Transcriptional mapping of the ribosomal RNA region of mouse L-cell mitochondrial DNA

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ABSTRACT

The map positions in mouse mitochondrial DNA of the two ribosomal RNA genes and adjacent genes coding several small transcripts have been determined precisely by application of a procedure in which DNA-RNA hybrids have been subjected to digestion by S1 nuclease under conditions of varying severity. Digestion of the DNA-RNA hybrids with S1 nuclease yielded a series of species which were shown to contain ribosomal RNA molecules together with adjacent transcripts hybridized conjointly to a continuous segment of mitochondrial DNA. There is one small transcript about 60 bases long whose gene adjoins the sequences coding the 5'-end of the small ribosomal RNA (950 bases) and which lies approximately 200 nucleotides from the D-loop origin of heavy strand mitochondrial DNA synthesis. An 80-base transcript lies between the small and large ribosomal RNA genes, and genes for two further short transcripts (each about 80 bases in length) abut the sequences coding the 3'-end of the large ribosomal RNA (~1500 bases). The ability to isolate a discrete DNA-RNA hybrid species approximately 2700 base pairs in length containing all these transcripts suggests that there can be few nucleotides in this region of mouse mitochondrial DNA which are not represented as stable RNA species.

INTRODUCTION

The most notable correlate to emerge from studies of the map positions of stable animal mitochondrial RNAs (mtRNA) is the location of the ribosomal RNA (rRNA) transcripts on the mitochondrial DNA (mtDNA) genome. In the mouse (1), human (2), *Xenopus laevis* (3) and rat (4) mtDNA systems the two rRNA cistrons map adjacent to each other at or near the D-loop origin of heavy strand (H-strand) DNA synthesis, with the small rRNA lying proximal to the D-loop. Transcription proceeds opposite the direction of H-strand DNA synthesis with the small rRNA gene transcribed before the large rRNA gene. A similar situation may prevail for *Drosophila* mtDNA as well (5,6). Since both rRNAs are encoded by the H-strand and these transcripts represent >90% of the RNA population, it is likely that this H-strand region contains a major promoter.

In order to understand the organization of this expressed region of the mouse mtDNA genome we have obtained a more detailed transcription map than has previously

been determined. These studies have utilized the available cloned mtDNA template strand as a hybridization substrate for mtRNA. When such DNA-RNA hybrids are subjected to digestion by S1 nuclease all single-stranded nucleic acid not hybridized is degraded, resulting in clean duplex fragments representing transcribed regions of the genome. We describe here the analysis of sets of S1 generated fragments carrying one or more transcripts hybridized conjointly to the same mtDNA segment. The location of these transcribed regions is derived by utilizing DNA template strands isolated from defined restriction fragments. The data are consistent with previous studies of human (2) and *Xenopus laevis* (7) mtDNA in which a low molecular weight RNA was mapped between the rRNAs, flanking the 5'-end of the small rRNA, and flanking the 3'-end of the large rRNA. In addition, we report here that more than one small molecular weight RNA is located near the 3'-end of the large rRNA and there is virtually no DNA sequence in this entire region that is not represented as a stable transcript; i.e., there may not be any non-transcribed, or transcriptionally non-conserved, spacers in a 2740 base mtDNA segment that includes the mouse mitochondrial rRNA cistrons.

MATERIALS AND METHODS

DNA preparations

E. coli C600 $r_k^- m_k^-$ containing the plasmid pAM1 or pAC133 were grown in M9 minimal-medium supplemented with 0.8% casamino acids, 0.4% glucose, thiamine (10 μ g/ml), ampicillin (25 μ g/ml). Propagation and isolation of plasmid DNAs were in accordance with P2-EK1 NIH guidelines. pAM1 contains the entire mouse mtDNA genome cloned in pACYC177 vector at a single *Hae*II site (8). Labeling of DNA with 3 H was achieved by adding [5-methyl- 3 H] thymidine (4 μ Ci/ml), unlabeled thymidine (0.1 μ g/ml) plus uridine (250 μ g/ml), over several divisions of growth. Cells were harvested at stationary phase, and plasmid DNA was prepared by the cleared-lysate procedure (1) (0.5% Triton, 62.5 mM EDTA, 25 mM Tris, pH 8), followed by two consecutive isolations as the closed circular DNA fraction from ethidium bromide (EthBr)-CsCl gradients. EthBr was removed by extraction with isopropanol/CsCl and the DNA was dialyzed against TE (10 mM Tris, pH 7.5, 1 mM EDTA) prior to precipitation by ethanol and storage in TE at -20° . The DNA had a specific activity of approximately 60,000 cpm/ μ g.

For strand separation, DNA was singly nicked by digestion with DNase I (1 μ g/ml) for 20 min at 20° in the presence of 300 μ g/ml EthBr in 10 mM NaCl, 10 mM Tris, pH 7.5, 10 mM $MgCl_2$ (9). After addition of EDTA to 25 mM, EthBr was removed by two phenol extractions. Following two ether extractions to remove phenol, the heavy (H) and light (L) strands of the duplex were resolved in and isolated from an alkaline

CsCl gradient (1).

For isolation of full-length mtDNA, pAM1 mtDNA was digested to completion with HaeII restriction endonuclease. The resultant digest was resolved on a 0.5% agarose gel and the 16 kb linear mtDNA band was excised from the gel, electroeluted, and concentrated by ethanol precipitation. The precipitated DNA was solubilized in TE and then subjected to alkaline CsCl gradient centrifugation to isolate the H- and L-strands as described above for pAM1 mtDNA.

The largest EcoRI-HindIII restriction fragments of mtDNA were prepared from pAC133 (1) by digestion with EcoRI-HindIII restriction endonucleases and subsequent resolution of digestion products in a 0.5% agarose gel. The two largest fragments of mtDNA were not well resolved and were removed from the gel together by electroelution. The H-strand was isolated from an alkaline CsCl gradient.

Gel electrophoresis of DNA

DNA was separated on horizontal native agarose gels 18.5 cm long x 14 cm wide x 0.4 cm thick (without wicks) utilizing 90 mM Tris, pH 8.4, 90 mM boric acid and 2 mM EDTA as the running buffer. Gels were stained for 20 min in running buffer plus 2 μ g/ml EthBr at 25°, then visualized under UV light for photography.

The size markers used for double-stranded DNA fragments (and DNA-RNA hybrids, see below) in this gel system were fragments of λ ci857 viral DNA produced by complete digestion with EcoRI and HindIII restriction endonucleases. Fragment sizes were taken from the data of Philippsen et al. (10) where length assignments are based on a comparison against sequenced ϕ X174 fragments.

Preparation of RNA

RNA was isolated from LA9 cells in mid-log phase (at $2-5 \times 10^5$ cells/ml). Cells were harvested and mitochondria were isolated by the "no-sucrose gradient" procedure of Bogenhagen and Clayton (11). The mitochondria were resuspended in 0.2 M NaCl, lysed with 1.0% Sarkosyl in the presence of phenol-chloroform, and nucleic acid was extracted as described by Battey and Clayton (1). This procedure yields approximately 500 μ g of crude mtRNA from 1 ml of packed cells. Gel analysis reveals that ~5% of the RNA is the small mitochondrial rRNA and ~5% is the large mitochondrial rRNA.

For *in vivo* 32 P labeling of RNA, a 500 ml culture was inoculated at 8×10^4 cells/ml. After 4 hr, 1 mCi of [32 P]_i was added to the culture flask. 2 and 4 mCi of [32 P]_i were added at 26 and 48 hr post-inoculation. Such cultures yield approximately 200 μ g of crude mtRNA (5×10^3 cpm/ μ g). The overall integrity of the RNA is similar to that isolated from cells not exposed to isotope (data not shown).

LiCl precipitation of RNA

RNA was dissolved in TE and added to an equal volume of 4 M LiCl, mixed and stored at -20° for at least 16 hr in a plastic tube (2 M LiCl does not freeze under

these conditions). The mixture was centrifuged to recover a precipitate and supernatant. The precipitate was washed once in 2 M LiCl, once in 70% ethanol containing 0.1 M sodium acetate, then dried and redissolved in TE. The supernatant fraction was adjusted to 0.3 M in sodium acetate and 2 vol ethanol were added. After at least 30 min at -70° , the precipitate was collected, dried and redissolved in TE. Gel analysis (see **RESULTS**) revealed that the LiCl pellet contained all the RNA species present in the original preparation (with no degradation) except the 5S cytoplasmic RNA and tRNAs. Conversely, the LiCl supernatant contained the 5S and tRNAs. The fractionation was judged to be at least 90% effective on the basis of electrophoretic analysis.

Gel analysis of RNA

Denaturing acrylamide-urea gels were used (4% acrylamide [20:1, acrylamide:bis-acrylamide], 7M urea) with 90 mM Tris, pH 8.4, 90 mM boric acid and 2 mM EDTA as the running buffer. Samples were denatured in 2 vol 10 M urea (containing 0.05% bromphenol blue and 0.05% xylene cyanole) at 95° for 60 sec before loading on the gel. After electrophoresis at 4° , gels were stained with 2 μ g/ml EthBr with occasional rocking to purge the gel of urea. RNAs were visualized under UV light for photography. For autoradiography of 32 P-labeled RNA, gels were dried and exposed to Kodak X-Omat R film at -70° using an intensifying screen.

DNA-RNA hybridization

Hybridization reactions were carried out in 0.5 M NaCl, 0.05 M Tris, pH 7.5, 5 mM EDTA at 65° for 15 min. The hybridization mixture was assembled at 0° and RNA was added last, just prior to heating to 65° . H-strand DNA was utilized as the single-stranded template. In most experiments the amounts of RNA and DNA added were adjusted to give a slight molar excess of the small rRNA species (calculated at about 5-10 μ g/ml) over its gene in the mtDNA preparation (about 3-5 μ g/ml). The total nucleic acid concentration in the hybridization mixture was approximately 200-400 μ g/ml. After incubation, the mixture was cooled in ice and two volumes of ethanol were added. After at least 20 min at -70° , nucleic acids were collected by centrifugation, dried, redissolved in TE and stored at -20° .

Digestion of hybrids by S1 nuclease

Two preparations of S1 nuclease were used. Boehringer S1 nuclease was dissolved in 0.1 M sodium acetate, pH 4.5, containing 0.1 mM ZnCl_2 , at a nominal concentration of 10^6 units/ml. Sigma S1 nuclease was supplied as a solution of nominally 10^5 units/ml in 50% glycerol containing ZnSO_4 and sodium acetate, pH 4.5. Since the Boehringer units and Sigma units were not defined in the same way, the data described in this work will be presented in terms of the volume of S1 preparations used per ml of S1 reaction mixture (μ l/ml). Most S1 reactions were carried out in a volume of 40 μ l, containing DNA-RNA hybrids at a total nucleic acid concentration of 60-90 μ g/ml.

The amount of hybrid used was adjusted so that each reaction contained about 70-100 ng of double-stranded small mitochondrial rRNA-rDNA hybrid.

The reaction mixture for Boehringer S1 nuclease contained 0.5 M NaCl, 0.07 M sodium acetate, 3 mM ZnCl₂, pH 4.8. Incubation was for 60 min at 20°. For Sigma S1 nuclease, the reaction mix contained 0.18 M NaCl, 0.06 M sodium acetate, 3 mM ZnCl₂, pH 4.5, and incubation was for 30 min at 37° (1, 12). In each case S1 digestions were terminated by addition of EDTA to 25 mM and sodium acetate to 0.3 M. The size of a DNA-RNA hybrid produced by S1 digestion was obtained from the lowest S1 concentration which resulted in a discrete species abundant enough to be measured with confidence. Further digestion with S1 gradually reduces the size of any particular hybrid by degradation at the duplex ends. The sizes given for the S1-derived DNA-RNA hybrids are the mean of at least 5 measurements from different gels. We assume DNA-RNA hybrids have the same size-mobility relationship as DNA-DNA duplexes; however, we find a systematic deviation (towards increased mobility) of about 3% (equivalent to about 70 bases) in duplexes containing both full-length small and large RNA bound to one DNA strand, compared with the predicted mobility based on summing individual sizes. DNA-RNA duplexes consisting of the large and small mitochondrial rRNA and their respective DNA complement migrate with the predicted mobility for a DNA-DNA duplex of 1500 and 950 bp, respectively, when measured against DNA restriction fragments of known length (J. Battey, unpublished observation).

RESULTS

The general map positions of the two mitochondrial rRNAs have been localized to one region of the mouse mtDNA genome (Figure 1). The procedure used in a previous study (1) relied on sizing the template DNA strand protected from S1-nuclease digestion by hybridization to complementary mitochondrial rRNAs. Fairly vigorous S1 digestion conditions were used. Since in that work (1) only protected DNA regions longer than 300 nucleotides were analyzed, the two rRNAs were individually mapped without regard for any small internal or flanking RNA transcripts which might be copied from this portion of the genome. Thus the map positions of these two rRNAs shown in Figure 1 could be considered accurate to ~100-200 nucleotides.

The principle underlying the experiments reported here was to refine the S1-digestion procedure for this system such that one could obtain the largest protected DNA templates subsequent to hybridization of mtDNA to the total mtRNA population. In order to establish such conditions, 5'-end labeled λ viral DNA restriction fragments were subjected to varying S1-nuclease digestion conditions. Two different 5'-end labeled populations were utilized; one in which the 5'-end is extended by three nucleotides due to digestion by HinfI restriction endonuclease, and the other which has a flush or

Figure 1. Simplified map of mouse mtDNA (l). The 16 kb genome is divided into 100 units, position 0 representing the origin of H-strand mtDNA replication. The clockwise arrow indicates the direction of elongation of the newly synthesized DNA H-strand. The previously mapped positions and direction of transcription of the small and large rRNAs are shown (l), together with the positions of restriction enzyme cleavage sites for HindIII, EcoRI, and HaeII.

"blunt" 5'-end due to restriction by AluI restriction endonuclease. By assaying the release of acid soluble radioactivity from 32 P-labeled 5' termini (13) it was found that Boehringer SI nuclease in the range 2-10 μ l/ml reaction mixture digested >90% of the staggered 5'-ends and <30% of the blunt, duplex 5'-ends under conditions of elevated salt (0.5 M NaCl), reduced temperature (20°) and at a pH of 4.8 (data not shown).

In order to define all of the transcripts from this region of the mtDNA genome (i.e. from map position 80-100), we formed DNA-RNA hybrids from full-length linear mtDNA isolated from pAMI DNA by restriction enzyme cleavage with HaeII and subsequent resolution of the resultant fragments by agarose gel electrophoresis. Full-length linear mtDNA was electroeluted from the gel and utilized for hybridization with total mtrRNA. These hybrid species were subjected to a series of SI-nuclease digestions in which the extent of digestion was progressively increased. The resultant DNA-RNA duplexes were analysed by agarose gel electrophoresis (Figure 2) and the size of each species was determined. It is evident that there are three families of hybrid duplexes which are uniquely degraded with increasingly aggressive SI-nuclease digestion. The

Figure 2. Neutral agarose gel electrophoresis of SI-digestion products of DNA-RNA hybrids formed between total mtRNA and full-length mtDNA H-strand from pAM1 cleaved at the HaeII site. The sizes of the bands in each of the three families are: largest family 2660, 2580, 2500 bp; intermediate family 1710, 1640, 1570, 1500 bp; smallest family 1090, 1020, 950 bp. Digestion conditions were Boehringer SI at 10 μ l/ml (lane A); Sigma SI at 3 (lane B), 9 (lane C) and 30 μ l/ml (lane D). The highly mobile, smeared material at the bottom of the gel are SI-digestion products of cytoplasmic rRNA.

largest family initially has three members which are not observed in the more extensive digestions. The intermediate family initially has four members of which only the two smallest remain after extensive digestion. In this case the relative abundance of the two smallest species increases with increasing digestion. The smallest family is seen to have three members of which only the smallest remains after extensive digestion. The relative abundance of this smallest member increases from a barely detectable

level to >90%.

It is already known that the major mtDNA transcripts are the two rRNAs and their sizes as determined by a similar technique (1) are consistent with those of the two smallest species in Figure 2, lane D (namely 1500 and 950 bp, respectively). This suggests that these two DNA-RNA hybrids represent rRNA-protected DNA sequences. The incremental size differences between duplexes within any one family are small, ranging from 60-80 bp. The size range of the largest family is inclusive of the sum of the two rRNAs. The disappearance of this family and the increasing abundance of the putative rRNAs with increasing S1 digestion is consistent with the concept that the largest family represents DNA-RNA hybrids in which both rRNA cistrons and any internal sequence on the DNA template are hybridized to RNA. The largest duplex in Figure 2, lane D, is 80 bases longer than the large rRNA. This S1-resistant species could represent the large rRNA-DNA hybrid with an additional 80-base transcript protected region immediately adjacent to either the 5'- or 3'-end of the mature, large rRNA.

In order to verify the proposition that the largest family shown in Figure 2, lane A, indeed contains both the large and small rRNAs, an experiment was carried out in which ^{32}P -labeled mtRNA was hybridized to mtDNA, and after S1 digestion and electrophoresis, DNA-RNA hybrids from the largest family were eluted from the gel for further analysis on a denaturing gel. In order to have sufficient material to perform this experiment it was necessary to prepare DNA-RNA hybrids using H-strand mtDNA isolated from pAM1 mtDNA that had not been digested with HaeII. These DNA-RNA hybrids after digestion with S1 nuclease yielded an even more complex pattern of bands than shown in Figure 2 for the largest and intermediate families (data not shown). The extra, larger bands could be attributed to the presence of S1-resistant DNA sequences in the pACYC177 vector very close to the HaeII site in which the mtDNA had been cloned (unpublished results). Nevertheless, it was possible to isolate in good yield the DNA-RNA hybrid band corresponding to the 2500 bp band seen in Figure 2, lane A. This hybrid was subjected to denaturing gel electrophoresis in order to determine the DNA and RNA species from which it had been formed (Figure 3, lanes A and F). The stained gel reveals three high molecular weight bands (lane A), the smaller two of which have the same mobilities as the large and small mitochondrial rRNAs, and which are labeled with ^{32}P (lane F). The unlabeled largest band therefore represents the segment of DNA, trimmed by S1 nuclease, to which both the large and small rRNAs had been bound.

If the only transcripts available for hybridization were the two rRNAs, one would expect to form one member of each of the two smallest families shown in Figure 2, and if the two rRNA coding sequences are immediately adjacent, one member

Figure 3. Denaturing gel electrophoresis of hybrids isolated after S1 digestion of DNA-RNA hybrids formed from ^{32}P -labeled mtDNA and the H-strand of mtDNA. Discrete species from various hybridizations (see below) were excised from preparative 1% agarose gels, electroeluted in the presence of carrier tRNA and concentrated by ethanol precipitation. After denaturation in 7 M urea at 95° for 1 min, the hybrids were analyzed on a 4% acrylamide gel in 7 M urea (see **MATERIALS AND METHODS**). Lanes A-E show EthBr stained gel; lanes F-I show autoradiogram of the same gel. The hybridization mixtures and the particular bands recovered (after digestion with Boehringer S1 at 10 $\mu\text{l/ml}$) were as follows: lanes A, F, total mtRNA x pAM1 (2500 bp); lanes B, G, LiCl precipitated mtRNA x pAM1 (2430 bp); lanes C, H total mtRNA x two largest EcoRI-HindIII fragments of mtDNA (1730 bp); lanes D, I LiCl precipitated mtRNA x two largest EcoRI-HindIII fragments of mtDNA (1680 bp). Lane E contains a marker track of total mtRNA (cf. Figure 4). The positions of the intact large and small mitochondrial rRNAs are indicated by arrows. For explanation of other bands see text.

of the largest family. Since this simple situation is clearly not the case one must invoke either the presence of other small transcripts (such as tRNAs) mapping in the vicinity of the rRNA genes, or some discrete impediment to S1 digestion brought about by secondary structure interactions in the mtDNA template strand itself. It has been previously demonstrated using electron microscopy that in HeLa cell mtDNA (2) and *Xenopus* mtDNA (7) three 4S RNA species hybridize very close to the rRNA genes, in the order: 4S-small rRNA-4S-large rRNA-4S. It is thus reasonable to suppose that a similar situation might obtain in mouse mtDNA, and that the multiplicity of bands in each of the three families shown in Figure 2 arises from simultaneous hybridization of short transcripts adjacent to and between the two rRNA genes.

If there are indeed short transcripts hybridizing in the region of the rRNA genes,

then by depleting the mtRNA preparations of these small transcripts by the technique of LiCl precipitation (14), before hybridization to mtDNA, a simplified pattern of SI-resistant hybrid bands should be observed. The effectiveness of selective LiCl precipitation of higher molecular weight RNA species is shown in Figure 4, lanes A-C.

Figure 4. Fractionation by LiCl of RNA isolated from crude mitochondria. ^{32}P -labeled RNA was separated on 4% acrylamide 7 M urea gels, and the dried gels were subjected to autoradiography. Lane A, total mtRNA; lane B, LiCl precipitated mtRNA; lane C, RNA soluble in LiCl and recovered by ethanol precipitation of 2 M LiCl supernatant. The nature and approximate size (in bases) of the major bands are indicated (see text). Abbreviations **CYTO** and **MITO** refer to cytoplasmic and mitochondrial, respectively.

The total ^{32}P -labeled mtRNA (Figure 4, lane A) is constituted primarily of the two mitochondrial rRNAs and tRNAs and a substantial amount of the two cellular rRNAs, cellular 5.8S RNA (16), cellular 5S RNA and cellular tRNAs which cannot be distinguished from mitochondrial tRNAs in this gel. In addition, there are a 240 and 165 nucleotide species which correspond to the cytoplasmic RNA species L and D, respectively (15). The minor, heterogeneous, higher molecular weight species are most likely cellular mRNAs or degradation products of larger, abundant RNA molecules. Upon LiCl-precipitation of this RNA population a partitioning of the RNA is apparent with the higher molecular weight species being precipitated (Figure 4, lane B) and the lower molecular weight species remaining soluble (Figure 4, lane C). These results indicate that >90% of the 5S RNA and tRNA species can be selectively removed from these crude mtRNA preparations prior to a hybridization experience. It is apparent that molecules of the size of cytoplasmic 5.8S RNA (160 bases) and larger are precipitated by LiCl.

The effect of using LiCl precipitated RNA for hybridization is shown in Figure 5. In the case of LiCl precipitated RNA, the final digestion products are the same as for total RNA (lane D compared to Figure 2, lane D). However, digestion under milder SI conditions reveals major digestion products of 2500 and 2430 bp in the largest family (lane A). The former major species (Figure 2, lane A) of 2660 bp is absent, and the 2580 bp band is very faint. In the case of the intermediate and smallest family, the former largest members are absent from any of the digests. In particular, the largest member is now reduced in size by ~80 bases in both the intermediate and smallest families. The simplest interpretation of these results is that there are one or more short transcripts mapping at the 5'-end region of the small rRNA gene and at the 3'-end of the large rRNA gene which are available for hybridization in total RNA preparations, but which are absent from the LiCl-precipitated RNA population. Thus, in the case of LiCl-precipitated mtRNA, it can be inferred that an 80-base transcript at the 5'-end of the small rRNA as well as another small transcript distal to the 3'-end of the large rRNA are reduced in the hybridization reaction, as evidenced by the most prominent species in the largest family of Figure 5, lane A, being shorter than the predominant species in the largest family of Figure 2, lane A, by a size of 160 bp. The predominant member (2500 bp) of the largest family in lane A of Figure 5 represents a duplex from the 5'-end of the small rRNA to the 3'-end of a second small transcript (~80 bases) proximal to the 3'-end of the large rRNA. The smaller, less predominant member (2430 bp) of the largest group in lanes A, B, C represents the region from the 5'-end of the small rRNA to the 3'-end of the large rRNA. In Figure 3 (lanes B and G) are shown the results of denaturing gel electrophoresis of the 2430 bp species obtained after SI digestion of hybrids formed from ^{32}P -labeled

Figure 5. Neutral agarose gel electrophoresis of SI-digestion products of DNA-RNA hybrids formed between LiCl precipitated mtRNA and full-length mtDNA H-strand from pAM1 cleaved at the *Hae*II site. The sizes of the species in each of the three families are: largest family 2580 (faint), 2500, 2430 bp; intermediate family 1640, 1570, 1500 bp; smallest family, 1020, 950 bp. Digestion conditions were Boehringer SI at 10 μ l/ml (lane A); Sigma SI at 3 (lane B), 9 (lane C) and 30 μ l/ml (lane D).

LiCl-precipitated mtRNA and pAM1 H-strand. This 2430 bp species clearly contains 32 P-labeled large and small rRNA molecules together with an unlabeled DNA strand to which they had both been hybridized.

The recovery of the 2500 bp species shown in Figure 5 suggests that the short transcript immediately adjacent to the 3'-end of the large rRNA is somewhat less soluble in 2 M LiCl than the two external short transcripts discussed above. Moreover, one can interpret the 1570 bp and 1020 bp bands to have arisen from a fourth short

transcript of ~80 bases which lies between the two rRNA genes. This internal transcript is clearly as insoluble in LiCl as the two rRNAs themselves. Further support for this conclusion is obtained from the following experiment.

In order to simplify and confirm the analysis of transcripts from the small rRNA region we employed a defined restriction fragment from this region of the mtDNA genome. This EcoRII-HindIII restriction fragment maps from positions 87 to 26 (through 0) (Figure 1). The EcoRI site is approximately mid-point in the large rRNA region and therefore the H-strand of this restriction fragment should be complementary to the 5'-end of the large rRNA, to any transcript between the two rRNAs, to the small rRNA and to any transcript adjacent to the 5'-end of the small rRNA. The SI-resistant duplexes obtained from hybridizing total mtRNA to this restriction fragment are shown in Figure 6, lanes A-D. As expected, the total number of species observed is less than when the entire mtDNA H-strand is available for hybridization (Figure 2). The largest family consists of one predominant band (1730 bp) at low SI-nuclease concentrations which is not observed at the highest SI-nuclease concentration. The intermediate family is composed of three members which are progressively degraded to the smallest member at higher SI-nuclease concentrations. In this case the smallest member is 940 bp, consistent with the size of the small rRNA. The larger members of this family are ~80 and 150 bp longer, suggesting that either (i) an 80-base transcript is adjacent to one end of the small rRNA and an 80-base transcript is adjacent to the other end or (ii) that two 80-base transcripts lie adjacent to each other very close to one end of the small rRNA gene. The smallest family has two members ~80 bp different in size. The smallest member is the predominant member at higher SI-digestion conditions and is ~650 bp in length. As will be shown below, this hybrid is formed from the portion of the large rRNA gene that lies clockwise from the EcoRI site, and its size gives a measure of the distance of the 5'-end of the large rRNA from the EcoRI cleavage site, namely 650 bp. The existence of a DNA-RNA hybrid species some 80 bp longer than the 650 bp duplex suggests that there is an ~80-base transcript immediately adjacent to the 5'-end of the large rRNA. This would correspond to one of the small transcripts adjacent to the small rRNA gene (see above). If there is only one small transcript of ~80 bases lying between the small and large rRNA gene, then the second 80-base transcript must lie at the 5'-end of the small rRNA gene.

Data supporting this conclusion were obtained by analysis of DNA-RNA hybrids formed from LiCl precipitated RNA and the EcoRI-HindIII restriction fragment (Figure 6, lanes E-H). It is seen that the overall pattern of surviving hybrid duplexes is simpler. The largest family is represented by a predominant member (1680 bp) whose length is ~50 bp shorter than the predominant member when total mtRNA is hybridized. The intermediate family has only two members, ~1020 and 940 bp in length, with only

Figure 6. Neutral agarose gel electrophoresis of S1-digestion products of DNA-RNA hybrids formed between H-strand of the two largest *EcoRI-HindIII* fragments of mtDNA and total mtRNA (lanes A-D) or LiCl precipitated mtRNA (lanes E-H). The sizes of the species (bp) in each of the three families are indicated on the figure at the left and right sides, respectively, for the sets of hybrids derived from total mtRNA and LiCl-precipitated mtRNA. Note that although the mtDNA preparation used for hybridization contained the second largest as well as the largest *EcoRI-HindIII* fragment of mtDNA, it is only the largest *EcoRI-HindIII* fragment which hybridizes to the rRNAs (see Figure 1). There were several faint bands visible on the original photographs of this gel which have not been investigated further.

the smallest member surviving extensive S1 digestion. Again, this 940 bp species is consistent with the size of the small rRNA. The smallest family is unchanged in terms of the relative amount of the two members during progressive S1 digestion (compare lanes D and H in Figure 6). We interpret these data to indicate that there is an ~80-base transcript in both total and LiCl precipitated mtRNA which is complementary to the mtDNA H-strand template between the 5'-end of the large rRNA and 3'-end of the small rRNA. In addition, there is an ~60 base transcript (measured at between 50 and 70 bases) present in total mtRNA and absent in LiCl precipitated mtRNA which is complementary to the mtDNA H-strand immediately adjacent to the

5'-end of the small rRNA. This assignment is consistent with the fact that the predominant member of the largest family in both total (1730 bp) and LiCl precipitated (1680 bp) mtRNA hybridizations contains the small rRNA and the 650 nucleotide portion of the large rRNA which spans the 5'-end of the large rRNA to the EcoRI cleavage site, as can be seen from the data presented in Figure 3, lanes C, D, H and I. In this experiment ^{32}P -labeled total mtRNA (lanes C, H) or LiCl precipitated mtRNA (lanes D, I) was hybridized to the EcoRI-HindIII DNA fragment and the largest SI-resistant duplexes were isolated and analyzed under denaturing gel electrophoresis conditions. Instead of the large rRNA being recovered from these hybrids, a new RNA molecule ~650 bases in length was observed. The small rRNA was present in these hybrids in an apparently unchanged form. In addition, the shortened unlabeled DNA fragment to which the rRNA molecules hybridized can be seen in lanes C and D.

To strengthen the conclusion that it is indeed short transcripts of the order of 80 bases in length which contribute to the appearance of the families of bands discussed, we examined the low molecular weight region of the denaturing gel on which ^{32}P -labeled RNA released from the DNA-RNA hybrids had been separated (Figure 7). This necessitated a prolonged exposure of the autoradiogram, so that spots in tracks E and F, corresponding to RNA of ~90 bases, could be seen. This small RNA in lane F corresponds to the transcript lying between the two rRNA genes (derived from the 1680 bp hybrid of Figure 6), while in lane E the single small species represents both this internal RNA as well as the short transcript mapping at the 5'-end of the small RNA (derived from the 1730 hybrid of Figure 6). It is also evident that ~90-base species of greater intensity are found in lanes A through D, where RNA released from DNA-RNA hybrids containing the complete small and large rRNA molecules was examined (obtained from hybridizations between ^{32}P -labeled mtRNA and pAM1 H-strand).

These results are consistent with the view that there are multiple short transcripts found in the various DNA-RNA hybrids analysed, and they all have similar mobilities, migrating to the same region as the cytoplasmic tRNAs (lane G). A diagrammatic representation of this region of the mtDNA genome is shown in Figure 8. The four short transcripts whose existence has been inferred from the results described above are denoted a, b, c and d.

DISCUSSION

The data reported here have permitted an accurate mapping of the mature mouse L-cell mitochondrial rRNA transcripts on the mtDNA genome (Figure 8). The major new observation is that there is little, if any, DNA sequence from the 5'-end of the small rRNA to the 3'-end of the large rRNA that is not represented by a

Figure 7. Autoradiogram of denaturing gel electrophoresis of hybrid bands formed from ^{32}P -labeled mtRNA and selected mtDNA sequences. The experiment was carried out in the same manner as that described in Figure 3, except that in this figure the autoradiogram was exposed for a longer period to show the short transcripts (indicated by arrow) released from the DNA-RNA hybrids after denaturation; consequently the relatively more intense bands at the top of the gel are considerably overexposed. The hybridization mixtures and the bands recovered after SI digestion and agarose gel electrophoresis are as follows: lane A, total mtRNA x pAM1 (2910 bp; this corresponds to the 2660 bp fragment produced when *Hae*II-cut linear mtDNA was used); lane B, total mtRNA x pAM1 (2500 bp); lane C, LiCl precipitated mtRNA x pAM1 (2770 bp; this corresponds to the 2500 bp fragment produced when *Hae*II-cut linear mtDNA was used); lane D, LiCl precipitated mtRNA x pAM1 (2430 bp); lane E, total mtRNA x two largest *Eco*RI-*Hind*III fragments of mtDNA (1680 bp); lane F, LiCl precipitated mtRNA x two largest *Eco*RI-*Hind*III fragments of mtDNA; lane G, total mtRNA as size markers (cf. Figure 4).

mtRNA transcript; i.e., there is no non-transcribed spacer region in this system. The transcripts are apparently subjected to a processing mode that does not discard a significant number of nucleotides. In fact, the inter-rRNA distance is only ~80 nucleotides and this region of the DNA codes for one small RNA species. In addition, there is one small transcript of ~60 nucleotides, immediately adjacent to the 5'-end of the small rRNA and two small transcripts each of ~80 nucleotides, immediately adjacent to the 3'-end of the large rRNA. Assuming these small transcripts are tRNAs,

Figure 8. Map of transcripts in the region of mouse mtDNA encoding rRNA genes and the four short transcripts denoted **a**, **b**, **c** and **d**. The sizes are given in bases (or bp). The transcript sizes were derived from mobilities of DNA-RNA hybrids in 1% agarose gels. It should be noted that for all transcripts in the largest group, there is a systematic discrepancy of ~3% (~70 bases) in the size inferred from the mobility on these gels. Thus the largest hybrid that can be generated according to this map should be 2750 bp in length; in fact, the longest hybrid (Figure 2, lane A) was measured at 2660 bp. The distance of 800 bp between the HaeII and EcoRI sites was measured by appropriate digests of pAM1 and pAC133 (data not shown). The distance of 1900 bp between the EcoRI site and the origin of H-strand replication (O_H) was taken from numerous unpublished restriction enzyme digests performed in this laboratory. The small transcript is thus inferred to lie about 200 bases from the origin of replication, that is from the 5'-end of the major D-loop H-strand initiation sequences (17,18).

these results are in close agreement with electron microscopic mapping of putative tRNAs on the HeLa (2) and *Xenopus laevis* (7) mtDNA genome. One would expect that all of these four small transcripts (Figure 8, species **a-d**) would be LiCl-soluble. In fact, only species **a** and **d** are clearly partitioned by this technique. The reason for this is not clear since recent efforts to sequence this region of the mtDNA genome have shown that genes for tRNA^{Val} and tRNA^{Leu} map at positions **b** and **c**, respectively (Van Etten and Clayton, manuscript in preparation).

A point of obvious interest is whether the inter-transcript regions are of zero, one or greater than one nucleotide. The precise answer to this question could be derived by actual sequencing of the RNA transcripts and subsequent alignment on the DNA template. Given the low abundance of the small RNAs (**a-d**) in total mtRNA isolates, the task would not be trivial. Since S1 nuclease may cleave DNA-RNA duplexes at the site of a single phosphodiester bond cleavage, these data do not necessarily eliminate the possibility that there are no internal DNA sequences that are not transcribed, nor conserved during processing. However, the differential accessibility of S1 nuclease to the various cleavage sites studied could be indicative that either the spaces between adjacent transcripts do differ in length, or that secondary structure effects prevent or facilitate the S1 nuclease from cleaving readily at a particular site.

Concerning the location of the 3'-end of the large rRNA relative to the HaeII site in mtDNA, it is noted in Figure 8 that the HaeII site lies a very short distance

inside the large rRNA gene. Recent efforts to position the 3'-end of the large mitochondrial rRNA on the mtDNA sequence have shown that the HaeII site is ~70 nucleotides from the 3'-end (Van Etten and Clayton, Walberg and Clayton, manuscripts in preparation). In this case the recovery of DNA-RNA hybrids containing both the large rRNA and transcript *c* arises because the large rRNA protrudes past the HaeII site to a sufficient extent to form a stable hybrid to the fragment of DNA to which transcripts *c* and *d* were hybridized.

We note that the 5'-end of species *a* maps within 200 nucleotides of the 5'-end of the largest mtDNA D-loop strand (17-18). This D-loop strand is an initially RNA-primed DNA initiation sequence which is elongated 5'-3' on the complementary L-strand template and in the opposite polarity with respect to transcription. Since the 5'-end of species *a* represents the first RNA transcript thus far detected in this region of the mtDNA genome, the exciting possibility that this point in the genome contains the major control region for both transcription and DNA replication becomes a hypothesis worthy of further investigation.

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