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REPRINTS

Evidence that the methylation inhibitor cycloleucine causes accumulation of a discrete ribosomal RNA precursor in hamster mitochondria

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Abstract

A novel RNA fraction, 'Cy RNA,' that accumulates in mitochondria when hamster cells are treated with the methylation inhibitor cycloleucine, has been characterized by high resolution acrylamide gel electrophoresis and DNA-RNA hybridization. Cy RNA ran in gels as a discrete band, with an apparent chain length of 2 600. It hybridized specifically to restriction fragments containing genes for the mitochondrial ribosomal RNAs. We infer that Cy RNA is a discrete polycistronic ribosomal RNA precursor transcript, whose processing is dependent on normal methylation.

Introduction

The methionine analogue cycloleucine (1-aminocyclopentane carboxylic acid) causes a marked preferential inhibition of nucleic acid methylation in mammalian cells (9). The nuclear precursors to cytoplasmic rRNA are especially heavily affected, and associated with the hypomethylation is a profound retardation of processing to cytoplasmic 18S and 28S rRNA (8). We have shown that cycloleucine also has effects on mitochondrial (mit) RNA. Prominent among these effects were inhibition of incorporation of ^{32}P into mature ('16S' and '12S') mit ribosomal RNA, coupled with substantial incorporation into a novel centrifugal fraction, 'Cy RNA,' that sedimented faster than 16S RNA and that was essentially unmethylated (10). We present here the results of experiments aimed at further characterizing the action of cycloleucine on mitochondria, and in particular at testing the idea that Cy RNA is a discrete precursor to the mit rRNAs, processing of which involves a methylation-dependent step.

Materials and methods

Hamster (BHK-21) cells were grown in S-MEM containing 40 μm methionine and 0.65 mM Pi (12). For labelling with uridine, cells were exposed to [5-^{3}H]uridine for 90 min, after 1 h pretreatment with cycloleucine (6 mg/ml). For labelling with ^{32}P (due to slower equilibration of added ^{32}Pi with RNA precursor pools), cells were labelled for 4 h, beginning 80 min prior to the addition of cycloleucine. Mit RNA and DNA were prepared as described in refs. 12 and 15. Poly A(-) RNA is defined as the moiety not adhering to oligo T in the presence of 0.4 M NaCl and poly A(+) RNA as the moiety adhering after three cycles of chromatography (14).

Restriction endonucleases were obtained from Bethesda Research Laboratories and used as recommended. Plasmid pBR322 containing the mit EcoRI B fragment (20) was constructed and handled as described in (15). Southern hybridization (21) was performed in 2 × SSC for 16 h at 65 °C (ref. 18); membranes were washed twice with 2 × SSC and twice with 20 mM Na_2HPO_4 at 20 °C. Gel electrophoresis was carried out in 7 M urea using 2.5–10% acrylamide gradients (11) or 3% acrylamide.

Molec. Biol. Rep. 11, 51–55 (1986).
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Results and discussion

We suggested that Cy RNA was a product of the mit genome on the basis of its localization in the mitochondrial fraction and its resemblance to mitochondrion-specific RNA in nucleotide composition, and we proposed that it might be a precursor to mit rRNA (10). To test these ideas, experiments were conducted in which labelled Cy RNA was hybridized to mit DNA. Figure 1 shows a density gradient pattern for the poly A(–) mit RNA from cells labelled with ^{32}Pi in the presence of cycloleucine, using the labelling schedule described in Methods; the prominent Cy RNA peak is apparent. The pool indicated in Fig. 1 was further purified by sedimentation in a 'standard salt' gradient (12) prior to use.

As target DNA, we used either a cloned EcoRI fragment of mit DNA (15) or genomic DNA. The EcoRI B fragment (20) has been found to extend from a residue approx. 1 250 upstream of the 12S RNA gene to one 330 downstream of the 16S RNA gene. A single HindIII site divides the fragment into a 2.6 kbp piece containing 160 residues of the cytochrome B gene, 3 tRNA genes, the 12S rRNA gene, and approx. 330 residues of the 16S RNA gene, and a 1.5 kbp piece containing the remainder (*ca* 1.2 kbp) of the 16S RNA gene, the tRNA^{val} gene, and 260 residues of URF1 (refs. 2, 6, 15 and our unpublished results). Under the hybridization conditions used, 12S RNA hybridized only to the 2.6 kbp fragment and 16S RNA only to the 1.5 kbp fragment (Fig. 2A). [This latter is likely a function of the limited representation of the 16S RNA fragment occurring on the 2.6 kbp piece, and its relatively low GC content (31%)]. Cy RNA hybridized to both fragments, and this hybridization was suppressed by unlabelled rRNA (Fig. 2B).

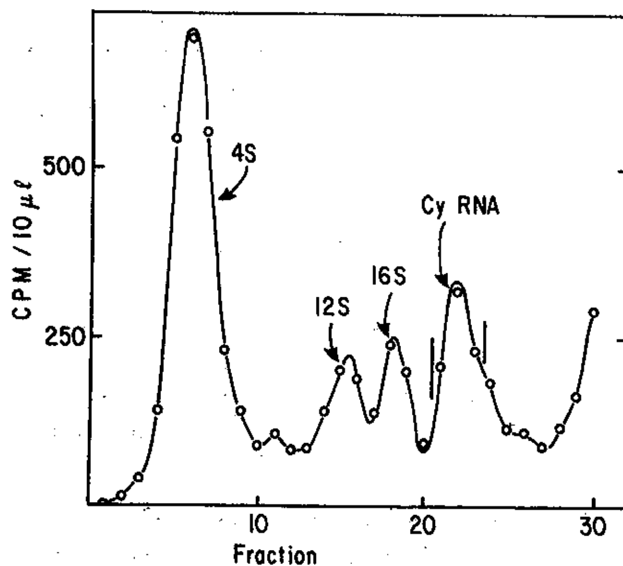

Fig. 1. Density gradient sedimentation pattern of ^{32}P-labelled mit RNA from cells treated with cycloleucine. Cells were labelled with ^{32}P (18 mci), and mit poly A(–) RNA was prepared and centrifuged through a low salt sucrose gradient, as described in Methods. Thirty fractions, counting from the top, were collected.

Figure 2C shows results for genomic mit DNA. The fluorescence pattern seen after digestion with EcoRI plus HindIII is in agreement with the earlier mapping studies (20). After hybridization to Cy RNA, autoradiography revealed only two labelled bands, whose positions were compatible with those of the 2.6 and 1.5 kbp segments.

Cy RNA from peaks such as that of Fig. 1 yielded, on electrophoresis through 2.5–10% acrylamide gradient urea gels, a single discrete band migrating somewhat slower than 16S RNA (data not shown). To further delineate the effects of cycloleucine, experiments were conducted in which both the poly A(–) and the poly A(+) fraction of mit RNA were analyzed on such gels. Figure 3 illustrates a protocol experiment. To monitor preexisting RNA, cells were labelled by prolonged growth in ^{32}Pi. Subcultures were then exposed to ^{3}H-uridine in the presence or the absence of cycloleucine. Levels of ^{3}H relative to ^{32}P were adjusted so that fluorography of dried gels (Panel A) registered primarily ^{3}H, and autoradiography (Panel B) registered only ^{32}P. The ^{3}H patterns demonstrate the effects of cycloleucine on poly A(–) RNA (lanes 2, 4): incorporation into 16S and 12S RNA was markedly inhibited and a new major band corresponding to Cy RNA appeared. The poly A(+) patterns resemble those found in HeLa mitochondria by Attardi & colleagues (e.g., refs. 3, 19), and we have numbered our putative mRNA bands following their designations. As shown by lanes 1 and 3, cycloleucine had little effect on the distribution of newly incorporated label among the poly A(+) species. The ^{32}P patterns (Panel B) show that cycloleucine had no discernible effect on preexisting mit RNA.

Careful examination of control patterns such as that of Figure 3 reveals the presence of minor bands

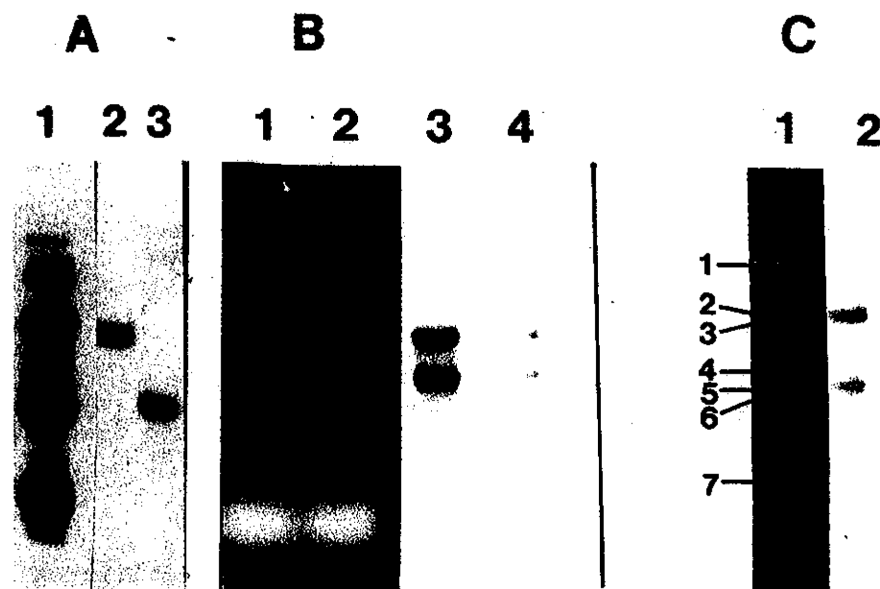

Fig. 2. Southern hybridization of Cy RNA. *Panel A.* Plasmid pBR322 containing a mit EcoRI B insert was digested with HindIII and EcoRI B. An aliquot was labelled using γ-^{32}P-ATP and T4-polynucleotide kinase, and subjected to electrophoresis (Lane 1) through 0.8% agarose in parellel with two aliquots (lanes 2, 3) of unlabeled digest. After transfer to nitrocellulose, one lane (No. 2) was hybridized to 5′-end labelled mit 12S RNA, and one (No. 3) to similarly labelled mit 16S RNA (ref. 5). The major labelled bands in Lane 1 are (from top down), linearized pBR322; the 2.6 kbp subfragment of mit EcoRI B; the 1.5 kbp subfragment; and contaminating bacterial RNA. *Panel B.* Similarly processed samples of unlabelled recombinant pBR322 were hybridized to ^{32}P-labelled cycloleucine RNA in the presence (lane 4) or the absence (lane 3) of highly purified mit 16S and 12S RNA (100 ng each). Lanes 1 and 2 show ethidium-induced fluorescence prior to transfer to nitrocellulose. *Panel C.* A sample of genomic DNA was processed as for Panel B and hybridized to Cy RNA (lane 2). Lane 1 shows ethidium-induced fluorescence; sizes of numbered bands were 4.7, 2.8, 2.6, 1.7, 1.5, 1.3 and 0.6 kbp, by comparison with markers run in parallel.

in the poly A(–) and the poly A(+) moieties that migrate indistinguishably from Cy RNA. We believe that Cy RNA, as well as polyadenylated versions of it, are indeed normal, albeit minor, constituents of mit RNA.

Electrophoresis through linear (3%) acrylamide gels was used to size Cy RNA. The resulting chain length, estimated by comparison to 16S and 12S RNA (cf. ref. 16), came to 2 600.

The present results show that Cy RNA is transcribed at least largely from the rRNA genes of hamster mit DNA; that it is a discrete species within the limits of resolution of acrylamide gel electrophoresis; that it occurs at low levels in normal cells; and that it is about 2 600 nucleotides long, compared to values of about 1 580 and 950 for mature mit rRNAs. These conclusions support the idea that Cy RNA is, or is closely related to, a ribosomal RNA precursor, whose processing is methylation dependent. Recent studies have described candidates for mit rRNA precursors in HeLa cells (19). So-called RNA 'u4a' is a minor poly A(–) species, which appears to originate from a promoter just upstream from a tRNA^{phe} gene adjacent to the 12S RNA gene, and to terminate at the end of the 16S RNA gene (2, 19). RNA 'u4' is proposed to be a partially processed moiety lacking the tRNA^{phe} stretch. The size of Cy RNA, and its being poly A(–), suggest that it corresponds to HeLa mit RNA u4 or u4a.

It is clear that the predominant effects of cycloleucine in mammalian cells result from S-adenosylmethionine depletion (9), and that this depletion interferes with methylation of RNA in all cellular compartments (1, 4, 8, 10). There is good evidence in particular that an array of undermethylated precursors to cytoplasmic rRNA accumulates in the nucleus as a consequence of impaired proces-

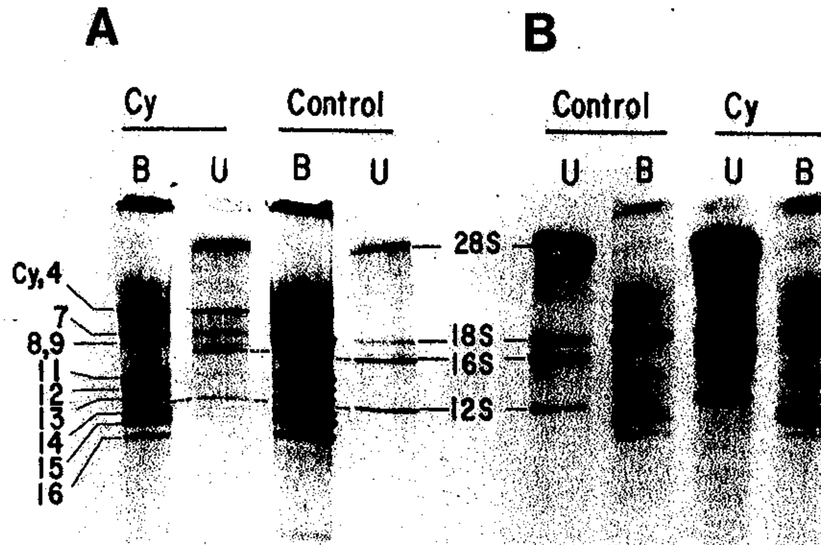

Fig. 3. Acrylamide gel analysis of mit RNA: the effect of cycloleucine. A 2-liter culture was labelled with 1 mci of ^{32}Pi for 18 h. One half was then labelled with [^{3}H]uridine (4 mci; final concentration, 1 μM) in the presence of cycloleucine, as described in Methods. The other half was labelled in paralellel, but not exposed to cycloleucine, RNA was prepared from each culture and differing aliquots were subjected to acrylamide gradient gel electrophoresed so that control and treated lanes would contain similar amounts of ^{3}H [approx. 14 000 CPM for poly A(-) RNA and 60 000 CPM for poly A(+) RNA]. Ratios of ^{3}H:^{32}P (DPM) were ~ 100:1. The gel was treated for fluorography (7) and exposed to film directly to detect primarily the ^{3}H (Panel A) and through a sheet of carbon paper to detect only the ^{32}P (Panel B). 'U,' oligo T unbound [Poly A(-)]; 'B,' oligo T bound [Poly A(+)]. The prominent 18S and 28S bands seen in Panel B arise from cytoplasmic ribosomes that contaminate the mit preparations. Labelling of these RNAs is minimal in short pulses, and essentially nil in cycloleucine-treated cells; hence, the relative intensities of these bands in Panel A provide a measure of the contribution of ^{32}P to the fluorographic exposure.

sing of these precursors, presumably because such processing is at least partially dependent on normal methylation of the precursors (8). It seems reasonable to infer that Cy RNA accumulates at the expense of mit rRNA because processing of the former, too, involves a methylation-dependent step. The pattern of presumed rRNA precursor accumulation in mitochondria is much simpler than that for cytoplasmic rRNA precursors in the nucleus. This may reflect simpler processing of the mit rRNA, and/or a single predominant methylation-dependent step. The putative mature products of Cy RNA have a number of methylated residues. Mit $tRNA^{val}$ (whose gene is between the rRNA genes) contains m^1A and m^2G (22) and 12S and 16S rRNAs contain 5 and 3 methylated residues, respectively (12, 13). The relative specificity of cycloleucine on poly A(-) *vs* poly A(+) patterns imply that one or more of the *rRNA* methylations are crucial.

Acknowledgments

This work was supported by Grant GM 14957 of the National Institutes of Health. D.L.P. was, and R.K.M. is, a pre-doctoral trainee under Institutional National Research Service Award CA 09069.

References

1. Amalric, F., Bachellerie, J. P., & Caboche, M., 1977. Nucl. Acids, Res. 4: 4357–4370.
2. Anderson, S., De Bruijn, M. H. L., Coulson, A. R., Eperon, I. A., Sanger, F. & Young, I. G., 1980. J. Mol. Biol. 156: 683–717.
3. Attardi, G., 1984. In: D. Apirion (ed.), Processing of RNA. CRC Press, Boca Raton, pp. 227–290.
4. Bachellerie, J. P., Amalric, F. & Caboche, M., 1978. Nucl. Acids Res. 5: 2927–2943.
5. Baer, R. J. & Dubin, D. T., 1980. Nucl. Acids Res. 8: 4927–4941.

6. Bibb, M. J., Van Etten, R. A., Wright, C. T., Walberg, M. W. & Clayton, D. A., 1981. Cell 26: 167-180.
7. Bonner, W. M. & Laskey, R. A., 1974. Eur. J. Biochem. 46: 83-88.
8. Caboche, M. & Bachellerie, J. P., 1977. Eur. J. Biochem. 74: 19-28.
9. Caboche, M. & Hatzfeld, J., 1978. J. Cell Physiol. 97: 361-370.
10. Dubin, D. T., Green, C. M. & Prince, D. L., 1982. In: Usdin, E., Borchardt, R. & Creveling, C. R., (eds.), Biochemistry of SAM & Related Compounds. Macmillan Press, London, pp. 289-296.
11. Dubin, D. T., Montoya, J., Timko, K. D. & Attardi, G., 1982. J. Mol. Biol. 157: 1-19.
12. Dubin, D. T. & Taylor, R. H., 1978. J. Mol. Biol. 121: 523-540.
13. Dubin, D. T., Taylor, R. H. & Davenport, L. W., 1978. Nucl. Acids Res. 5: 4385-4397.
14. Hirsch, M. & Penman, S., 1973. J. Mol. Biol. 80: 379-381.
15. Kotin, R. M. & Dubin, D. T., 1984. Biochim. Biophys. Acta 782: 106-108.
16. Lehrach, H., Diamond, D., Wozney, J. M. & Boedtker, H., 1977. Biochemistry 16: 4743-4751.
17. Lombardini, J. B., Coulter, A. W. & Talalay, P., 1970. Molec. Pharmacol. 6: 481-489.
18. Maniatis, T., Fritsch, E. F. & Sambrook, J., 1982. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor, New York.
19. Montoya, J., Gaines, G. L. & Attardi, G., 1983. Cell 34: 151-159.
20. Nass, M. M. K., 1981. Biochim. Biophys. Acta 655: 210-220.
21. Southern, E. M., 1975. J. Mol. Biol. 98: 503.
22. Sprinzl, M., Moll, J., Meissner, F. & Hartmann, T., 1985. Nucl. Acids Res. r1-r50.

Received 7.8.1985.