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# POST-TRANSCRIPTIONAL MODIFICATION OF ANIMAL MITOCHONDRIAL RNA: A METHYLATION-DEPENDENT STEP IN RIBOSOMAL RNA PROCESSING

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## INTRODUCTION

The RNA of animal mitochondria undergoes substantial post-transcriptional modification, including formation of ψ, hypermodified residues, and methylated residues. The methylated ribosides are among the most abundant modified ribosides, and are the most varied. Table 1 summarizes compositional results of ours on methylated ribosides of unfractionated tRNA from mosquito and hamster mitochondria. Analyses of individual tRNA sequences are in accord with the overall compositional results, as shown in the Table. The methylated ribosides of mitochondrial (mit) tRNA [aside from the interesting special case of the methylated hypermodified riboside, $ms^2i^6A$ (see ref. 2), which we have not included in the Table] constitute a subset of those of cytoplasmic (cyt) tRNA; $m^5U$ and $m^7G$ are abundant in cyt tRNA, but are conspicuously absent from mit tRNA. The positions of most of the methylated residues of mit tRNA correspond to common positions for these residues in eukaryotic tRNA (Table 1); thus, it is reasonable to think that the same enzymes, or at least products of the same genes, effect these methylations for both mit and cyt tRNA. This latter has indeed been shown to be the case for $m_2^2G$ and $m^5U$ in yeast (4). In contrast, the abundant residue $m^1A$ occurs in a unique position in the animal mit tRNA sequences.

Table 2 summarizes the methylation status of animal mit rRNA. The methylated residues of 16S RNA have clear homologues in cyt rRNA. In the case of 12S RNA, this holds only for $m_2^6A$; in fact, $m^4C$, $m^5C$ and $m^5U$ are absent from 18S RNA.

It is likely that a different methyltransferase is specific for each type of methylated residue and for each position within a sequence (except perhaps for neighboring $m^5C$'s in tRNA and $m_2^6A$'s in 12S RNA). Thus, roughly 10 methyltransferases would be required for tRNA methylation and 5 for rRNA methylation in mosquito or hamster mitochondria. As a step towards eventually characterizing these enzymes, and assessing the roles of the methylated residues, we have examined effects of the methionine analogue cycloleucine on mit RNA, using the hamster (BHK-21) cell system. Cycloleucine has been shown to selectively suppress macromolecule methylation by competitively inhibiting ATP:methionine adenosyltransferase, a cytoplasmic enzyme that is presumed to supply mitochondria, along with other subcellular compartments, with S-adenosylmethionine (10).

TABLE 1. METHYLATED RIBOSIDES OF ANIMAL MITOCHONDRIAL TRANSFER RNA

Results for unfractionated tRNA were obtained following Ref. 1. Sequence positions are from the work of B. Roe, E. and K. Randerath and their colleagues, and from our work [see Sprinzl *et al* (2), whose numbering system we use]. We indicate whether a particular localization is typical of eukaryotic "(E)" or prokaryotic "(P)" tRNA, both "(B)", or neither "(N)", following Dirheimer *et al* (3).

| | Unfractionated tRNA, Ribosides per 100 nucleotides | | tRNA Sequence Position | |
|---|---|---|---|---|
| | Mosquito | Hamster | Mosquito | Mammalian |
| $m^3C$ | 0.23 | 0.03 | - | 32(E) |
| $m^5C$ | | 0.16 | - | 48,49(E) |
| $m^1A$ | 0.81 | 0.64 | 9(N) | 9(N) |
| $m^1G$ | 0.56 | 0.21 | 9(E) | 9(E),37(B) |
| $m^2G$ | 0.34 | 0.87 | 10(E) | 10,26(E) |
| $m_2^2G$ | 0.06 | 0.22 | - | 26(E) |
| Nm* | 0.03 | <0.03 | | |
| Cm | | | 39(E) | - |
| Ψm | | | 39(E) | - |

*Ribose-methylated residues were assayed as a group in unfractionated tRNA. We have found Cm in mosquito mit $tRNA^{ser}$ and Ψm in mosquito mit $tRNA^{Gly}$.

TABLE 2. METHYLATED RIBOSIDES OF ANIMAL MITOCHONDRIAL RIBOSOMAL RNA

The mosquito and hamster mit values are from our work [summarized in (5)]; *E. coli* values from (6,7); 18S RNA (*Xenopus*) from (8); and 28S RNA (yeast) from (9). We indicate sequence position numbers for the *E. coli* and eukaryotic RNAs tabulated. Homologies that are in doubt due to position, or nature, of modified residue are bracketed.

| Mosquito | Hamster | *E. coli* Homologue | Eukaryotic Homologue |
|---|---|---|---|
| 12S RNA* | | | |
| $m^4C$ | $m^4C$ | $m^4Cm$,1402 | [Cm,1661] |
| $m^5C$ | $m^5C$ | [$m^5C$, 1407] | - |
| 2 $m_2^6A$'s | 2 $m_2^6A$'s | $m_2^6A$, 1518,1519 | $m_2^6A$, 1807,1808 |
| - | $m^5U$ | - | - |
| 16S RNA* | | | |
| UmGm | UmGm | UmG, 2552 | UmGm, 2918,2919 |
| - | GmG | GmG, 2251 | GmG, 2616 |

*We yield to majority usage in designating the small and large ribosomal subunit RNAs of animal mitochondria 12S and 16S RNA, rather than 13S and 17S RNA or SSU and LSU RNA, as used earlier and/or in Fig. 1.

EFFECT OF CYCLOLEUCINE ON TRANSFER RNA.

Short (3h) periods of cycloleucine treatment were found to inhibit synthesis of both mit and cyt tRNA by about 50%, as estimated by incorporation of label from $^{32}Pi$ or $^{3}H$-uridine (11). The mature-sized tRNA which did accumulate was, as expected, undermethylated, as estimated by relative incorporation of $^{3}H$ from [methyl-$^{3}H$]methionine and $^{32}P$ from $^{32}Pi$. The methylated residues of cyt tRNA occurred for the most part at 40-50% of their normal levels, with extremes being $m^1G$ (70% of normal) and ribose-methylated residues (20%). The methylated residues of mit tRNA occurred at 10-50% of normal levels, with $m^1A$ (again) a striking exception: its formation was markedly and preferentially spared (calculated from Tables 1 and 2 of ref 11). This, coupled with the unique position of $m^1A$ residues in the mit sequences, leads us to predict that the mit and cyt $m^1A$-forming enzymes will prove to be fundamentally different.

EFFECT OF CYCLOLEUCINE ON HIGH MOLECULAR WEIGHT RNA.

The most striking effect of cycloleucine was observed when mit RNA from treated cells was fractionated by sucrose density gradient centrifugation: a new major peak appeared that sedimented beyond 16S rRNA. This peak, which we designated Cy RNA, was polyA(-), resembled mit RNA in GC content (low), and was essentially unmethylated (11). We have now further characterized Cy RNA, using high resolution gel electrophoresis and DNA-RNA hybridization, and have begun to characterize an RNA species found in normally growing cells that is similar or identical to Cy RNA. Figure 1 shows a typical "low salt" (20 mM $Na^+$) density gradient pattern for the polyA(-) moiety of mit RNA labeled with uridine for 1h during cycloleucine treatment. A prominent Cy RNA peak is seen, sedimenting ~20% faster than 16S RNA. Such a peak is not seen in mit RNA from cells similarly pulse-labeled during normal growth, nor, as shown in the figure, in uniformly labeled mit RNA. When Cy RNA from the centrifugal peak was subjected to electrophoresis through a 2.5-10% acrylamide gradient-urea gel, it yielded a single sharp band running behind 16S RNA. The corresponding cut from uniformly labeled mit RNA yielded, albeit as a minor component, a similarly migrating band (Fig. 1, Insert). When such cuts were run in "standard salt" (120 mM $Na^+$) gradients, they yielded peaks (again minor) whose sedimentation rate (30% faster than 16S RNA) was indistinguishable from that of Cy RNA (cf. ref. 11, Fig. 2); and material from these peaks "ran true" on re-sedimentation. Most of the labeled RNA from the original gradient cuts migrated faster than Cy RNA in gels (Fig. 1, Lane 2) and slower in standard salt gradients. This is as expected for 16S RNA and for partial degradation products of contaminating cyt rRNA (12), which we indeed believe are the major constituents of such cuts. We infer from the above results that (1) Cy RNA is a discrete species within the limits of resolution of gradient

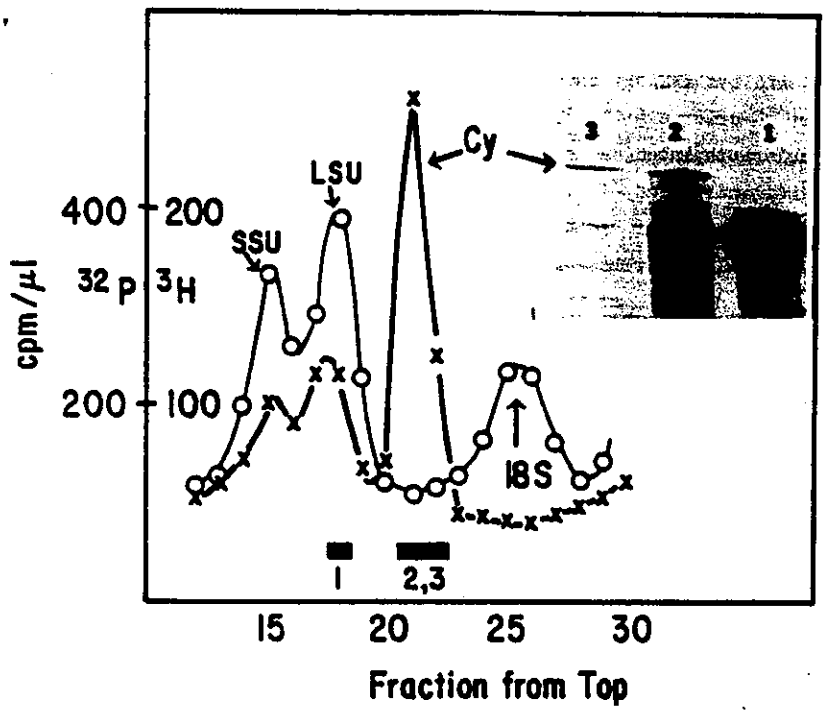


Fig. 1. Centrifugal and Electrophoretic Analysis of Cy-RNA. One culture of BHK cells was treated with cycloleucine, 6 mg/ml, for 3h in medium (12) containing 40 uM methionine; $^{3}H$-uridine was present during the last hour of treatment. A second culture was labeled with $^{32}P$ for 24h of normal growth. PolyA(-) mit RNA was prepared from each culture, and was centrifuged through low salt sucrose gradients, as in (12); (X), $^{3}H$; (O), $^{32}P$. Portions of pool 1 (from the $^{32}P$-labeled culture) and 2 and 3 (from the $^{32}P$ and $^{3}H$-labeled culture, respectively) were run on a 2.5-10% acrylamide gradient gel (21) (Insert).

acrylamide gels; and (2) normal cells contain a species similar or identical to Cy RNA, albeit at very low levels (0.2-0.5% of 16S RNA). We shall refer to this putative normal equivalent as Cy* RNA.

Cy RNA was sized by gel electrophoresis through 3% acrylamide-urea gels by comparison with 16S and 12S RNA. The resulting chain length came to 2.6 kb, estimated by plotting logarithm of mobility vs. square root of chain length (13).

Cycloleucine was found to have little effect on the polyA(+) fraction of mit RNA as assessed by density gradient analysis (11). However, this fraction consists of a multiplicity of species - mainly mRNAs - resolution of which requires gel electrophoresis. Fig. 2 illustrates an experiment aimed at detailed comparison of effects of cycloleucine on the polyA(+) vs the polyA(-) moieties of pulse-labeled mit RNA. Parallel cultures were labeled with uridine as for Fig.

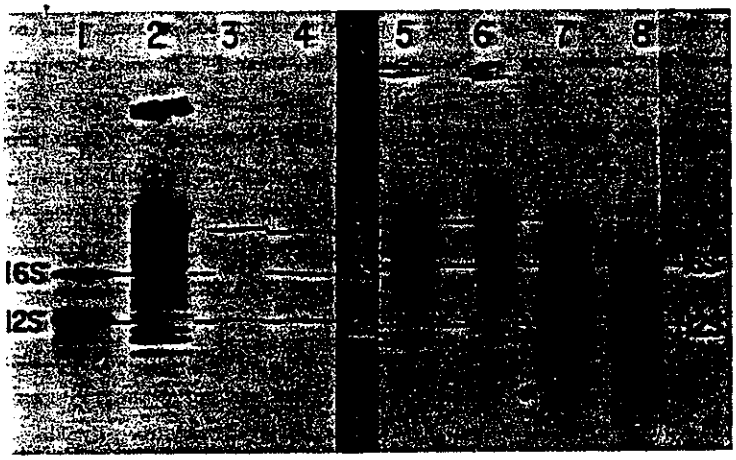


Fig. 2. Gel Electrophoretic Analysis of The Effect of Cycloleucine on PolyA(-) and polyA(+) mit RNA. Cultures were labeled with uridine in the presence or absence of cycloleucine, and samples of mit RNA were subjected to electrophoresis, as for Fig. 1. Lanes 3 and 4 represent 3% samples of the polyA(-) RNA from treated and control cells, respectively; lane 2 represents a sample (60%) of the polyA(+) RNA from the treated culture. Lanes 1 and 8 contain marker 16S and 12S RNA. Lanes 5 and 6 show patterns for polyA(+) RNA samples from control and treated cells, respectively, run in parallel. Lane 7 represents a uniformly labeled sample similar to that of Fig. 1, Lane 2.

1, in the presence or absence of cycloleucine. When equivalent aliquots of total polyA(-) RNA were run in 2.5-10% gels, the preferential incorporation of radioactivity from uridine into Cy RNA and the inhibition of incorporation into 16S and 12S RNA in the treated cells were apparent (lanes 3, 4); in contrast, the pattern of mit polyA(+) RNA was relatively normal (lane 2). A closer comparison of mit polyA(+) patterns from normal and treated cells is provided by lanes 5 and 6, in which samples from each culture were run in parallel. A minor polyA(+) band migrating like Cy RNA can be seen in pulse-labeled mit RNA from normal cells; the prominence of this band, which we believe to be a polyadenylated version of Cy RNA, was enhanced by cycloleucine treatment. Minor bands that we believe to be polyadenylated versions of 16S and 12S RNA also occur in the polyA(+) fraction; labeling of these bands was regularly suppressed by cycloleucine. The remaining polyA(+) bands are presumably mit mRNAs, and most appear to correlate well with those identified in HeLa mitochondria by G. Attardi and his colleagues (e.g., ref. 14): e.g., their #'s 11, 12, 13 (between 16S and 12S RNA); and 14, 15, 16 (below 12S RNA). The only effect of cycloleucine on these species was possible mild inhibition of labeling of bands 12 and 16.

The patterns of Lanes 3 and 4, Fig. 2, suggest that Cy RNA is a precursor to 16S and 12S rRNA. More definitive evidence on this point was sought by Southern hybridization experiments. We used as target either restricted genomic DNA or a cloned EcoRI fragment corresponding to "EcoRI B" of M. Nass (15), whose mapping results indicated that it contained the 16S and 12S rRNA genes and no more than small portions of genes for other high molecular weight transcripts. This proved to be the case; sequencing studies have shown that the segment is homologous to murine mit DNA (18) residues 15115 (1250 bp upstream from the 12S rRNA gene) to 3007 (332 bp downstream from the 16S RNA gene). Further, a unique HindIII site within the fragment (15) separates it into a 2.6 kbp moiety containing the 12S RNA gene and extending about 0.3 bp into the 16S RNA gene; and a 1.5 kbp moiety containing the remainder (1.3 kbp) of the 16S RNA gene. Hybridization conditions were used that resulted in hybridization between 16S RNA and only the 1.5 kbp DNA fragment; 12S RNA of course hybridized only with the other fragment. Cy RNA hybridized to both fragments, and this hybridization was suppressed by highly purified rRNA (Fig. 3, left). Further, when genomic mit DNA was used, only 2 bands, whose positions were compatible with the 2.6 and 1.5 kbp fragments, yielded positive signals (Fig. 3, right). We infer that Cy RNA is encoded at

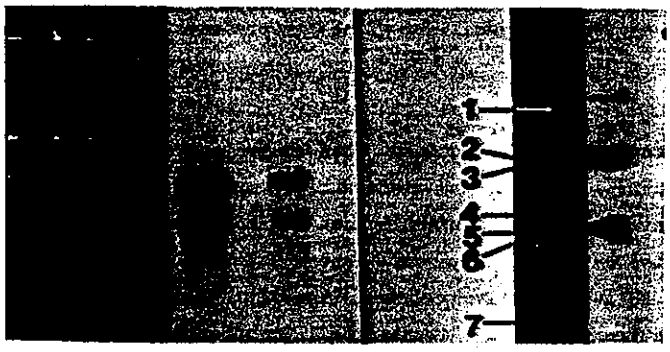


Fig. 3. Southern Hybridization of Cy RNA. Left Panel: Plasmid pBR322 (~100 ng) containing mit EcoRI B (17) was digested with EcoRI plus HindIII followed by electrophoresis through a 0.8% agarose gel and transfer to nitrocellulose. Duplicate strips were hybridized to $^{32}$P-labeled Cy RNA [purified by centrifugation through low and then standard salt (12)] in the absence (3rd lane) or presence (4th) of highly purified mit rRNA (100 ng each of 16S and 12S RNA). Hybridization (18,19) was in 2X SSC for 16h at 65° followed by washing twice each with 2X SSC and 20 mM $Na_2HPO_4$, at 20°. The first two lanes show ethidium-induced fluorescence of (from top down) linearized vector DNA, the 2.6 kbp fragment of the insert, and the 1.5 kbp fragment. Right Panel: Mit genomic DNA was processed as above. The first lane shows ethidium-induced fluorescence. Sizes of numbered bands as estimated by comparison with markers were (from the top down, in kbp) 4.7, 2.8, 2.6, 1.7, 1.5, 1.3 and (barely visible) 0.6 (cf. ref. 15). The second lane shows the results of autoradiography after hybridization with Cy RNA.

least in part by the rRNA genes, and not by genes encoding mRNAs.

We have begun $S_1$ protection experiments aimed at more precisely defining the extent of the Cy RNA. In this work, we used a HpaII-SauI subfragment of the EcoRI B fragment, extending from about 600 residues upstream of the 5' end of 12S RNA to 100 residues downstream of it (Fig. 4). Template strand 5'-endlabeled at the SauI site was hybridized to 3 RNA fractions: highly purified 12S RNA, 16S RNA and Cy* RNA. Stretches protected against nuclease $S_1$ degradation were examined by gel electrophoresis in parallel with sequencing reactions from the same DNA strand. As shown in Fig. 5A, results for 12S RNA were in accord with expectation: a small array of protected DNA fragments was centered about the known 12S RNA gene end. Cy* RNA yielded a very similar pattern (Fig. 5B); that this was not due to contaminating 12S RNA is shown by the fact that 16S RNA, which is more likely to be affected by such contamination, yielded little protection.

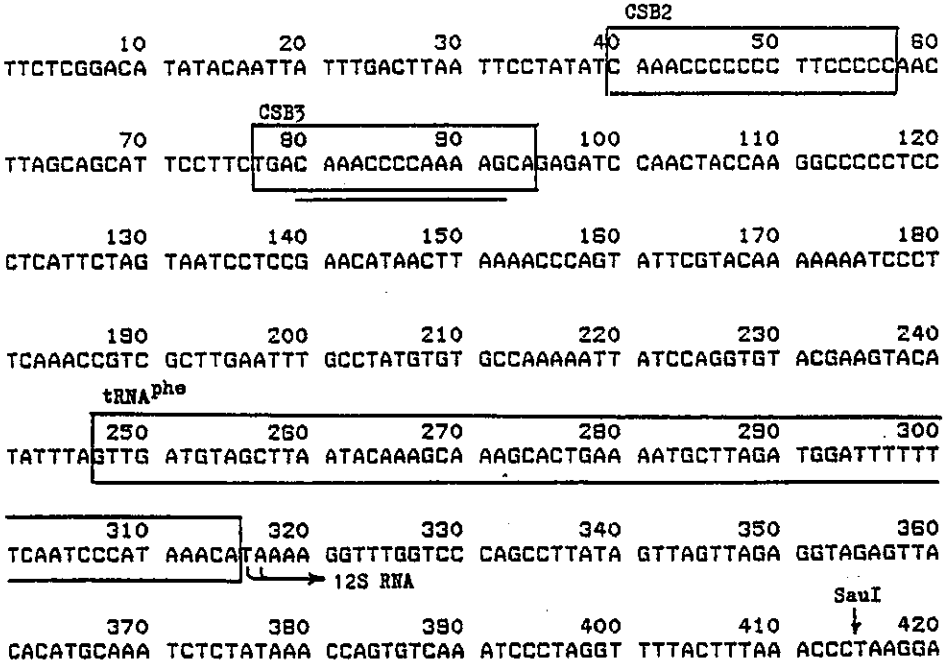


Fig. 4. Sequence of hamster mit DNA expected to contain promoters. Numbering begins 303 residues from the HpaII site of the HpaII-SauI fragment used in the $S_1$ analyses. We have underlined a portion of conserved sequence box ("CSB") 3 that corresponds to the "consensus" human mit promoter [CANACC(G)CC(A)AAAGAYA ] (ref. 20) minus the last 3 residues.

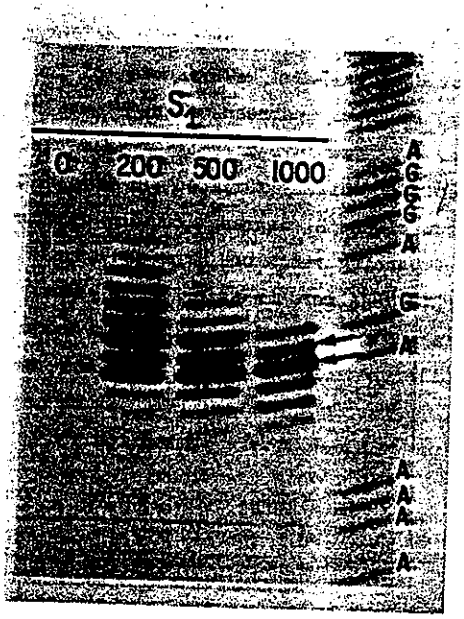


Fig. 5A. $S_1$ Protection: 12S RNA. DNA (~30 fmoles) labeled at the SauI site (Fig. 4 and Text) was hybridized to ~150 fmoles of 12S RNA followed by digestion with $S_1$ nuclease, 0-1000 units/ul as noted, and gel electrophoresis (21). A sample of the same DNA subjected to the "A+G" reaction (22) was run in parallel. Arrows indicate $S_1$ bands corresponding to 12S RNA 5'-termini (ref. 23 and Fig. 4).

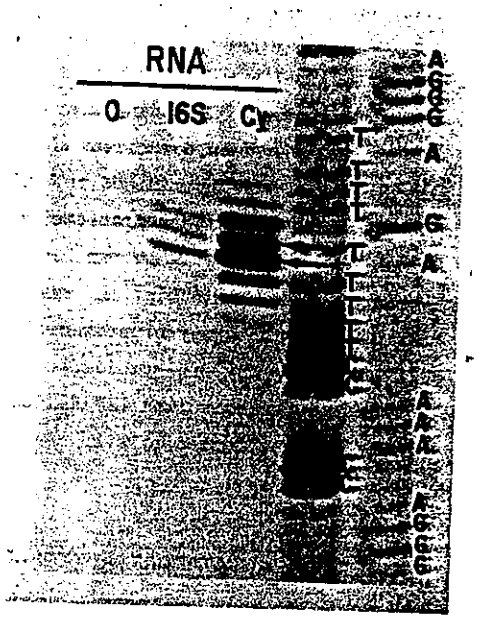


Fig. 5B. $S_1$ Protection: $Cy^*$ RNA. Samples of $Cy^*$ RNA (~30 fmoles) and (as a control) 16S RNA (~150 fmoles), purified by 2 cycles of centrifugation as for Fig. 3, were hybridized to mit DNA and treated with $S_1$ as for Panel A; 1000 units/ml of $S_1$ were used. No protected bands were obtained in the absence of RNA ("0"). The C+T and A+G reactions are shown.

We infer that the 5' terminus of $Cy^*$ RNA (and presumably Cy RNA) corresponds to the 5'-end of 12S RNA. Its apparent chain length would bring its 3' terminus to the region of the 3' end of the 16S RNA gene; more precise definition of this end is being sought. These results support the view that Cy RNA is a discrete polycistronic precursor to rRNA, containing sequences corresponding to 12S rRNA, $tRNA^{val}$ and 16S rRNA, and whose processing is dependent on methylation. The nature of the methylated residues involved, and the nature of their involvement, is a matter for speculation; however, prime candidates are the $m^6_2A$'s of 12S RNA, which occur near the 3' end of the mature molecule; and the $m^1A$ destined to be formed near the 5'-end of the $tRNA^{val}$ (cf. Table 1). The latter possibility is rendered less likely by the apparent relatively normal patterns of mRNA, many of which are also encoded by genes adjacent to potential $m^1A$-containing tRNAs.

Montoya et al (14) have described a minor polyA(-) RNA fraction, "u4", in

human (HeLa cell) mitochondria, whose kinetics of labeling indicated that it was, or at least was closely related to, an rRNA precursor. It seems likely that u4 RNA and Cy* RNA are homologous species. We have not obtained evidence in the hamster Cy* RNA system for a slightly larger species observed in the HeLa system ("u4a"), with 5'-terminus(i) just upstream of the $tRNA^{phe}$ gene, within the major H-strand promoter (see refs. 14, 20, 24). The putative consensus sequence defining the human mit promoters (20) is absent from the corresponding hamster mit DNA region (Fig. 4). Thus the major hamster promoter is likely to be different, and/or differently located, from the human, and may in fact correspond to the end of the 12S RNA gene.

We have not accomplished our initial goal of accumulating substantial chemical levels of undermethylated mit RNA for use in detecting mitochondrion-specific methylases, due to apparent decay of the cycloleucine effect beyond about 4 hours (cf. ref. 10). However, we continue to believe this system has potential to this end, and trials of modified treatment regimens are planned.

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