

## Markedly Different ATP Requirements for rRNA Synthesis and mtDNA Light Strand Transcription *Versus* mRNA Synthesis in Isolated Human Mitochondria\*

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In isolated mitochondria from HeLa cells, the ATP requirements for mitochondrial DNA (mtDNA) transcription and RNA processing can be satisfied by either endogenous synthesis, mainly through oxidative phosphorylation, or by exogenous supply. The pattern of RNA synthesis changes dramatically depending upon the level of ATP available. At the low intramitochondrial ATP levels produced from endogenous ADP in the presence of an oxidizable substrate and phosphate, the mRNA species are labeled to a substantial extent, whereas there is only a marginal labeling of the rRNA species and light (L) strand transcripts. By contrast, high ATP levels, either provided exogenously or produced endogenously in the presence of an oxidizable substrate, phosphate, and exogenous ADP, strongly stimulate rRNA synthesis (about 10-fold) and light (L) strand transcription (>10-fold), with only a slight increase in mRNA synthesis.

similar optimum ATP concentration has been found in an open *in vitro* transcription system from HeLa cells utilizing a solubilized mitochondrial RNA polymerase preparation and an exogenous template (11). It is interesting that the concentration of ATP in rapidly isolated HeLa cell mitochondria has been estimated to be close to this optimum for *in vitro* mtDNA transcription (19). The correspondence of the physiological concentration of ATP in mitochondria with that needed for maximal RNA synthesis in the two *in vitro* systems mentioned above suggests that a high ATP concentration may be required for maximal rate of mtDNA transcription *in vivo*. The present work has, unexpectedly, revealed a strikingly different dependence of mRNA synthesis *versus* rRNA synthesis and light (L) strand transcription upon the concentration of ATP, either provided exogenously or generated by oxidative phosphorylation.

### MATERIALS AND METHODS

The methods used were essentially as described earlier (18) 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<sub>2</sub>, 2 mg of bovine serum albumin (BSA)/ml, 10% glycerol, and 5–10 μCi of [ $\alpha$ -<sup>32</sup>P]UTP (400–600 Ci/mmol) per sample (0.5 ml). Each sample contained the mitochondrial fraction from ~0.5 g of HeLa cells and was incubated for 30 min at 37 °C, unless otherwise specified. Addition of other chemicals to the incubation medium was done just prior to resuspension of the mitochondrial pellet. Phosphate was added, whenever appropriate, in the form of Na<sub>2</sub>HPO<sub>4</sub> at 10 mM. The electrophoresis of labeled nucleic acids was carried out through 5% polyacrylamide, 7 M urea gels, unless otherwise specified. Hybridization of the *in vitro*-labeled RNA species with single-stranded M13-cloned human mtDNA fragments and S<sub>1</sub> nuclease treatment were carried out as described earlier (18). The S<sub>1</sub>-resistant products were analyzed by electrophoresis through a 5% polyacrylamide, 7 M urea gel, followed by autoradiography.

### RESULTS

**mtDNA Transcription in Isolated Organelles**—In the present work, isolated mitochondria were incubated with [ $\alpha$ -<sup>32</sup>P]UTP in basic incubation medium with various additions, as specified below, and the extracted RNA was then analyzed in a 5% polyacrylamide, 7 M urea gel. As shown in Fig. 1a, the characteristic set of *in vitro* and *in vivo* synthesized transcripts previously described (17, 20) can be recognized. Although the relative mobility of the various RNA species in a polyacrylamide/urea gel is somewhat different from that previously observed in a CH<sub>3</sub>HgOH-agarose gel (20), their order of migration is conserved, as verified by S<sub>1</sub> mapping experiments.<sup>1</sup> In Fig. 1 and the following figures, the RNA species

A fairly complete picture is available of mtDNA transcription in human cells (1). The discrete transcription products have been mapped, and their structural and metabolic properties characterized (2–5). The main initiation sites for transcription of the heavy (H) and light (L) strands have been identified both *in vivo* (6–8) and *in vitro* (9–11), and the analysis of the enzymatic machinery involved in transcription (12) and RNA processing (13) has begun. By contrast, very little is known about the regulation of these processes (14, 15). The location of the mtDNA transcription and RNA processing apparatus in the unique environment of mitochondria and the association of mtDNA with the inner mitochondrial membrane (16) suggest that this apparatus may be sensitive to the energetic state of the organelles. The recent development of a highly efficient and faithful mtDNA transcription system utilizing isolated mitochondria from HeLa cells has offered the opportunity of dissecting out the energetic requirements of mtDNA transcription and RNA processing by changing the environment of the organelles in a controlled way (17, 18).

Previous transcription studies with isolated mitochondria have shown that RNA synthesis in this system is strongly stimulated by the addition of ATP to the incubation mixture, with a maximum effect occurring at about 1 mM (18). A

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<sup>1</sup> J. Montoya, G. Gaines, M. King, C. Rossi, and G. Attardi, manuscript in preparation. The temporary unavailability of CH<sub>3</sub>HgOH of satisfactory quality from commercial sources forced the adoption of polyacrylamide/urea as a denaturing gel system.

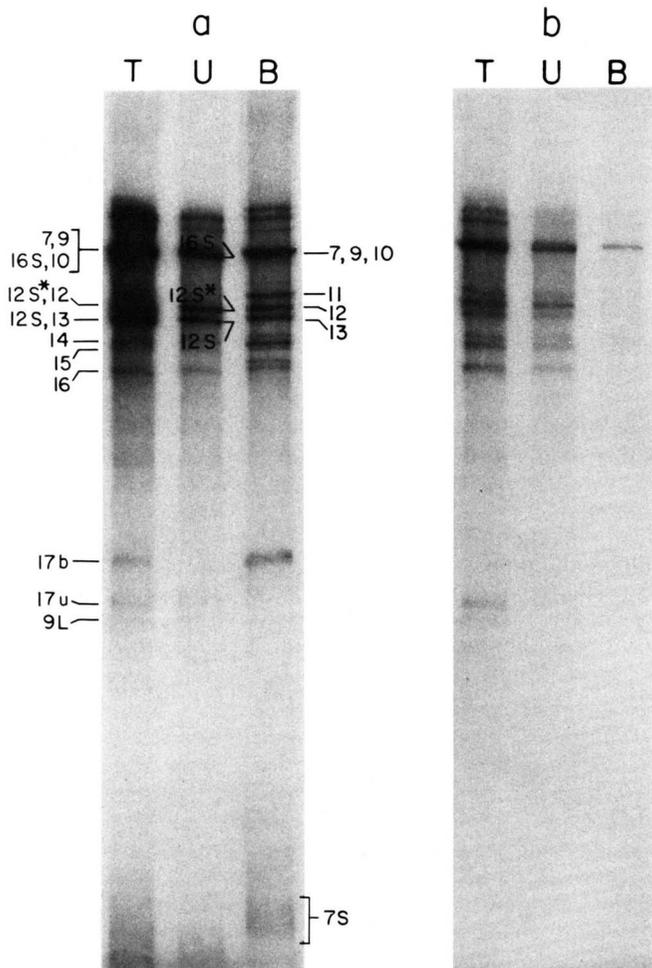


FIG. 1. Electrophoretic patterns of total, oligo(dT)-cellulose-unbound and oligo(dT)-cellulose-bound RNA from mitochondria labeled with  $[\alpha\text{-}^{32}\text{P}]\text{UTP}$  in the presence of ATP and phosphate, or pyruvate and phosphate. Mitochondrial RNA was labeled with  $[\alpha\text{-}^{32}\text{P}]\text{UTP}$  in isolated organelles in the presence of 10 mM  $\text{NaPO}_4$  and either 1 mM ATP (a) or 2 mM sodium pyruvate (b), extracted, and fractionated on an oligo(dT)-cellulose column. Samples of the total RNA (T) and the oligo(dT)-cellulose-unbound (U) and -bound (B) fractions were electrophoresed through a 5% polyacrylamide, 7 M urea gel and autoradiographed. The portion of oligo(dT)-cellulose-bound RNA analyzed, in terms of mitochondria equivalents, was 10 times greater than the portion of unbound RNA. Equal amounts of mitochondrial nucleic acids were run through the corresponding lanes in (a) and (b). In the oligo(dT)-cellulose-unbound fraction of the RNA sample analyzed in Fig. 1a, the bands designated 16 S, 12 S\*, and 12 S presumably contain a relatively small amount of mRNAs 7 + 9, 12, and 13, respectively, which were not retained on the oligo(dT)-cellulose column due to the lack or short length of the poly(A) tail (17, 18). 17b and 17u, oligo(dT)-cellulose-bound and -unbound forms of mRNA 17; 9L, leader of mRNA 9. See text for details.

are designated according to Amalric *et al.* (20) and Montoya *et al.* (7). 12 S and 16 S RNAs are the two rRNAs, 12 S\* is a 12 S rRNA precursor carrying the adjacent  $\text{tRNA}^{\text{Phe}}$  and the leader at its 5'-end (17) (Fig. 2), the band designated 4 and 4a contains transcripts covering the whole rDNA region, including the rRNA precursors u4a and u4 (7), RNA 5, 7, 9, and 11–17 represent the mRNAs corresponding to the various reading frames of the heavy (H) strand (2, 21), RNA 6 is a precursor of mRNA 9 (2), RNA 10 is a polyadenylated form of 16 S rRNA (2), RNA 9L is the leader segment of RNA 6 (22, 31), and 7 S RNA is an L-strand transcript mapping in

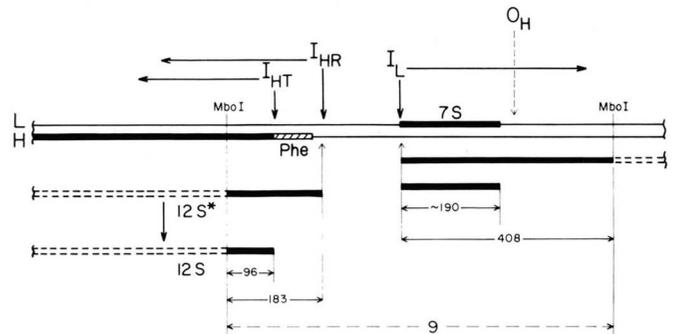


FIG. 2. Portion of the HeLa cell mtDNA genetic and transcription maps illustrating the region near the origin of H-strand synthesis ( $O_H$ ) involved in initiation of transcription. The leftward and rightward heavy arrows indicate the direction of H- and L-strand transcription, respectively, and the downward heavy arrows, the initiation sites for H-strand ( $I_{HR}$  and  $I_{HT}$ ) and L-strand transcription ( $I_L$ ). According to the dual transcription model, H-strand transcription from  $I_{HR}$  yields transcripts of the rDNA region terminating at the 3'-end of the 16 S rRNA gene (RNA u4a) and producing the bulk of the rRNA, while transcription from  $I_{HT}$  yields polycistronic transcripts of the total H-strand destined to be processed to give a polyadenylated rDNA transcript (RNA b4) and the mRNAs and most of the tRNAs encoded in the H-strand (7). The *MboI* fragment 9 utilized in the  $S_1$  protection experiments, the segments of the 12 S and 12 S\* RNAs protected by the H-strand of this fragment cloned in M13 (M8.9), and the 7 S RNA and the segment of the longer L-strand transcript protected by the L-strand of the same fragment cloned in M13 (M9.9) are also shown.

the 5'-end proximal segment of the L-strand transcription unit (23) (Fig. 2).

**Oxidizable Substrates Stimulate Labeling of Mitochondrial RNA**—The role of the respiratory activity of the organelles was first investigated. It is known that the mitochondrial respiratory chain can be stimulated in isolated organelles by the addition of oxidizable substrates (24). As shown in Fig. 3, the labeling of RNA in mitochondria incubated in basic medium was marginal and was not stimulated by addition of 10 mM  $\text{Na}_2\text{HPO}_4$ . The labeling was only slightly to moderately increased by the addition of an oxidizable substrate at 1 mM, with citrate being the most effective, followed by pyruvate and succinate. In contrast, the labeling of RNA was increased about 10-fold relative to that observed in the basic medium, as determined from the acid precipitable radioactivity, by the addition of both phosphate and one of the oxidizable substrates. The increase in labeling appeared to affect mRNAs and rRNAs to a similar extent. A striking feature of the RNA labeled under all these conditions was the low level of labeling of the rRNAs relative to the mRNAs, as judged from the relative intensities of the bands corresponding to mRNAs 14–16 and to 12 S rRNA and 12 S\* RNA. It is known that, *in vivo*, the rate of synthesis of the rRNAs in HeLa cell mitochondria is 15- to 60-fold that of the mRNAs (5). In the electrophoretic run shown in Fig. 3, components with the mobility of 7 S RNA would have run out of the gel. However, in other experiments, after shorter electrophoretic runs (see below), no labeled 7 S component was found in the RNA from mitochondria incubated in the presence of phosphate and either succinate or pyruvate.

**Stimulation of RNA Labeling by Oxidizable Substrates is Blocked by Respiration Inhibitors or Oligomycin**—The experiments described above suggested an involvement of mitochondrial respiration and oxidative phosphorylation in the stimulation of RNA labeling. Direct evidence for this involvement was provided by the use of specific inhibitors. Antimycin is known to inhibit the oxidation of both pyruvate and succinate by blocking the electron transport chain at site 2 (25,

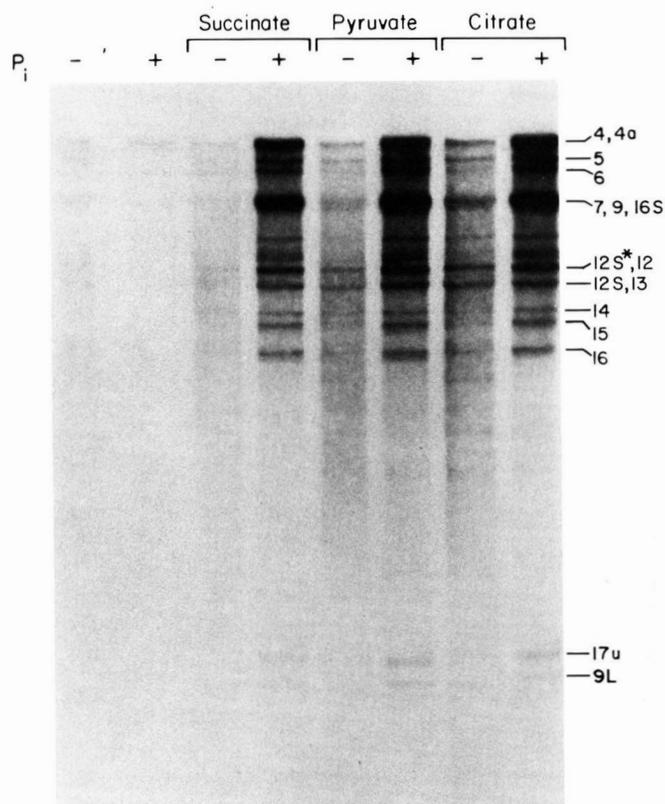


FIG. 3. Effects of phosphate and oxidizable substrates on the labeling of RNA in isolated mitochondria. Mitochondria were labeled with [ $\alpha$ - $^{32}$ P]UTP in the presence or absence of 10 mM phosphate ( $P_i$ ) and/or 1 mM sodium succinate, or 1 mM sodium pyruvate, or 1 mM sodium citrate, as indicated. The total labeled RNA was electrophoresed through a 5% polyacrylamide, 7 M urea gel and autoradiographed. Equal amounts of mitochondrial RNA were applied onto each lane.

26), while rotenone inhibits only the oxidation of pyruvate by blocking the electron transport chain at site 1 (27, 28). As predicted, stimulation of RNA labeling in isolated mitochondria by pyruvate and phosphate was blocked by both rotenone and antimycin, whereas stimulation by succinate and phosphate was blocked only by antimycin (Fig. 4). The stimulatory effect of the oxidizable substrates in the presence of phosphate was presumably due to oxidative phosphorylation of endogenous ADP to ATP, as indicated by the observation that the effect was strongly reduced by oligomycin, a drug which selectively inhibits the mitochondrial  $H^+$ -ATPase, and therefore blocks oxidative phosphorylation of ADP (Fig. 4).

**High Levels of ATP Stimulate Preferentially Labeling of rRNA and L-Strand Transcripts**—In order to test whether, in the experiments carried out in the presence of an oxidizable substrate and phosphate, the endogenous ADP was limiting, the effects of exogenous ADP on mitochondrial RNA labeling were investigated. The RNA labeled in the presence of 1 mM ADP alone gave an electrophoretic pattern resembling that observed for the RNA labeled in the presence of an oxidizable substrate and phosphate, as concerns the low level of radioactivity in the 12 S\* and 12 S rRNA bands (not shown). Increasing concentrations of ADP, in the presence of a constant amount of phosphate (10 mM) and succinate (2 mM), stimulated progressively the labeling of the different RNA species (Fig. 5a). However, the most striking result was the differential increase in labeling of the rRNAs versus the mRNAs. The labeling of the mRNAs (for example, mRNAs 14–16), in the presence of 0.2 mM ADP, was ~2-fold greater

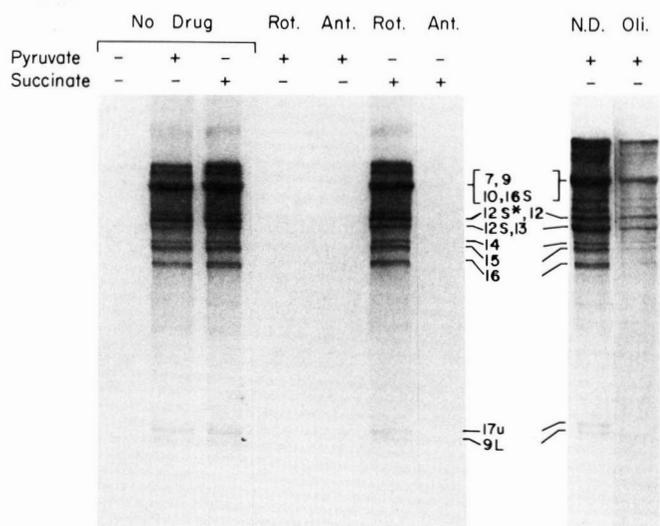


FIG. 4. Effects of antimycin, rotenone, and oligomycin on mitochondrial RNA labeling in the presence of phosphate and either pyruvate or succinate. RNA was labeled in isolated mitochondria in the presence of 10 mM phosphate and either 2 mM pyruvate or 2 mM succinate. 20  $\mu$ M rotenone (*Rot.*), or 2  $\mu$ M antimycin (*Ant.*), or 1.65  $\mu$ g/ml oligomycin (*Oli.*) was added to the indicated incubation mixtures prior to the resuspension of the mitochondrial pellets. The RNA samples were treated as in Fig. 3. *N.D.*, no drug.

than in the presence of only succinate and phosphate, as estimated from densitometric tracings. In contrast, the labeling of the bands containing the 12 S\* RNA and 12 S RNA was stimulated about 10-fold. Despite the comigration of the 12 S\* and 12 S RNAs with mRNAs 12 and 13, respectively, and assuming that mRNAs 12 and 13 behave as mRNAs 14–16 (Ref. 5; see also Fig. 1a), most of the increase in labeling in the 12 S\* and 12 S bands in the presence of ADP must have involved the rRNA species. The increase in the labeling of 12 S rRNA was especially marked, suggesting a very efficient processing of the 12 S\* RNA to 12 S RNA under these conditions. No estimate was made of the effects of the addition of ADP on the labeling of 16 S rRNA, which is not resolved from mRNAs 7 and 9 in the polyacrylamide/urea gel system. However, by the same argument made above, most of the large increase in radioactivity of the thick composite band containing RNAs 7, 9, 10, and 16 S must have been due to the 16 S rRNA. A fractionation of the RNA in a  $CH_3HgOH$ -agarose gel, in which the 16 S rRNA is resolved from the other RNA species, revealed in fact an equivalent stimulation of labeling of the 12 S rRNA species and of 16 S rRNA (data not shown) (see also Fig. 1a). Note also in Fig. 5a the increase in labeling of the band containing the rRNA precursors u4a and u4 (7). Fig. 5a further shows the strong stimulation of labeling of 7 S RNA in mitochondria incubated in the presence of 1 mM ADP, succinate, and phosphate.

As shown in Fig. 5, a and b, increasing concentrations of ATP, in the presence of succinate and phosphate, have effects comparable to, and possibly even greater than those of ADP, as concerns the preferential stimulation of labeling of the rRNA species, including the 16 S rRNA (as verified in  $CH_3HgOH$ -agarose gels (not shown)), and 7 S RNA (Fig. 5a; in Fig. 5b, the 7 S RNA band is barely recognizable against the background, due to the low exposure of the autoradiogram). Also in the absence of succinate and phosphate, ATP stimulates RNA labeling in a way qualitatively similar to that observed with ATP, succinate, and phosphate, although to a somewhat lower extent (Fig. 5c). In Fig. 5, b and c, the low exposure of the autoradiograms allows one to appreciate more

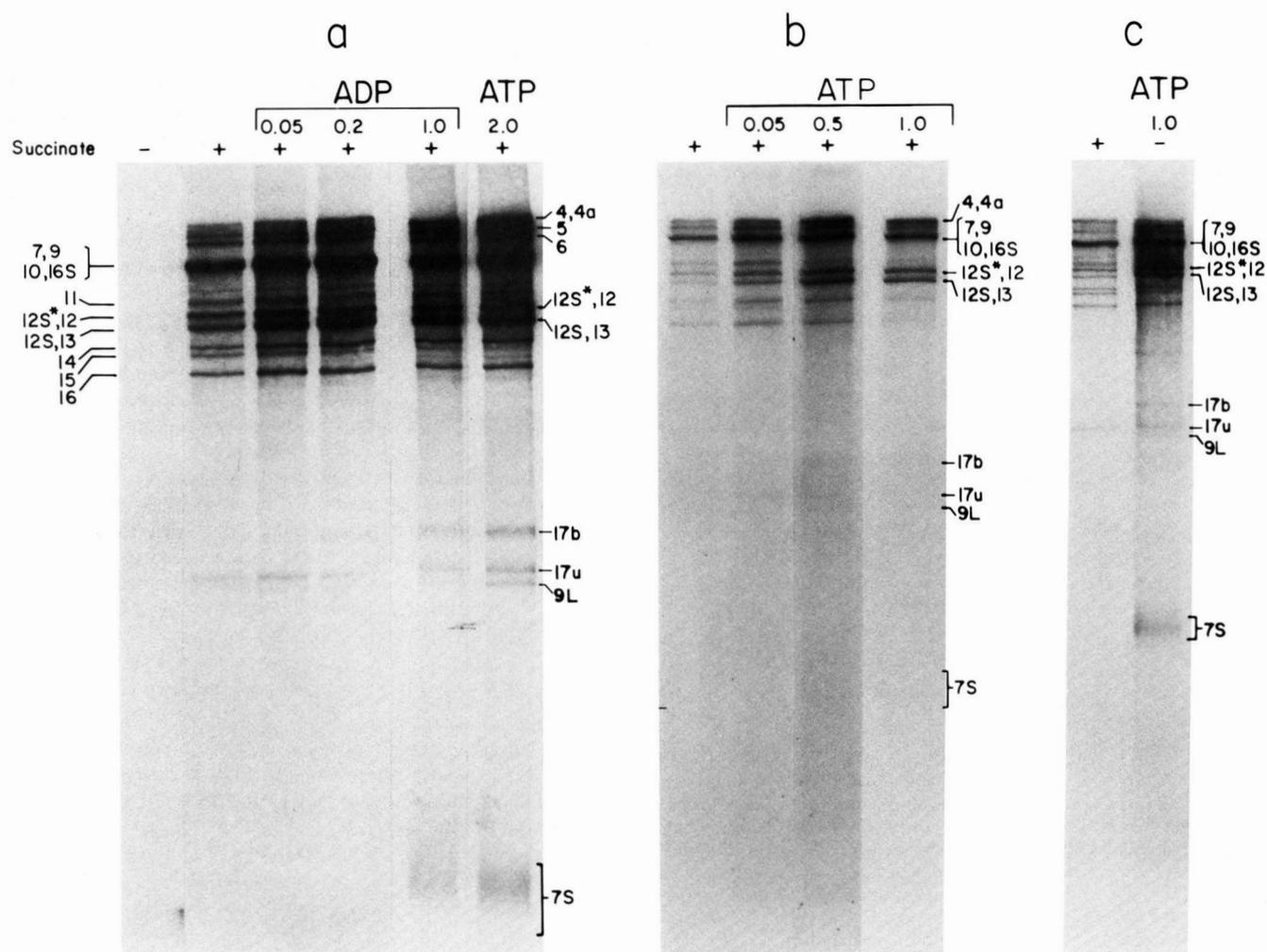


FIG. 5. **Effects of ADP or ATP on mitochondrial RNA labeling in the presence or absence of succinate and phosphate.** Mitochondria were labeled with [ $\alpha$ - $^{32}$ P]UTP in the presence of 10 mM phosphate and in the presence (+) or absence (-) of 2 mM sodium succinate, with varying concentrations of ADP or ATP, as indicated in millimolars at the top of the lanes. In panel c, no phosphate was added to the ATP-containing sample, and the succinate sample contained 10 mM sodium succinate. The RNA samples were treated as in Fig. 3. Within each panel, all the lanes were autoradiographed for the same length of time.

easily the uniform labeling of the mRNAs and the near absence of rRNA labeling in the samples lacking ATP and the strong stimulation of rRNA labeling by high ATP levels.

A quantitation of the overall effects of exogenous ADP or ATP on mitochondrial RNA labeling is shown in Fig. 6. The addition of 1 mM ADP to the basic medium greatly increases (~20-fold) the RNA labeling. The combination of 1 mM pyruvate, 1 mM ADP, and 10 mM phosphate causes a further stimulation of RNA labeling to about 50-fold the basic medium level. A similar stimulation of labeling is produced by 1 mM ATP alone. The addition of 1 mM ATP, 1 mM pyruvate, and 10 mM phosphate to the incubation medium increases the labeling above that seen with ATP alone by approximately the same difference as observed between the sample incubated in the presence of ADP and that incubated in the presence of ADP, pyruvate, and phosphate. Since roughly 50% of the added 1 mM ATP, as measured in the medium, is dephosphorylated to ADP and AMP during a 30-min incubation at 37 °C (18), the stimulation of labeling by pyruvate and phosphate over that observed with ATP alone may be a consequence of the subsequent phosphorylation of ADP, which raises the intramitochondrial ATP concentration.

*Effects of Inhibitors or Uncouplers of Oxidative Phosphorylation on the Stimulation of RNA Labeling by ADP or ATP*—The stimulation of RNA labeling in isolated mitochondria by high concentrations of ADP or ATP was further analyzed by using inhibitors of the respiratory chain or of the [ $H^+$ ]ATPase and an uncoupler of oxidative phosphorylation. As shown in Fig. 6, labeling of RNA in the presence of ADP or ATP alone was essentially unaffected by either antimycin or oligomycin. In contrast, the addition of antimycin or oligomycin to the transcription system in the presence of ADP plus pyruvate and phosphate inhibited almost completely the stimulation of labeling observed under these conditions over the level observed with ADP alone. This inhibition was especially marked for the labeling of the rRNAs and 7 S RNA (not shown). Similarly, the stimulation of RNA labeling produced by pyruvate and phosphate in the presence of ATP over the level observed with ATP alone was inhibited by both antimycin and oligomycin.

The uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP<sup>2</sup>) had no appreciable effect on the mitochondrial RNA

<sup>2</sup> The abbreviation used is: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

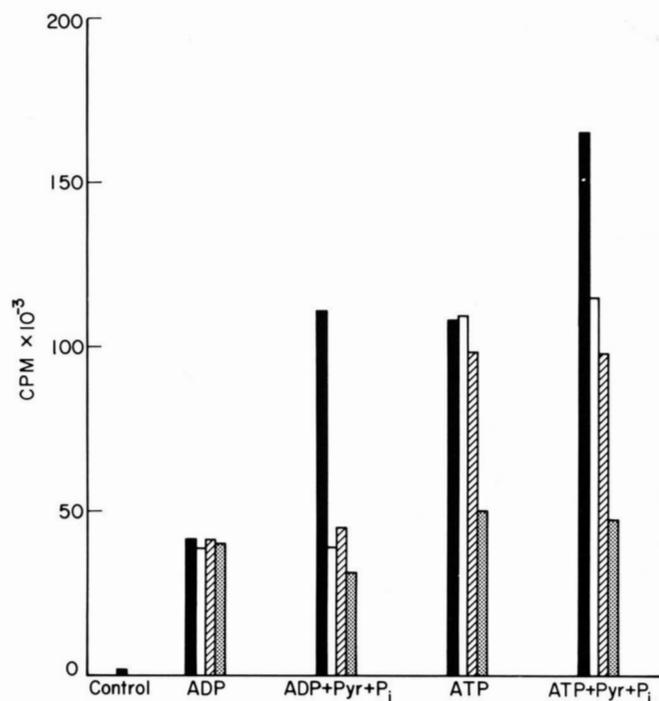


FIG. 6. Levels of RNA labeling in mitochondria incubated in the presence of ADP or ATP, with or without pyruvate and phosphate, and effects of antimycin, oligomycin, and CCCP. Mitochondria were labeled with [ $\alpha$ -<sup>32</sup>P]UTP in medium containing 1 mM ADP with or without 1 mM pyruvate (*Pyr*) and 10 mM phosphate (*P<sub>i</sub>*), or 1 mM ATP with or without 1 mM pyruvate and 10 mM phosphate, in the absence of drugs, or in the presence of 2  $\mu$ M antimycin, or 1.65  $\mu$ g/ml oligomycin, or 1.7  $\mu$ g/ml CCCP. 10% of each sample of extracted RNA was added to 1 ml of 1 N HCl, 50 mM NaH<sub>2</sub>PO<sub>4</sub>, containing 20  $\mu$ g of salmon sperm DNA, and incubated at 0°C for 20 min. The precipitates were collected on glass fiber filters, and the filters were washed and counted in scintillation fluid. No drug, solid columns; antimycin, open columns; oligomycin, hatched columns; CCCP, dotted columns. Control: basic incubation medium.

labeling observed in the presence of ADP, except for an indication of inhibition of the processing of 12 S\* RNA to 12 S rRNA (not shown). In contrast, this drug dramatically inhibited the labeling of all RNA species in the presence of ADP plus pyruvate and phosphate and reduced it to a level comparable to that observed with ADP alone. CCCP substantially inhibited RNA labeling in the presence of ATP, and reduced the RNA labeling observed in the presence of ATP plus pyruvate and phosphate to a level considerably lower than that found with ATP alone.

*Changes in Precursor Uptake Do Not Account for the Effects on RNA Labeling*—Experiments were carried out to test to what extent an effect on the uptake of the [ $\alpha$ -<sup>32</sup>P]UTP into the organelles could be responsible for the observed stimulation of RNA labeling by an oxidizable substrate and phosphate or by ATP. Mitochondria from 0.5 g of cells were incubated for 30 min at 37°C in basic medium containing 5  $\mu$ Ci [ $\alpha$ -<sup>32</sup>P]UTP in the presence or absence of 1 mM pyruvate and 10 mM phosphate or of 1 mM ATP, then pelleted, washed once in buffer, and counted. A radioactivity of  $1.07 \times 10^5$ ,  $1.44 \times 10^5$ , and  $2.05 \times 10^5$  cpm was measured in the samples incubated in basic medium, in basic medium plus pyruvate and phosphate and in basic medium plus ATP, respectively. Very similar values were obtained after two washes of the mitochondria, micrococcal nuclease digestion, a further wash, then pronase digestion and phenol extraction. A considerable portion of this radioactivity was due to unincorporated precursor. These tests thus excluded a major role of differences in

precursor uptake in the RNA-labeling data.

*The Effects on RNA Labeling Reflect Changes in Rate of Synthesis of the Different Species*—In order to test whether the different RNA-labeling patterns observed under different incubation conditions reflected changes in the rate of synthesis or in the stability of the various classes of RNA molecules synthesized *in vitro*, a short pulse-labeling experiment was performed. To allow time for equilibration of the mitochondria with the exogenous ATP or pyruvate and to exhaust any endogenous ADP, the organelles had been preincubated for 6 or 15 min in different media before addition of the label. As shown in Fig. 7 for the experiment with 6-min preincubation, the patterns observed after a 3-min pulse were similar to those obtained after 30-min labeling without a preincubation step. In particular, the pyruvate sample showed minimal labeling of the rRNAs and 7 S RNA. In the samples incubated in the presence of ATP or ATP plus pyruvate, the relative abundance of 12 S\* RNA and the near-absence of mature 12 S rRNA and of polyadenylated mRNA 17 were presumably due to the short duration of the pulse. Identical results were obtained in an experiment with 15-min preincubation (not shown).

In other experiments, after a 15-min [ $\alpha$ -<sup>32</sup>P]UTP pulse in the presence of pyruvate and phosphate, or ATP and phosphate, or pyruvate, ATP, and phosphate, the mitochondria were incubated up to 120 min in the presence of a dose of actinomycin D (6.4  $\mu$ g/ml) sufficient to block further RNA synthesis almost completely (17). No obvious difference was observed in the relative rates of decline of radioactivity in the

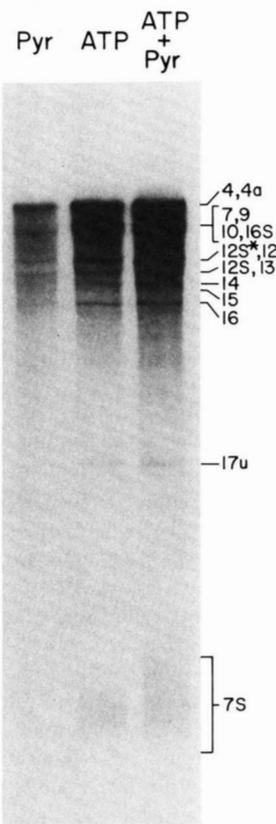


FIG. 7. Short pulse labeling of RNA in isolated mitochondria in the presence of pyruvate and phosphate, or ATP and phosphate, or ATP, pyruvate, and phosphate. Mitochondria were incubated for 6 min in basic incubation medium in the presence of 10 mM NaPO<sub>4</sub> and either 2 mM sodium pyruvate or 1 mM ATP or 2 mM pyruvate and 1 mM ATP, and then labeled with [ $\alpha$ -<sup>32</sup>P]UTP for 3 min. Equal portions of the total RNA from the different samples were run in a polyacrylamide/urea gel.

rRNA and mRNA species in each of the three media mentioned above (not shown). These experiments and the pulse experiments discussed above clearly showed that the effects on the RNA-labeling patterns observed in different media reflected mainly, if not exclusively, differences in the rates of synthesis of the various RNA species.

**Quantitation by S<sub>1</sub> Protection Analysis of the Differential ATP Effects on rRNA, mRNA, and L-Strand Transcript Labeling**—In order to examine quantitatively the dependence of the RNA-labeling pattern upon the composition of the medium, and to avoid the ambiguities arising from the comigration of RNA components in polyacrylamide/urea gels, an S<sub>1</sub> nuclease protection analysis was performed. Two single-stranded M13 clones containing either the H-strand (M8.9) or the L-strand (M9.9) of the MboI fragment 9 of human mtDNA, which encompasses the origin region (from position 0(=16569) to 739) (21), were used in this analysis. Fig. 2 shows the expected sizes of the RNA fragments protected by these clones. In addition, a single-stranded M13 clone containing the H-strand fragment between positions 7662 and 8291 (OP-10), which was expected to protect 629 nucleotides of mRNA 16 (2, 4), was used to monitor the labeling of this RNA species. As shown in Fig. 8, the clone M8.9 protected two RNA fragments of ~96 nucleotides and ~183 nucleotides in length (lane 3), which correspond to those expected from protection of the 12 S and 12 S\* RNA, respectively (Fig. 2). The L-strand counterpart, M9.9, yielded a specific band at ~408 nucleotides, which corresponds to the L-strand transcript extending from the initiation site (*I<sub>L</sub>* in Fig. 2) to the 5'-end of the mtDNA fragment in the M13 clone, and a group of bands between 160 and 200 nucleotides, which correspond to the heterogeneous 7 S RNA (lane 4) (23). Finally, the OP-10 clone protected the majority of mRNA 16, giving a band at ~629 nucleotides (lane 5). The two bands corresponding to sizes of 350–370 nucleotides and the high molecular weight bands, which are observed in all lanes, presumably result from formation of RNA-RNA hybrids (2).

The relative levels of labeling of the rRNAs, mRNAs, and L-strand transcripts during incubation of mitochondria in the presence of either 1 mM ATP and 10 mM phosphate or 2 mM pyruvate and 10 mM phosphate were estimated by using an excess (>10-fold) of the OP-10 clone and either the M8.9 or the M9.9 clone in the same hybridization mixture for S<sub>1</sub> protection of the labeled RNA. The results of this experiment are shown in the right half of Fig. 8 (sixth to eighth lanes and tenth to twelfth lanes). In the experiment carried out in the presence of ATP and phosphate, the ratio of the sum of radioactivity in the two bands corresponding to the 12 S and 12 S\* RNA fragments protected by M8.9 to the radioactivity in the band corresponding to the mRNA 16 fragment protected by OP-10 was about 1.5, as determined by densitometry of the autoradiograms. Also, the ratio of the sum of radioactivity in the bands resulting from protection of 7 S RNA and the longer L-strand transcript by M9.9 to the radioactivity in the band corresponding to the protected mRNA 16 fragment was approximately 7. In contrast, when RNA labeled in the presence of pyruvate and phosphate was hybridized to OP-10 and either M8.9 or M9.9, and S<sub>1</sub> nuclease treated, the ratio of the sum of the intensities of the bands corresponding to the 12 S and 12 S\* RNAs to the intensity of the mRNA 16 band was about 0.2, and the ratio of label in the bands corresponding to 7 S RNA and the longer L-strand transcript to that in the mRNA 16 band was approximately 0.1. Hence, the relative labeling of the different classes of RNA changed dramatically depending upon the type of energetic substrates present.

In the experiments described above, RNA u4a, the rRNA

precursor (7), if present, would have yielded a protected 183-nucleotide fragment, as the 12 S\* RNA, and RNA u4, a processing intermediate (7), a protected 96-nucleotide fragment, as the 12 S rRNA. Similarly, RNA b4, the polyadenylated rDNA transcript which belongs to the entire H-strand transcription unit starting at *I<sub>HT</sub>* (7) (Fig. 2), may have contributed to the formation of the 96-nucleotide S<sub>1</sub>-resistant fragment. However, this contribution would be expected to be very small, considering the rate of labeling and the length of RNA b4 (7). An interesting finding in the above experiments is the considerably greater labeling of the protected fragment of 12 S\* RNA (183 nucleotide) as compared to the protected fragment of 12S rRNA (96 nucleotide), which is in contrast to the equal amounts of label observed in the full-length 12 S rRNA and 12 S\* RNA species in the same experiments (not shown). In the ATP sample, the ratio of radioactivity in the 183- and 96-nucleotide long protected fragments was about 5.6, while the ratio of labeling of the 12 S\* RNA and 12 S rRNA bands (mostly represented by the rRNA species) was ~1.0. In another similar experiment, the ratio of radioactivity in the 183- and 96-nucleotide protected fragments was 2.1, whereas the ratio of labeling of the 12 S\* RNA and 12 S rRNA bands was ~0.7.

**Polyadenylation of Mitochondrial RNA Requires High ATP Levels**—Fig. 1 shows a comparison of the total (*T*), oligo(dT)-cellulose unbound (*U*) and oligo(dT)-cellulose bound (*B*) RNA labeled in isolated mitochondria in the presence of 1 mM ATP and 10 mM phosphate (*a*) or 2 mM sodium pyruvate and 10 mM phosphate (*b*). The preferential stimulation of labeling of the rRNAs and 7 S RNA in 1 mM ATP, over the level observed in the pyruvate sample, is again recognizable in the total and the oligo(dT)-cellulose unbound RNA patterns. Equally striking is the difference in the labeling of the oligo(dT)-cellulose bound RNA in the two samples. It is clear that a poly(A) stretch of greater than 20 nucleotides, as required for binding to oligo(dT)-cellulose, is much more efficiently added in the presence of ATP and phosphate than in the presence of pyruvate and phosphate. The extent of this process under different conditions is most clearly appreciated by an examination of the bands corresponding to mRNA 17. The non-polyadenylated (17u) and polyadenylated (17b) forms of RNA 17 are easily separable in this gel system (22, 31). In the presence of an oxidizable substrate and phosphate (Figs. 1b, 3–5), the labeling of mRNA 17b is essentially nil. However, when a high concentration of ADP with succinate or pyruvate and phosphate (Fig. 5), or of ATP, with or without succinate or pyruvate and phosphate (Figs. 1a and 5), is added to the labeling mixture, a high proportion of the newly synthesized mRNA 17 is polyadenylated.

#### DISCUSSION

This work has provided insights into the energetic requirements for mtDNA transcription in isolated HeLa cell mitochondria. The observation, made in the present and previous experiments (18), that RNA labeling in isolated mitochondria was only marginal in basic medium implied that the large intramitochondrial ATP pool (19) must have been to a large extent depleted as a result of hydrolysis or export during cell fractionation or incubation of the organelles. The evidence presented in this paper shows that the presumably depleted ATP pool can be rebuilt to different levels either by exogenous ATP, or by ATP produced endogenously, via oxidative phosphorylation, when an oxidizable substrate, phosphate, and ADP are added to the basic medium.

The patterns of mitochondrial RNA labeling observed under different conditions clearly indicated that the oxidizable

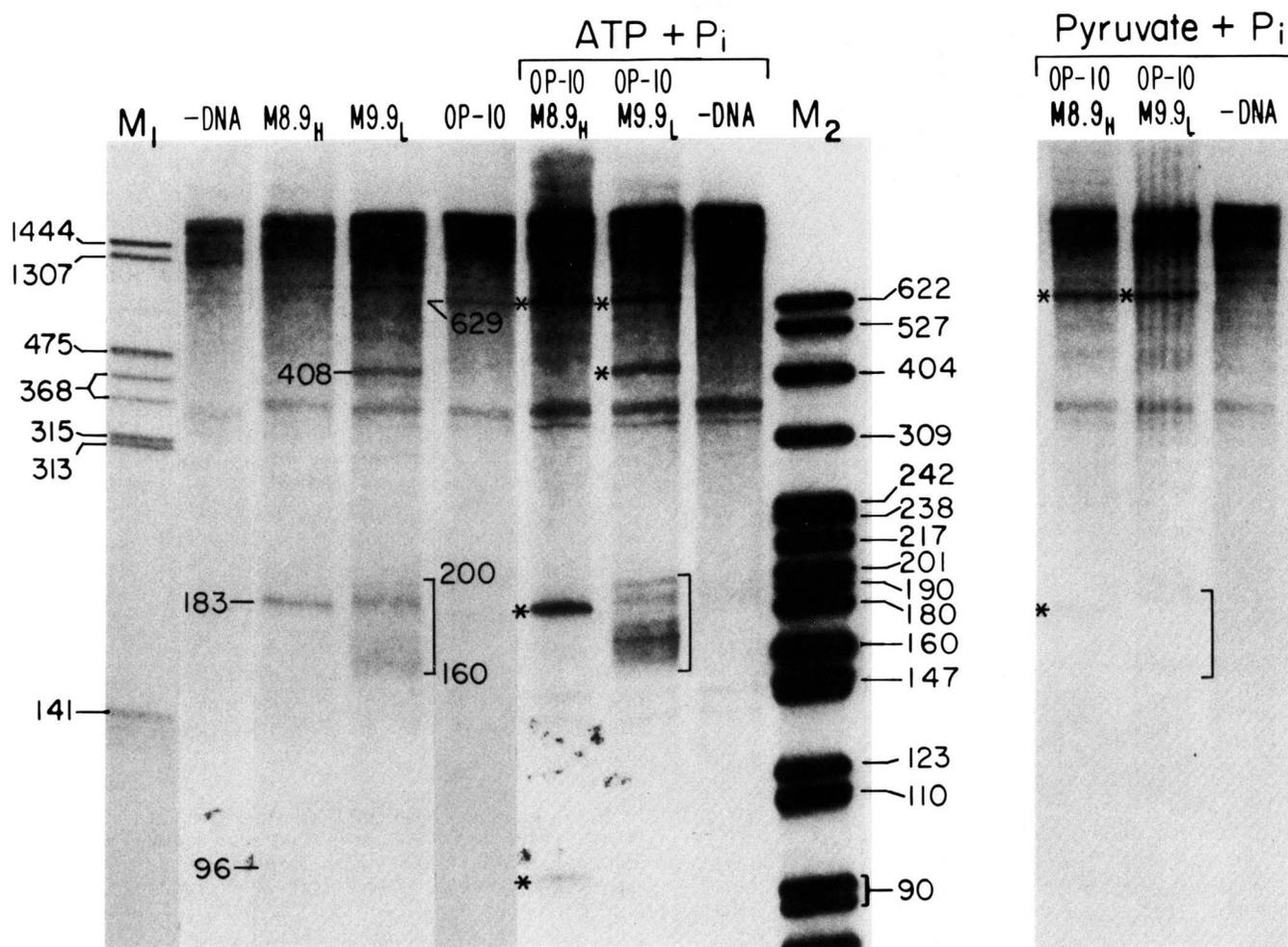


FIG. 8.  $S_1$  nuclease protection analysis of RNA labeled in isolated mitochondria in the presence of ATP and phosphate, or pyruvate and phosphate. Samples of RNA (from 0.05 ml of packed cells), labeled with  $[\alpha\text{-}^{32}\text{P}]\text{UTP}$  in the presence of 1 mM ATP and 10 mM phosphate ( $\text{ATP} + \text{P}_i$ ) (sixth to eighth lanes), or 2 mM sodium pyruvate and 10 mM phosphate ( $\text{Pyruvate} + \text{P}_i$ ) (tenth to twelfth lanes), were hybridized with a mixture of 10  $\mu\text{g}$  each of single-stranded M13 clones, as indicated. The clones are designated M8.9<sub>H</sub> (containing the H-strand of the *Mbo*I fragment 9 between positions 5 and 744 in human mtDNA), M9.9<sub>L</sub> (containing the L-strand of the *Mbo*I fragment 9 between positions 0 and 739), and OP-10 (containing the H-strand of the fragment between the *Bcl*I and *Xba*I sites at positions 7662 and 8291) (21). From the known amount of the individual RNA species in each sample (<0.1  $\mu\text{g}$  (5)), the amount of DNA used for hybridization was estimated to contain a large excess (>10-fold) of the homologous sequences. The samples were  $S_1$  nuclease-treated, and electrophoresed through a 5% polyacrylamide, 7 M urea gel. The second to fifth lanes show control experiments in which RNA samples (labeled in the presence of ATP and phosphate) were hybridized with the indicated individual M13 clones and then treated as described above, for the identification of the protected RNA fragments. The asterisks indicate the protected fragments. The markers are *Taq*I-digested pBR322 DNA ( $M_1$ ) and *Msp*I-digested pBR322 DNA ( $M_2$ ), labeled with  $[\alpha\text{-}^{32}\text{P}]\text{dGTP}$  and  $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ .

substrates tested, as well as phosphate and ADP, were present in limiting amounts in the isolated organelles. The low level of RNA labeling observed in the presence of ADP alone, which was antimycin, oligomycin, and CCCP insensitive, was probably due to substrate level phosphorylation (29).

The marked increase in RNA labeling caused by addition of ATP alone to the basic incubation medium was, as expected, substantially insensitive to antimycin and oligomycin; by contrast, it was inhibited to a considerable extent by CCCP, presumably due to the uncoupling effects and stimulation of ATPase activity by this drug. The antimycin- and oligomycin-insensitive increase in RNA labeling, which was observed in the presence of ATP, an oxidizable substrate and phosphate, over the level attained in the presence of ATP alone, was presumably due to respiration-driven phosphorylation of ADP arising from hydrolysis of ATP during incubation.

The results of direct measurements, as well as the evidence of differential effects of ATP on the labeling of the various RNA classes, excluded that the stimulation of RNA labeling produced in isolated mitochondria by the increased ATP concentration was due to changes in the specific activity of the mitochondrial UTP pool or to an increased phosphorylation of  $[\alpha\text{-}^{32}\text{P}]\text{UMP}$  or  $[\alpha\text{-}^{32}\text{P}]\text{UDP}$  resulting from enzymatic hydrolysis of  $[\alpha\text{-}^{32}\text{P}]\text{UTP}$  before, during, or after uptake into the organelles. Furthermore, the results of the short pulse and actinomycin D chase experiments described above argued against the possibility that the qualitative effects on the RNA-labeling patterns produced by increasing the level of available ATP resulted from changes in the relative rates of decay of the various classes of newly synthesized molecules, clearly pointing, on the contrary, to changes in their relative rates of synthesis. The labeling behavior of the various RNA species,

as well as the S1 protection data, also excluded that there is an accumulation of the rRNA precursors u4a and u4 (7) in low ATP, and that the high ATP requirement is for processing rather than for synthesis of the rRNAs.

The main conclusion of the present study is that rRNA synthesis and L-strand transcription, in isolated human mitochondria, exhibit a much higher ATP requirement than does mRNA synthesis. At the low ATP levels expected to be produced in the presence of pyruvate or a tricarboxylic acid cycle intermediate and phosphate and in the absence of adenine nucleotides, there is a substantial synthesis of the mRNAs, while the synthesis of the rRNAs is marginal and at best comparable to that of the mRNAs. At the high intramitochondrial ATP levels attained in the presence of 1 mM ATP (or ADP) plus 1 or 2 mM pyruvate (or succinate) and 10 mM phosphate, there is a more than 10-fold increase in the rRNA labeling and only a 2-fold increase in the labeling of the mRNAs (the latter being accountable in good part by the moderate increase in precursor uptake), relative to the labeling observed in the presence of an oxidizable substrate and phosphate. Similarly, the synthesis of 7 S RNA is substantially absent at low ATP levels and very pronounced at high ATP levels. Although mRNA synthesis occurs at a substantial level at low ATP levels, the polyadenylation of the mRNAs does require high ATP, in agreement with previous observations (18).

There is good evidence that a substantial fraction of the rRNA synthesized in isolated mitochondria is represented by chains initiated *in vitro* (18). In the S<sub>1</sub> protection experiments reported here, the molar ratio of the 5'-end proximal 183-nucleotide protected fragment of the 12 S\* RNA to the 5'-end proximal 96-nucleotide protected fragment of 12 S rRNA (Fig. 2), at high ATP levels, was two to three times higher than the molar ratio of the newly synthesized complete 12 S\* and 12 S rRNA species in the same experiment. This suggests that removal of the tRNA<sup>Phe</sup> and leader sequences from the rRNA precursors may require a prior processing of the precursors at the 3'-end of the 12 S rRNA sequence, and that, at high ATP levels, there is, in the total RNA preparation, a large amount of nascent or incomplete released rRNA chains carrying at their 5'-end the tRNA<sup>Phe</sup> and the leader. The finding that mRNAs encoded in the 5'-end proximal half (as mRNAs 12, 13, and 16) and in the terminal portion (as mRNA 11) of the total H-strand transcription unit which controls the synthesis of the H-strand encoded mRNAs (Fig. 2) were labeled to a similar extent both in the presence of an oxidizable substrate and phosphate and in the presence of high ATP levels strongly suggests that a large portion of the mRNAs synthesized under both conditions also resulted from new initiation events.

As to the L-strand transcription unit, the S<sub>1</sub> protection experiments have revealed an RNA extending from the initiation site (I<sub>L</sub>) to more than 200 nucleotides beyond the 3'-end of 7 S RNA (Fig. 2), which probably represents the initial portion of the L-strand polycistronic transcript. Nothing conclusive is known about the kinetic relationship between this transcript and 7 S RNA, although the possibility of a precursor to product relationship has been suggested (18, 22, 30). The two transcripts appeared to be labeled with similar efficiency in the presence of 1 mM ATP and 10 mM phosphate; in the presence of 2 mM pyruvate and 10 mM phosphate, both species were much less labeled.

It seems possible that the high ATP demand for rRNA synthesis and L-strand transcription observed in isolated mitochondria reflects a requirement for initiation of transcription. However, no evidence has been obtained concerning

the nature of the possible ATP-requiring step. We have previously presented a model of regulation of the synthesis of mitochondrial rRNAs and mRNAs involving two modes of transcription of the rDNA region under the control of two distinct promoters, leading to the synthesis of the two classes of RNA (7). This model was based on the evidence for the existence *in vivo* of two initiation sites for H-strand transcription (6, 8) (Fig. 2) and has been supported by the observation, both *in vivo* and in isolated mitochondria, of two types of rDNA transcripts with different kinetic properties (7) and different sensitivity to intercalating drugs, low temperature, Ca and Mg ions (17, 18), which can be correlated with the two initiation sites. The findings reported here concerning the uncoupling of the synthesis of the two classes of RNA at low ATP levels are consistent with a model of independent control of the transcriptional initiation events leading to the synthesis of rRNAs and mRNAs in mitochondria.

We do not know whether and how the different ATP requirements for the three transcription units of human mtDNA observed in isolated organelles can be related to a control mechanism operating in the intact cell. Nothing is known in fact about the physiological fluctuations in ATP concentration within mitochondria *in vivo* in different cell types. However, it is possible that the differential sensitivity to the ATP level of the synthesis of rRNAs and mRNAs, which has been detected here, represents a device which becomes of value to the cell under certain physiological or stress conditions.

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