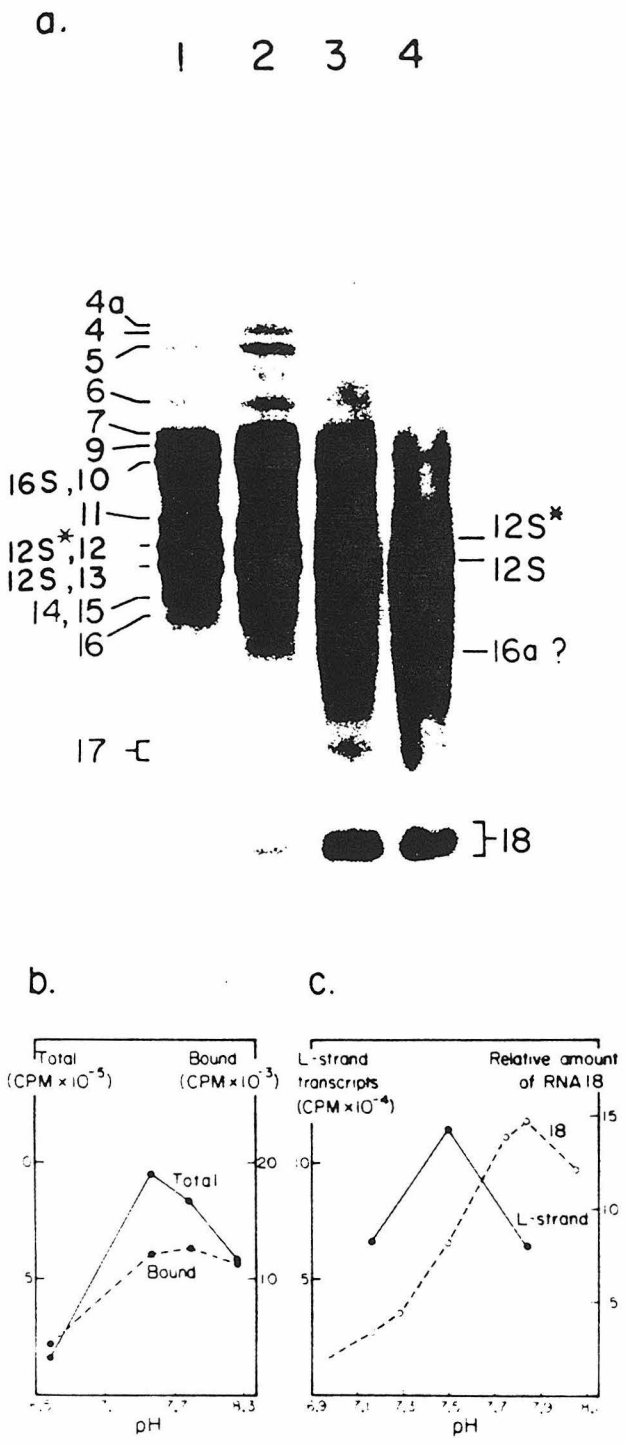


FIG. 5. Effects of pH on mtDNA transcription in isolated organelles. (a) Autoradiogram after electrophoresis through an agarose-CH_3HgOH gel of mtDNA transcription products labeled in vitro in transcription assays carried out at pH 6.95, 7.3, 7.75, and 8.05, respectively, in lanes 1 through 4. Equal amounts of RNA were run in all four lanes. (b) Radioactivity incorporated into total RNA or oligo(dT)-cellulose-bound RNA at different pHs. (c) Effect of pH on the in vitro labeling of L-strand transcripts, as determined by solution hybridization with separated mtDNA strands, and of RNA species 18 (7S RNA), as determined by densitometric measurements on the autoradiograms (arbitrary units). The L-strand transcripts were analyzed in a different experiment from that shown in (b). The pHs indicated in the graphs were measured at 37°C.

labeled RNA (Fig. 7c). Particularly significant is the observation that, with the ATP concentration in the medium increasing from 1 to 5 mM, the labeling of L-strand transcripts decreased by a factor of ~2, whereas the labeling of 7S RNA decreased by a factor of ~5.

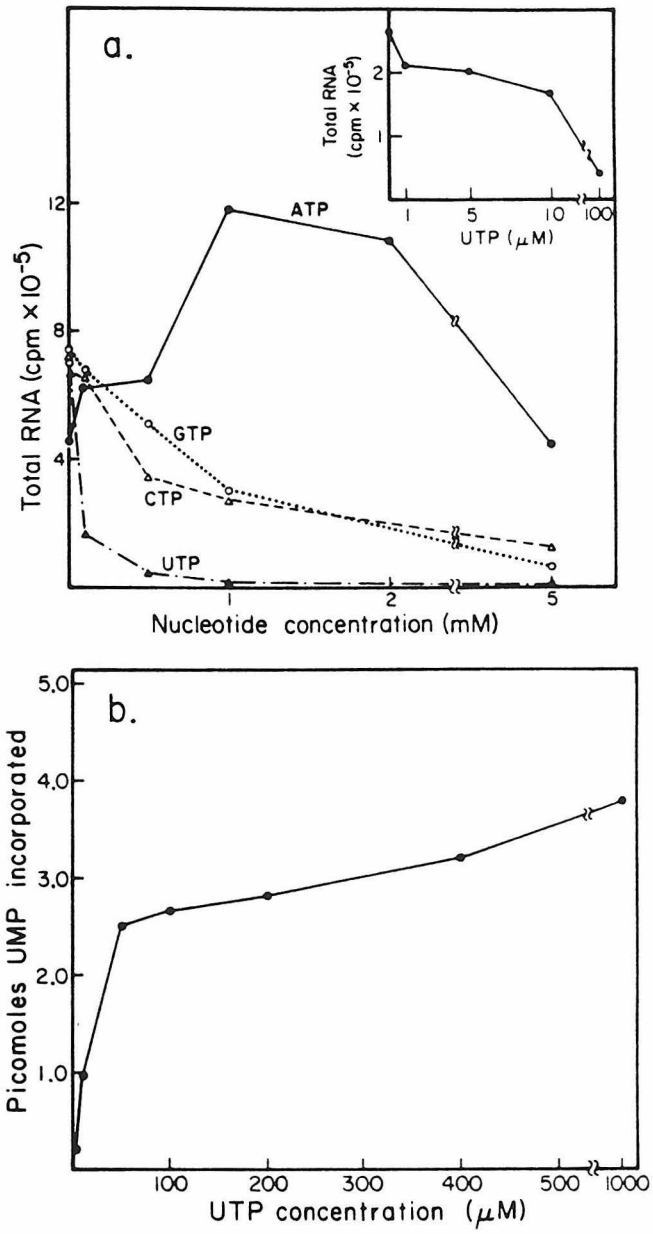


FIG. 6. Effects of NTP concentration on mtDNA transcription in isolated organelles. (a) Experiments testing the effects of various amounts of unlabeled UTP, CTP, or GTP in the presence of 1 mM ATP or the effects of different ATP concentrations in the absence of the other NTPs on RNA labeling with [α-^{32}P]UTP (20 μCi/ml); the insert shows in expanded form the effects of low concentrations of UTP on RNA labeling in an experiment similar to that illustrated in the main portion of the panel. (b) In vitro incorporation of UMP into mitochondrial RNA during 30 min of incubation of mitochondria in the presence of different UTP concentrations in the medium. The data have been calculated from the radioactivity incorporated into total RNA on the basis of the precursor specific activity at each UTP concentration and normalized to 10^6 cell equivalents ([α-^{32}P]UTP was present in all assays at 17.8 μCi/ml).

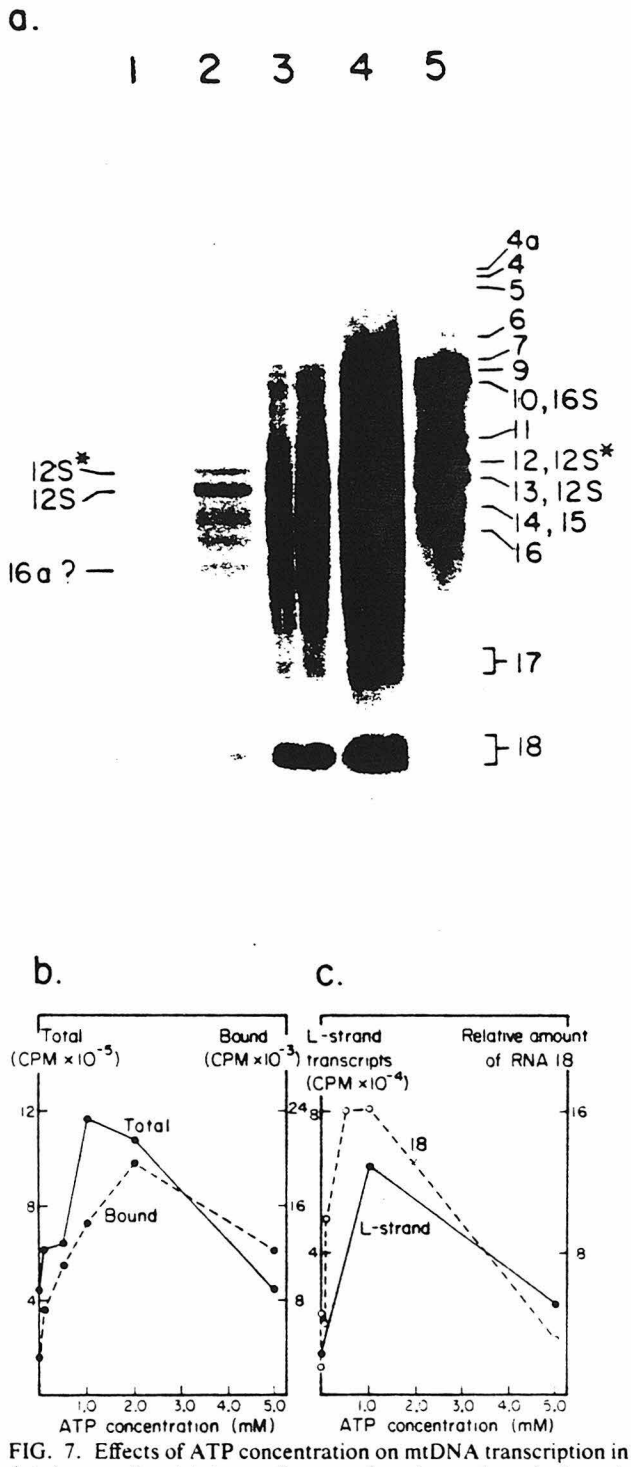


FIG. 7. Effects of ATP concentration on mtDNA transcription in isolated organelles. (a) Autoradiogram after electrophoresis through an agarose-CH_3HgOH gel of transcription products labeled in the presence of 0, 0.01, 0.1, 1, and 5 mM ATP, respectively, in lanes 1 through 5. Equal amounts of material were run in the five lanes. (b) Radioactivity incorporated into total RNA or oligo(dT)-cellulose-bound RNA in the presence of different ATP concentrations. (c) Effects of various ATP concentrations on the in vitro labeling of L-strand transcripts and of RNA species 18 (7S), determined as explained in the legend for Fig. 5. The L-strand transcripts were analyzed in a different experiment from that shown in (b).

The effect of varying the mitochondrial concentration on in vitro labeling of mitochondrial RNA is shown in Fig. 8a. The data are expressed in terms of counts per minute per milligram of protein; a relative mitochondrial concentration of 1 (i.e., the mitochondria from 1 g of packed cells resuspended in 1 ml of medium) equals about 1.3 mg of mitochondrial protein per ml. The incorporation of label into RNA per mitochondrion decreased slightly (~30%) as the relative mitochondrial concentration increased from 0.1 to 1; further increases in mitochondrial concentration caused an almost proportional decrease in RNA labeling per mitochondrion. This decrease in labeling could not be due to an increase in overall protein concentration, since a BSA concentration more than sevenfold greater than the unit mitochondrial protein concentration used had no inhibitory effect (Fig. 8b); on the contrary, addition of BSA to 1 mg/ml increased labeling approximately twofold, suggesting a possible stabilization of mitochondria by nonspecific protein.

At 37°C, the incorporation of [^{32}P]UMP into RNA was linear for ca. 30 min and then plateaued (Fig. 9a). In another experiment (data not shown), RNA labeling reached a plateau at ca. 45 min. In an experiment in which mitochondria were exposed to [α-^{32}P]UTP during a 30-min interval starting immediately after centrifugal separation or after a 15- or 30-min preincubation in the absence of labeled precursor, the relative amounts of radioactivity incorporated into the three RNA samples were 9, 3.5, and 1, respectively. At 21°C, RNA labeling in isolated organelles proceeded at a much lower rate but remained approximately linear for at least 2 h. An analysis of the labeling of gel fractionated mitochondrial RNA species after different times of incubation of mitochondria at 37°C (10, 20, and 30 min) showed a parallel increase in the radioactivity incorporated into the various species (data not shown). Likewise, the labeling patterns of the RNA from mitochondria exposed to [α-^{32}P]UTP after different times of preincubation in the absence of labeled precursor, as mentioned above, were very similar (data not shown). Particularly significant was the constancy in the relative labeling of the rRNAs, mRNAs, and 7S RNA, which are the products of three distinct transcription units (19, 20, 25); also remarkable was the similarity in the relative labeling, at different times, of mRNAs 5, 7, 9, 11, and 14 through 16, which are encoded

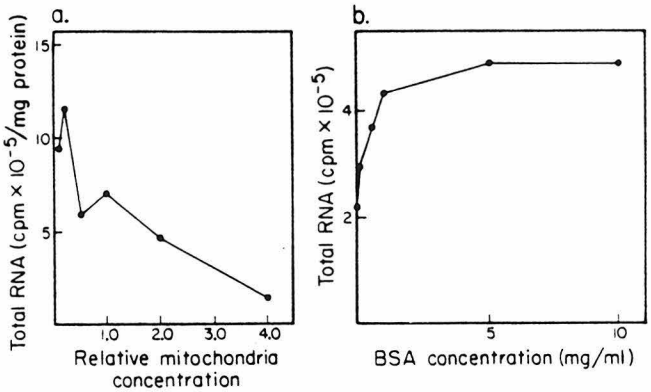


FIG. 8. Effects of mitochondrial or BSA concentration on mtDNA transcription in isolated organelles. (a) Radioactivity incorporated into total RNA in in vitro assays utilizing various concentrations of mitochondria. The radioactivity data have been normalized to 1 mg of mitochondrial protein. (b) Radioactivity incorporated into total RNA in the presence of different BSA concentrations in the incubation medium.

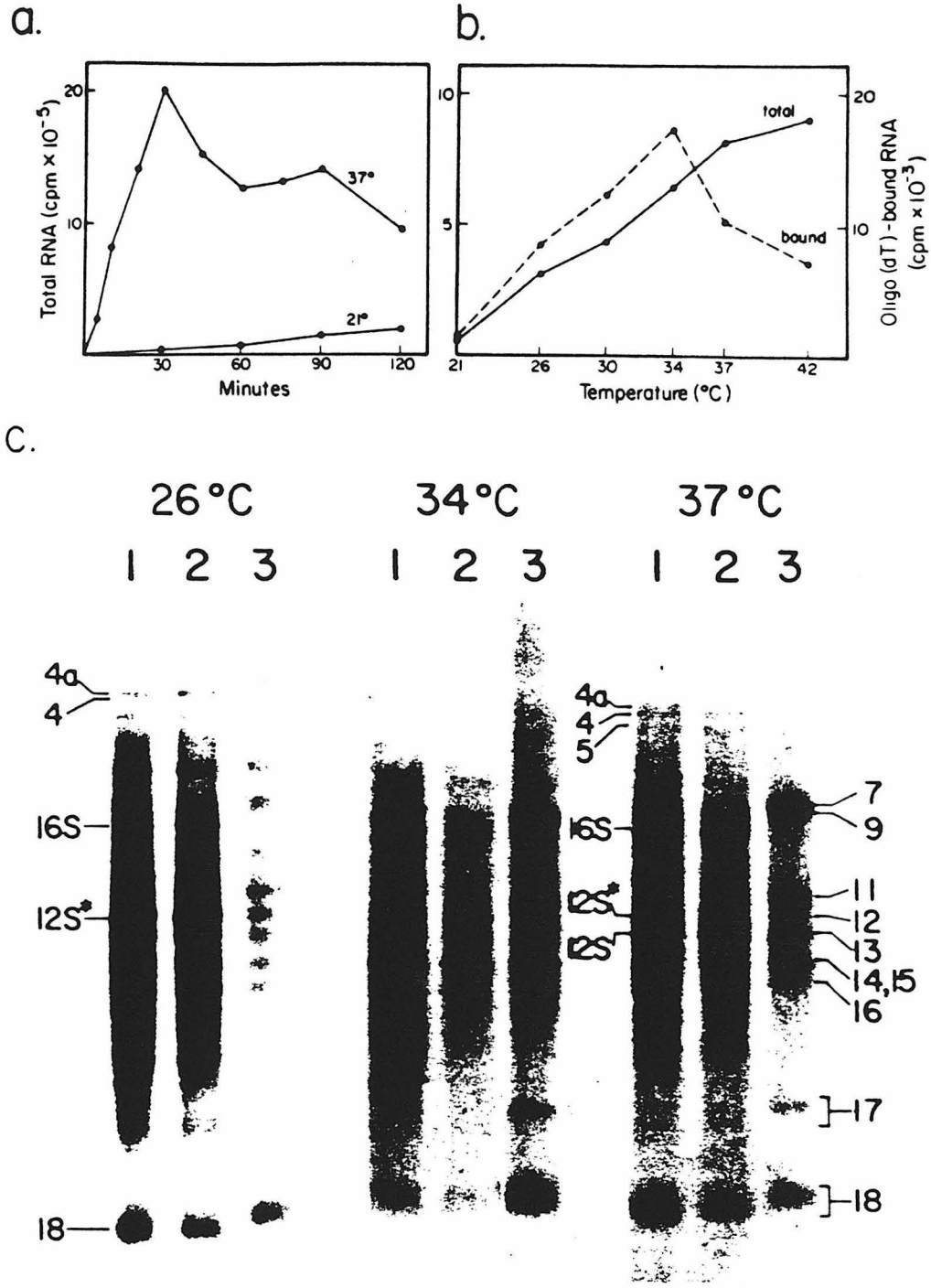


FIG. 9. Effects of incubation temperature on mtDNA transcription in isolated organelles. (a) Radioactivity incorporated into total RNA after different times of incubation at 37 or 21°C. (b) Radioactivity incorporated into total RNA or oligo(dT)-cellulose-bound RNA after 30 min of incubation at different temperatures. (c) Autoradiogram after electrophoresis through an agarose-CH_3HgOH gel of total RNA (lane 1), oligo(dT)-cellulose-unbound RNA (lane 2), and oligo(dT)-cellulose-bound RNA (lane 3) labeled at 26, 34, or 37°C. Relative amounts of material electrophoresed in lanes 1 through 3 were 1, 1, and 10, respectively, in the left panel and 1, 1, and 40, respectively, in the middle and right panels.

in different regions of the H strand. Fractionation on oligo(dT)-cellulose of mitochondrial RNA labeled in vitro at different temperatures revealed that the proportion of labeled RNA which was retained on oligo(dT)-cellulose increased below 37°C, with a maximum at 34°C (Fig. 9b and c). As previously observed (12), incubation at temperatures below 37°C resulted in an increased proportion of label in 12S* RNA relative to 12S RNA and in RNA u4a relative to RNA u4 (Fig. 9c), a phenomenon which reflects the cold sensitivity of the step involved in the removal of the $tRNA^{Phe}$ and 5′

leader from the rRNA precursors (Fig. 1). The significance of the pronounced labeling of band 7 in the oligo(dT)-cellulose-bound RNA labeled at 34 or 37°C (Fig. 9c) is not clear. Experiments are in progress to ascertain whether this observation reflects a more efficient polyadenylation of mRNA 7 or the comigration with the latter RNA of another as yet unidentified RNA species.

In vitro mtDNA transcription rate. As discussed above, dilution of [α-^{32}P]UTP with unlabeled UTP does not cause a proportional decrease in the incorporation of label and thus results in an increase in the measured amount of total UMP incorporated. The curve measuring the apparent incorporation of total UMP into RNA as a function of external UTP concentration shows near-saturation at ca. 1 mM UTP. One can derive a minimum estimate of the rate of RNA synthesis in vitro from the highest level of the total UMP incorporation curve (~4 pmol/30 min per 10^6 cell equivalents), assuming a negligible dilution by the intramitochondrial UTP pool of the exogenous UTP imported into the organelles. Considering that the H- and L-strand transcripts are labeled in vitro to a similar extent (the observed ratio of label in H- and L-strand transcripts was ~1.3) and that the average transcript therefore has close to 27% U content (average of the T content of the H and L strands [2]), one can calculate a minimum rate of incorporation into RNA of ~0.5 pmol of NTS per min/10^6 cell equivalents.

In vitro initiation of transcription. The results described above did not provide any information as to whether initiation of transcription occurs in the present in vitro system and, if so, to what extent it accounts for the observed labeling patterns. To answer these questions, experiments utilizing the four [γ-^{32}P]NTPs as labeled precursors were done. These failed to show any specific labeling of discrete RNA species. A very low level of labeling of the normal set of mitochondrial RNA species was observed with all four [γ-^{32}P]NTPs, as well as with $^{32}P_i$, suggesting the existence of intra- or extra-mitochondrial kinase activity. As an alternative approach to investigate the occurrence of initiation of transcription of the rRNA genes in isolated mitochondria, the labeling of the 5'-end-proximal segments of 12S* and 12S RNA encoded in the *Mbo*I-9 fragment of human mtDNA (188 and 92 NTs long, respectively) was compared with that of their 3'-end-proximal segments encoded in the *Mbo*I-4 fragment (374 NTs long) (Fig. 1). The S1 protection technique was used to isolate these RNA segments. As expected, hybridization with mp8-M9H (an M13 vector containing the H strand of the *Mbo*I-9 fragment) protects from S1 digestion an ~188-NT segment of 12S* RNA (lane 1) and an ~92-NT segment of 12S RNA (Fig. 10a, lane 3) (a 188-NT band in the latter hybridization reflects contamination of 12S RNA by 12S* RNA); mp8-M4H (an M13 vector carrying the H strand of the *Mbo*I-4 fragment) protects an ~374-NT segment of both 12S* and 12S RNA (lanes 2 and 4).

To quantitate the labeling of the protected RNA segments, an experiment similar to that shown in Fig. 10a was carried out in which 12S* and 12S RNA, labeled with [α-^{32}P]UTP for 30 min at 37°C and purified by two successive CH_3HgOH-agarose gel runs, were each hybridized with a large excess of an equimolar mixture of mp8-M9H and mp8-M4H; after S1 digestion, the protected fragments were isolated on a polyacrylamide-urea gel, eluted, and counted.

Exclusive in vitro labeling of 12S* and 12S RNA due to de novo initiation would result in the labeling of their 5'-end- and 3'-end-proximal segments in proportion to their U content (46 and 28U, respectively, in the 12S* and 12S RNA segments protected by *Mbo*I-9H [12S*/*Mbo*I-9H and 12S/*Mbo*I-9H] and 81 U in 12S*/*Mbo*I-4H or 12S/*Mbo*I-4H) (2). A different situation would prevail if 100% of the in vitro labeling of 12S* and 12S RNA were due to elongation of chains initiated in vivo. This is illustrated schematically for 12S* RNA in Fig. 10b. Assuming an equal spacing of the RNA polymerase molecules along the transcribed DNA segment, the ratio of radioactivity in 12S*/*Mbo*I-9H and 12S*/*Mbo*I-4H would be 0.062 (see the legend of Fig. 10 for details). The proportion of labeling of 12S* RNA due to de novo initiation or elongation can be calculated by solving the two equations:

$$\text{cpm in } 12^*\text{S}/Mbo\text{I-9H} = 0.062\,x + 0.57\,y$$

$$\text{cpm in } 12\text{S}^*/Mbo\text{I-4H} = x + y$$

where x and y are, respectively, the radioactivity of the 3'-terminal segment due to elongation and initiation, 0.062 is the value described above, and 0.57 is the ratio of 46 to 81 U. The ratio of 0.57 y to 0.062 x then gives the ratio of radioactivity from initiation to that from elongation in the 5'-terminal segment. The experimental value for the latter ratio was ~7.6. Considering that the specific activity of the elongated 5' segments is one-half that of the de novo-initiated segments (Fig. 10b), one can calculate that ~80% of the 12S* RNA molecules completed in vitro are due to de novo initiation. A similar calculation for 12S RNA shows that ~30% of the 12S RNA molecules completed in vitro are initiated de novo. In this experiment, the 12S RNA molecules represented ~50% of the total 12S RNA gene transcripts and were derived presumably from processing of 12S* RNA molecules. Under the reasonable assumption that these 12S RNA molecules were the "oldest," i.e., including all those already started in vivo, it is not surprising that the proportion of de novo-initiated 12S RNA molecules was significantly lower than that of 12S* RNA molecules. With the caution imposed by the constraints placed in the above calculations, in particular by the assumption of an equal spacing of the RNA polymerase molecules along the transcribed DNA segments, the available evidence indicates that a major portion (>50%) of the transcription of the 12S RNA gene in vitro reflects de novo initiation events.

DISCUSSION

The most important outcome of the present work has been the demonstration of the capacity of isolated HeLa cell mitochondria to support DNA transcription and RNA processing in a way closely reproducing the in vivo events and at rates approaching the in vivo rates. Such a capacity has allowed the analysis of the role of different parameters on these processes without the constraints imposed by the nucleo-cytoplasmic compartment. As described in this paper, the efficiency and fidelity of mitochondrial RNA synthesis in isolated organelles should make this a valuable system for future studies on the control by the nucleus and cytoplasm of mitochondrial gene expression.

Requirements for RNA synthesis in isolated mitochondria. Although the energetic requirements of the in vitro system described here have not been analyzed in detail, it is clear that RNA synthesis in this system is dependent to a considerable extent on an external ATP source. In different experiments, isolated mitochondria were capable of supporting only 10 to 35% of their RNA synthetic capacity in the absence of any added ATP. It seems likely that, in the absence of exogenous ATP, isolated organelles utilize ATP produced endogenously through oxidative phosphorylation. Addition to the incubation medium of a respiratory substrate

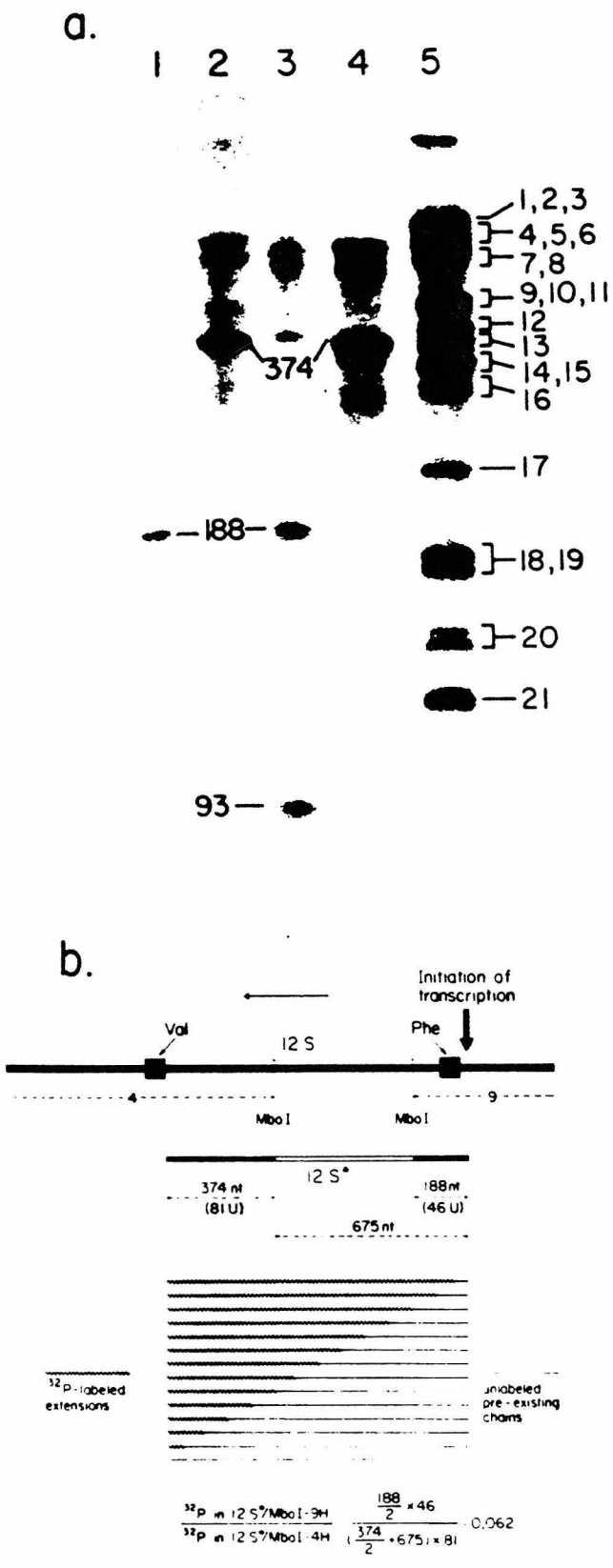


FIG. 10. Initiation of mtDNA transcription in isolated organelles. (a) Isolation by S1 protection of the 5'-end-proximal and 3'-end-proximal segments of 12S* and 12S RNA. 12S* and 12S RNA, in vitro labeled with [α-^{32}P]UTP, were isolated by two consecutive electrophoretic runs and each hybridized with an excess of *Mbo*I-9H or *Mbo*I-4H (Fig. 1); after S1 digestion, the protected segments were separated by electrophoresis through an 8% polyacrylamide–8 M urea gel. Lanes 1 and 2, 12S* RNA segments protected by *Mbo*I-9H and *Mbo*I-4H, respectively; lanes 3 and 4, 12S RNA segments protected by *Mbo*I-9H and *Mbo*I-4H, respectively; lane 5, 3'-end-labeled mtDNA *Hpa*II digest marker. (b) Scheme illustrating the rationale for estimating the relative levels of labeling of the 5'-end-proximal and 3'-end-proximal segments of 12S* RNA under the assumptions of no in vitro initiation and uniform spacing of RNA polymerase along the transcribed mtDNA segment. In particular, under these assumptions the radioactivity in the 5'-end-proximal segment of 12S* RNA would be a function of the average length of the labeled NTP stretches corresponding to this segment multiplied by the number of U's that it contains (ignoring any inhomogeneity in U distribution); similarly, the radioactivity in the 3'-end-proximal segment would be a function of the average length of the labeled stretches corresponding to this segment plus the length of the portion of 12S* upstream of it (the latter component is accounted for by the RNA polymerase molecules sitting on this portion of 12S* at the time of addition of label) multiplied by the number of U's in the 3'-end-proximal segment.

(pyruvate, citrate, α-ketoglutarate, or succinate), alone or in combination with ADP and P_i, in place of ATP, has produced variable stimulation of RNA labeling, in some cases up to the level produced by exogenous ATP (unpublished data).

The optimum incubation conditions found for the various RNA synthesis and processing steps occurring in isolated mitochondria probably reflect in good part the ionic, pH, and NTP requirements of the corresponding individual enzymatic reactions. In fact, the membrane potential generated by ATP hydrolysis or respiration was probably adequate to support import of cations or NTs into the organelles in proportion to their external concentration (16, 17). It is interesting that the optimum values for the concentrations of NaCl, KCl, Mg^{2+}, and ATP as well as for pH found in the present work for the overall RNA synthetic capacity of isolated organelles are reasonably close to those determined for mtDNA transcription in an in vitro soluble system derived from HeLa cell mitochondria (D. Shuey and G. Attardi, manuscript in preparation). By contrast, the divalent cations Ca^{2+} and Mn^{2+} had at comparable concentrations a much more drastic inhibitory effect on RNA synthesis in isolated organelles than in the soluble system. This may reflect the high uptake capacity for Ca^{2+} and Mn^{2+} which mitochondria possess due to the existence of a specific translocation system (29), and therefore the higher effective concentrations of these ions reached inside the organelles.

The relatively high ATP optimum concentration found for RNA synthesis in isolated organelles (1 to 2 mM) does not appear to be due to its rapid degradation in the in vitro system, since about 50% of the exogenous ATP could be recovered as such from the medium even after 30 min of incubation at 37°C. Based on the reported distribution of ATP in different subcellular fractions of HeLa cells (5) and on the fraction of the cell volume occupied by mitochondria in these cells (~10.5%) (28), the concentration of ATP in HeLa cell mitochondria can be estimated to be ca. ~0.8 mM, i.e., close to the optimum ATP concentration observed here. It is interesting that a relatively high ATP optimum concentration for mitochondrial RNA synthesis (0.5 to 1 mM), with a sharp decrease in synthesis below 0.1 mM, has also been found in the soluble system mentioned above from HeLa cell mitochondria (Shuey and Attardi, in preparation). It is not possible at present to say whether the high ATP optimum

concentration observed for RNA synthesis in isolated organelles reflects the requirements of the enzymatic machinery involved or a regulatory role of ATP.

The inhibitory effects of CTP, GTP, and UTP on mitochondrial RNA labeling are rather intriguing. It seems possible that they reflect competition by the three NTPs for a common uptake system distinct from the ADP-ATP carrier. Alternatively, the three NTPs may act at the level of the transcriptional machinery. The increase in UTP uptake into HeLa cell mitochondria with increasing concentrations of the NTP in the medium has been found in the present work to offset in part the effect of dilution of the label. The near-saturation exhibited by the curve measuring the apparent incorporation of total UMP into RNA may indicate that at ca. 1 mM exogenous UTP, the uptake capacity of mitochondria for this NTP is close to its maximum or that the amount of imported NTP has become larger than the size of the mitochondrial UTP pool, or both. It is interesting that on the basis of the intracellular distribution of UTP (5) and the mitochondrial volume (28), the mitochondrial UTP concentration can be estimated to be ~215 μM, which falls within the near-horizontal portion of the UMP incorporation curve. This estimate makes the second alternative mentioned above more plausible. An increase in the measured levels of UMP incorporation into RNA with increasing concentration of exogenous UTP has been previously reported for isolated HeLa cell mitochondria (Novitski, Ph.D. thesis) and also observed for isolated yeast organelles (6).

The rate of in vitro mitochondrial RNA synthesis approaches the in vivo rate. The rate of RNA synthesis in isolated HeLa cell mitochondria observed in the present work is unusually high for an in vitro system. A rate of incorporation of ~0.5 pmol of NTPs per min/10^6 cell equivalents was estimated from the UMP incorporation in the presence of 1.0 mM UTP and from the postulated U content of the average mtDNA transcript. If the observed inhibitory effect of high UTP concentrations on RNA labeling were due to action on the RNA polymerase(s), a correction for this inhibition would raise the actual rate of nucleotide incorporation to ~0.8 pmol of NTPs per min/10^6 cell equivalents. These rates have to be compared with that of 5 to 7 pmol of NTPs per min/10^6 cell equivalents estimated for the in vivo synthesis of HeLa cell mitochondrial RNA (4). Considering that only 50 to 75% of the total mitochondrial transcriptional activity was recovered in the centrifugal fraction analyzed in the present work and that no correction was made for possible pool effects, one can estimate that the in vitro synthetic capacity of the present in vitro system is at least 10 to 15% of the in vivo capacity. An appreciable portion of the decrease in mitochondrial RNA synthetic capacity in isolated organelles appears to be due to depression of rRNA synthesis, which accounts for only 10 to 20% of the total H-strand transcription (as estimated from densitometric measurements), instead of 85 to 90%, as is the case in vivo (4). The observed absolute rate of UMP incorporation into RNA of isolated HeLa cell mitochondria is comparable to the rates reported for isolated yeast mitochondria (6, 14, 22).

Under optimal conditions, in vitro labeling of mitochondrial RNA continues at a constant rate for about 30 min at 37°C and then plateaus. The overall kinetics of incorporation of [^{32}P]UMP into RNA probably reflect the changes with time in the rate of RNA synthesis, the rate of RNA decay, and the intramitochondrial UTP pool specific activity. In an experiment in which, after either a 15- or a 30-min [α-^{32}P]UTP pulse at 37°C, the cells were further incubated in the presence of a dose of actinomycin D (8.1 μM) sufficient to block RNA synthesis almost completely (12), no obvious decline of radioactivity in the rRNA and mRNA species was observed over a period of 105 min (unpublished data). Under these conditions, therefore, the half-life of the mitochondrial RNA species labeled in vitro appeared to be considerably longer than that previously measured in HeLa cells after a cordycepin block of RNA synthesis (13); thus, RNA decay probably did not play a significant role in determining the shape of the [^{32}P]UMP incorporation curve. On the other hand, as mentioned earlier, the in vitro labeling of RNA during a 30-min exposure of mitochondria to [α-^{32}P]UTP after a 30-min preincubation in nonradioactive medium was only 10% of that found without preincubation. It seems likely that in the linear portion of the RNA labeling curve, a progressive decrease in RNA synthetic activity is compensated for by the concomitant increase in precursor pool specific activity.

No appreciable change in the pattern of labeling of the various RNA species was observed after up to 60 min of incubation of isolated mitochondria at 37°C. This observation indicates that the different transcription and RNA processing events occur in vitro at the same relative rates throughout that period and furthermore that abnormal degradation processes do not play a significant role in generating the patterns observed after up to 60 min of incubation at 37°C.

In vitro initiation of mitochondrial RNA synthesis. A substantial fraction of the RNA synthesized in isolated mitochondria is represented by chains initiated in vitro. Direct determinations of the in vitro labeling of the 5'- and 3'-end portions of the 12S rRNA gene transcripts (12S* and 12S RNA) indicate that >50% of these transcripts were generated de novo in the isolated organelles. Although similar determinations were not made for the polycistronic transcripts that start at the downstream H-strand transcription initiation site and produce the mRNAs and most of the tRNAs encoded in the H strand (20), their high rate of labeling, lasting for about 30 min at 37°C, indicates that a large fraction of them must have been initiated in vitro. Consistent with this conclusion is the observation that the mRNAs encoded in the 5'-end-proximal half (mRNA 9) and the 5'-end-distal half (such as mRNAs 16, 7, and 11) of the total H-strand transcription unit are labeled to a similar extent. Furthermore, their relative labeling does not change even after 30 min of preincubation in unlabeled medium. Similar observations on the relative labeling of the L-strand-coded 7S RNA after different times of in vitro incubation strongly suggest that a large fraction of these molecules have been initiated in vitro.

Differential behavior in vitro of rRNA and mRNA synthesis. A distinguishing feature of the in vitro system described in this work, as compared with the in vivo situation, is the considerably lower efficiency and greater lability of rRNA synthesis relative to mRNA synthesis. In view of the evidence discussed in the previous section, it seems likely that the reduced efficiency of rRNA synthesis reflects at least in part a lower rate of initiation of transcription at the rRNA specific promoter under the in vitro conditions. However, the relatively low level of in vitro labeling of 16S RNA also points to defects in chain elongation or termination, or both. The ratio of labeling of 16S RNA and (12S plus 12S*) RNA (0.1 to 0.5) observed under optimal conditions is substantially lower than the value of ca. 1 most frequently found for the labeling ratio of in vivo synthesized 16S and 12S RNA. (In the latter case, the deviation from the value of ~1.6 expected for an equimolar synthesis of the two rRNA species is due to

the particular tendency to degradation of 16S RNA [3].) The lower ratio of labeling of 16S RNA to (12S plus 12S*) RNA under the in vitro conditions does not appear to be due to instability of the newly formed 16S RNA, since the same ratio was found also after a short period of in vitro labeling (10 min). Rather, it may reflect a high probability of premature termination of the rDNA transcripts within the 16S RNA gene or a failure of these transcripts to recognize the termination signal at the 3′ end of this gene, or both (11).

The defects in rRNA formation appear to be accentuated under suboptimal conditions for in vitro RNA synthesis. Thus, in previous work (12) actinomycin D, proflavine, and ethidium bromide were found to preferentially inhibit the labeling of the two rRNA species, in particular of 16S RNA, over that of the mRNAs in isolated mitochondria. The same phenomenon has been observed in the present work in the presence of Mn^{2+} or Ca^{2+}. In the work cited above, the preferential inhibition of 16S RNA labeling in the presence of actinomycin D was accompanied by a progressive increase in the relative labeling of a component with the same electrophoretic mobility as that of the RNA species 16a previously observed in in vivo-labeled mitochondrial RNA from actinomycin D-treated cells (Fig. 2) (1). The latter RNA has been shown to correspond to a 16S RNA molecule prematurely terminated at about one-third the distance from the 5′ end of the 16S rRNA gene (12; J. Montoya, G. Gaines, and G. Attardi, unpublished data). An accumulation of a similarly migrating component, concomitant with a decrease in 16S RNA labeling, has also been observed in the present work in mitochondria incubated at high pHs. Experiments are in progress to determine whether the component with the electrophoretic mobility of RNA 16a observed in vitro does indeed represent prematurely terminated 16S RNA.

The relative abundance in the in vitro-synthesized RNA of the RNA species 12S* and of the rRNA precursor u4a, both of which still carry at their 5′ end the $tRNA^{Phe}$ and leader sequences (9, 12), indicates a slowing down of the processing step leading to the removal of these sequences. This slowing down in rRNA processing had been previously observed in isolated mitochondria and found to be accentuated at low temperatures or in the presence of proflavine or ethidium bromide (12). In the present work, an inhibition of the 5′-end processing step was produced by high concentrations of Ca^{2+} or Mn^{2+}. The 12S* RNA species probably also occurs in vivo as a short-lived intermediate. In the electrophoretic pattern of the oligo(dT)-cellulose-unbound mitochondrial RNA from cells labeled in the presence of actinomycin D, the band corresponding to the position of 12S* RNA has often been found to be of a somewhat higher intensity than can be accounted for by the expected amount of comigrating mRNA 12: this observation suggests the presence of a small pool of 12S* RNA molecules in the mitochondria of actinomycin D-treated cells. In exponentially growing cells, there is evidence indicating that processing of the primary rDNA transcripts occurs while they are still in the nascent state (4a, 20). However, it is not yet known whether this processing involves the formation of 12S* RNA as an obligatory intermediate.

The lower efficiency of rRNA formation relative to mRNA synthesis in isolated mitochondria and its particular sensitivity to the incubation conditions probably reflect the more stringent requirements of the processes involved, whether at the level of the initiation reaction, of chain elongation and termination, or of RNA processing. A special role could be played in these processes by the ribosomal proteins. It seems conceivable that the cessation of import of these proteins, or at least of some critical ones among them, from the cytoplasm may be the main factor responsible for the decrease in the rate of initiation of rRNA synthesis, the inefficiency of 5′-end processing, and possibly the defects in chain elongation. Ribosomal proteins may also play a critical role in the attenuation phenomenon leading to the specific termination of the rDNA transcripts at the 3′ end of the 16S RNA gene (4, 11).

The observations reported in the present and previous work (12) concerning the preferential reduction of rRNA synthesis, especially 16S RNA synthesis, in isolated mitochondria relative to the synthesis of the mRNAs encoded in the downstream regions of the H strand clearly point to independent controls of the two overlapping transcription units which are involved in the synthesis of the rRNAs and mRNAs (19, 20). Thus, these results give strong support to the similar conclusion derived from S1 mapping and kinetic experiments carried out with in vivo-synthesized RNA (20).

Polyadenylation reaction in vitro less efficient than in vivo. In vivo the bulk of the individual mRNAs possess a polyadenylate tail 55 to 60 NTPs long (18, 23) and are thus retained on oligo(dT)-cellulose (1). By contrast, only 5 to 20% of each of the mRNAs synthesized in isolated organelles are retained on oligo(dT)-cellulose due to either the absence or the shorter length of the polyadenylate tail. High concentrations of ATP (>1 mM) or temperatures of incubation below 37°C (34°C or lower) do increase the extent of polyadenylation of mitochondrial RNA, but this still remains below the level observed in vivo. Therefore, the polyadenylate-synthesizing apparatus appears to be relatively more affected under the in vitro conditions than the mtDNA transcription machinery. This implies that the addition of the 55- to 60-NTP-long polyadenylate tail can be dissociated from the processing step which produces the individual mRNAs from the putative polycistronic transcript. However, the present results do not exclude the possibility that the addition of one or a few A's at the 3′ end of the mRNAs and rRNAs is obligatorily coupled with the processing event (4, 27).

Dissociation of 7S RNA formation from overall L-strand transcription. The strikingly different behavior of 7S RNA labeling and overall L-strand transcription under different conditions of incubation of isolated mitochondria was an unexpected observation in the present work. This RNA species, ~200 NTPs long, maps in the region of the genome immediately preceding the origin of replication in the direction of L-strand transcription and possesses a putative ribosome attachment site (25). The function of this unusual RNA species is unknown, although a role in connection with the initiation of mtDNA replication or with the complete transcription of the L strand has been postulated (4). A main L-strand transcription initiation site has been recently localized in human mtDNA near the 5′ end of the 7S RNA coding sequence (25) both by S1 mapping of in vitro-capped mitochondrial RNA and in vivo-synthesized nascent chains (19) and by in vitro transcription studies (30; Shuey and Attardi, in preparation). Furthermore, the in vivo- and in vitro-synthesized transcripts starting at this initiation site have been found to extend beyond the 3′ end of 7S RNA for up to more than 600 NTPs (Shuey and Attardi, in preparation). These results have suggested the possibility that these transcripts may be a part of the putative fast-turning-over whole L-strand polycistronic transcripts destined to yield by processing the eight tRNA species and the mRNA for the unidentified reading frame (URF) 6 encoded in the L strand (26). According to this view, 7S RNA may also derive from

processing of these polycistronic transcripts; alternatively, this RNA species may be independently transcribed, starting at a site very close to or even coinciding with the initiation site so far identified.

The results obtained in this work support the idea that 7S RNA formation can be independently regulated from whole L-strand transcription. The dramatic drop in 7S RNA labeling at a high ATP concentration (5 mM) could conceivably be due to an accelerated turnover of this species. By contrast, in view of the apparently long half-life of the in vitro-synthesized 7S RNA and the short duration of the experiment, it seems unlikely that the large increase of 7S RNA labeling at high pH, which was not accompanied by a proportional increase in L-strand transcription, is due to an increase in stability of this RNA species. Rather, it seems probable that the increase in 7S RNA labeling reflects an increase in 7S RNA formation. However, these experiments cannot distinguish between an effect of the high pH on a processing event involving a larger precursor, possibly the whole L-strand polycistronic transcript, and an effect on an independent transcription event. As to the mechanism of this phenomenon, it is not possible to say whether the high pH modifies the internal ionic environment of the organelle or affects the energetic state of the inner mitochondrial membrane.

ACKNOWLEDGMENTS

These investigations were supported by Public Health Service grants GM-11726 and T32 GM-07616 from the National Institutes of Health.

We are greatly indebted to Michael King for supplying us with M13 clones of human mtDNA. We thank Arger Drew for excellent technical assistance.

LITERATURE CITED

1. **Amalric, F., C. Merkel, R. Gelfand, and G. Attardi.** 1978. Fractionation of mitochondrial RNA from HeLa cells by high resolution electrophoresis under strongly denaturing conditions. J. Mol. Biol. **118**:1–25.
2. **Anderson, S., A. T. Bankier, B. G. Barrell, M. H. L. deBruijn, A. R. Coulson, J. Drouin, I. E. Eperon, D. P. Nierlich, B. A. Roe, F. Sanger, P. H. Schreier, A. J. H. Smith, R. Stader, and I. G. Young.** 1981. Sequence and organization of the human mitochondrial genome. Nature (London) **290**:457–465.
3. **Attardi, B., and G. Attardi.** 1971. Expression of the mitochondrial genome in HeLa cells. I. Properties of the discrete RNA components from the mitochondrial fraction. J. Mol. Biol. **55**:231–249.
4. **Attardi, G., P. Cantatore, A. Chomyn, S. Crews, R. Gelfand, C. Merkel, J. Montoya, and D. Ojala.** 1982. A comprehensive view of mitochondrial gene expression in human cells, p. 51–71. *In* P. Slonimski, P. Borst, and G. Attardi (ed.), Mitochondrial genes. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

4a. **Attardi, G., G. Gaines, and J. Montoya.** 1983. Regulation of ribosomal RNA and messenger RNA synthesis in human mitochondria, p. 107–125. *In* R. J. Schweyen, K. Wolf, and F. Kaudewitz (ed.), Mitochondria. Walter de Gruyter & Co., Berlin.

5. **Bestwick, R. K., G. L. Moffett, and C. K. Mathews.** 1982. Selective expansion of mitochondrial nucleoside triphosphate pools in antimetabolite-treated HeLa cells. J. Biol. Chem. **257**:9300–9304.
6. **Boerner, P., T. L. Mason, and T. D. Fox.** 1981. Synthesis and processing of ribosomal RNA in isolated yeast mitochondria. Nucleic Acids Res. **9**:6379–6390.
7. **Cantatore, P. and G. Attardi.** 1980. Mapping of nascent light and heavy strand transcripts on the physical map of HeLa cell mitochondrial DNA. Nucleic Acids Res. **8**:2605–2625.
8. **Casey, J. and N. Davidson.** 1977. Rates of formation and thermal stabilities of RNA:DNA and DNA:DNA duplexes at high concentrations of formamide. Nucleic Acids Res. **4**:1539–1552.
9. **Crews, S. and G. Attardi.** 1980. The sequence of the small ribosomal RNA gene and the phenylalanine tRNA gene are joined end to end in human mitochondrial DNA. Cell **19**:775–784.
10. **Crews, S., D. Ojala, J. Posakony, J. Nishiguchi, and G. Attardi.** 1979. Nucleotide sequence of a region of human mitochondrial DNA containing the precisely identified origin of replication. Nature (London) **277**:192–198.
11. **Dubin, D. T., J. Montoya, K. D. Timko, and G. Attardi.** 1982. Sequence analysis and precise mapping of the 3′ ends of HeLa cell mitochondrial ribosomal RNAs. J. Mol. Biol. **157**:1–19.
12. **Gaines, G., and G. Attardi.** 1984. Intercalculating drugs and low temperature inhibit synthesis and processing of ribosomal RNA in isolated human mitochondria. J. Mol. Biol. **172**:451–466.
13. **Gelfand, R., and G. Attardi.** 1981. Synthesis and turnover of mitochondrial ribonucleic acid in HeLa cells: the mature ribosomal and messenger ribonucleic acid species are metabolically unstable. Mol. Cell. Biol. **1**:497–511.
14. **Groot, G. S. P., N. van Harten-Loosbroek, G.-J. B. van Ommenn, and H. L. A. Pijst.** 1981. RNA synthesis in isolated yeast mitochondria. Nucleic Acids Res. **9**:6379–6390.
15. **Hare, J. F., E. Ching, and G. Attardi.** 1980. Isolation, subunit composition and site of synthesis of human cytochrome c oxidase. Biochemistry **19**:2023–2030.
16. **Harold, F. M.** 1972. Conservation and transformation of energy by bacterial membranes. Bacteriol. Rev. **36**:172–230.
17. **Henderson, P. J. F.** 1971. Ion transport by energy-conserving biological membranes. Annu. Rev. Microbiol. **25**:393–428.
18. **Hirsch, M., and J. Penman.** 1973. Mitochondrial polyadenylic acid-containing RNA: localization and characterization. J. Mol. Biol. **80**:379–391.
19. **Montoya, J., T. Christianson, D. Levens, M. Rabinowitz, and G. Attardi.** 1982. Identification of initiation sites for heavy strand and light strand transcription in human mitochondrial DNA. Proc. Natl. Acad. Sci. U.S.A. **79**:7195–7199.
20. **Montoya, J., G. L. Gaines, and G. Attardi.** 1983. The pattern of transcription of the human mitochondrial rRNA genes reveal two overlapping transcription units. Cell **34**:151–159.
21. **Montoya, J., D. Ojala, and G. Attardi.** 1981. Distinctive features of the 5′ terminal sequences of the human mitochondrial mRNAs. Nature (London) **290**:465–470.
22. **Newman, D., and N. Martin.** 1982. Synthesis of RNA in isolated mitochondria from *Saccharomyces cerevisiae*. Plasmid **7**:66–76.
23. **Ojala, D., and G. Attardi.** 1974. Expression of the mitochondrial genome in HeLa cells. XIX. Occurrence in mitochondria of polyadenylic acid sequences, "free" and covalently linked to mitochondrial DNA-coded RNA. J. Mol. Biol. **82**:151–174.
24. **Ojala, D., and G. Attardi.** 1980. Fine mapping of the ribosomal RNA genes of HeLa cell mitochondrial DNA. J. Mol. Biol. **138**:411–420.
25. **Ojala, D., S. Crews, J. Montoya, R. Gelfand, and G. Attardi.** 1981. A small polyadenylated RNA (7S RNA), containing a putative ribosome attachment site, maps near the origin of mitochondrial DNA replication. J. Mol. Biol. **150**:303–314.
26. **Ojala, D., C. Merkel, R. Gelfand, and G. Attardi.** 1980. The tRNA genes punctuate the reading of genetic information in human mitochondrial DNA. Cell **22**:393–403.
27. **Ojala, D., J. Montoya, and G. Attardi.** 1981. The tRNA punctuation model of RNA processing in human mitochondria. Nature (London) **290**:470–474.
28. **Posakony, J. W., J. M. England, and G. Attardi.** 1977. Mitochondrial growth and division during the cell cycle in HeLa cells. J. Cell Biol. **74**:468–491.
29. **Saris, N.-E., and K. E. O. Akerman.** 1980. Uptake and release of bivalent cations in mitochondria. Curr. Top. Bioenerg. **10**:103–179.
30. **Walberg, M. W., and D. A. Clayton.** 1983. In vitro transcription of human mitochondrial DNA. J. Biol. Chem. **258**:1268–1275.

CHAPTER 3

Identification and Characterization of the Excised Leader RNA (9L) for Cytochrome c Oxidase Subunit 1 mRNA

Abstract

An RNA species, 9L, has been identified and shown to be transcribed from the heavy strand of mitochondrial DNA of the region surrounding the light-strand origin of replication. This RNA 9L probably results from the excision of the 5' proximal ~320 nucleotides from RNA 6, leaving RNA 9 - the functional RNA for translation of the cytochrome c oxidase subunit I. Although RNA 9L contains the sequence complementary to four tRNAs, its most stable structure, as determined by computer analysis, is one with several very long stems and loops, not cloverleafs. This high degree of very stable secondary structure probably causes the anomalous behavior of the RNA 9L when electrophoresed through denaturing polyacrylamide gels. Furthermore, this secondary structure is possibly responsible for RNA 9L's stability and processing characteristics seen *in vivo*.

Introduction

The tRNA punctuation model for RNA processing in mammalian mitochondria states that the tRNA sequences interspersed between the rRNA and protein coding sequences in the polycistronic transcripts of the heavy (H) strand mtDNA act as processing signals for the formation of the rRNAs, mRNAs and tRNAs (1,2). This model is based upon the extremely compact nature of the mitochondrial genome, with its tRNA genes being butt-joined to the adjacent genes (3,4), on the evidence for transcription of the H strand in the form of polycistronic RNA molecules from two initiation sites (5) and on metabolic data of the mature RNAs (6). In addition, an RNase P-like activity capable of processing the *E. coli* suppressor $tRNA^{Tyr}$ precursor at the same site as the *E. coli* enzyme has been identified and partially purified from HeLa cell mitochondria (7). The identification in the RNA synthesized *in vivo* and in isolated organelles (9) of the 12S rRNA precursor $12S^{*}$ and its maturation to rRNA 12S via specific removal of the $tRNA^{Phe}$ and leader has provided direct evidence of an intermediate in the processing events. Examination of the mature RNA species derived from the H strand shows that, in the majority of cases, the processing of the primary transcript occurs at the boundary between a H-strand encoded tRNA and an mRNA, rRNA, or tRNA.

The formation of COI mRNA (RNA 9) appears to be a unique situation in the maturation of mRNAs in human mitochondria. This RNA does not derive directly from the processing of the primary transcript, rather via an intermediate (RNA 6) by the removal of a 5' segment which is complementary to the four tRNAs encoded in the L strand and to the template sequence corresponding to the origin of L-strand synthesis (4) (Fig. 1). Using a transcription system in isolated mitochondria, we have identified an RNA species (designated 9L) which corresponds to the leader portion of RNA 6, and which also occurs *in vivo*. The

existence and the properties of RNA 9L have given new insights into the processing of RNA in human mitochondria.

Materials and Methods

Labeling and isolation of mitochondria RNA

In vivo labeled mitochondrial RNA was prepared from HeLa cells incubated for 4 hours in the presence of 0.1 μg/ml actinomycin D and ^{32}P-orthophosphate as described previously (10). Labeling of RNA in isolated mitochondria was accomplished using techniques described previously (9, 11), with slight alterations. Specifically, mitochondria from 0.5 grams of HeLa cells were incubated in 10% glycerol, 25 mM NaCl, 40 mM Tris HCl (pH 7.4), 5 mM $MgCl_2$, 2 mg/ml bovine serum albumin (BSA), 10 mM H_2PO_4 (pH 7.0), 1 mM ATP, and 10-50 μCi [α-^{32}P]UTP (400-600 Ci/mM Amersham) for 30 minutes at 37°C. Isolation and separation on oligo(dT)-cellulose of total mitochondrial nucleic acids from the mitochondria was carried out as described previously (10, 11).

Fractionation and analysis of labeled RNA

Mitochondrial nucleic acids from either labeling procedure were fractionated by electrophoresis through 5% polyacrylamide-7M urea gels and the gels dried for autoradiography. Bidimensional electrophoresis was carried out by first running the sample in a narrow (2.5-3.0 mm) lane of a 5% polyacrylamide-7M urea gel, cutting the lane and immediately cementing the lane with 1% agarose horizontally across the top of a prepared 10% polyacrylamide-7M gel. The nucleic acids were then electrophoresed into the 10% acrylamide-7M urea gel and the gel dried for autoradiography. Bidimensional purification of RNA 9L was carried out by electrophoresing a sample of labeled RNA into a 5% polyacrylamide-7M urea

gel, autoradiographing the gel overnight and cutting the appropriate region in the gel. This slice was then layered and cemented on the top of a lane in the 10% polyacrylamide-7M urea gel and electrophoresed in the second dimension. The gel was autoradiographed, the appropriate region was cut and the RNA eluted from the gel slice by sequential soakings in SDS buffer—0.5% SDS, 0.15 M NaCl, 10 mM Tris HCl (pH 7.4), 1.0 mM EDTA. The RNA was then ethanol precipitated and phenol extracted prior to further use.

S_1 protection, RNase H, and primer extension analysis

Labeled RNA species purified by either single or bidimensional gel electrophoresis for S_1 protection analysis were hybridized to single- or double-stranded M13 clones in high formamide conditions favoring RNA-DNA hybridization over DNA-DNA reassociation (12) as previously described (9-11). The S_1 nuclease (Sigma) was used at 100-200 U in an 0.2 ml volume for 30 minutes at 41°C. Electrophoretic analysis of the protected RNA fragments were performed under denaturing conditions in polyacrylamide gels.

RNase H analysis was carried out on bidirectional-electrophoretically purified RNA 9L labeled in isolated mitochondria. An excess of an oligodeoxynucleotide corresponding in sequence to the H strand of mitochondrial DNA between nucleotides 5699 and 5720 was heated with labeled RNA 9L in 20 µl of high formamide, S_1 hybridization buffer (1) at 68°C for 15 minutes and then allowed to hybridize at 49°C for 1 hour. 200 µl of RNase H buffer (20 mM Tris HCl (pH 7.5)), 10 mM $MgCl_2$, 100 mM KCl, 0.1 mM DTT, and 5% glycerol) and two units of RNase H were added and the samples were incubated at 37°C for 30 minutes. The reaction was stopped by the addition of 300 µl SDS buffer and ethanol precipitation. The samples were then subjected to denaturing gel electrophoresis. The primer extension analysis was carried out by mixing

bidimensional-electrophoretically purified RNA 9L with the oligodeoxynucleotide used in RNase H experiments which had been previously 5'-end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (13), with 10 units RNasin (Promega Biotec), 0.5 mM of each of the dNTPs, eight units of AMV reverse transcriptase, all in a final concentration of 50 mM KCl, 80 mM Tris HCl (pH 8.3) and 10 mM $MgCl_2$ at 42°C for 60 minutes. The reaction was stopped with SDS buffer and ethanol precipitated for analysis on a 15% polyacrylamide-7M urea gel. The DNA sequence ladder was generated by using the same oligodeoxynucleotide (unlabeled) as a primer and a single-stranded M13 clone containing the L-strand of the mitochondrial DNA between positions 5275 and 6204 as template, the Klenow fragment of *E. coli* DNA polymerase I, [α-^{32}P]dCTP as label, and the three other unlabeled deoxynucleoside triphosphates in the presence of one of the four dideoxynucleotides, A,C,G,T (14).

SP6 and T7 synthesis and gel analysis of RNA

The mitochondrial DNA fragment between the EcoRI site at 5274 and the HindIII site at 6203 was cloned into PGEM1 (Promega Biotec), a plasmid containing opposing promoters for SP6 and T7 RNA polymerases. The new plasmid, pGLO I (light-strand origin) was digested with either StuI, HhaI, HindIII, or EcoRI and the appropriate RNA polymerase reactions were carried out, using the conditions suggested by Promega Biotec. The reactions were stopped by the addition of SDS buffer, phenol extraction and ethanol precipitation. The samples were then resuspended in an 8 M urea loading buffer, heated at 68°C for 15 minutes and electrophoresed through a 5 or 10% polyacrylamide-7M urea gel.

Results

Transcription of mitochondrial DNA (mtDNA) in isolated HeLa cell mitochondria has been shown to faithfully reproduce the *in vivo* process (9, 11). Figure 2 shows the autoradiographic patterns of *in vivo* ^{32}P labeled mitochondrial RNA and of RNA labeled in isolated organelles using [α-^{32}P]UTP as precursor, fractionated by electrophoresis through 5% polyacrylamide-7M urea gels. Although the relative migration of the RNA species in this gel system is different from that observed in a CH_3HgOH-agarose gel (15), the components indicated have been identified by S_1 mapping experiments (8). Notice, in particular, the presence in the pattern of *in vivo* labeled oligo(dT)-cellulose unbound RNA of a minor band corresponding to RNA 12S* (9), previously not recognized. Differences in the migration and relative intensities of some bands between the *in vivo* and *in vitro* patterns appears to be due to the incomplete polyadenylation and processing occurring in isolated mitochondria (11). Of special interest are the three bands migrating in the region of RNA 17 in the *in vitro* labeled RNA (which are also present in the *in vivo* labeled RNA, though much less intense due to the shorter exposure of the autoradiogram). The slowest migrating of these bands was retained on oligo(dT)-cellulose and was therefore presumably polyadenylated, while the two faster migrating species were not retained. In an attempt to identify these species, S_1 protection analysis was performed with the *in vitro* labeled RNA species, as shown in Figs. 3 and 4. Initially, an M13 cloned H-strand mtDNA fragment corresponding to RNA 17 was used. *In vitro* labeled RNA was fractionated on a 5% polyacrylamide-7M urea gel, the appropriate bands were cut, eluted and then hybridized to the single-stranded M13 cloned mtDNA fragment. After S_1 digestion, the samples were electrophoresed through denaturing 5% polyacrylamide gels. It is clear from Fig. 3 that the two slowest migrating RNA species map in the region corresponding to RNA 17, and are, therefore, the

polyadenylated and non-polyadenylated forms of RNA 17 (designated as 17b and 17u). By contrast, the fastest moving RNA appeared to be unrelated to RNA 17. The size of this RNA species (~320 nt) indicated that it might be the leader of COI mRNA excised from RNA 6. Hence, a second set of S_1 protection experiments was performed using the M13 cloned EcoRI-HindIII H-strand mtDNA fragment which corresponds to a 3'-end proximal portion of RNA 12, the $tRNA^{Trp}$, and a 5'-end proximal portion of RNA 6 (Fig. 1). Figure 4a shows the results from this experiment, which indicate clearly that the RNA in question is an H-strand transcript from the above-mentioned region. Further S_1 protection analysis utilizing subfragments of the EcoRI-HindIII fragment (Fig. 1) showed that the RNA corresponds precisely to the H-strand segment complementary to the four tRNA genes surrounding the origin of L-strand synthesis (Fig. 1 and 4b). This RNA was therefore designated 9L (for RNA 9 leader).

During the mapping of RNA 9L, an anomalous electrophoretic behavior of this RNA species was noted. RNA 9L migrates much slower in a 10% polyacrylamide-7M urea gel than expected for its size. This was most clearly seen when the mitochondrial RNA was fractionated in bidimensional (2-D) denaturing gel electrophoresis, with the first and second runs being in 5% and 10% polyacrylamide-7M urea gels, respectively. One such experiment is shown in Fig. 5. Total *in vitro* labeled mitochondrial RNA was electrophoresed through a 5% polyacrylamide-7M urea gel, a lane was then cut and immediately layered horizontally over a 10% polyacrylamide-7M urea gel, and the RNA components were electrophoresed in the second dimension. As shown in Fig. 5a, all the components including RNAs 17b and 17u, migrated along the diagonal, except RNA 9L which migrated in the second dimension much slower than expected. When the oligo(dT)-cellulose bound and unbound fractions of the *in vitro* labeled RNA were analyzed in the same way, it was found that the majority of the slower

migrating component was in the unbound fraction (Fig. 5b, panels 2 and 4). When the *in vivo* labeled RNA was analyzed, the slower migrating component was also observed, in approximately equal amounts in the bound and unbound fractions (Fig. 5b, panels 1 and 3; see legend). By comparing the relative labeling *in vivo* of RNA 9L and RNA 17b, and assuming equivalent rates of synthesis, one can estimate that RNA 9L has an approximate half life of 30 minutes, i.e., similar to that of the mRNAs (6).

A further set of experiments was done to analyze in more detail the electrophoretic behavior of the RNA species migrating in the region of RNA 17. The analysis was accomplished by first electrophoresing the RNA sample through a 5% polyacrylamide-7M urea gel, cutting the appropriate gel band after autoradiography, directly placing this band upon a 10% polyacrylamide-7M urea gel and electrophoresing the RNA into the second gel. Figure 6b shows the results of such an experiment done with *in vivo* labeled oligo(dT)-cellulose bound RNA. The RNA from the region corresponding to 17b, 17u, and 9L (Fig. 6a) was cut and run in a 10% gel (lane B); the radioactivity was found to be associated with a major band at the expected position for RNA 17b, and a minor, much slower moving band representing the polyadenylated form of RNA 9L. When the analogous experiment was carried out with *in vivo* labeled oligo(dT)-cellulose unbound RNA (lane U), a minor part of the radioactivity was found to migrate as a band slightly faster than the polyadenylated RNA 9L, representing the non-polyadenylated form of this RNA. The majority of the radioactivity was found in a doublet of bands representing RNA 17u and a faster moving species, the nature of which is not known. A similar analysis was carried out on *in vitro* labeled RNA components. The total *in vitro* labeled RNA was electrophoresed through a 5% gel, as shown in Fig. 6a, and specific bands cut and rerun in a 10% gel. RNA 17b ran as a single band to the expected position on the 10% gel (Fig. 6b), lane 1).

Most of the radioactivity from the 17u band (5% gel) also migrated to the expected position for RNA 17u (Fig. 6b, lane 2; however, a minor band was observed at the position of RNA 9L, presumably representing polyadenylated RNA 9L (given the estimated size of RNA 9L of 320 nt (see below), its polyadenylated form was expected to overlap the 17u band (346 nt). Most of the 9L band migrated in a 10% gel as a band slightly faster than the putative polyadenylated RNA 9L, representing the non-polyadenylated form. A minor fraction of the radioactivity did migrate as a fairly broad band slightly faster than RNA 17u, corresponding to the faster moving doublet in the *in vivo* non-polyadenylated RNA fraction. The unusual electrophoretic behavior of RNA 9L provided a convenient method for its purification.

In order to test the possibility that incomplete denaturation was responsible for the behavior of RNA 9L, a sample of RNA 9L was eluted from a 5% polyacrylamide-7M urea gel, boiled for 5 minutes in 50 mM CH_3HgOH and electrophoresed immediately through a 10% polyacrylamide-7M urea gel. The anomalous behavior in the 10% polyacrylamide-7M urea gels persisted despite this harsh treatment: this indicated that the unusual structure of RNA 9L withstood denaturation or reformed easily upon removal of the CH_3HgOH, despite the presence of 7M urea.

Anomalous migration in 2-D denaturing polyacrylamide gel electrophoresis has been associated with branched or lariat RNA structures (16-18). On the other hand, the high degree of secondary structure expected in the RNA 9L could conceivably have been responsible for its unusual electrophoretic properties. Several experiments were done to examine the structure of RNA 9L. An oligodeoxynucleotide was synthesized which was complementary to a 22 nt segment of RNA 9L comprised between 119 and 141 nt from the 5' end (Fig. 7a). This was used for RNase H and primer extension experiments, as illustrated schematically

in Fig. 7b. The oligodeoxynucleotide was hybridized to *in vitro* labeled electrophoretically purified RNA 9L, deriving mostly from the oligo(dT)-cellulose unbound fraction, and subjected to RNase H treatment. Figure 8b shows the results obtained. Two major bands corresponding to RNA segments of about 183 and 120 nt were observed: this result excluded model 4 of Fig. 7b. A fainter band corresponding to a segment of 177 nt was also observed.

A primer extension experiment using the same oligodeoxynucleotide labeled at its 5' end, AMV reverse transcriptase, unlabeled deoxynucleoside triphosphates, and unlabeled electrophoretically purified RNA 9L as template was carried out. The results are shown in Fig. 8b. After electrophoresis of the extended primer through a denaturing 15% polyacrylamide gel, a major band is observed representing a product whose 3' end corresponds to the T or A at positions 5580 or 5581, respectively. Upon longer exposures, a minor band is visible, representing a product whose 3' end corresponds to the C at position 5586. These primer extension results rule out model 2 (Fig. 7b). Hence, the experimental evidence strongly favors a linear structure for RNA 9L, indicating that the high degree of secondary structure suspected for this RNA probably accounts for its anomalous electrophoretic behavior. In order to rule out the possibility of a lariat structure with its branch point within the stretch complementary to the synthetic oligodeoxynucleotide (model 3, Fig. 7b), the following experiment was done. A fragment of mtDNA including the 9L coding sequence and about 300 nt on either side was cloned into PGEM1 (Promega Biotec), as diagrammed in Fig. 9a. After appropriate restriction enzyme cuts of the plasmid, transcripts of the insert were made *in vitro* using the SP6 or T7 promoter and the corresponding RNA polymerase, and then electrophoresed through 5% and 10% polyacrylamide-7M urea gels. As shown in Fig. 9b, any transcript which included a major portion of the 9L region, independently of the coding strand, migrated anomalously in a 10% denaturing gel.

Discussion

We have identified a mitochondrial RNA species which corresponds to the H-strand complement of the four tRNAs encoded in the L strand around the origin of L-strand synthesis (4). This RNA, designated 9L, most probably results from the processing of RNA 6 into a leader 9L and RNA 9 (COI). S_1 protection analysis and primer extension experiments have localized the 5' ends of the great majority (~90%) of the molecules to the T or A at position 5580 or 5581, respectively (and the remainder to the C or T at position 5586 or 5587). The 3' ends were judged to be mostly at 5900 (A) and some at 5891 (C) by RNase H and S_1 analysis.

The finding of an RNA species with ends mapping at the positions indicated above provides some insights into the mechanism of RNA processing in mammalian mitochondria. First of all, the existence of the RNA 9L itself indicates that the processing machinery does not recognize efficiently the anti-tRNA sequences as processing signals. The 5' end of the great majority of the RNA 9L molecules appears to derive from the cleavage of the polycistronic transcript at the 3' end of the H strand encoded $tRNA^{Trp}$ rather than at the 5' end of the anti-$tRNA^{Ala}$, situated seven nucleotides downstream. (On the other hand, the 3' end of the minor primer extension product at position 5586 corresponds to the 5' end of the anti-$tRNA^{Ala}$ sequence, again suggesting that this sequence may function as a processing signal with low efficiency.) Other H-strand transcripts previously analyzed provide additional evidence concerning the nature of the processing signals recognized in mitochondria. For example, the mature oligoadenylated RNA 9 (COI) contains the anti-$tRNA^{Ser}$ sequence in the 3'-untranslated region (2, 4), again indicating that, in general, the complements of tRNAs are not recognized by the processing enzyme(s). However, clearly at least one of the anti-sense tRNA sequences can act as a processing signal, that of anti-$tRNA^{Glu}$. Cleavage on the 3' end and possibly, also on the 5' end of the anti-

$tRNA^{Glu}$ is required for the formation of RNA 11 and RNA 5 (19). It seems likely that the structure of the individual anti-tRNA and the surrounding sequences determines their efficiency as signals for the mitochondrial RNase P.

RNA 9L has an interesting behavior when electrophoresed through denaturing polyacrylamide gels. This anomalous behavior is particularly evident when this RNA species is separated by 2-D electrophoresis in 5% and 10% denaturing polyacrylamide gels. This behavior has recently been associated with lariat or branched RNA molecules. Three different strategies were used to determine the structure of RNA 9L: RNase H digestion of the RNA 9L molecule after hybridization with an oligodeoxynucleotide complementary to an internal segment, primer extension using the same oligodeoxynucleotide as primer and RNA 9L as template, and *in vitro* synthesis of transcripts containing the 9L sequence or the complementary sequence using an SP6/T7 system. The evidence obtained strongly favors a linear structure, thus indicating that the anomalous electrophoretic behavior of RNA 9L is due to its capacity to form an unusually stable secondary structure which impedes its progress through a 10% "denaturing" polyacrylamide gel. The most stable secondary structure of the molecule as determined by the Nussinov-Jacobson program (20) is shown in Fig. 10. To our knowledge, this is the first time that an unusual electrophoretic behavior in denaturing gels of a linear RNA molecule due to secondary structure has been reported.

The mapping position, structure and relative metabolic stability of RNA 9L leads one to believe that this molecule may have a physiological role. The possibility that RNA 9L may be involved in regulating the initiation of L-strand synthesis is suggested by its complementarity to the template sequence for the priming of L-strand synthesis. Alternatively, RNA 9L may act as an inhibitor of translation for COI. It is as yet unknown whether or not RNA 6 can act as a

translationally competent RNA. Another possibility is that RNA 9L is the RNA component of the mitochondrial RNase P, and therefore is involved in the processing of the H-strand transcripts.

Acknowledgements

We thank Anne Chomyn for the computer analysis and Jan Hoh for help with the sequencing experiments. The technical assistance of Ms. Arger Drew is gratefully acknowledged.

References

1. Ojala, D., Merkel, C., Gelfand, R., and Attardi, G. 1980. The tRNA genes punctuate the reading of genetic information in human mitochondrial DNA. *Cell* **22**, 393-403.
2. Ojala, D., Montoya, J., and Attardi, G. 1981. The tRNA punctuation model of RNA processing in human mitochondria. *Nature* **290**, 470-474.
3. Crews, S., and Attardi, G. 1980. The sequence of the small ribosomal RNA gene and the phenylalanine tRNA gene are joined end to end in human mitochondrial DNA. *Cell* **19**, 775-784.
4. Anderson, S., Bankier, A. T., Barrell, B. G., de Bruijn, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J. H., Staden, R., and Young, I. G. 1981. Sequence and organization of the human mitochondrial genome. *Nature* **290**, 457-465.
5. Montoya, J., Christianson, T., Levens, D., Rabinowitz, M., and Attardi, G. 1982. Identification of initiation sites for heavy strand and light strand transcription in human mitochondrial DNA. *Proc. Natl. Acad. Sci. USA* **79**, 7195-7199.
6. Gelfand, R., and Attardi, G. 1981. Synthesis and turnover of mitochondrial ribonucleic acid in HeLa cells; the mature ribosomal and messenger ribonucleic acid species are metabolically unstable. *Molec. Cell Biol.* **1**, 497-511.
7. Doersen, C.-J., Guerrier-Takada, C., Altman, S., and Attardi, G. 1985. Characterization of an RNase P activity from HeLa cell mitochondria. *J. Biol. Chem.* **260**, 5942-5949.

8. Montoya, J., Gaines, G., King, M., and Attardi, G. Manuscript in preparation.

9. Gaines, G., and Attardi, G. 1984. Intercalating drugs and low temperatures inhibit synthesis and processing of ribosomal RNA in isolated human mitochondria. *J. Mol. Biol.* **172,** 451-466.

10. Montoya, G., Gaines, G., and Attardi, G. 1983. The pattern of transcription of the human mitochondrial rRNA genes reveals two overlapping transcription units. *Cell,* **34,** 151-159.

11. Gaines, G., and Attardi, G. 1984. Highly efficient RNA-synthesizing system that uses isolated human mitochondria: new initiation events and *in vivo*-like processing patterns. *Molec. Cell Biol.* **4,** 1605-1617.

12. Casey, J., and Davidson, N. 1977. Rates of formation and thermal stabilities of RNA:DNA and DNA:DNA duplexes at high concentrations of formamide. *Nucl. Acids Res.* **4,** 1539-1552.

13. Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 122-123.

14. Martin, C. Personal communication of techniques.

15. 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.

16. Padgett, R. A., Konarska, M. M., Grabowski, P. J., Hardy, S. F., and Sharp, P. A. 1984. Lariat RNAs as intermediates and products in the splicing of messenger RNA precursors. *Science* **205,** 898-903.

17. Ruskin, B., Krainer, A. R., Maniatis, T., and Green, M. R. 1984. Excision of an intact intron as a novel lariat structure during pre-mRNA splicing *in vitro*. *Cell* **38,** 317-331.

18. Brody, E., and Abelson, J. 1985. The "Spliceosome": Yeast pre-messenger RNA associates with a 40S complex in a splicing-dependent reaction. *Science* **228**, 963-967.

19. Attardi, G. 1984. RNA synthesis and processing in mitochondria. **In** Processing of RNA. D. A Pirion (ed.). CRC Press, Inc., Boca Raton, Florida, pp. 227-290.

20. Chomyn, A., Mariottini, P., Cleeter, M. W. J., Ragan, C. I., Matsuno-Yagi, A., Hatefi, Y., Doolittle, R. F., and Attardi, G. 1985. Six unidentified reading frames of human mitochondrial DNA encode components of the respiratory-chain NADH dehydrogenase. *Nature* **314**, 592-597.

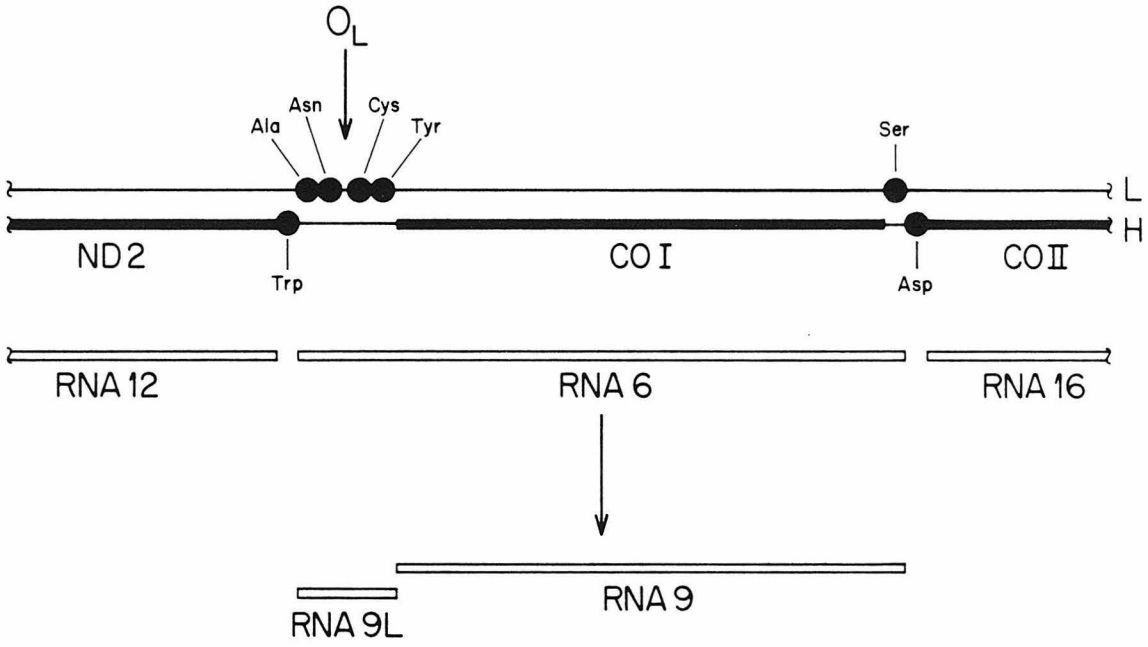

O_L
Ala
Asn
Cys
Tyr
Ser
L
H
ND2
Trp
CO I
Asp
CO II
RNA 12
RNA 6
RNA 16
RNA 9
RNA 9L

Fig. 1. Segment of the HeLa cell mitochondrial DNA genetic and transcription map of the cytochrome oxidase subunit I gene and surrounding regions (4). The 3'-proximal portion of the gene for a subunit of NADH dehydrogenase (ND2—formerly URF2 (20)), and the 5'-proximal portion of the gene for cytochrome oxidase subunit II and their corresponding RNAs are noted. O_L is the origin of L-strand DNA replication. The mapping position of RNA 9L, as determined by S_1 mapping and primer extension experiments is shown.

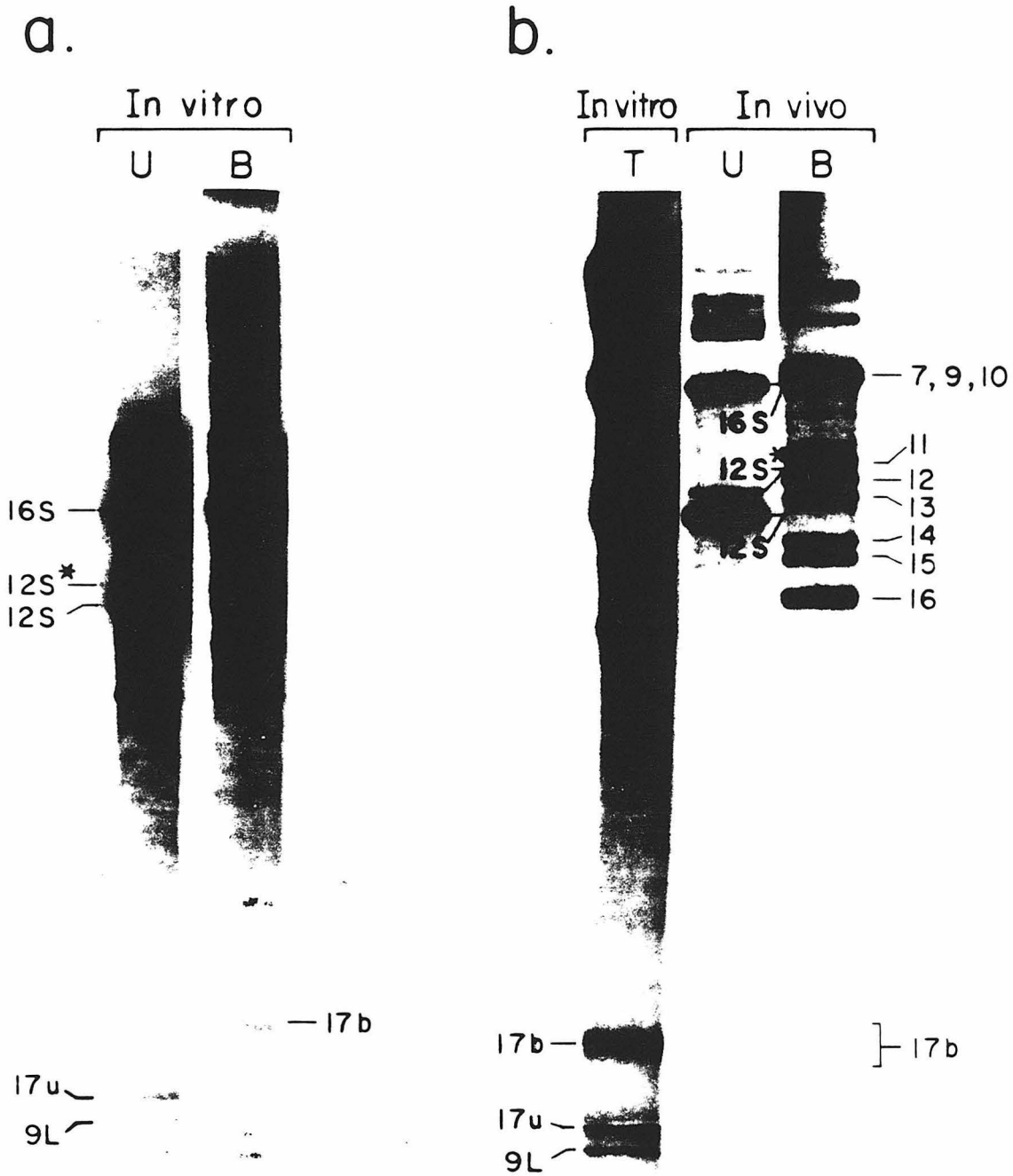

a.
In vitro
U
B
16S
12S*
12S
17b
17u
9L
b.
In vitro
In vivo
T
U
B
7, 9, 10
16S
11
12S*
12
13
14
15
16
17b
17b
17u
9L

Fig. 2. Separation on a 5% polyacrylamide-7M urea gel of oligo(dT)-cellulose fractionated RNA labeled in isolated mitochondria or in *in vivo* in the presence of 0.1 μg/ml actinomycin D. T represents the total RNA; U, the RNA which did not bind to oligo(dT)-cellulose; and B, the RNA which bound and was eluted from oligo(dT)-cellulose. In a., the RNA in lane B was derived from 2.5 times the amount of mitochondria as in lane U; in b., the RNA in lane B was derived from 10 times the amount of cells as in lane U.

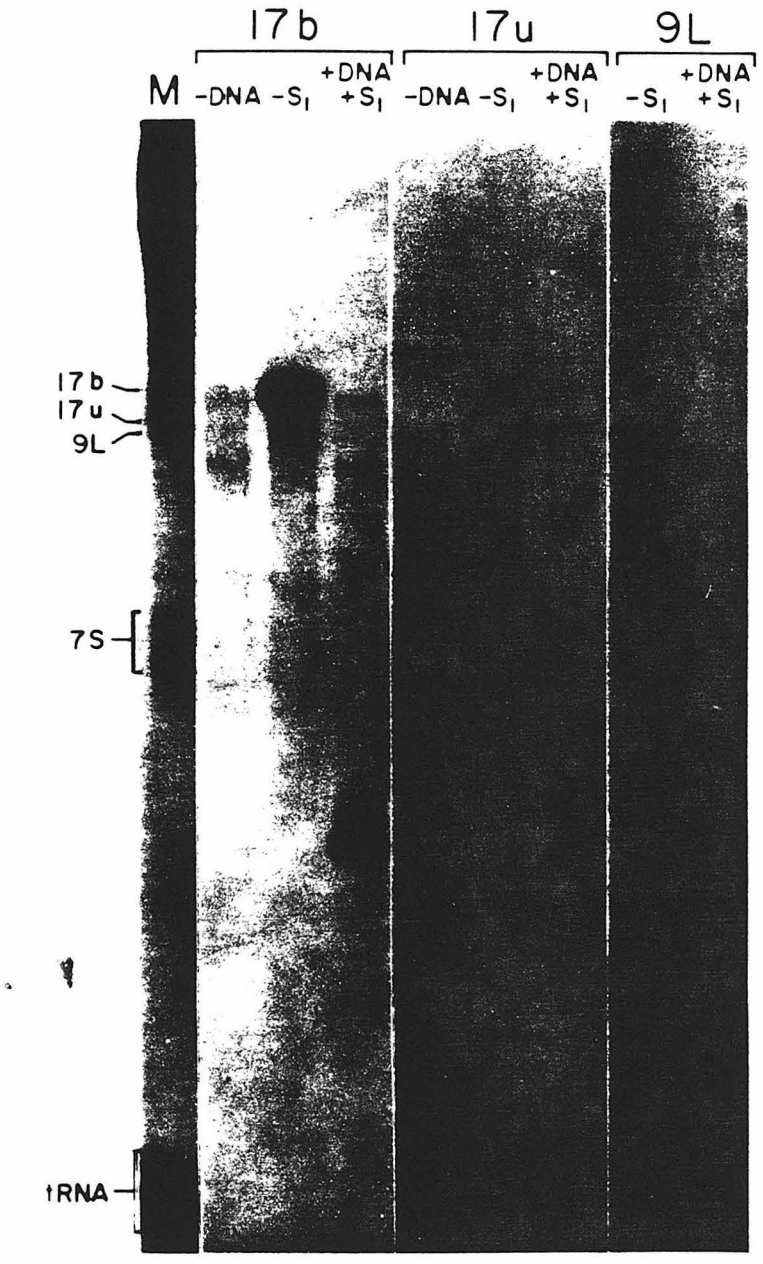

17b
17u
9L
M
-DNA
-S1
+DNA +S1
-DNA
-S1
+DNA +S1
-S1
+DNA +S1
17b
17u
9L
7S
tRNA

Fig. 3. S_1 protection analysis of the three bands migrating in the regon of RNA 17. Total RNA labeled in isolated mitochondria was electrophoresed through a 5% polyacrylamide-7M urea gel and the bands marked 17b, 17u, and 9L were cut and eluted. The RNA was hybridized to a single-strand M13 clone containing the H strand of mitochondrial DNA between 5274 and 6203. S_1 digestion was performed and the digested samples were electrophoresed through a 5% polyacrylamide-7M urea gel. M is total RNA labeled in isolated mitochondria.

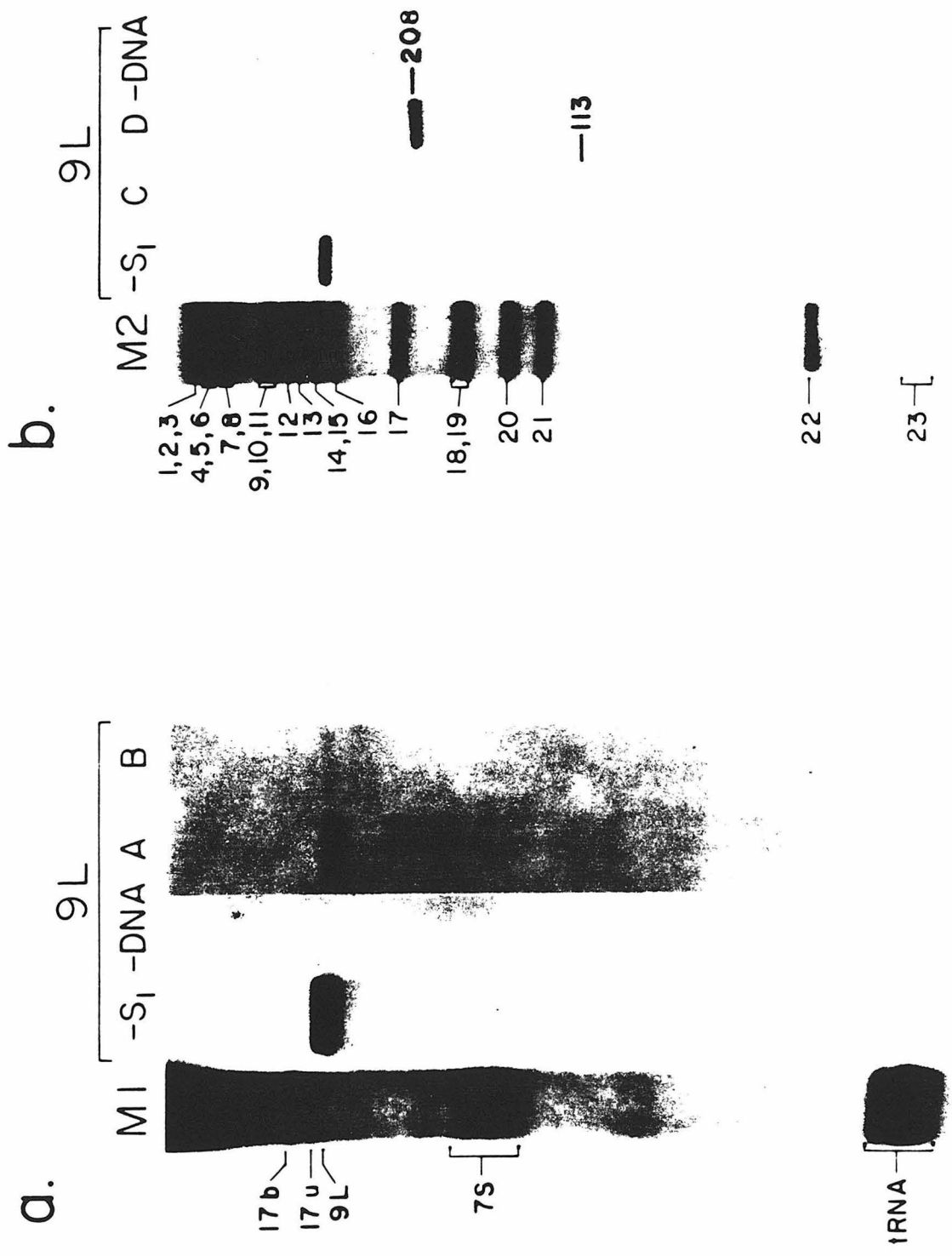

a.
9L
M1 -S1 -DNA A B
17b
17u
9L
7S
tRNA
b.
9L
M2 -S1 C D-DNA
1,2,3
4,5,6
7,8
9,10,11
12
13
14,15
16
17
18,19
20
21
22
23
208
113

Fig. 4. S_1 protection analysis of RNA 9L using DNA from the region surrounding the origin of L-strand synthesis. a) Labeled RNA 9L was purified as in Fig. 3 and hybridized to single-stranded M13 clones containing mitochondrial H strand (A) or L strand (B) DNA from the EcoRI site at 5274 to the HindIII site at 6203. These samples were then digested with S_1 nuclease and electrophoresed through a 5% polyacrylamide-7M urea gel. Lane M1 shows the electrophoretic pattern of total RNA labeled in isolated mitochondria. b) Labeled RNA 9L was hybridized to double-stranded fragments between the EcoRI site at 5274 and the HpaI site at 5693 (C) or the HpaI site and the HindIII site at 6203 (D), digested with S_1 nuclease and electrophoresed through a 5% polyacrylamide-7M urea gel. Lane M2 shows the electrophoretic pattern of the HpaII digest of HeLa cell mitochondrial DNA 3'-end labeled with [α-^{32}P]dGTP and dCTP.

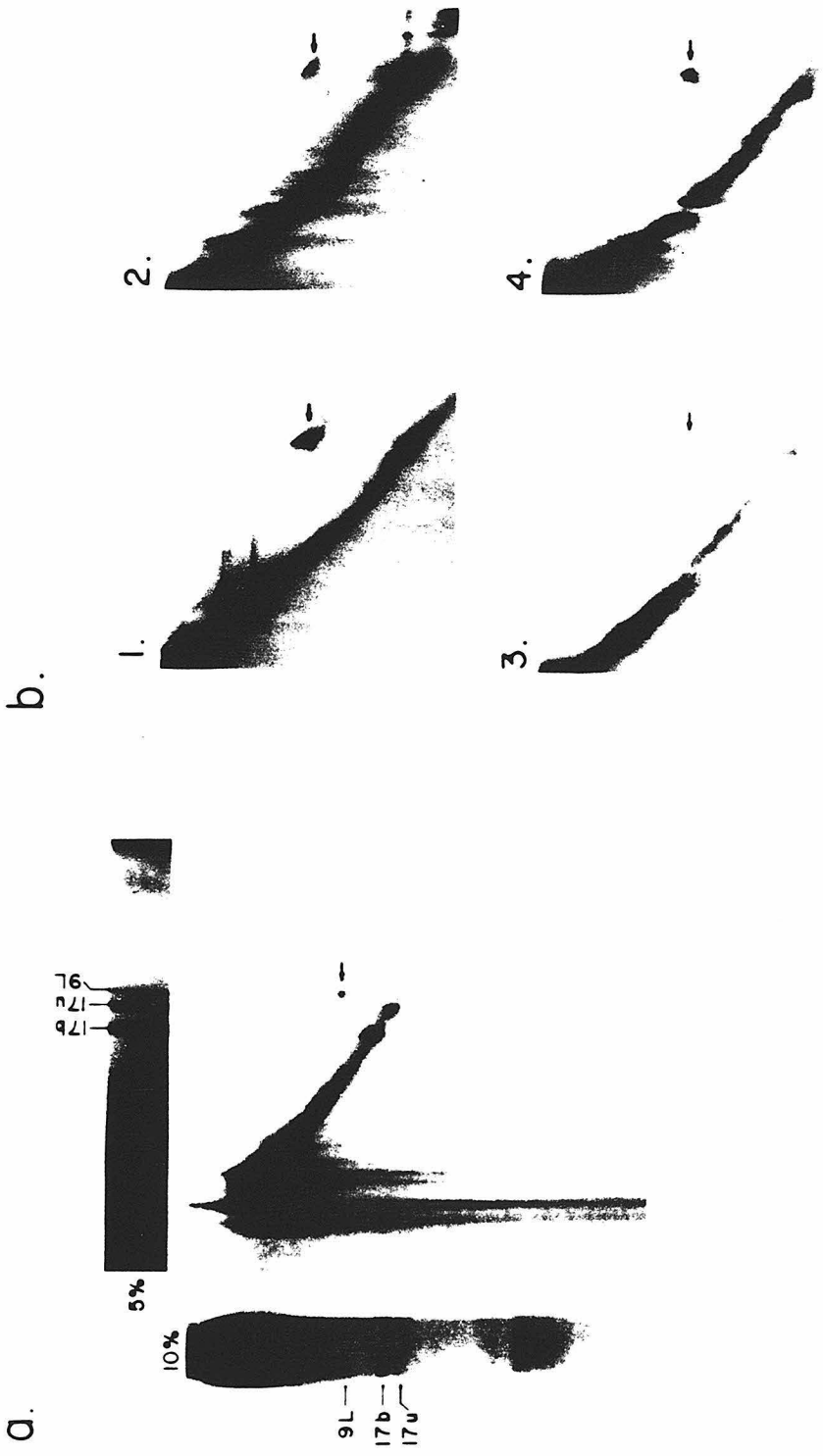
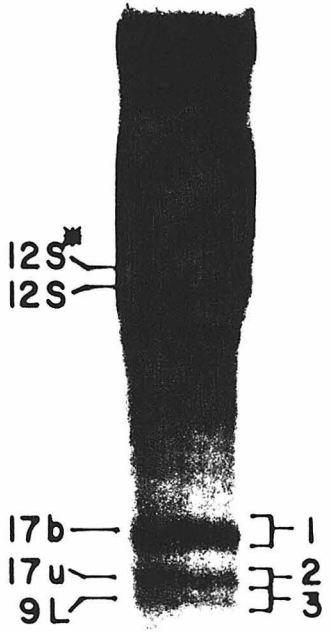

a.
9L
17u
17b
5%
10%
9L
17b
17u
b.
1.
2.
3.
4.

Fig. 5. Bidimensional polyacrylamide gel electrophoresis of *in vivo* labeled mitochondrial RNA or RNA labeled in isolated mitochondria. a) Two samples of total RNA labeled in isolated mitochondria were run in a 5% polyacrylamide-7M urea gel and one lane was electrophoresed into a 10% polyacrylamide-7M urea gel. An additional sample was electrophoresed in parallel through a 10% polyacrylamide-7M urea gel. The two samples electrophoresed in one dimension are indicated. b) Samples of oligo(dT)-cellulose bound (panels 1, 2) or unbound (panels 3, 4) RNA, labeled *in vivo* (panels 1, 3) or in isolated organelles (panels 2, 4) were electrophoresed bidirectionally. Panel 1 represents the RNA from 10 times the amount of cells as in panel 3, while panels 2 and 4 represent the equivalent amounts of mitochondrial RNA. In both a) and b), the arrows point to RNA 9L.

a.

5% PAGE



b.

10% PAGE

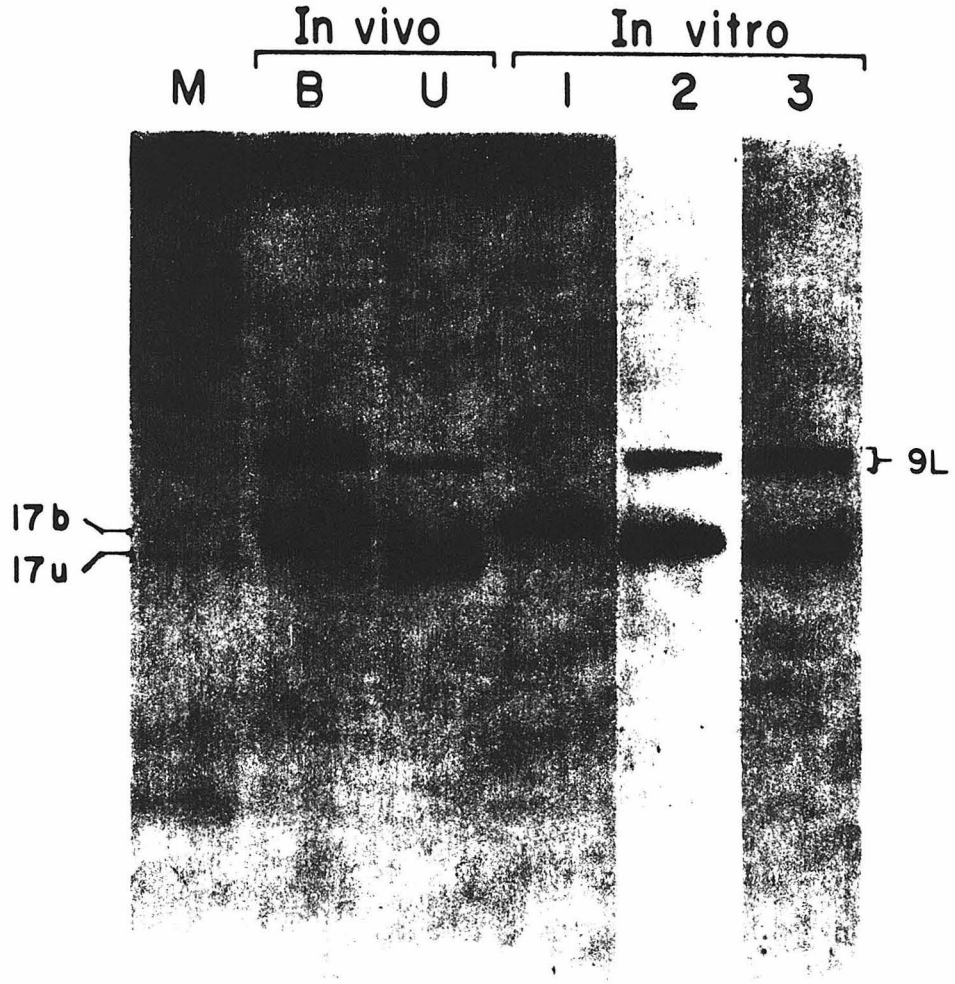


Fig. 6. Analysis of total, bound and unbound RNA 9L labeled in isolated mitochondria and *in vivo*. a) RNA labeled in isolated organelles electrophoresed through a 5% polyacrylamide-7M urea gel. Shown are the slices 1, 2, and 3 which were cut and applied to the top of a 10% polyacrylamide-7M urea gel and electrophoresed. b) In addition, *in vivo* labeled RNA was separated into oligo(dT) bound (B) and unbound (U) fractions and electrophoresed into a 5% polyacrylamide-7M urea gel. A slice containing RNA 17b, 17u, and 9L was cut from each lane and applied to the 10% acrylamide-7M urea gel and electrophoresed. M represents a sample of total RNA labeled in isolated mitochondria.

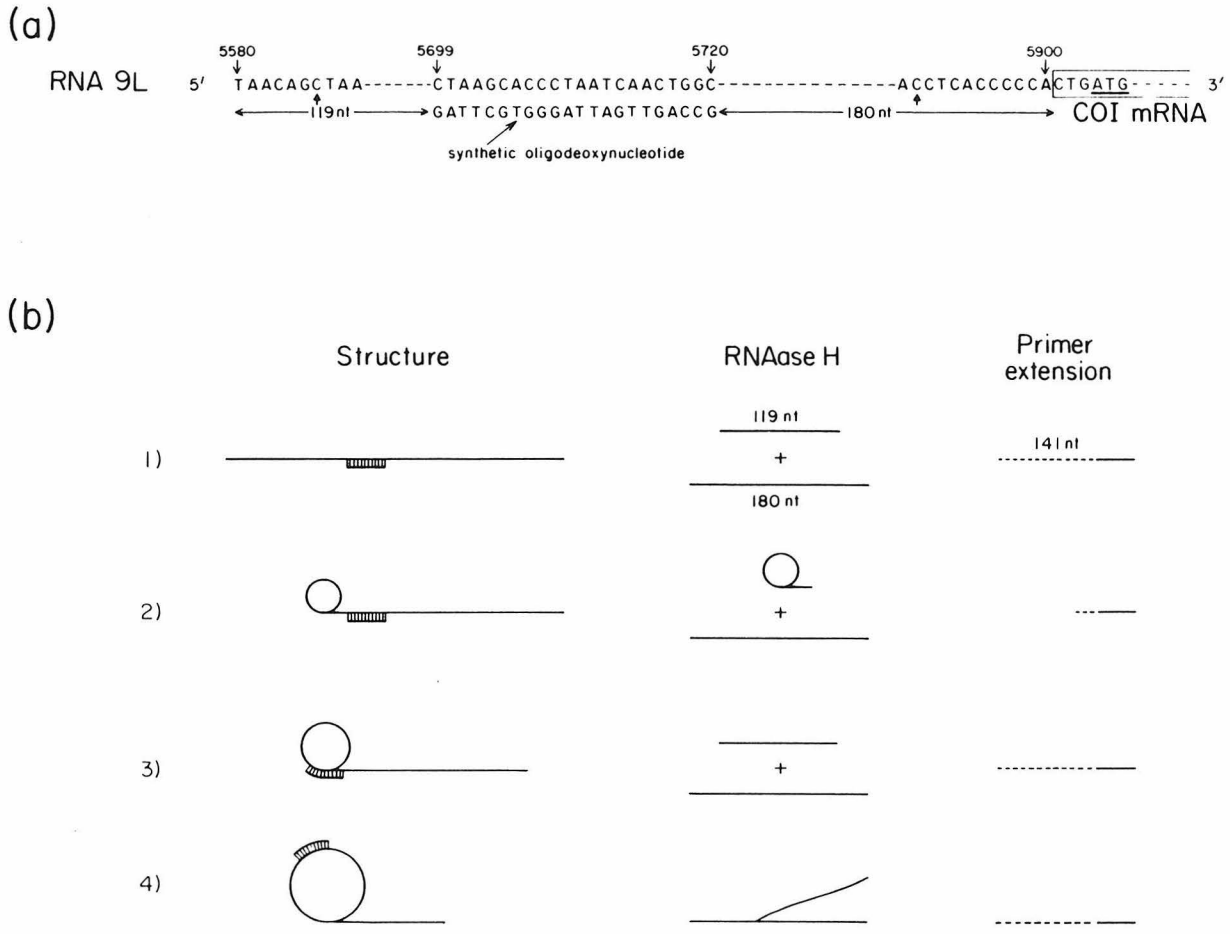
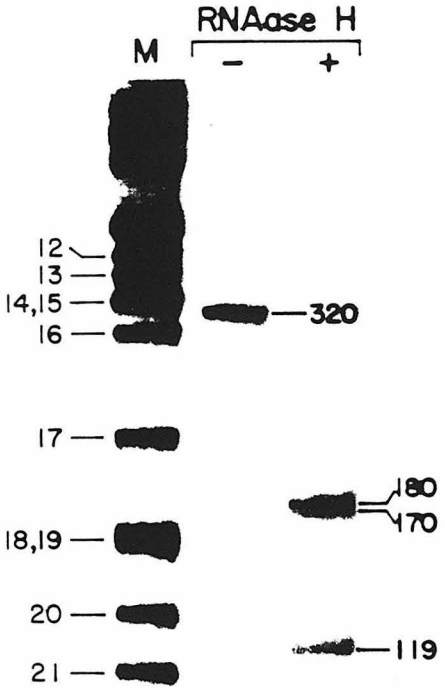

(a)
RNA 9L
5580
5699
5720
5900
5′ TAACAGCTAA------CTAAGCACCCTAATCAACTGGC----------ACCTCACCCCCACTGATG----- 3′
119nt
GATTCGTGGGATTAGTTGACCG
180nt
COI mRNA
synthetic oligodeoxynucleotide
(b)
Structure
RNAase H
Primer extension
119 nt
141 nt
180 nt
1)
2)
3)
4)

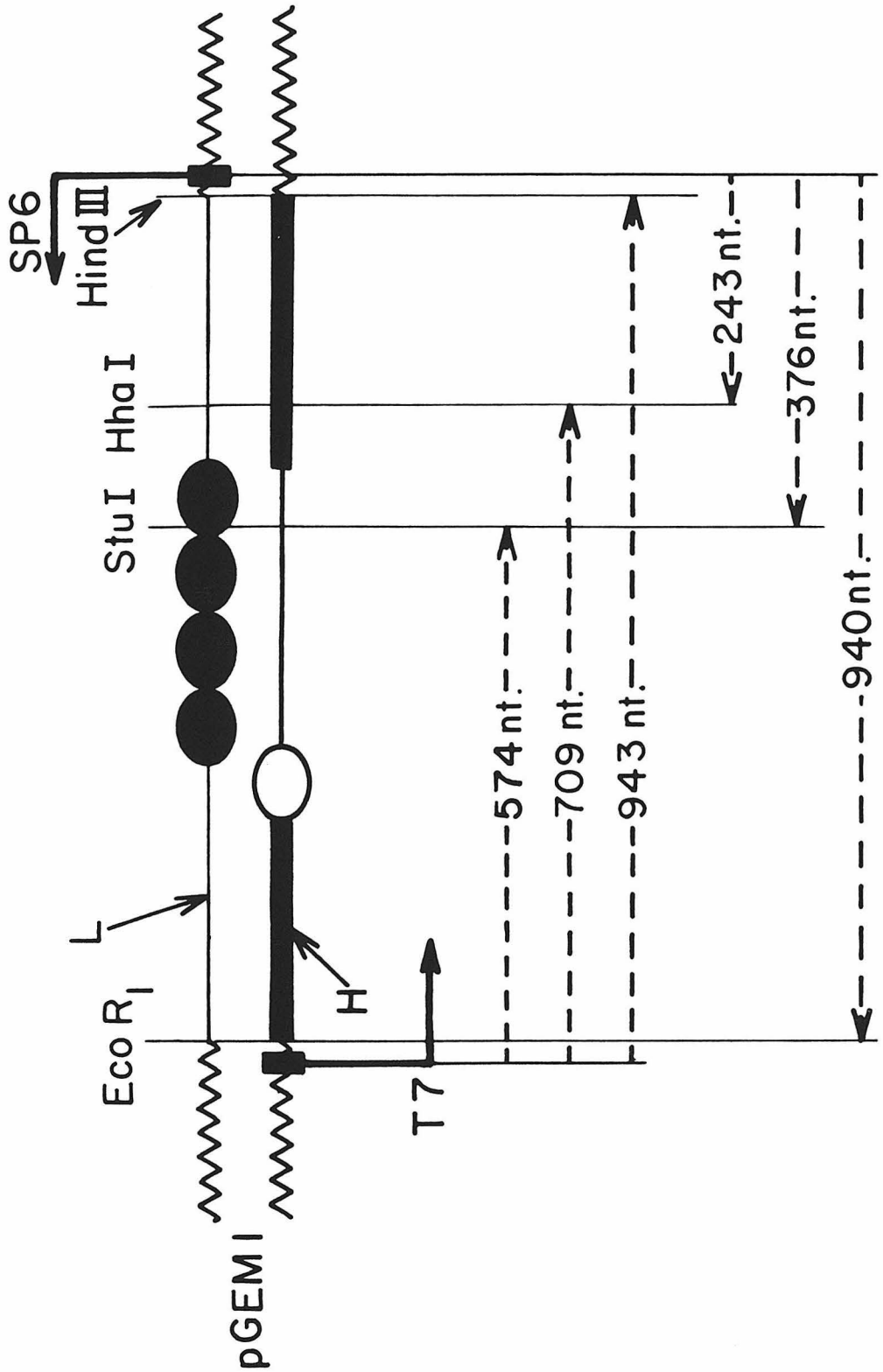
Fig. 7. Schematic of the rationale to determine the structure of RNA 9L. a) Partial sequence of RNA 9L and complete sequence of the synthetic oligodeoxynucleotide used in the RNase H and primer extension experiments. The small upward arrows indicate the approximate positions of the 5' and 3' ends of alternative 9L species detected in primer extension and S_1 experiments. b) Diagrammatic representation of the possible structures of RNA 9L and of the predicted results in RNase H and primer extension experiments.

a.


RNAase H
M
−
+
12
13
14,15
16
17
18,19
20
21
320
180
170
119

b.


A
C
G
T
TAATTTCTGTAACAGCTAAGGACT


SP6
Hind III
Stu I
Hha I
Eco R I
L
H
T7
pGEM I
574 nt.
709 nt.
943 nt.
243 nt.
376 nt.
940 nt.

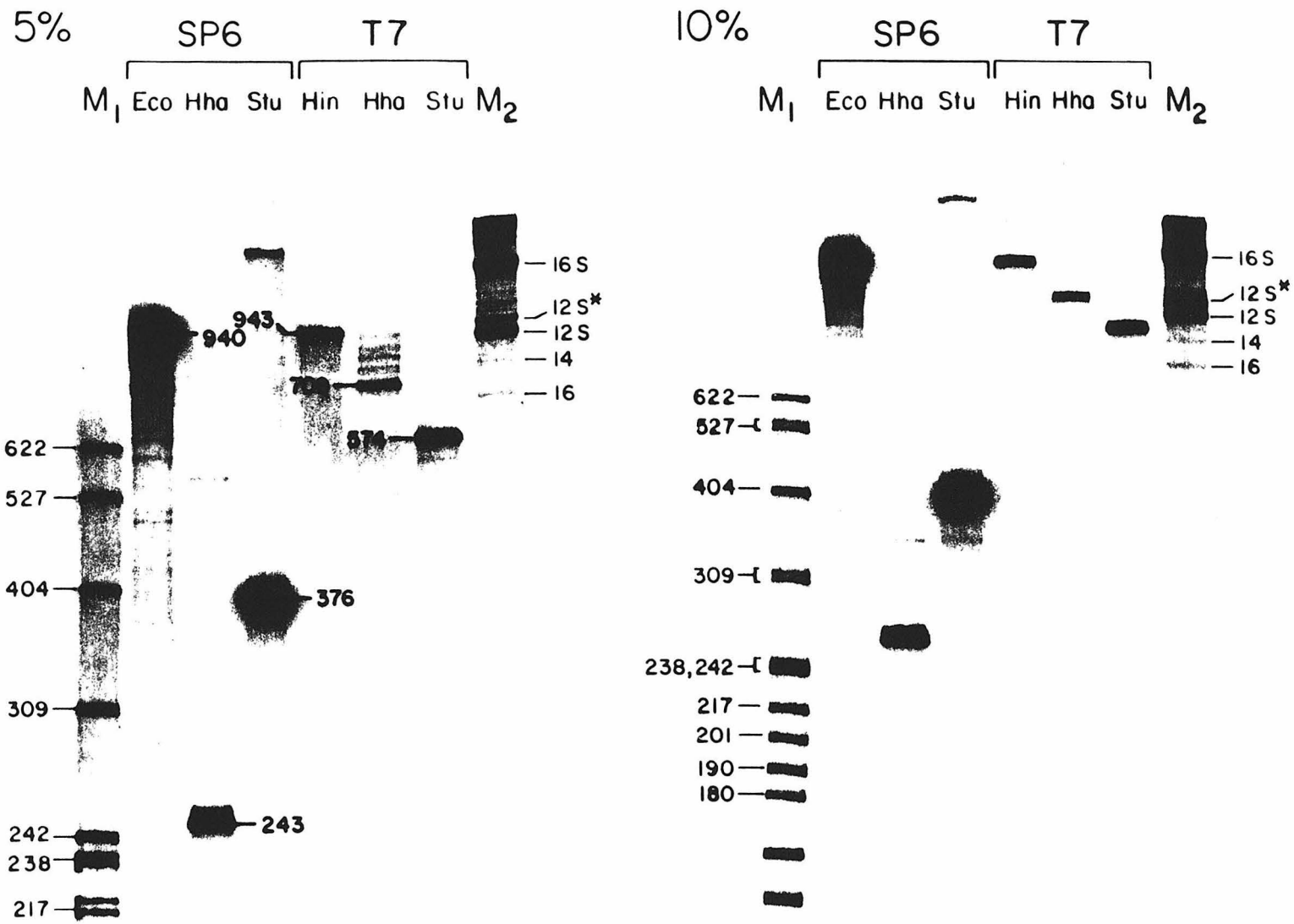

5%
SP6
T7
M1 Eco Hha Stu Hin Hha Stu M2
16 S
12 S*
12 S
14
16
943
940
622
527
404
376
309
243
242
238
217
10%
SP6
T7
M1 Eco Hha Stu Hin Hha Stu M2
16 S
12 S*
12 S
14
16
622
527
404
309
238,242
217
201
190
180

Fig. 9. Electrophoretic behavior of RNA species which contain part or all of RNA 9L or its antisense sequence. a) Diagram of plasmid pGLO I which contains the SP6 and T7 promoters and mitochondrial DNA between 5274 and 6203. Shown are the restriction sites used in the analysis. b) Autoradiographic patterns of RNA species synthesized by either the SP6 or T7 RNA polymerase using the pGLO I plasmid cut with restriction enzymes as a template. Lane M1, the electrophoretic patterns of 3'-end labeled, Msp digested pBR322 fragments, and lane M2, the electrophoretic patterns of RNA labeled in isolated mitochondria.

3'
5'
5580
5600
5620
5640
5660
5680
5700
5720
5740
5760
5780
5800
5820
5840
5860
5880
5890

Fig. 10. Computer approximation of the structure of RNA 9L as determined by the Nussinov-Jacobson program.

CHAPTER 4

Energetic Control of Mitochondrial Transcription

Abstract

HeLa cell mitochondrial DNA transcription occurs from three units, two heavy strand and one light strand. Transcription of these three units are separable in an isolated mitochondrial system. Transcribing one of the heavy-strand units results in the synthesis of the ribosomal RNA species 12S and 16S, and the other unit results in the synthesis of the mRNAs. Stimulation of all three transcription units, especially the rRNA and light strand, is caused by increasing the ATP concentration in the system, either by exogenous addition or by oxidative phosphorylation. A partial stimulation, resulting in the synthesis of heavy-strand mRNAs, but not a corresponding increase in the rRNAs or light-strand RNA synthesis, is caused by the addition of TCA cycle intermediates or pyruvate, without ADP or ATP. The addition of respiratory and ATP synthase inhibitors blocks any stimulation in transcription resulting from these processes. These studies strongly suggest an energy dependent control pathway for mitochondrial DNA transcription exists and is mediated by the level of oxidative phosphorylation occurring in the organelle and the concentration of ATP in the reaction system.

Introduction

The HeLa cell mitochondrial genome has been sequenced (1) and the mitochondrially encoded RNAs have been precisely mapped (2-5). These studies have shown that this genome is amazingly compact (6-8), with both rRNAs, 14 tRNAs, and all but one of the protein-coding RNAs transcribed from the heavy (H) strand of the DNA. The other RNA coding for a protein, the remaining eight tRNAs, and the 7S RNA are transcribed from the light (L) strand of the DNA. In addition, the major initiation sites for both H- and L-strand transcription are located within a region of the genome proximal to the D-loop (Fig. 1). As shown *in vivo* by Montoya and coworkers (9) and confirmed *in vitro* by others (10-12), the two major sites for the initiation of transcription of the H strand occur at the 5' end of the 12S rRNA and a site about 20 nucleotides 5' to the $tRNA^{phe}$. The major initiation site for L-strand transcription has been mapped *in vivo* (9) and *in vitro* (10-12, for comparison, 13) to the 5' end of the 7S RNA.

Detailed studies on the synthetic rates *in vivo* of the two rRNAs, 12S and 16S, and the majority of mRNAs (14) has shown that there appears to be two distinct RNA synthesis events occuring from the H strand. The rRNAs are synthesized at a rate 10-50 times that of the H-strand mRNAs. In addition, two RNAs, u4a and b4, mapping in the same region as the 12S and 16S rRNAs were identified (15) and shown to have synthetic rates similar to the rRNAs and the mRNAs, respectively. Since initiation of H-strand transcription occurs only 5' to the 12S rRNA, two overlapping transcription events must occur to allow for differences in synthetic rates of the mRNAs and rRNAs. One transcription event results in the synthesis of the rRNAs and terminates at the 3' end of 16S, and a second, less frequent event would continue beyond the termination point and synthesize the mRNAs of the H strand. The control of these two transcription events has been postulated to occur at the site of initiation (15). The initiation of

transcription at I_{HR} (Fig. 1) would result in the synthesis of the $tRNA^{phe}$, the 12S rRNA, the $tRNA^{val}$, and the 16S rRNA. Initiation at I_{HT} (Fig. 1) would result in the almost complete transcription of the H strand and hence, the synthesis of the H-strand mRNAs (including b4) and the remaining 12 H-strand tRNAs.

Previous experiments using an isolated mitochondrial transcription system has shown that the relative labeling of the rRNAs and the H-strand mRNAs can be altered in the presence of intercalating drugs (16). These changes are probably due in part to the different sensitivities of the two initiation events to actinomycin D, ethidium bromide or proflavin, and in part to the interference by the drugs of the two classes of RNA with other molecules, leading to the premature termination of transcription. New findings, presented here, suggest that the transcription activities from the two H-strand units can be dissociated in isolated organelles without the use of drugs.

Studies with an isolated mitochondrial transcription system has indicated that labeling of RNA is stimulated by the addition of ATP (17). Similarly, stimulation by ATP was shown *in vitro* using a solubilized mitochondrial RNA polymerase and an exogenously added template (10). Maximum stimulation of labeling *in vitro* and in isolated organelles occurred at a concentration of ATP of about 1 mM; a similar concentration of ATP was found in mitochondria rapidly isolated from cells (18). The correlation of physiological concentrations of ATP in mitochondria with those needed for maximal labeling of RNA in the two *in vitro* systems indicates that the concentration of ATP within mitochondria may have an effect on the rate of mitochondrial transcription. Results presented here suggest that the concentration of ATP, either added exogenously or generated by oxidative phosphorylation, plays a crucial role in the extent of labeling of the mitochondrial RNA.

Materials and Methods

The details of the methods used were essentially the same as described earlier (16, 17) with minor modifications. In particular, the basic incubation medium consisted of 40 mM Tris-hydrochloride (pH 7.5 at 25° C), 25 mM NaCl, 5 mM $MgCl_2$, 2 mg of bovine serum albumin (BSA) per ml, 10% glycerol, and 5 to 50 μCi of [α-^{32}P]UTP (400 to 600 Ci/mMol). Each sample contained the mitochondrial fraction from ~0.5 grams of HeLa cells and was incubated for 30 minutes at 37°C. Addition of other chemicals was done prior to resuspension of the mitochondrial pellet. The electrophoresis of labeled nucleic acids was done through a 5% polyacrylamide-7M urea gel, followed by drying and autoradiography. Hybridization of the *in vitro* labeled RNA species with the single-stranded M13 cloned human mitochondrial DNA and S_1 nuclease treatment was carried out as described in Fig. 6. The S_1-resistant products were analyzed by electrophoresis through a 5% polyacrylamide-7M urea gel, followed by autoradiography.

Results

Figure 1 shows a representation of the region of mitochondrial DNA which contains the origin of H-strand replication and the three initiation sites for mitochondrial transcription. The sites of initiation for the three transcription units (9) are marked; I_{HR} is the initiation site for the bulk of rRNA synthesis, I_{HT} is the second initiation site for H-strand transcription located at the 5' end of the 12S rRNA gene and I_L is the initiation site for L-strand transcription. As reported earlier (16), the two H-strand transcription units could be separated, as shown by mitochondrial RNA labeling in isolated organelles. The ratio of the rRNA to mRNA labeling was considerably reduced in the presence of actinomycin D, ethidium bromide and proflavin. In addition, the overall labeling was significantly

decreased in the presence of the drugs. Several results presented here suggest the separation of the two H-strand and single L-strand transcription events may occur during changes of the energetic state of the mitochondria.

The mitochondrial respiratory chain in isolated organelles can be stimulated by the addition of pyruvate or intermediates of the tricarboxylic acid (TCA) cycle (19). Furthermore, phosphorylation of ADP to ATP will also increase with respiration in coupled mitochondria if phosphate (P_i) is present. Figure 2 shows the effect of the addition of ATP or ADP with or without P_i and pyruvate on mitochondrial transcription. Isolated mitochondria were incubated in the presence or absence of 1 mM ATP, 1 mM ADP, 10 mM P, and 1 mM Pyruvate, and the incorporation of [α-^{32}P]UTP into nucleic acids was analyzed by acid precipitation. As seen previously (17), 1 mM ATP stimulates the labeling of RNA. Interestingly, the addition of 1 mM ADP increases labeling, but not to the extent as that of ATP. However, the combination of 1 mM pyruvate, 1 mM ADP and 10 mM P_i causes an increase in labeling to about the same level as ATP alone or with P_i. In a parallel fashion, the combination of 1 mM ATP, 1 mM Pyruvate, and 10mM P_i in the incubation medium increases the labeling above that seen with ATP and P_i by approximately the difference of labeling between ADP and P_i, and pyruvate, ADP, and P_i. Since roughly 50% of the 1 mM ATP is dephosphorylated to ADP and AMP during a 30 minute incubation at 37°C (17), the stimulation of labeling by pyruvate and P_i over that of ATP alone may be a consequence of the subsequent rephosphorylation of the ADP by oxidative phosphorylation.

The increase in labeling by ADP, pyruvate and P_i over that of ADP and P_i might be caused by a stimulation in the respiratory activity of the isolated mitochondria by the addition of an oxidizable substrate. To further investigate this possibility, pyruvate and two intermediates of the TCA cycle, succinate and citrate were tested in the transcription assay. The results are shown in Fig. 3.

Overall labeling of RNA is increased by a factor of about 10 in the presence of both phosphate and one of the oxidative substrates. Addition of either P_i or one substrate alone has no effect on the basal labeling. Furthermore, the increase in labeling appears to uniformly affect mRNAs and rRNAs, as seen in the relative intensities of bands corresponding to mRNAs 14 and 16 and rRNAs 12S* and 12S.

In order to determine the relative contribution to the increase in transcription by pyruvate, P_i and ADP shown in Fig. 2 and Fig. 3, constant concentrations of pyruvate and P_i were added to the isolated mitochondria transcription mixtures with increasing amounts of ADP. The results are shown in Fig. 4. Increasing concentrations of ADP in the presence of P_i and pyruvate stimulates the labeling of mRNA (for example, mRNAs 14, 15, 16) ~2 fold, while increasing the labeling of the rRNA bands (as typified by 12S* and 12S) ~10 fold. In fact, the increase in rRNA labeling is considerably more than 10 fold, since no attempt to estimate the contribution of RNA 12 and RNA 13 to the bands 12S* and 12S, respectively.

The results described above suggest that the stimulation of transcription can be separated into two levels, based on the differential sensitivities of the H-strand transcription events. The labeling of mRNAs is stimulated at low ATP concentrations, while labeling of rRNAs to their high levels requires high ATP concentrations. The stimulation of respiration by the TCA intermediates or pyruvate and P_i probably causes the phosphorylation of the endogenous ADP (C. Rossi, unpublished results), which results in an increase in the intramitochondrial pool of ATP. Alternatively, exogenously added ADP without oxidative substrates can be transformed into ATP and AMP by adenylate kinase, or phosphorylated through substrate level phosphorylation (20). When ADP, P_i and a substrate is present, mitochondrial respiration and ATP synthase activities are increased resulting in high levels of endogenous ATP, and labeling of mitochondrial RNA is equivalent to that seen in the presence of exogenously added ATP.

As reported before (17), the extent of oligoadenylation of mRNAs labeled in isolated mitochondria was dependent upon the concentration of ATP present in the incubation mixture. Figure 5 shows a comparison of RNA labeled in isolated mitochondria in the presence of (a) 1 mM ATP and 10 mM P_i or (b) 2 mM Na pyruvate and 10 mM P_i, from the total (T), unbound (U)—not binding to oligo(dT) cellulose, and bound (B)—binding to oligo(dT) cellulose, fractions. The addition of 1 mM ATP results in about a 2-fold greater labeling of the mRNA species than mitochondria in 2 mM pyruvate. However, the intensities of the bands corresponding to 12S, 12S*, and 16S rRNAs are about 10-fold higher in the ATP stimulated patterns compared with those of pyruvate, as seen in Fig. 3. The bound patterns are also very different; an oligo(A) stretch of greater than 20 nucleotides as required for bindng to oligo(dT) cellulose is much more efficiently added in the presence of 1 mM ATP than pyruvate and P_i. This is most easily seen in a comparison of the bands corresponding to RNA 17. The non-oligoadenylated (17u) and oligoadenylated (17b) forms of RNA 17 are easily separable in this gel system (Chapter 3, this work; J. Montoya and G. Gaines, unpublished results). In the presence of any of the three oxidizable substrates and P_i alone (Fig. 3), the labeling of RNA 17b is essentially nonexistent. However, when increasing concentrations of ADP are added to an incubation mixture with pyruvate and P_i or increasing concentrations of ATP are added to any labeling mixture, the mitochondria polyadenylate an increasing percentage of the labeled RNA 17. Therefore, both processes of labeling and oligoadenylation of the RNAs require ATP, either added exogenously or produced by oxidative phosphorylation. Interestingly, however, the oligoadenylation of the H-strand RNAs continues to increase with higher concentrations (greater than 2 mM) of exogenously added ATP (17), whereas, labeling of the mRNA species decreases.

The results shown in Fig. 3 and Fig. 5 indicate that mitochondria in the presence of pyruvate and P_i synthesize a higher percentage of the total RNA from the mRNA transcription unit than do mitochondria in the presence of pyruvate, P_i and ADP or ATP. In order to test this quantitatively and to avoid the ambiguities of overlapping bands in polyacrylamide-urea gels, S_1 nuclease protection analysis was performed on RNA labeled in mitochondria incubated in media with 1 mM ATP and 10 mM P_i, or 2 mM Na pyruvate and 10 mM P_i. Figure 1 shows the expected sizes of RNA protected with single-stranded M13 DNAs containing either the H strand (M8.9) or the L strand (M9.9) of the MboI fragment of mitochondrial DNA from the origin region (1-740). In addition, a single-stranded M13 DNA containing the H-strand fragment between 7661-8290 (OP-10), which protects 629 nucleotides of RNA 16 was used in these experiments. Figure 6 shows the electrophoretic patterns of S_1 analysis performed on RNAs labeled in the two media and protected with the three M13 clones as indicated. Each M13 clone specifically protects RNA molecules of distinct sizes, as shown in the first four lanes after the marker (M_1).

When the H-strand clone of the origin region, M8.9, is used for hybridization, bands of 96 nt and 183 nt specifically appear corresponding to the protection of the 12S and 12S* RNAs, respectively. Protection with the L-strand counterpart, M9.9, shows a specific band at 408 nt corresponding to the L-strand transcript from the 5' end of 7S RNA to the end of the mitochondrial DNA in the clone and a group of bands between 155 and 210 nt corresponding to the heterogeneous 7S RNA and possibly the L-strand transcript from which 7S has been cleaved (7S extension). Finally, the OP-10 clone protects the majority of RNA 16, giving a band at 629 nt. An assessment of the relative amounts of labeling of RNA from the three different transcription units, two H strand and one L strand, was achieved by using both the OP-10 clone and either the M8.9 or M9.9

clone in the same sample to hybridize total mitochondrial RNA labeled in the presence of 1 mM ATP and P_i or 2 mM pyruvate and P_i, and then protect the RNA during S_1 digestion. The results of this experiment are shown in Fig. 6. In the presence of ATP, the ratio of the sum of the radioactivity in the bands corresponding to the two rRNAs protected by M8.9 to the band corresponding to RNA 16 protection by OP-10 is about 1.5, as determined by densitometry. Also, the relative labeling of the bands corresponding to the protection by M9.9, 7S RNA and 7S extension RNA, compared with that of the band corresponding to RNA 16 protection, is approximately 7. In contrast, when RNA labeled in isolated mitochondria in the presence of pyruvate and P_i is hybridized to either M8.9 or M9.9 and OP-10 and S_1 nuclease treated, the ratio of the intensity of the bands for 12S and 12S* to RNA 16 is about 0.2 and 7S and 7S extension to RNA 16 is approximately 0.1. Hence, the labeling of the RNAs from the three different transcription units can change relative to each other depending upon the type of energetic substrates present.

It is difficult to quantitate the actual contribution of each H-strand transcription unit to the bands protected by M8.9. Clearly the great majority of the two bands in the ATP labeled RNA is due to either 12S* (183 nt band) or 12S (96 nt band). Compared with 12S, the contribution of b4, representing the mRNA transcription unit would be very small, considering its labeling efficiency and its length (15). Therefore, the nearly parallel reduction in radioactivity of both the 183 nt and 96 nt bands in comparing the ATP and pyruvate labeled patterns represents the greatly reduced labeling of the two rRNA species while the contribution made by 4b to the intensity of the 96 nt band remains constant. Separate similar experiments show that the relative intensities of the bands at 183 and 96 nt fluctuate depending upon the efficiency of processing in the mitochondria for 12S*→12S (data not shown).

The use of antimycin and rotenone in the transcription mixture further supported the involvement of mitochondrial respiration and oxidative phosphorylation in the observed stimulation of transcription by the TCA cycle intermediates and pyruvate. Antimycin, an antibiotic, inhibits the oxidation of both pyruvate and succinate by inhibiting the electron transport chain at site 2 (21, 22). Rotenone, an insecticide, inhibits only the oxidation of pyruvate by inhibiting the electron transport chain at site 1 (23). Hence, the increase in transcription caused by succinate should be inhibited only by antimycin, while the increase with pyruvate should be inhibited by both drugs. Figure 7 shows the results of labeling RNA in the presence of either pyruvate or succinate and inhibiting the respiratory chain at specific sites using antimycin or rotenone. As predicted, stimulation of RNA labeling in isolated mitochondria by pyruvate is blocked with both rotenone and antimycin, whereas, stimulation by succinate is only blocked with antimycin.

The second phase in stimulation of RNA labeling, that resulting in a large increase in rRNA synthesis, was suggested to be due to a substantial change in the ATP concentration, either generated by oxidative phosphorylation or exogenous addition. The drug oligomycin selectively inhibits the ATPase in mitochondria, and therefore blocks oxidative phosphorylation of ADP. Figure 8 shows the acid precipitable radioactivity of transcription reactions in isolated mitochondria stimulated by ADP or ATP with or without pyruvate and P_i. The addition of antimycin or oligomycin to the transcription system blocks the further stimulation of labeling observed with ADP, pyruvate, and P_i over that of ADP alone. Labeling of RNA in the presence of ATP is essentially unaffected by either drug, however, antimycin does decrease the additive effect of pyruvate and P_i. The results further indicate that stimulation of RNA labeling in isolated mitochondria by ADP, pyruvate, and P_i requires both electron transport and phosphorylation

activities suggesting that this stimulation is caused by the increased production of ATP. In addition, the need for ATP can be supplied by the exogenous addition of the nucleotide.

Discussion

Evidence is presented here which indicates that the labeling of RNA in isolated mitochondria is increased by either the addition of exogenous ATP, as shown before (17), or the stimulation of oxidative phosphorylation. The increase caused by the addition of exogenous ATP is unaffected by inhibitors of respiration and only slightly affected by the inhibitor of mitochondrial ATPase, oligomycin. On the other hand, stimulation of labeling by an increase in the respiratory capacities of the mitochondria through the addition of TCA cycle intermediates or pyruvate is blocked by drugs which inhibit electron transport and the mitochondrial ATPase (not shown). Furthermore, the additional increase in RNA labeling by stimulating respiration with pyruvate and P_i in the presence of ADP was blocked by oligomycin and antimiycin.

The stimulation of labeling of RNA in isolated mitochondria most likely represents the increase in transcription by these organelles. The uptake of radioactive precursor by mitochondria in the presence of 1 mM ATP was roughly twice that of mitochondria in buffer without ATP (data not shown). Therefore, the increase in labeling caused by the stimulation of oxidative phosphorylation or exogenous addition of ATP was not due to a large change in uptake. The possibility does exist, however, that the radioactivity is taken up by mitochondria as [α-^{32}P]UMP or [α-^{32}P]UDP, and then phosphorylated to [α-^{32}P]UTP, thereby entering the pool of nucleotides used by the RNA polymerase. If this were the case, stimulation of a UMP or UDP kinase activity by ATP would cause an increase in labeling without specifically changing the rate of transcription. This

mechanism would not explain the different sensitivities of the three transcription units to ATP levels, either added or generated. Hence, although some of the increase of RNA labeling may be due to an increase in uptake of nucleotide precursor and/or its introduction into the pool used by the RNA polymerase, the majority and specificity of the stimulation in labeling is most probably due to changes in the transcriptional activities of the mitochondria. It appears likely that the two H-strand transcription events are differentially sensitive to the concentration of ATP in the organelle. These differences probably reflect a mechanism for selection by the polymerase of either the I_{HT} or I_{HR} initiation sites. Further work will elucidate the details of this mechanism.

The assumption that the rate of transcription of one mRNA, RNA 16, used in the S_1 protection analysis, represents the rate of all H-strand mRNAs synthesized is based upon the kinetic properties of mitochondrial RNAs previously described in this laboratory (14). In addition, the relative labeling of all the mRNAs separable in denaturing polyacrylamide gels, regardless of the presence or absence of oxidizable substrates or nucleotides in the incubation mixtures, remained constant. There was noted, however, a difference in the oligoadenylation of different mRNA species depending upon the incubation conditions. In the presence of 1 mM ATP, isolated mitochondria oligoadenylate up to 50% of the newly synthesized RNA 17, whereas about 10-15% of the bulk of the mRNAs are bound by oligo(dT) cellulose (17). The reason for this heterogeneity remains unclear.

Although the majority of this work was intended to study the control of H-strand transcription in mitochondria, several observations were made involving that of the L strand. First, the synthesis of an RNA corresponding to an L-strand transcript extending from the 5' end of 7S RNA to beyond the MboI site at 1 (1) was shown by S_1 protection analysis. This RNA, 7S extension, appeared to label

with similar efficiencies as the 7S RNA in the two different transcription assays (one with 1 mM ATP and one with 2 mM pyruvate). Several possibilities exist for this RNA 7S extension; it may represent the kinetic precursor to RNA 7S or it may be unrelated and allow the light-strand transcription to proceed beyond to origin region in a function analogous to RNA b4 (15). It is as yet unknown whether the3' end of 7S is formed through processing, termination or a combination of both. Considering 7S RNA's potential role in replication (24), the relationship between these two RNA species may determine the potential coordination between control of L-strand transcription and DNA synthesis.

Acknowledgements

We thank Mike King for his generous gifts of the M13 clones used in this study. We also thank Ram S. Puranam for his critical reading of the manuscript. The technical assistance of Ms. Arger Drew is gratefully acknowledged.

References

1. Anderson, S., Bankier, A. T., Barrell, B. G., deBruijn, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. H. H., Staden, R., and Young, I. G. 1981. Sequence and organization of the human mitochondrial genome. *Nature* **290**, 457-465.
2. Montoya, J., Ojala, D., and Attardi, G. 1981. Distinctive features of the 5'-terminal sequences of the human mitochondrial mRNAs. *Nature* **290**, 465-470.
3. Ojala, D., Crews, S., Montoya, J., Gelfand, R., and Attardi, G. 1981. A small polyadenylated RNA (7S RNA), containing a putative ribosome attachment site, maps near the origin of human mitochondrial DNA replication. *J. Mol. Biol.* **150**, 303-314.
4. Cantatore, P., and Attardi, G. 1980. Mapping of nascent light and heavy strand transcripts on the physical map of HeLa cell mitochondrial DNA. *Nucl. Acids Res.* **8**, 2605-2625.
5. Ojala, D., and Attardi, G. 1980. Fine mapping of the ribosomal RNA genes of HeLa cell mitochondrial DNA. *J. Mol. Biol.* **138**, 411-420.
6. Crews, S., and Attardi, G. 1980. The sequence of the small ribosomal RNA gene and the phenylalanine tRNA gene are joined end to end in human mitochondrial DNA. *Cell* **19**, 775-784.
7. Attardi, G. 1981. Organization and expression of the mammalian mitochondrial genome: a lesson in economy. *Trends in Biochemical Science* **6**, Part I, 86-89; Part II, 100-103.

8. Attardi, G., Cantatore, P., Chomyn, A., Crews, S., Gelfand, R., Merkel, C., Montoya, J., and Ojala, D. 1982. A comprehensive view of mitochondrial gene expression in human cells. In Mitochondrial Genes. G. Attardi, P. Borst, and P. Slonimski (eds.). Cold Spring Harbor, New York, pp. 51-71.

9. Montoya, J., Christianson, T., Levens, D., Rabinowitz, M., and Attardi, G. 1982. Identification of initiation sites for heavy strand and light strand transcription in human mitochondrial DNA. *Proc. Natl. Acad. Sci. USA* **79**, 7195-7199.

10. Shuey, D. J., and Attardi, G. 1985. Characterization of an RNA polymerase activity from HeLa cell mitochondria, which initiates transcription at the heavy strand rRNA promoter and the light strand promoter in human mitochondrial DNA. *J. Biol. Chem.* **260**, 1952-1958.

11. Bogenhagen, D. F., Applegate, E. F., and Yoza, B. K. 1984. Identification of a promoter for transcription of the heavy strand of human mtDNA: *In vitro* transcription and deletion mutagenesis. *Cell* **36**, 1105-1113.

12. Chang, D. D., and Clayton, D. A. 1984. Precise identification of individual promoters for transcription for each strand of human mitochondrial DNA. *Cell* **36**, 635-643.

13. Yaginuma, K., Kobayashi, M., Taira, M., and Koike, K. 1982. A new RNA polymerase and *in vitro* transcription of the origin of replication from rat mitochondrial DNA. *Nucl. Acids. Res.* **10**, 7531-7542.

14. Gelfand, R., and Attardi, G. 1981. Synthesis and turnover of mitochondrial ribonucleic acid in HeLa cells; the mature ribosomal and messenger ribonucleic acid species are metabolically unstable. *Molec. Cell Biol.* **1**, 497-511.

15. Montoya, J., Gaines, G., and Attardi, G. 1983. The pattern of transcription of the human mitochondrial rRNA genes reveals two overlapping transcription units. *Cell* **34**, 151-159.

16. Gaines, G., and Attardi, G. 1984. Intercalating drugs and low temperatures inhibit synthesis and processing of ribosomal RNA in isolated human mitochondria. *J. Mol. Biol.* **172**, 451-466.

17. Gaines, G., and Attardi, G. 1984. Highly efficient RNA-synthesizing system that uses isolated human mitochondria: new initiation events and *in vivo*-like processing patterns. *Molec. Cell Biol.* **4**, 1605-1617.

18. Bestwick, R. K., Moffett, G. L., and Mathews, C. K. 1982. Selective expansion of mitochondrial nucleoside triphosphate pools in anti-metabolite-treated HeLa cells. *J. Biol. Chem.* **257**, 9300-9304.

19. Chance, B., and Williams, G. R. 1955. Respiratory enzymes in oxidative phosphorylation. I. Kinetics of oxygen utilization. *J. Biol. Chem.* **217**, 383-393.

20. Lehninger, A. 1975. Biochemistry, 2nd edition. Worth Publishers, Inc., New York, pp. 417-459.

21. Chance, B. 1958. The kinetics and inhibition of cytochrome components of the succinic oxidase system III cytochrome b. *J. Biol. Chem.* **233**, 1223-1229.

22. Rieske, J. S. 1971. Changes in oxidation-reduction potential of cytochrome b observed in the presence of antimycin A. *Arch. Biochem. Biophys.* **145**, 179-193.

23. Storey, B. T. 1981. Inhibitors of Mitochondrial Function. M. Erecinska, and D. F. Wilson (eds.). Pergamon Press, Oxford, pp. 101-108.

24. Chang, D. D., and Clayton, D. A. 1985. Priming of the human mitochondrial DNA replication occurs at the light-strand promoter. *Proc. Natl. Acad. Sci. USA* **82**, 351-355.

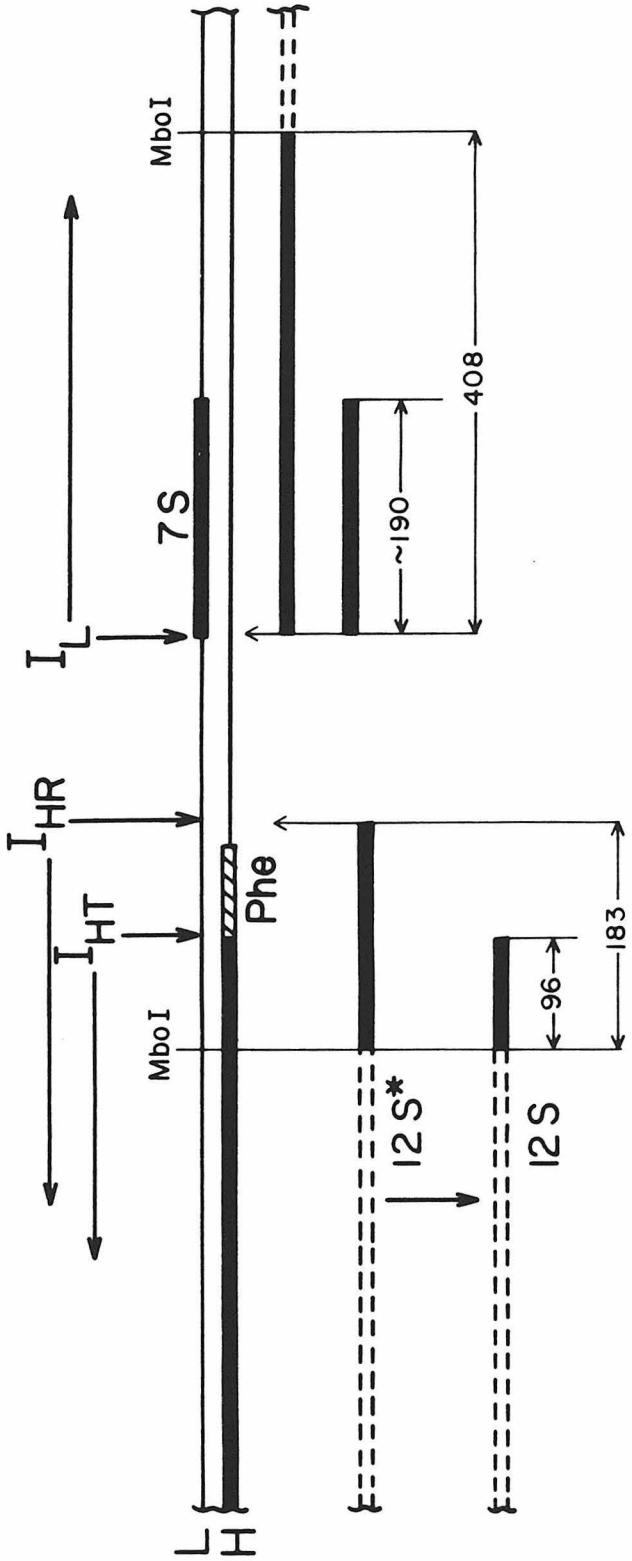

MboI
408
~190
7S
I_L
I_{HR}
I_{HT}
Phe
183
96
MboI
12S*
12S
L
H

Fig. 1. Portion of the HeLa cell mtDNA genetic and transcription maps illustrating the region near the origin of H-strand replication involved in initiation of transcription (1, 4, 9). The leftward and rightward heavy arrows indicate the direction of H- and L-strand transcription, respectively, and the downward heavy arrows indicate the initiation sites for H- (I_{HR} and I_{HT}) and L-strand transcription (I_L). The expected sizes of S_1 protection experiments are shown for the 12S and 12S* RNAs of the H-strand transcripts and the 7S and 7S extension of the L-strand transcripts. The two MboI sites in this region are shown.

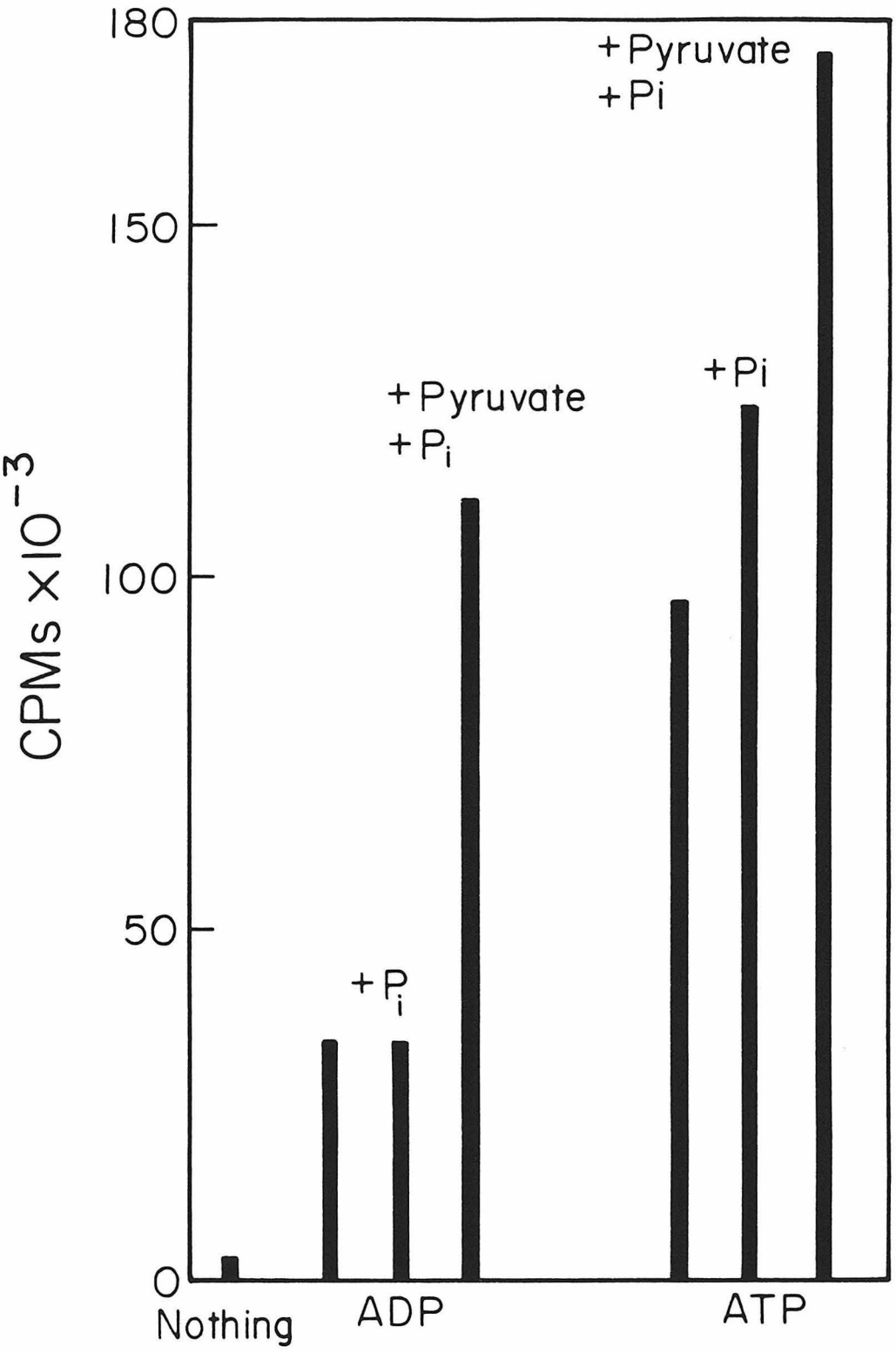

180
150
100
50
0
CPMs X10^{-3}
+Pyruvate
+Pi
+Pyruvate
+Pi
+Pi
+Pi
Nothing
ADP
ATP

Fig. 2. Comparison of the effects of ADP, ATP, pyruvate and phosphate on the acid precipitable radioactivity upon labeling of RNA in isolated mitochondria. 10% (2 μ) of each sample of labeled RNA was added to 1 ml of 1 M HCl, 50 mM NaH_2PO_4, and 2 μg of salmon sperm DNA, incubated at 0°C for 20 minutes, precipitated onto glass fiber filters, and the filters counted in scintillation fluid. Shown is the radioactivity from mitochondrial RNA labeled in the presence or absence of 1 mM ADP, 1 mM ATP, 1 mM sodium pyruvate, and 10 mM NaH_2PO_4 (P_i) (pH 7.6).

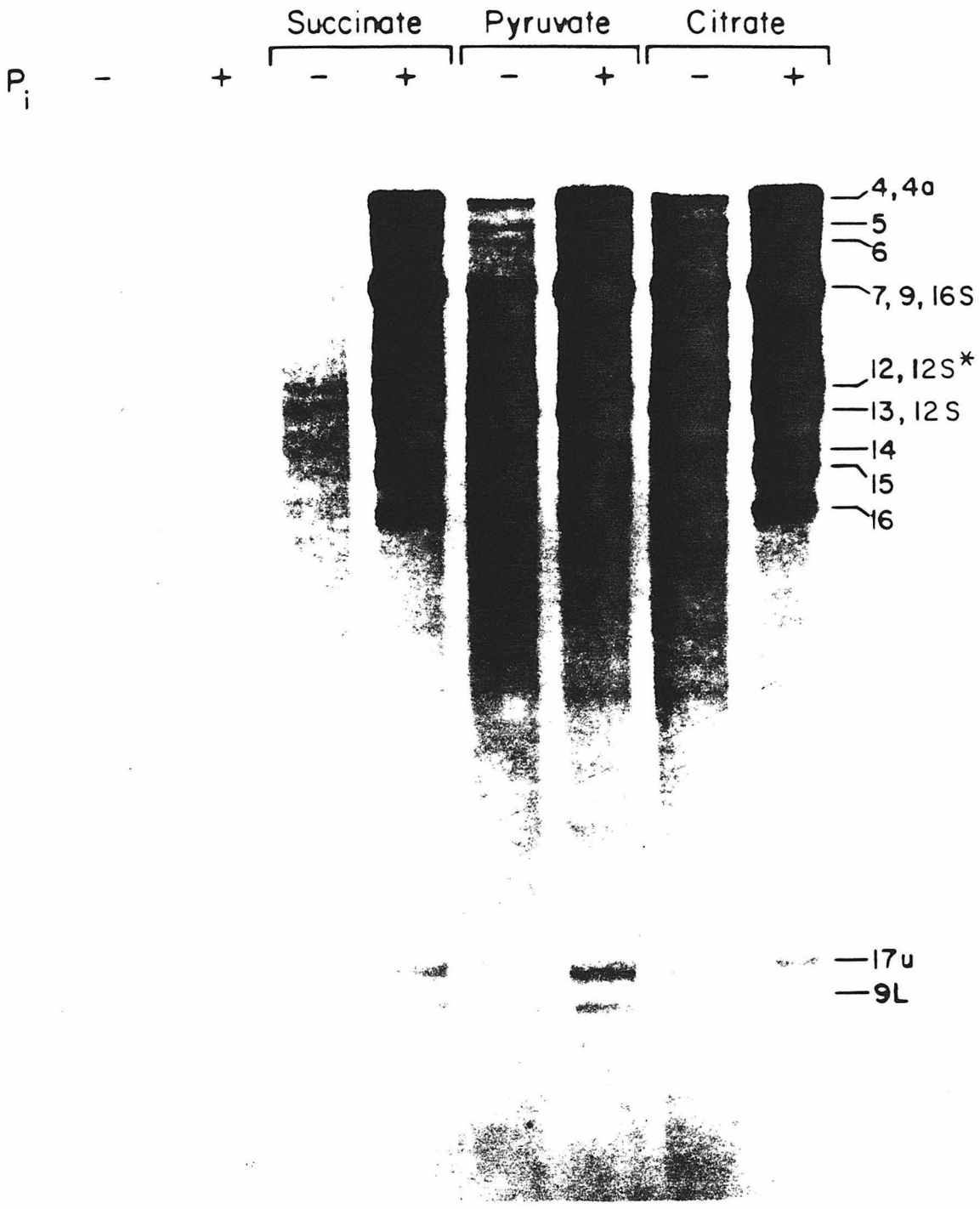

Succinate
Pyruvate
Citrate
P_i - + - + - + - +
4, 4a
5
6
7, 9, 16S
12, 12S*
13, 12S
14
15
16
17u
9L

Fig. 3. Comparison of the effects of phosphate, succinate, pyruvate, and citrate upon *in vitro* mitochondrial RNA labeling. Mitochondria were incubated as usual in the presence and absence of 10 mM Na_2HPO_4 (pH 7.6), 1 mM sodium succinate, sodium pyruvate, or sodium citrate as marked. The total labeled RNA was electrophoresed through a 5% polyacrylamide-7M urea gel and autoradiographed. Equal amounts of mitochondrial RNA was applied to each lane, the major RNA bands are marked.

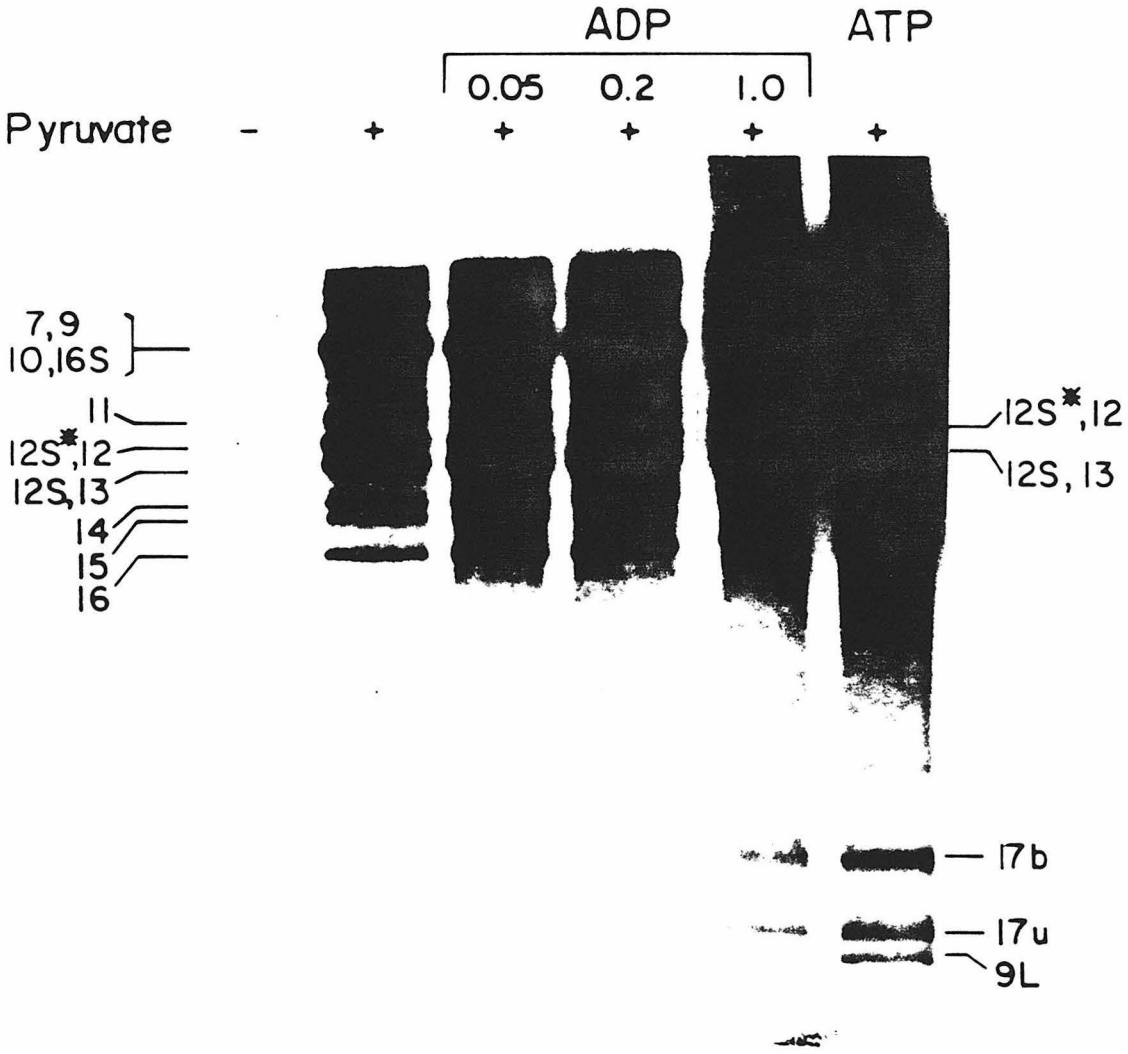

ADP
ATP
0.05
0.2
1.0
Pyruvate
-
+
+
+
+
+
7,9
10,16S
11
12S*,12
12S,13
14
15
16
12S*,12
12S, 13
17b
17u
9L

Fig. 4. Electrophoretic analysis of the effect of ADP on mitochondrial RNA labeling in the presence of pyruvate and P_i. RNA was labeld with [α-^{32}P]UTP in isolated mitochondria in the presence of 10 mM P_i and 2 mM pyruvate (+) with varying concentrations of ADP, as noted in MM at the top of the lanes. The ATP concentration used was 2.0 mM in the lane on the right. The RNA was electrophoresed through a 5% polyacrylamide-7M urea gel. The important RNA species are shown.

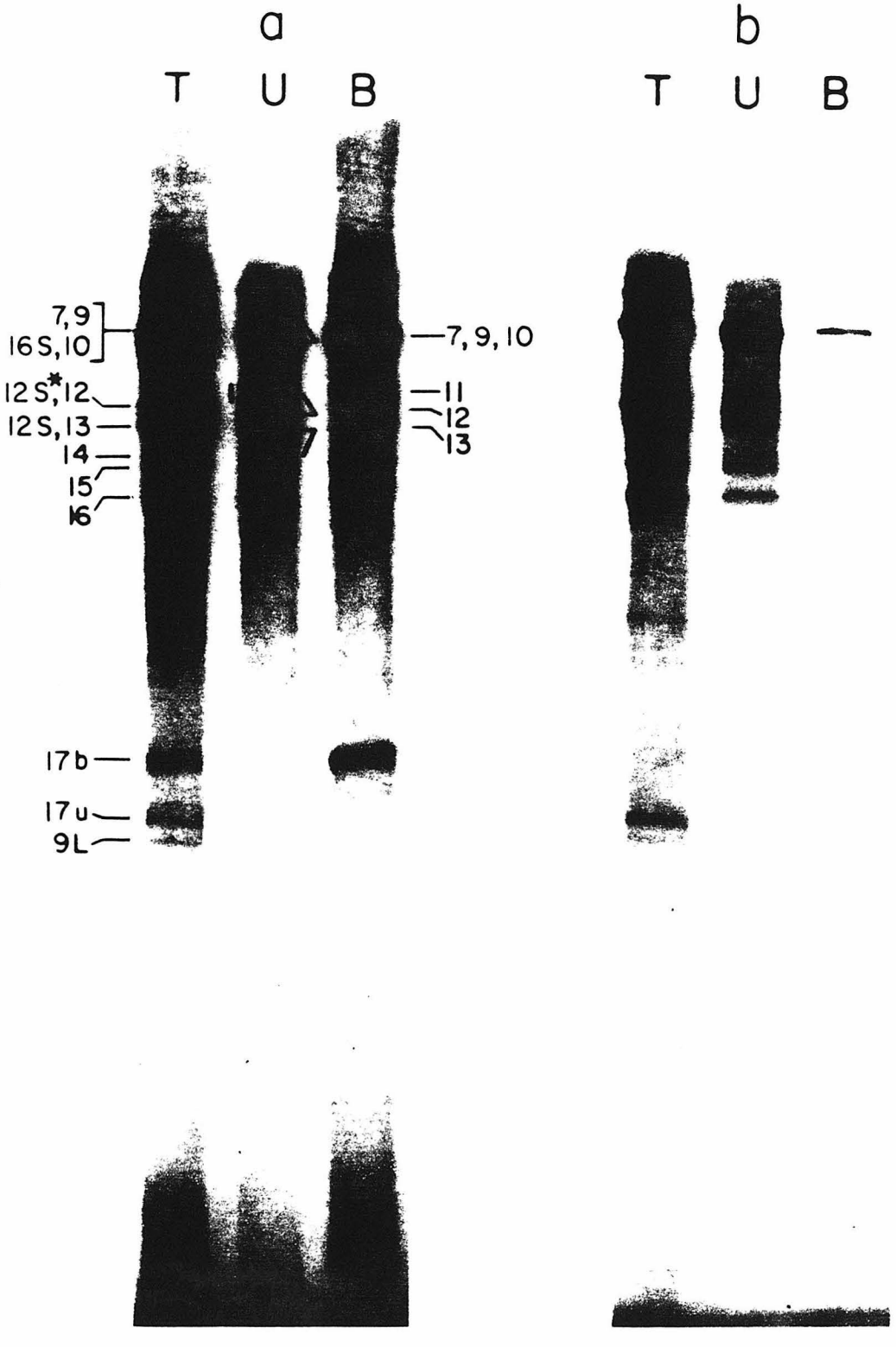

a
T
U
B
b
T
U
B
7,9
16S,10
12S*,12
12S,13
14
15
16
17b
17u
9L
7,9,10
11
12
13

Fig. 5. Comparison of HeLa cell mtDNA transcription in isolated organelles incubated in the presence of 1 mM ATP or 2 mM sodium pyruvate. Mitochondrial RNA was labeled with [α-^{32}P]UTP in isolated organelles in the presence of 10 mM Na_2HPO_4 (pH 7.6) with either (a) 1 mM ATP or (b) 2 mM sodium pyruvate, the RNA extracted and fractionated on an oligo(dT)-cellulose column. The total RNA (T), unbound (U) and bound (B) fractions were separated electrophoretically through a 5% polyacrylamide-7M urea gel. Tenfold more mitochondrial RNA was applied to the bound (B) lanes than the rest. Equal amounts of mitochondrial nucleic acids were applied to the corresponding lanes in a and b. The important RNA species are marked.

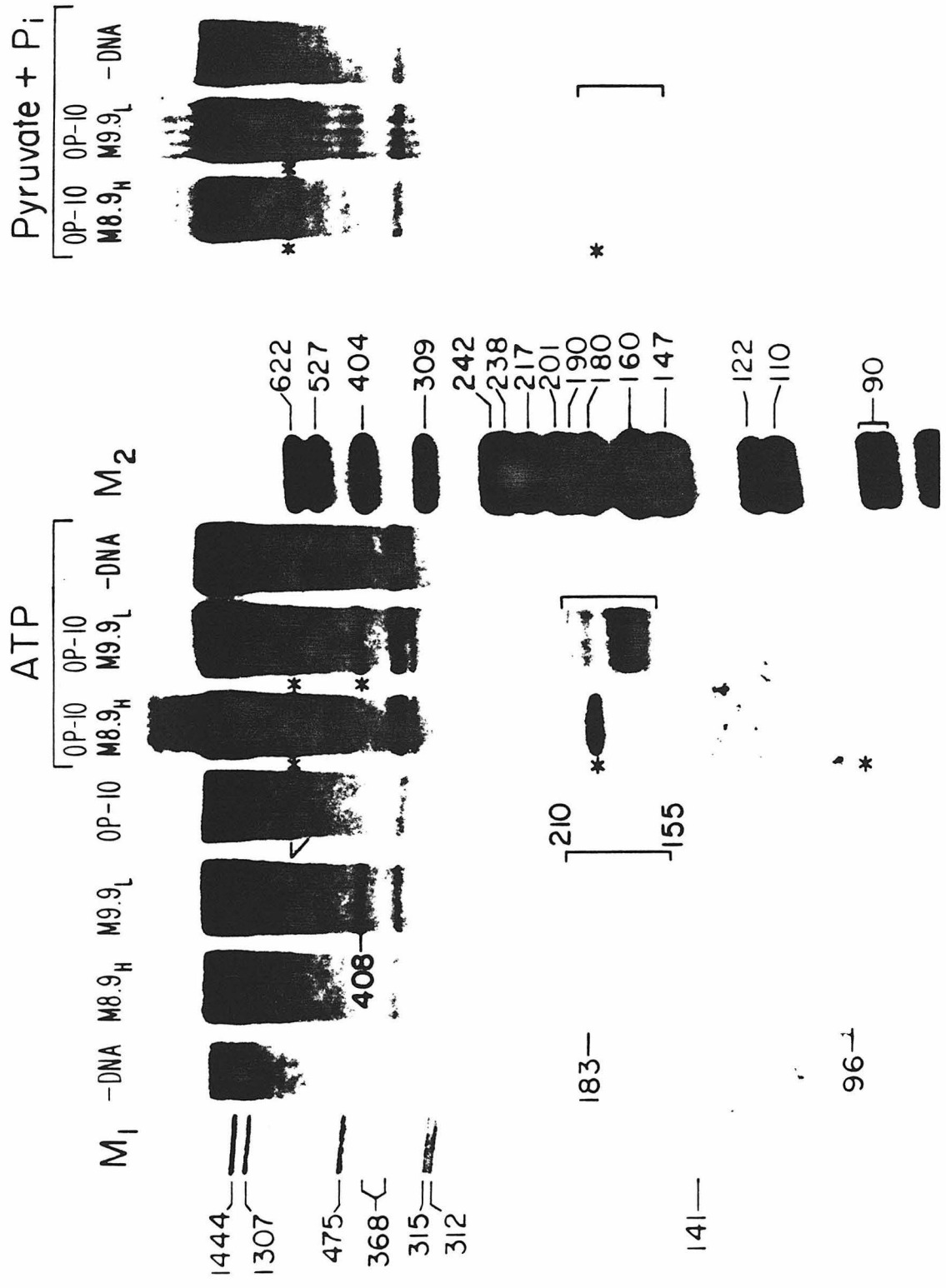

ATP
Pyruvate + Pi
M1
M2
-DNA
M8.9H
M9.9L
OP-10
1444
1307
475
368
315
312
408
210
155
183
141
96
622
527
404
309
242
238
217
201
190
180
160
147
122
110
90

Fig. 6. S_1 nuclease analysis of mtRNA *in vitro* labeled in the presence of 1 mM ATP or 2 mM sodium pyruvate to determine the relative amounts of H-strand mRNA and rRNA and L-strand RNA labeling. mtRNA was labeled with [α-^{32}P]UTP by incubation of isolated mitochondria in the presence of 1 mM ATP and 10 mM Na_2HPO_4 (pH 7.6) (P_i) or 2 mM sodium pyruvate and 10 mM P_i and purified by the standard techniques mentioned in the Materials and Methods. The RNA was mixed with single-stranded M13 clones (a generous gift of Mike King) designated M8.9$_H$ [containing the H strand of the MboI fragment between nucleotides 4 and 743 in mtDNA (1)], M9.9$_L$ [containing the L strand of the MboI fragment between 0 and 739 in mtDNA, and OP-10 (containing the BclI to XbaI sites at 7661-8290 of the H strand of mtDNA]. The second through fifth lanes shows labeled RNA hybridized to one M13 clone, as noted, S_1 nuclease treated as described earlier (17), electrophoresed in a 5% polyacrylamide-7M urea gel and the gel autoradiographed. The RNA in the lanes marked ATP was labeled in the presence of 1 mM ATP and 10 mM P_i, purified and hybridized to the clones indicated and treated as the second through fifth lanes. The RNA in the lanes marked pyruvate + P_i was labeled in the presence of 2 mM sodium pyruvate and 10 mM P_i, hybridized to the clones indicated and treated as in lanes 2-5. The expected bands are marked. When protected with M8.9$_H$, the RNA has two specific bands remaining, one of 183 nucleotides corresponding to the 5'-end protection of 12S* and u4a, and one of 96 nucleotides corresponding to the 5'-end protection of 12S and b4 (Fig. 1). When protected with M9.9$_L$, the samples show one band at 408 nucleotides corresponding to a continuation of L-strand transcription beyond the MboI site, and a group of bands at 155 to 210 corresponding to the protection of 7S RNA and possibly a processing fragment from the continuation of the L-strand transcription. When protected with OP-10 the specific band at 629 nucleotides occurs corresponding to the almost complete protection of RNA 16. The markers

are digested pBR322 (M_1) and Msp digested pBR322 (M_2) labeled with [α-^{32}P]dGTP and [α-^{32}P]dCTP. The asterisks show the corresponding protected RNA fragments in the samples protected with more than one clone.

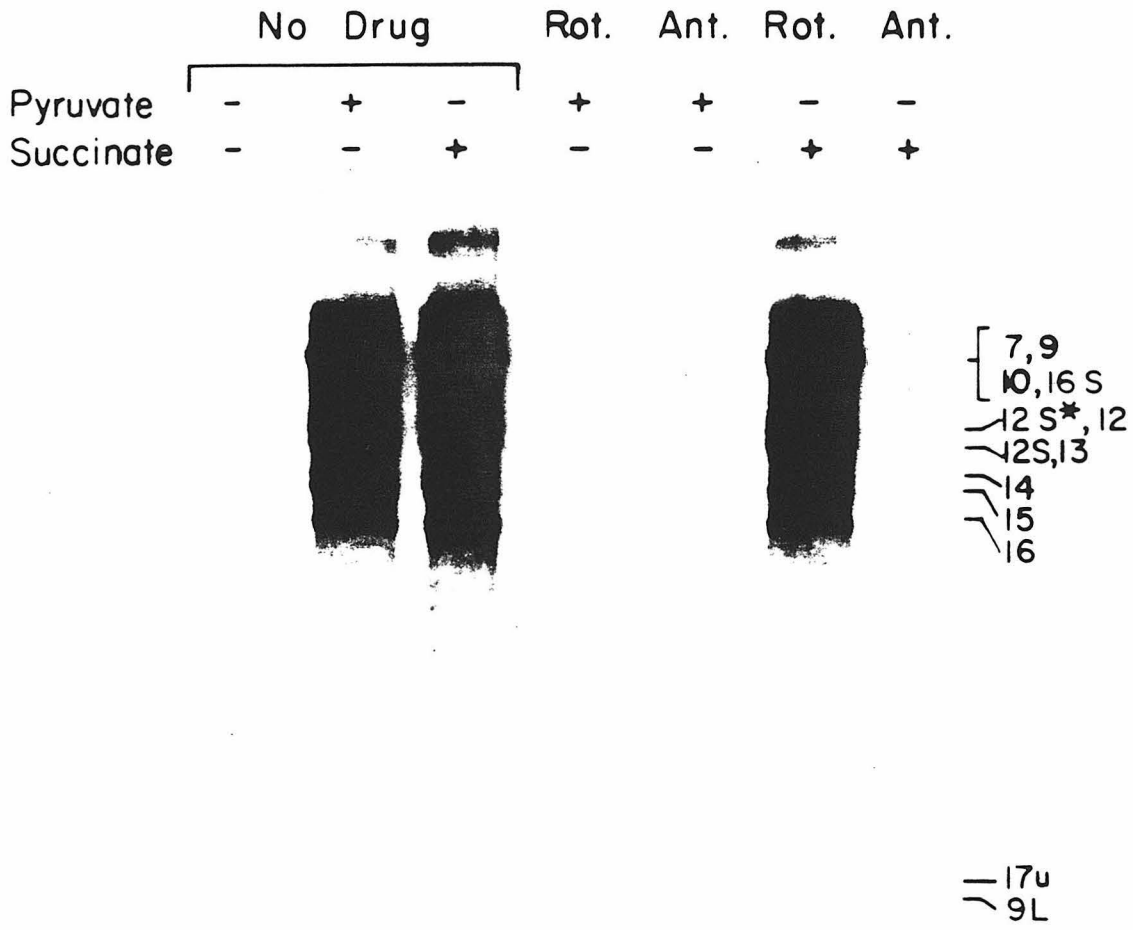

No Drug
Rot.
Ant.
Rot.
Ant.
Pyruvate - + - + + - -
Succinate - - + - - + +
7, 9
10, 16 S
12 S*, 12
12S, 13
14
15
16
17u
9L

Fig. 7. Blocking of the stimulation in labeling caused by pyruvate and succinate. RNA was labeled in isolated mitochondria in the presence of 10 mM P_i and either 2 mM pyruvate or 2 mM succinate. 20 μM rotenone and 2 μM antimycin was added to the incubation mixtures prior to the resuspension of the mitochondrial pellet. The RNA was electrophoresed through 5% polyacrylamide-7M urea gel. The important RNA species are noted.

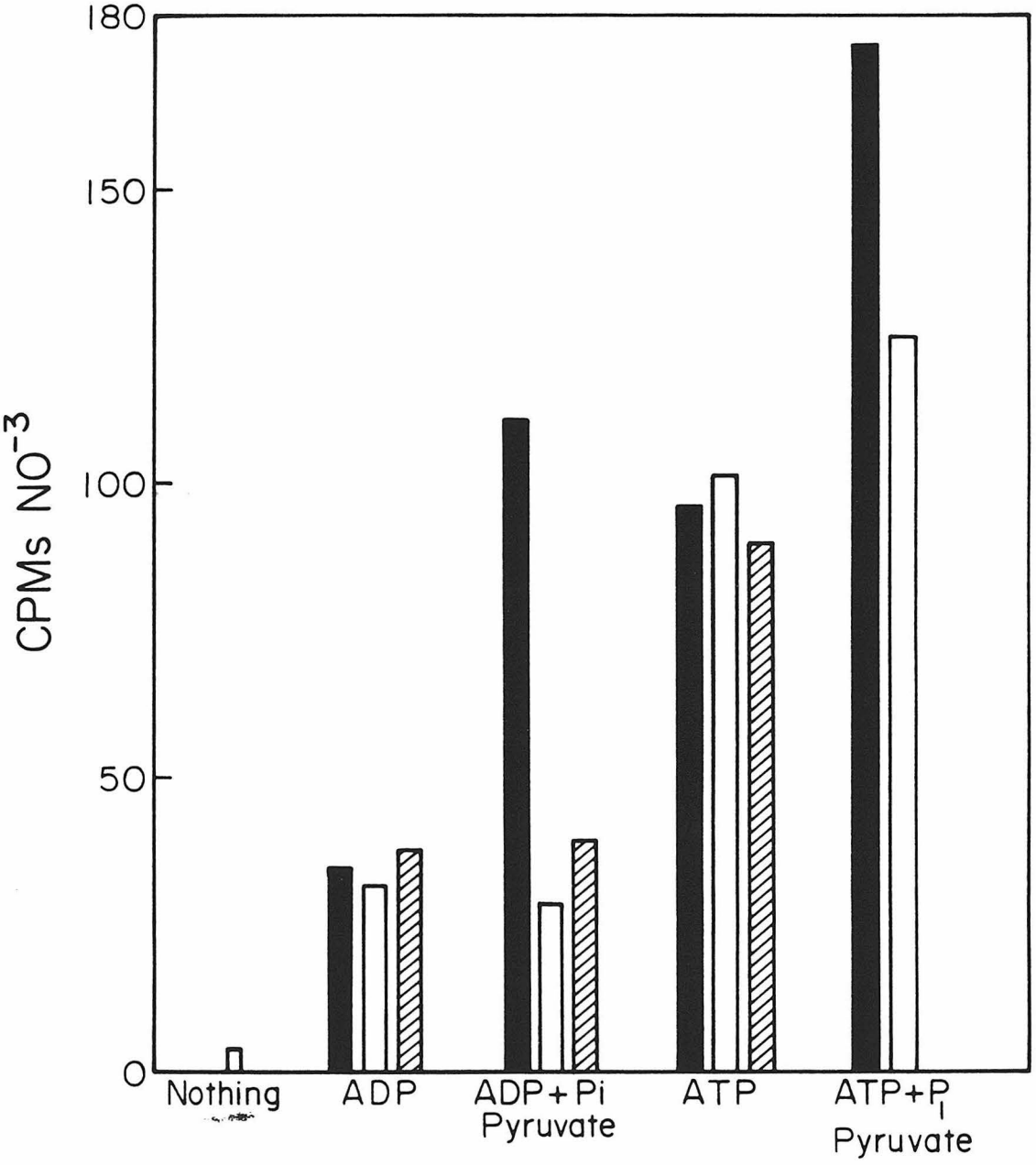

180
150
100
50
0
CPMs NO^{-3}
Nothing
ADP
ADP+Pi
Pyruvate
ATP
ATP+P$_i$
Pyruvate

Fig. 8. Comparison of the effects of antimycin and oligomycin upon the acid precipitable radioactivity from labeling of RNA in isolated mitochondria. 10% (2 μl) of each sample of labeled RNA was treated as shown in Fig. 2. The mitochondrial samples were incubated in the presence of 1 mM ADP, 1 mM ATP, 1 mM sodium phosphate, and 10 mM P_i as marked. The columns represent the counts from mitochondria with no drug (solid columns), 2 μM antimycin (open columns), and 2 μg/ml oligomycin (slashed columns). CPMs NO^{-3} should read CPMs × 10^{-3}.

CHAPTER 5

Isolation and partial characterization of a low molecular weight cytoplasmic factor(s) which effects RNA labeling in isolated mitochondria

Abstract

A low molecular factor(s) from HeLa cell cytoplasm was partially purified and shown to inhibit the overall labeling of RNA and reduce the relative labeling of rRNA to mRNA in isolated mitochondria. The extent of these reductions is very dependent upon the concentrations of the factor(s). Furthermore, the factor(s) block the increases in overall and especially ribosomal RNA synthesis caused by addition of ATP. A purification scheme using sequential Amicon Centricon microconcentrator centrifugations and a final chromatographic step on G-25 Sephadex has shown that the reduction in the ratio of rRNA to mRNA labeling is caused by a factor(s) about the size of a nucleotide.

Introduction

The control of mitochondrial transcription depends upon the nuclear-cytoplasmic gene expression systems and the energetic state of the mitochondria. All of the proteins involved in mitochondrial RNA synthesis are encoded by the nucleus (1); hence, intuitively, the amount of expression of these genes will determine at a first level the extent of mitochondrial transcription. A second level of control has been discovered in an isolated mitochondrial transcription system which appears to be determined by the presence of ATP or oxidizable substrates for respiration (Chapter 4, this thesis). Regulation of RNA synthesis can be observed in this system in both overall levels and the relative levels of rRNA$_S$and mRNAs. These levels of RNA synthesis depend upon the extent of stimulation of each of the transcription units (2). Mitochondrial heavy (H) strand transcription occurs in two transcription units, one which synthesizes the phenylalanine and valine tRNAs and the 12S and 16S rRNAs, and a second one which continues through the first unit and around the remainder of the H strand. The second unit gives rise to the remaining H-strand tRNAs and mRNAs (2). The light (L) strand DNA is also transcribed, allowing for the synthesis of eight tRNAs, several long, oligoadenylated species and the 7S RNA, possibly involved in translation (3) and replication (4). Using an isolated mitochondrial transcription system (5, 6) as an assay, we have characterized and partially purified a small cytoplasmic factor(s) which causes a reduction of overall labeling of mitochondrial RNA, and a further preferential reduction of rRNA synthesis.

Materials and Methods

HeLa cells were grown as described previously (6). The cells were harvested and washed 2x in NKM (0.13M NaCl, 0.005 M KCl, 0.0075 M $MgCl_2$). The cell pellet was resuspended in half the cell volume of 1/10x IB (4 mM Tris (pH 7.4), 2.5

mM NaCl, 0.5 mM $MgCl_2$) and the cells were broken by homogenization. 10x IB (400 mM Tris (pH 7.4), 250 mM NaCl, 50 mM $MgCl_2$) was added until the concentration of the homogenized solution was about 1x IB (40 mM Tris (pH 7.4), 25 mM NaCl, 5 mM $MgCl_2$). The unbroken cells and nuclei were removed by centrifuging at 2000 RPM for 3 minutes, and recentrifuging the supernatant a second time. The remaining supernatant was aliquoted into 1.5 ml Eppendorf tubes (usually 0.5 ml per tube), and centrifuged at full speed (12.7K RPM) in a microfuge for 1 minute. The supernatant was saved as the cytoplasmic fraction, and the pellet containing the mitochondria was suspended in IB #2 (10% glycerol, 40 mM Tris (pH 7.4), 25 mM NaCl, 5 mM $MgCl_2$, 2 mg/ml bovine serum albumin (BSA)) and recentrifuged for 1 minute. The resulting pellet was then resuspended in an incubation media containing 1 mM ATP for the transcription assay.

The transcriptional assay, including the analysis of the labeled nucleic acids, was performed as described previously (6), with the following modifications. Cytoplasm in various concentrations was used in place of buffer and the transcription reactions were carried out for 30 minutes at 37° C unless otherwise noted. ATP was added to 1 mM in all samples just prior to the addition of 5-10 μCi [α-^{32}P]UTP and the start of labeling.

The purification of the cytoplasmic factor(s) was started by centrifuging roughly 20 ml of cytoplasm twice at 27,000 RPM in a SW41 rotor for 1 hour. The supernatant (S-100 fraction) was then centrifuged in Centricon-30 microconcentrators (Amicon Corp., Danvers, Massachusetts) until about 10 ml of the S-100 fraction had passed the filters. The flowthrough (< 30 kd fraction) was centrifuged through Centricon-10 microconcentrators until about 5-6 ml had passed the filters. The flowthrough (< 10 kd fraction) was then lyophilized and resuspended at various volumes for use, or further purification.

Results

Different concentrations of ATP or oxidizable substrates in the isolated mitochondrial transcription system results in different relative labelings of mitochondria mRNA and rRNA encoded on the H strand of the DNA, as well as a change in overall transcription (Chapter 4, this thesis). Compared with buffer, the presence of a crude cytoplasmic fraction has profound effects upon the incorporation of [α-^{32}P]UTP into the RNA by isolated mitochondria. As shown in Fig. 1, RNA labeled in isolated mitochondria in either IB #2 or cytoplasm was separated into the total (t) oligo(dT)-cellulose unbound (u) and bound (b) fractions and electrophoresed through a 1.4% agarose CH_3HgOH gel and the gel autoradiographed. The total amount of labeling is dramatically decreased when cytoplasm is substituted for buffer during the incubation, partly due to the high concentration of UTP in cytoplasm (200 μM) (7). Cytoplasm also reduces the labeling of rRNAs to a level at or below those of the mRNAs. The actual level of 12S and 12S* labeling is somewhat less than the intensities of the bands indicated because of the comigration of mRNAs 13 and 12 with the two small rRNAs, respectively. The L-strand transcription is practically nonexistent in the presence of cytoplasm, as exemplified by the lack of RNA 18 labeling. However, additional experiments using buffer IB #2 as the incubation medium gave inconsistent RNA 18 labeling. The reason for this variation is unknown. Despite the extreme effect cytoplasm has on transcription, little change was seen for processing and polyadenylation (Fig. 1). In both circumstances, roughly 10% of the mRNAs were bound to oligo(dT) cellulose, a phenomenon previously noted (6).

Susceptibility to dilution

The ability of the cytoplasmic fraction to effect *in vitro* mitochondrial RNA labeling was extremely sensitive to dilution, as shown by Fig. 2. The labeled RNA

from isolated mitochondria incubated in various concentrations of cytoplasm and IB #2 was extracted and electrophoresed through a 1.4% agarose CH_3HgOH gel, and the gel autoradiographed. The reduction in specific activity of the [α-^{32}P]UTP by the cytoplasmic pool of UTP results in the decrease in total labeling of RNA at low concentrations of cytoplasm. A further decrease in labeling occurs in high concentrations of cytoplasm which cannot be explained by the specific activity of [α-^{32}P]UTP. (The effect is very noticeable considering the 10-fold greater amount of mitochondrial RNA applied to the 100% cytoplasm lane compared with the 0 and 20% lanes.) Furthermore, the ratio of mRNA to rRNA changes dramatically at the high concentrations of cytoplasm (> 60%). Hence, high concentrations of cytoplasm decrease overall labeling and dramatically decrease the labeling of 12S, 12S* and 16S rRNAs. However, a dilution of cytoplasm of 40% nearly removes these effects. The greater sensitivity of the rRNA labeling in high concentrations of cytoplasm is evident in the ratio of the sums of intensities of bands corresponding to mRNAs 14, 15, and 16 to rRNAs 12S* (12) and 12S (13). Again, no attempt was made to subtract the contribution of mRNAs 12 and 13 to the ribosomal bands. As seen in Fig. 1, the labeling of the rRNAs in 100% cytoplasm is the same or less than that of the mRNAs. Because of inconsistencies in breakage of cells, the intensities of these two effects varied slightly between experiments. In general, a dilution of 40-60% of the cytoplasm with IB #2 removed both effects. Addition of glycerol and BSA to the cytoplasm has no effect on the labeling patterns, suggesting that addition of IB #2 to the incubation does not provide a component for increased rRNA and total RNA synthesis.

Purification of the factors responsible for the activities

The extreme dependence upon concentration of the cytoplasm required the use of novel methods for purification of the factors involved in the control of the relative levels of transcription of mRNA and rRNA. No dilution greater than approximately 2 fold could be tolerated in the transcription assay. Therefore a strategy using Centricon microconcentrators was tried. The results are shown in Fig. 3. Isolated mitochondria were incubated in the presence of either a 100% or a 50% concentration (diluted with IB #2) of four different cytoplasmic fractions separated by microconcentration. The labeled RNA from these transcription reactions was electrophoresed through a 5% polyacrylamide-7M urea gel and the gel autoradiographed. The S-100 fraction from cytoplasm contained the activities causing both the reduction in overall labeling and the pronounced reduction in rRNA labeling. Furthermore, all fractions separated by sequential centrifugation through exclusion filters (Centricon 30 and 10) contain both activities as assayed by mitochondrial transcription. Since the Centricon microconcentrators only concentrate material which does not pass the filter, and both activities are found in all fractions, including the flowthrough of the Centricon 10, the activities are caused by molecules of less than 10,000 daltons.

Further purification of these activities required a concentration step. As shown in Fig. 4, the < 10 kd fraction from sequential centrifugation through membranes could be concentrated by lyophilization and resuspension. The concentration of the < 10 kd fraction by lyophilization and resuspension was 4 fold, and subsequent dilutions showed that this material could be mixed with three volumes of buffer to regain both the activities seen in the unconcentrated, undiluted < 10 kd fraction. Here again, a 2-fold dilution of either unconcentrated material or an 8-fold dilution of 4-fold concentrated material resulted in the loss of the reduction in overall labeling and the preferential inhibition of the rRNA

synthesis. Concentration of the < 10 kd fraction increased both activities. A concentration equivalent to 200% of the original < 10 K fraction (lane 1/1) resulted in no labeling in the transcription assay. mRNA but no rRNA labeling occurs in a concentration equivalent to 133% of the original < 10 K fraction (lane 1/2). Clearly, both the overall inhibitory activity and the preferential inhibition of the rRNA transcription unit can be concentrated. This characteristic of the factors responsible for these activities was then exploited in a further purification step.

The results of G-25 Sephadex column chromatography of the lyophilized < 10 kd fraction is shown in Fig. 5a. The profiles of OD_{230} of the < 10 kd fraction and radioactivity of an [α^{32}P]UTP marker are shown in Fig. 5a. Fractions from the G-25 Sephadex column separation were lyophilized and resuspended to a concentration of < 10 kd constituents equivalent to that found in cytoplasm, assuming no dilution by the column separation. These fractions were used to resuspend the mitochondria in transcription assays, and the resulting labeled RNA patterns are shown in Fig. 5b. Since fractions 1-9 had no effect on the labeling of RNA (data not shown), lanes 4 and 7 can be considered equivalent to control buffer lanes. The overall reduction in labeling corresponds exactly to the peak of radioactivity, added as a marker, and is due mostly to a reduction in specific activity of the [α-^{32}P]UTP used in labeling by the endogenous UTP of the < 10 kd fraction. Some loss in the activity which specifically blocks the labeling of rRNA has occurred, probably due to a dilution of the factor(s) during column filtration, as exemplified by the radioactivity elution profile (Fig. 5a). As seen in Fig. 2, this dilution is sufficient to lose the activity which results in the further reduction in labeling not explained by the decrease in the specific activity of the [α-^{32}P]UTP. The second activity, causing a change in the relative levels of rRNA to mRNA is maximally found in fractions 13 and 14. Here again, the small reduction in

concentration of these chromatographed molecules reduces the activity considerably. It is clear, however, that the substance which preferentially reduces the rRNA labeling has G-25 Sephadex filtration characteristics very similar to UTP.

Discussion

The interactions between the products of gene expression from the two cellular genomes, nuclear and mitochondrial, is evident. However, the mechanisms involved in these interactions are unknown. Furthermore, there exists a group of small molecules present in cytoplasm which act at a level independent of direct action by the nucleus. Several groups have begun to study these molecules; primarily those involved in mitochondrial protein synthesis and import of proteins into the organelle (8-11). Finzi and Beattie (12) have reported a stimulation of yeast mitochondrial protein synthesis in isolated organelles by a cytoplasmic factor(s) of < 2000 daltons. This factor(s) is not species specific; a rat liver cytoplasmic factor(s) can replace the yeast factor(s) in stimulation of yeast mitochondrial protein synthesis. Several other groups have described cytoplasmic factor(s) involved in import of proteins (9-11), including one of < 5000 daltons (9). In this report, we describe a small molecular weight factor(s) which both reduces the overall labeling of RNA in isolated mitochondria and changes the relative rates of labeling between the rRNAs and mRNAs encoded on the heavy strand of mtDNA.

The control of the two heavy strand transcription units in mitochondria may play an important part in the biogenesis and function of the organelle. As noted previously (4) (Chapter 4, this thesis) the rRNA synthesis which normally occurs at about 10-50 times the rate of the majority of mRNA synthesis *in vivo* (13) can be reduced to near or below that of the mRNA synthesis *in vitro*. Cytoplasm used instead of buffer results in an overall reduction in labeling below that expected

due to the reduced specific activity (activity #1) and a further decrease in the labeling of the rRNA species (activity #2). The labeling of RNA based solely on the specific activity of [α-^{32}P]UTP should be about 2-4 times greater than that observed in mitochondria incubated in cytoplasm (7). At least two possible explanations could account for activity #1. First, some component in cytoplasm could reduce the transcription within the mitochondria, presumably by effecting the rate of initiation or elongation. Secondly, this component in the cytoplasm could effect the uptake of the UTP, thereby reducing the labeling independently of an actual change in transcription. Activity #2 is independent of the specific activity of the [α-^{32}P]UTP. Unfortunately, since the mechanism of overall reduction in labeling is unknown, the direction of the change in transcription rates, i.e., rRNA decreased or mRNA increased, is unknown.

In vivo, the greater labeling of rRNA over that of mRNA is achieved through a mechanism which controls the amount of termination at the 3'-end of the 16S rRNA (2), and may be determined by the point of initiation of the H-strand transcription. Several studies (2, 5) have suggested that initiation at the upstream site, I_{HR} (Fig. 1, Chapter 2), results in the termination of the transcription at the 3' end of 16S, whereas, initiation at the downstream site, I_{HT}, results in an almost complete transcription of H strand. Currently, nothing is known about the mechanism of action of this small cytoplasmic factor(s) which causes the change the change in the relative levels of labeling in isolated organelles of these two classes of mitochondrial RNA. Most likely, the factor(s) changes the relative rates of initiation at the two H-strand sites, due probably to differential sensitivities of the two promoter mechanisms. Alternatively, the factor(s) may cause all polymerase molecules to continue through the 16S termination site and around the genome, regardless of the initiation site utilized. Both these models, differential sensitivities and anti-termination, are based on the premise that both

transcriptional events are possible simultaneously within each mitochondrion. Another explanation for the change in relative labeling rates could be that two pools of mitochondria exist; one pool is synthesizing RNA strictly from the rRNA transcription unit and the other, probably smaller, pool synthesizing RNA from the complete H strand of DNA. If this were the case, the small cytoplasmic factor(s) which reduces the relative amounts of labeling of rRNA to mRNA could be inhibiting the transcription or the uptake of label ([α-^{32}P]) within only the rRNA-synthesizing mitochondria, or increasing the labeling of the mRNA synthesizing mitochondria in an opposing manner. Parallel studies introducing the purified factor(s) into broken mitochondrial transcription systems and *in vivo* footprinting of mitochondrial DNA in intact organelles in the presence of this factor(s) may provide insights about its mechanisms of action.

Several experimental observations are suggestive of the nature of the physiological roles played by these factors. The first is that both activity #1 and activity #2 were extremely dilution sensitive. A dilution of 50% of the cytoplasm or the equivalent amount of the low molecular weight fraction resulted in a loss of both activities. This dilution sensitivity is characteristic of regulatory pathways which undergo "sensitivity amplification" (14). Classical sensitivity amplifications occur in adaptive systems where the constitutive level of an activity is high compared to the transitory changes, or in "futile cycle" regulatory systems which have two pathways; one for synthesis and one for degredation of the same molecules. Both cases require a large response to a small change in signal over a background noise. Sensitivity amplification seen here may be used in the cell to coordinate mitochondrial RNA synthesis with the cell cycle or the production of nuclear proteins required for mitochondrial biogenesis, such as mitochondrial ribosomal proteins. For example, previous studies have shown cell cycle dependence of the mitochondrial RNA labeling *in vivo* (15), however, no attempt

was made to characterize the factors involved in this control. The factor(s) characterized in this work negates both the stimulation of overall RNA and preferential rRNA labeling induced by the addition of exogenous ATP to the isolated mitochondrial transcription system (Chapter 4). This block in stimulation does not appear to be caused by increased hydrolysis of ATP by the cytoplasm itself (data not shown); however, removal of ATP from the accessible pool by binding or other means has not been ruled out.

The extreme sensitivity to dilution of the cytoplasmic activities required the development of a novel method of purification. Centricon microconcentrators were used to remove the bulk of the proteins and large molecular weight materials. Further purification was achieved first by concentrating the < 10 kd fraction with lyophilization and then chromatographing the concentrated solution on a G-25 Sephadex column. The results from the chromatography indicate that the factor(s) which cause a change in relative rRNA and mRNA labeling are roughly the size of a nucleotide triphosphate. Several nucleotide derivatives, including ppGpp, AppppA, and ITP have not shown any effect on the labeling of RNA in isolated mitochondria. Experiments are planned to attempt to identify the chemical nature of these molecules, and their possible relationship to the published small molecular weight cytoplasmic factor(s).

References

1. Tzagaloff, A. 1982. Mitochondria. Plenum Press, New York and London, pp. 235-266.
2. Montoya, J., Gaines, G. L., and Attardi, G. 1983. The pattern of transcription of the human mitochondrial rRNA genes reveals two overlapping transcription units. *Cell* **34**, 151-159.
3. Ojala, D., Crews, S., Montoya, J., Gelfand, R., and Attardi, G. 1981. A small polyadenylated RNA (7S RNA), containing a putative ribosome attachment site, maps near the origin of human mitochondrial DNA replication. *J. Mol. Biol.* **150**, 303-314.
4. Chang, D. D., and Clayton, D. A. 1985. Priming of the human mitochondrial DNA replication occurs at the light-strand promoter. *Proc. Natl. Acad. Sci. USA* **82**, 351-355.
5. Gaines, G. L., and Attardi, G. 1984. Intercalating drugs and low temperatures inhibit synthesis and processing of ribosomal RNA in isolated human mitochondria. *J. Mol. Biol.* **172**, 451-466.
6. Gaines, G., and Attardi, G. 1985. Highly efficient RNA synthesizing system using isolated human mitochondria: New initiation events and *in vivo* like processing patterns. *Mol. Cell. Biol.* **4**, 1605-1617.
7. Bestwick, R. K., Moffett, G. L., and Mathews, C. K. 1982. Selective expansion of mitochondrial nucleoside triphosphate pools in antimetabolite-treated HeLa cells. *J. Biol. Chem.* **257**, 9300-9304.
8. Poyton, R. O., and Kavanagh, J. 1976. Regulation of mitochondrial protein synthesis by cytoplasmic proteins. *Proc. Natl. Acad. Sci. USA* **73**, 3947-3951.

9. Argan, C., Lusig, C. J., and Shore, G. C. 1983. Membrane and cytosolic components affecting transport of the precursor for ornithine carbamyl-transferase into mitochondria. *J. Biol. Chem.* **258,** 6667-6670.

10. Miura, S., Mori, M., and Tatibana, M. 1983. Transport of ornithine carbamoyltransferase precursor into mitochondria. *J. Biol. Chem.* **258,** 6671-6674.

11. Ohta, S., and Schatz, G. 1984. A purified precursor polypeptide requires a cytosolic protein fraction for import into mitochondria. *Embo J.* **3,** 651-657.

12. Finzi, E., and Beattie, D. 1984. The stimulation of yeast mitochondrial protein synthesis by low molecular weight cytoplasmic factors: requirement for intact mitochondria and lack of effect by folate derivatives. *Int. J. Biochem.* **16,** 177-182.

13. Gelfand, R., and Attardi, G. 1981. Synthesis and turnover of mitochondria ribonucleic acid in HeLa cells: The mature ribosomal and messenger ribonucleic acid species are metabolically unstable. *Mol. Cell. Biol.* **1,** 497-511.

14. Koshland, D. E., Jr., Goldbetter, A., and Stock, J. B. 1982. Amplification and adaptation in regulatory and sensory systems. *Science* **217,** 220-225.

15. Attardi, G., Constantino, P., England, J., Lynch, D., Murphy, W., Ojala, D., Posakosy, J., and Storrie, B. 1976. The biogenesis of mitochondria in the HeLa cells: A molecular and cellular study. **In** Genetics and Biogenesis of Mitochondria and Chloroplasts. C. W. Birky, P. Perlman, and T. J. Byers (eds.). Ohio State University Press, Columbus, Ohio, pp. 3-65.

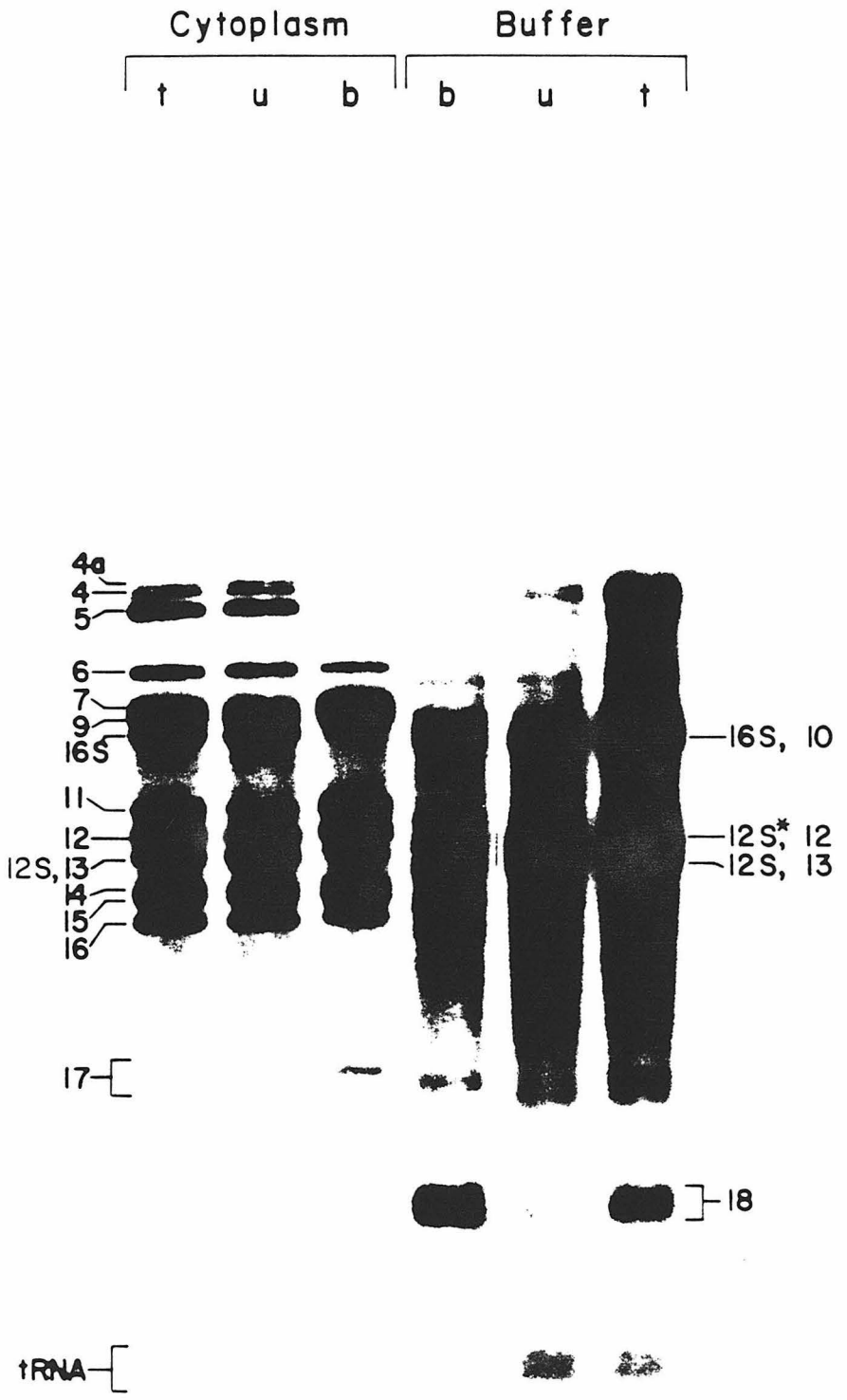

Cytoplasm
Buffer
t u b
b u t
4a
4
5
6
7
9
16S
11
12
12S, 13
14
15
16
17
18
tRNA
16S, 10
12S*, 12
12S, 13

Fig. 1. Comparison of HeLa cell mtRNA labeling in isolated organelles incubated in cytoplasm or in IB #2. The transcription reactions were carried out for 90 minutes at 30°C. The labeled mitochondrial RNA was fractionated on an oligo (dT)-cellulose column and the total (t) RNA , and the unbound (u) and bound (b) fractions were separated electrophorectically on a 1.4% agarose/CH_3HgOH slab gel. The relative amounts of mitochondrial RNA applied to each lane were as follows: cytoplasm (t)-12, cytoplasm (u)-20, cytoplasm (b)-100, buffer (b)-10, buffer (u)-1, buffer (t)-1.

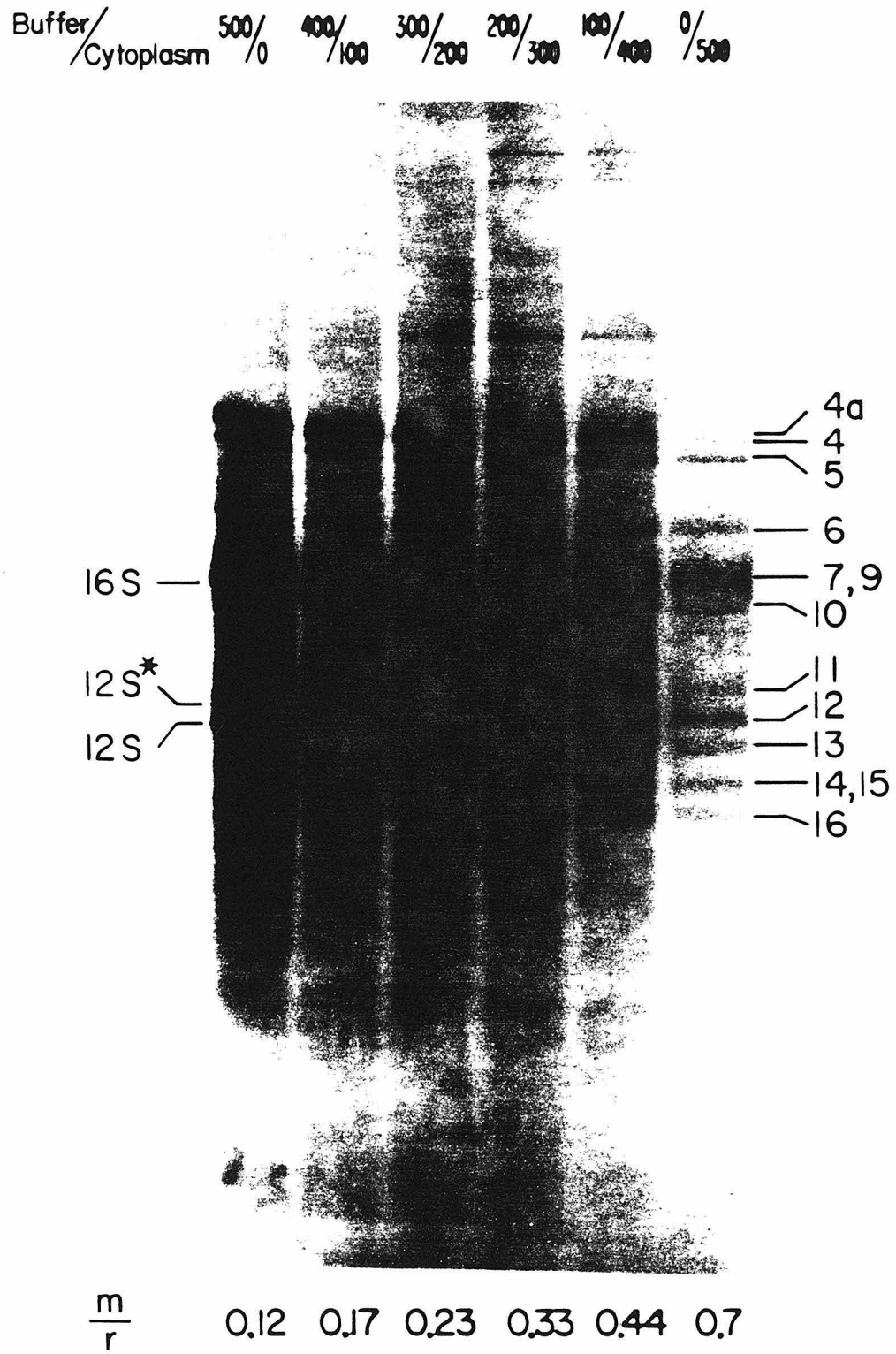

Buffer/Cytoplasm
500/0
400/100
300/200
200/300
100/400
0/500
16S
12S*
12S
4a
4
5
6
7,9
10
11
12
13
14,15
16
m/r
0.12
0.17
0.23
0.33
0.44
0.7

Fig. 2. Effect of dilution of cytoplasm on mtRNA labeling in isolated organelles. Varying amounts of IB #2 were mixed with the corresponding amounts of cytoplasm (as indicated at the tops of the lanes) and added to isolated mitochondria. The transcription reactions were carried out for 90 minutes at 30°C. The total RNA was separated electrophoretically as in Fig. 1. The amounts electrophoresed were 2, 2, 4, 8, 15, and 20 µl, respectively, left to right. The autoradiogram was scanned with a Joyce-Loebe double-beam densitometer and the areas of the peaks were determined by measuring the peak heights and bases. m is the sum of band intensities at 14, 15 and 16, r is the sum of band intensities at 12S* (12) and 12S (13), for each lane.

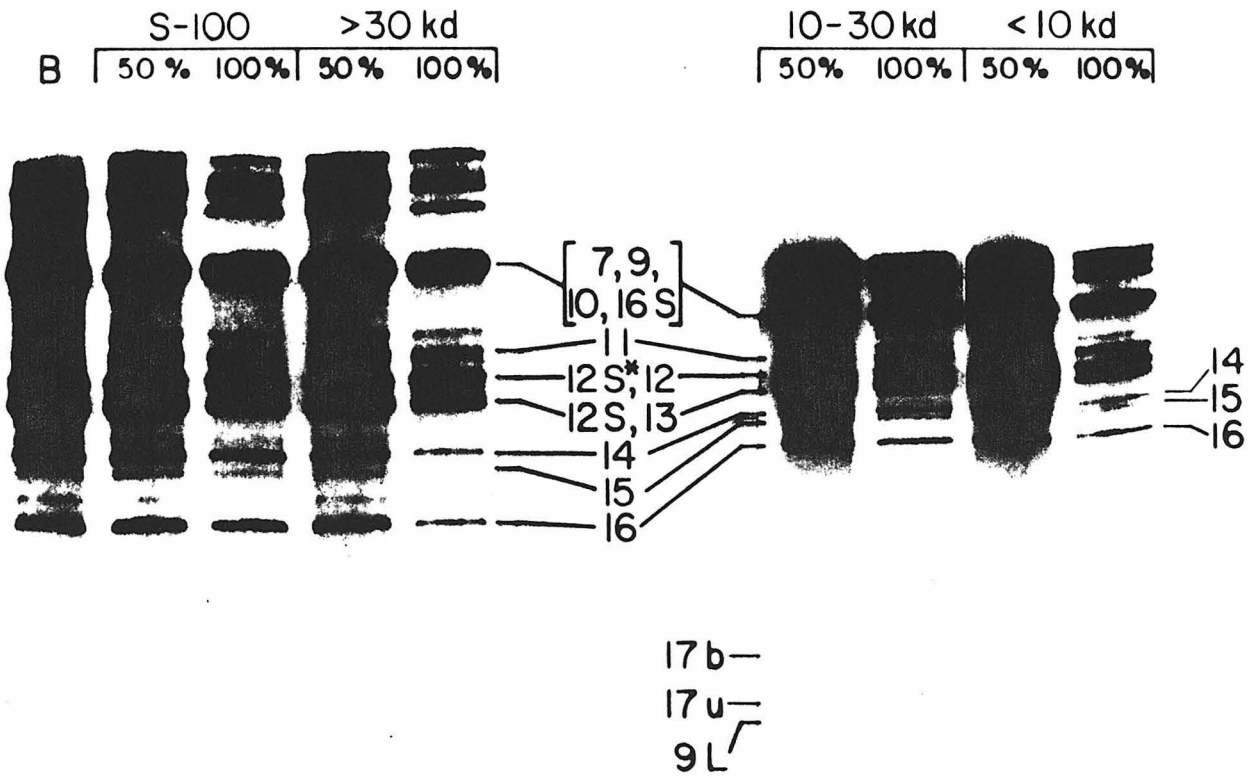

B
S-100
>30 kd
50 %
100%
50 %
100%
10-30 kd
<10 kd
50%
100%
50%
100%
7, 9,
10, 16 S
11
12 S*, 12
12 S, 13
14
15
16
14
15
16
17 b
17 u
9 L
17 b
17 u
9 L

Fig. 3. Purification of the two inhibitory activities by microconcentration through sequential Amicon microconcentrators. RNA labeled in isolated mitochondria incubated in the presence of either 50 or 100% of the cytoplasmic fractions, noted at the tops of the lanes, was extracted and electrophoresed through 5% polyacrylamide-7M urea gels. The gels were then dried and autoradiographed. The gel on the left was electrophoresed for about 5 hours at 500 volts, the gel in the right was electrophoresed for about 3 1/2 hours at 500 volts. The designations S-100 and < 10 kd are explained in the Materials and Methods, the > 30 kd fraction represents the S-100 material that did not pass the Centricon-30 filter and the 10-30 kd fraction represents the < 30 kd that did not pass the Centricon-10 filter. Lane B shows the autoradiographic pattern from labeled RNA made in isolated mitochondria resuspended in 100% IB #2. The relative amounts of mitochondrial RNA electrophoresed are 1, 5, and 10 for the B, 50%, and 100% lanes, respectively.

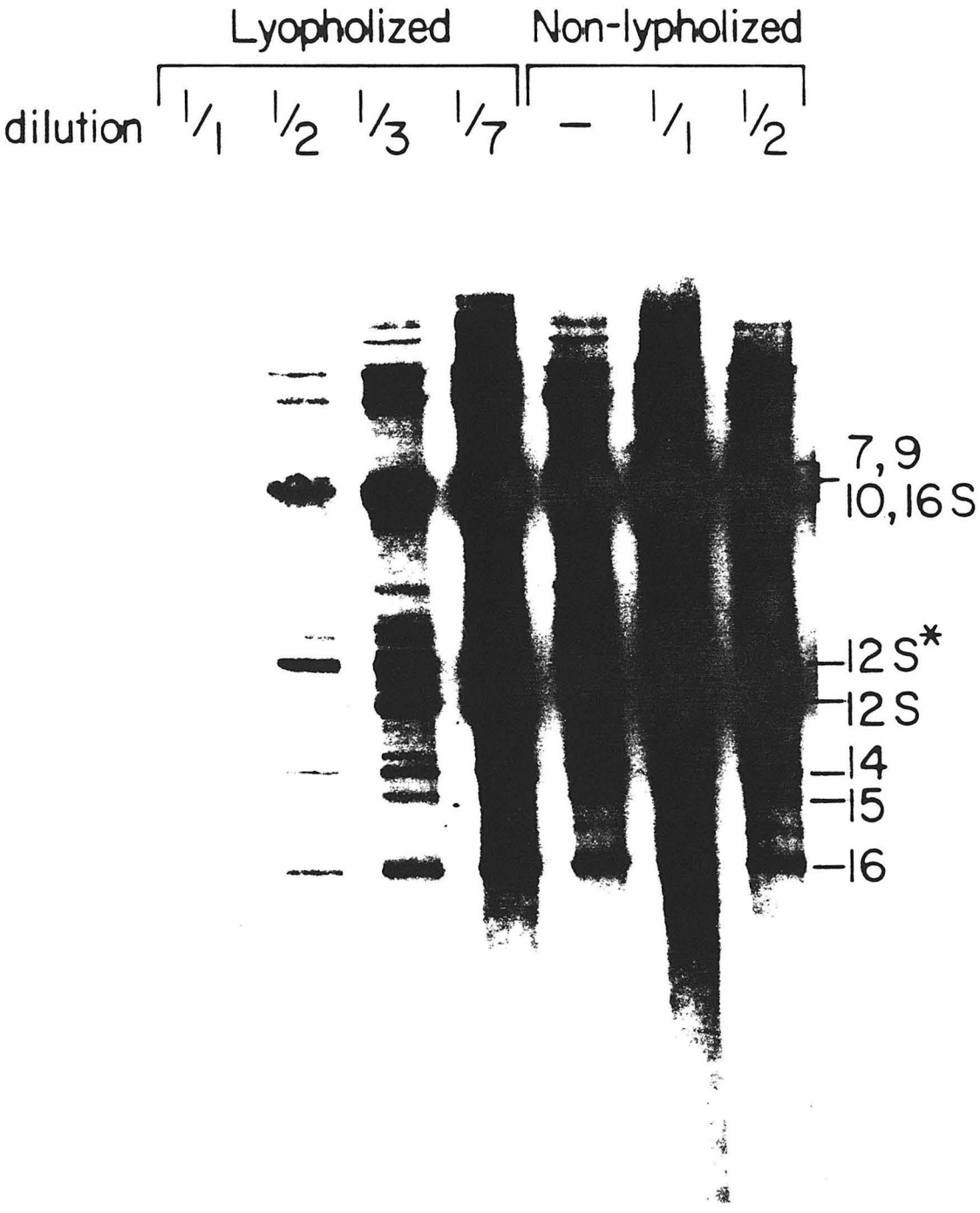
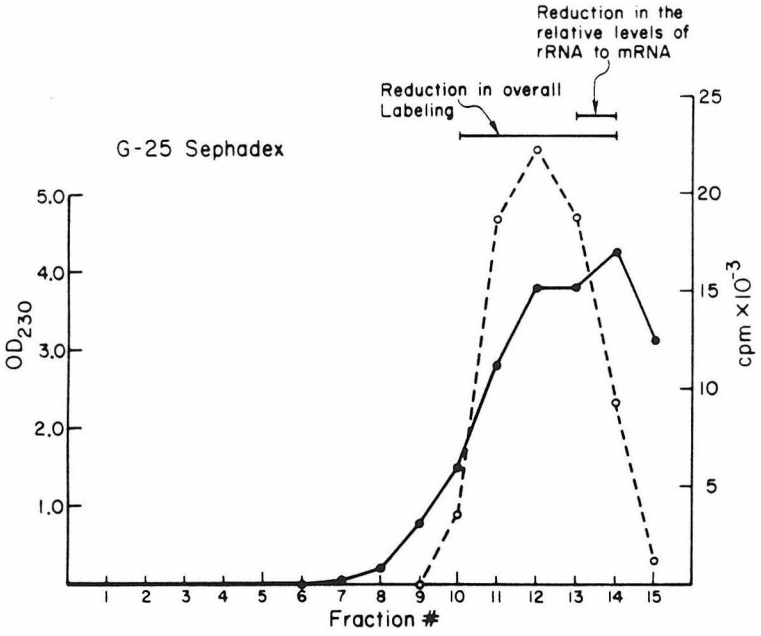
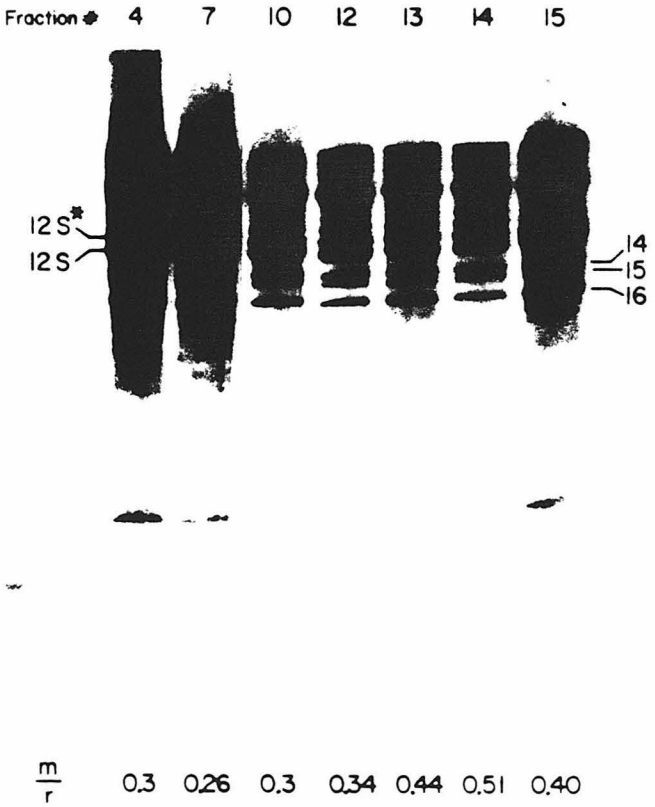

Lyopholized
Non-lypholized
dilution
1/1
1/2
1/3
1/7
–
1/1
1/2
7, 9
10, 16 S
12 S*
12 S
14
15
16

Fig. 4. Concentration of the < 10 kd fraction by lyophilization. Cytoplasm from exponentially grown cells was collected without readjusting the salt concentration to 1 × IB after breakage centrifuged, and fractionated through sequential microconcentrator centrifugations. Half of the < 10 kd fraction was then lyophilized to dryness and resuspended to 1/4 the prelyophilization volume with 10% glycerol, 24 mM Tris (pH 7.4) 15 mM NaCl, 3 mM $MgCl_2$ and 2 mg/ml BSA. This material was then appropriately diluted with IB #2 minus ATP and used to resuspend freshly prepared mitochondrial pellets. 100 mM ATP was added to make the final concentration 1 mM, the samples mixed and 10 μCi of [α-^{32}P]UTP was added just prior to the start of the incubation. The remainder of the < 10 kd fraction was adjusted to approximately the same concentration of constituents as IB #2 minus ATP with concentrated stocks, diluted appropriately with IB #2 minus ATP and used to resuspend freshly prepared mitochondrial pellets. The samples were then treated in the same manner as the lyophilized samples. The labeled RNA from these incubations was electrophoresed through a 5% polyacrylamide-7M urea gel, and the autoradiograph of that gel is shown. The dilution refers to the volumes of lyophilized or nonlyophilized < 10 kd fractions/the volumes of IB #2 minus ATP. The relative amounts of mitochondrial RNA electrophoresed are 10, 10, 5, 2.5, 5, 2.5, and 1.0 per lane, respectively. RNA species are indicated.

a.



b.



$\frac{m}{r}$	0.3	0.26	0.3	0.34	0.44	0.51	0.40

Fig. 5. Elution profiles of the lyophilized < 10 kd fraction applied to a G-25 Sephadex column and subsequent transcription assays of the fractions. a) The elution profiles for OD_{230} (o—o) and [α-^{32}P]UTP radioactivity (o--o), added prior to applying the concentrated sample to a column equilibrated in 1/10 × IB, are shown. Each fraction was 1.3 ml and about 5.0 ml was allowed to pass before the first fracton was collected. b) Each fraction was lyophilized and resuspended to 0.5 ml in 10% glycerol, 30 mM Tris (pH 7.4), 18.5 mM NaCl, 3.7 mM $MgCl_2$ and 2 mg/ml BSA, to make the final concentration of added materials equivalent to IB #2. A 125 µl portion of each fraction was diluted with an equal volume of IB #2 minus ATP and used to resuspend a freshly prepared mitochondrial pellet from about 250 µl of packed cells (~0.3 mg mitochondrial protein). After resuspension, ATP (1 mM final concentration) and [α-^{32}P]UTP was added and the incubations were carried out as in Fig. 4. The labeled RNA was purified as in Materials and Methods and electrophoresed through a 5% polyacrylamide-7M urea gel and the gel autoradiographed. Shown are the results from incubations in fractions noted. The relative amounts of mitochondrial RNA electrophoresed per lane was 1, 1, 1, 5, 5, 5, and 5, from left to right.