11. J. R. Cox and O. B. Ramsay, *Chem. Rev.* **64**, 317 (1964).
12. N. Bjerrum, *Z. Phys. Chem.* **106**, 219 (1923).
13. J. G. Kirkwood and F. H. Westheimer, *J. Chem. Phys.* **6**, 506 (1938); F. H. Westheimer and J. G. Kirkwood, *ibid.*, p. 513; F. H. Westheimer and M. W. Shookhoff, *J. Am. Chem. Soc.* **61**, 555 (1939).
14. M. Kutake, Ed., *Constants of Organic Compounds* (Asakura, Tokyo, 1963).
15. J. Kumamoto and F. H. Westheimer, *J. Am. Chem. Soc.* 77, 2515 (1955).
16. J. Pinnow, *Z. Electrochem.* **24**, 21 (1918).
17. F. C. Knowles and A. W. Benson, *Trends Biochem. Sci.* **8**, 178 (1983).
18. J.-M. Crafts, *Bull. Soc. Chim. France Ser. 2* **14**, 99 (1870); C. D. Baer, J. O. Edwards, P. H. Rieger, *Inorg. Chem.* **20**, 905 (1981).
19. V. E. Bel'skii and G. Z. Motygullin, *Bull. Acad. Sci. USSR* **1967**, 2427 (1967); E. T. Kaiser, M. Panar, F. H. Westheimer, *J. Am. Chem. Soc.* **85**, 602 (1963).
20. Ebelman, *Ann. Chem.* **57**, 334 (1846); R. Aelion, A. Loebel, F. Eirich, *Recl. Trav. Chim. Pays-Bas* **69**, 61 (1950); *J. Am. Chem. Soc.* **72**, 5705 (1950).
21. D. Barby, T. Griffiths, A. R. Jacques, D. Pawson, *Spec. Publ. Chem. Soc.* **31**, 320 (1977).
22. D. L. Miller and F. H. Westheimer, *J. Am. Chem. Soc.* **88**, 1507 (1966).
23. F. Lippmann, in *Phosphorus Metabolism*, W. D. McElroy and H. B. Glass, Eds. (Johns Hopkins Press, Baltimore, 1951), vol. 1, p. 521.
24. A. J. Zaug and T. R. Cech, *Science* **231**, 470 (1986).
25. C. Guerrier-Takada, K. Gardiner, T. Marsh, N. Pace, S. Altman, *Cell* **35**, 849 (1983).
26. H. B. White III, *J. Mol. Evol.* 7, 101 (1976); C. M. Visser and R. M. Kellog, *ibid.* **11**, 163, (1978); *ibid.*, p. 171; see also L. E. Orgel, *J. Mol. Biol.* **38**, 381 (1968).
27. B. L. Bass and T. R. Cech, *Nature (London)* **308**, 820 (1984).
28. C. Guerrier-Takada and S. Altman, *Science* **223**, 285 (1984); S. Altman, *Cell* **36**, 237 (1984).
29. F. H. Westheimer, *Nature (London)* **319**, 534 (1986).
30. A. J. Zaug and T. R. Cech, *Cell* **19**, 331 (1980); *Biochemistry* **25**, 4478 (1986); T. R. Cech, *Sci. Am.* **255**, 64 (November 1986).
31. J. Eigner, H. Boedtker, G. Michaels, *Biochim. Biophys. Acta* **51**, 165 (1961).
32. F. M. Richards and H. W. Wyckoff, in *The Enzymes*, P. D. Boyer, Ed. (Academic Press, New York, ed. 3, 1971), vol. 4, p. 647.
33. A. J. Kirby, *Adv. Phys. Org. Chem.* **17**, 183 (1980).
34. J. M. Sturtevant, J. A. Gerlt, F. H. Westheimer, *J. Am. Chem. Soc.* **95**, 8168 (1973).
35. F. H. Westheimer, *Acc. Chem. Res.* **1**, 70 (1968); K. Taira, T. Fanni, D. Gorenstein, *J. Am. Chem. Soc.* **106**, 1521 (1984).
36. L. Thelander and P. Reichard, *Annu. Rev. Biochem.* **48**, 133 (1979); M. A. Ator and J.-A. Stubbe, *Biochemistry* **24**, 7214 (1985).
37. W. Butcher and F. H. Westheimer, *J. Am. Chem. Soc.* 77, 2420 (1955); P. W. C. Barnard *et al.*, *Chem. Ind. (London)* **1955**, 760 (1955).
38. F. H. Westheimer, *Chem. Rev.* **81**, 313 (1981).
39. J. P. Guthrie, *J. Am. Chem. Soc.* **99**, 3991 (1977).
40. M. C. Bailly, *Bull. Soc. Chim. France Ser. 5* **9**, 314 (1942).
41. J. Rebek and F. Gavina, *J. Am. Chem. Soc.* **97**, 1591 (1975); D. Samuel and B. Silver, *J. Chem. Soc.* **1961**, 4321 (1961); A. J. Kirby and A. G. Varvoglis, *ibid.* **1968B**, 135 (1968); F. Ramirez and J. F. Maracek, *Tetrahedron* **35**, 1581 (1979); A. C. Satterthwait and F. H. Westheimer, *J. Am. Chem. Soc.* **103**, 1177 (1981); K. C. Calvo and F. H. Westheimer, *ibid.* **105**, 2827 (1983).
42. D. J. Harven *et al.*, *J. Am. Chem. Soc.* **101**, 7409 (1979); M. Henchman, A. A. Viggiano, J. F. Paulson, A. Freedman, J. Wormhoudt, *ibid.* **107**, 1453 (1985).
43. S. L. Buchwald, J. M. Friedman, J. R. Knowles, *ibid.* **106**, 4911 (1984); J. Friedman and J. R. Knowles, *ibid.* **107**, 6126 (1985); J. Knowles, personal communication.
44. W. P. Jencks, *Acc. Chem. Res.* **13**, 161 (1980).
45. J. R. Knowles, *Annu. Rev. Biochem.* **49**, 877 (1980); S. L. Buchwald, D. E. Hansen, A. Hassett, J. R. Knowles, *Methods Enzymol.* **87**, 279 (1982); K. C. Calvo, *J. Am. Chem. Soc.* **107**, 3690 (1985).
46. A. J. Kirby and A. G. Varvoglis, *J. Am. Chem. Soc.* **89**, 415 (1967); K. C. Calvo and F. H. Westheimer, *ibid.* **106**, 4205 (1984); K. C. Calvo and J. M. Berg, *ibid.*, p. 4202.
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Research Articles

A Mammalian Mitochondrial RNA Processing Activity Contains Nucleus-Encoded RNA

DAVID D. CHANG AND DAVID A. CLAYTON

Ribonuclease mitochondrial RNA processing, a site-specific endoribonuclease involved in primer RNA metabolism in mammalian mitochondria, requires an RNA component for its activity. On the basis of copurification and selective inactivation with complementary oligonucleotides, a 135-nucleotide RNA species, not encoded in the mitochondrial genome, is identified as the RNA moiety of the endoribonuclease. This finding implies transport of a nucleus-encoded RNA, essential for organelle DNA replication, to the mitochondrial matrix.

ALTHOUGH MITOCHONDRIA CONTAIN THEIR OWN GENETIC information, distinct from nuclear DNA (*1–3*), most proteins required for mitochondrial biogenesis are encoded in the nucleus and transported into mitochondria (*4–6*). Although present in all eukaryotes, mitochondria of metazoan species and higher plants show great variation in morphology and cellular distribution depending on particular developmental stages (*7, 8*) and tissue types (*9, 10*), suggesting a dynamic interaction between the nucleus and mitochondria. Understanding this coordinated interplay between the two distinct intracellular genetic compartments, nuclear DNA and mitochondrial DNA (mtDNA), is a central issue in cell biology. Since the enzymes involved in replication and transcription of mtDNA (*11, 12*), which control genomic expression, are also nucleus-encoded, their identities and modes of regulation may provide some insights into nuclear-mitochondrial interactions. We recently characterized a site-specific endoribonuclease that cleaves RNA near one of the transition sites of primer RNA synthesis to DNA synthesis at the leading-strand origin of mtDNA replication (*13*). We now show that this endoribonuclease, termed MRP (for mitochondrial RNA processing), contains an RNA component that is essential for its enzymatic activity. A partial sequence analysis of this endogenous RNA indicates that it is a nuclear gene product, which necessitates a translocation of a highly charged nucleic acid through the hydrophobic phospholipid bilayer into the mitochondrial matrix.

Micrococcal nuclease sensitivity of the endonuclease. Most biological catalyses are mediated by enzymes composed of polypep-

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tide chains. However, a small number of enzymatic reactions, especially ones involving RNA processing or protein synthesis, requires RNA either as a catalytic core or for the structural integrity of the enzyme (*14*). The possibility that ribonuclease (RNase) MRP might require an RNA component for its catalytic reaction was tested by treating the enzyme with micrococcal nuclease (MN), a nuclease that absolutely requires Ca^{2+} as a cofactor (Fig. 1A). After digestion, MN was inactivated by chelating Ca^{2+} with EGTA, and the activity of RNase MRP was assayed with the 270-nucleotide (nt) 3′ end labeled RNA substrate as described (*13*). RNase MRP cleaves this 270-nt RNA at a single position, generating a 130-nt product. Prior incubation of the RNase MRP fraction for up to 20 minutes in the presence of Ca^{2+} alone or MN alone had no significant effect on the endonuclease activity. Adding MN in the presence of chelated calcium had no effect on the enzymatic activity (Fig. 1A, lane 5), but adding MN and free calcium together led to complete inactivation within 5 minutes (Fig. 1A, lane 6). Similarly, the endonuclease was also inactivated by prior treatment with pancreatic RNase A, but not with deoxyribonuclease (DNase) I, indicating that the observed MN sensitivity of the cleavage reaction was due to degradation of an essential RNA component.

We next examined the thermal sensitivity of RNase MRP (Fig. 1B). Although the endonuclease was stable at 37°C and can carry out the cleavage reaction at 42°C (see Fig. 6), increasing the temperature to 50°C or above resulted in a significant reduction in activity. In addition, the endonuclease could be inactivated by 1 m*M* *N*-ethylmaleimide (*13*), a sulfhydryl-reducing agent, or by proteinase K digestion.

These results demonstrate the requirement for both an RNA species and a protein component for the cleavage reaction. Furthermore, RNase MRP is always recovered as a single entity during chromatographic purification procedures (*13*) and has an unusually high sedimentation coefficient of 15*S* on a glycerol density gradient, a result consistent with the endonuclease being a ribonucleoprotein. Whether the RNA moiety serves as a catalytic subunit or has some other auxilliary function, such as cleavage site selection or the maintenance of structural integrity of the enzyme complex, remains to be determined.

Copurification of a 135-nt RNA with the endonuclease. To identify the RNA moiety of RNase MRP, we initially examined the complexity of the RNA species present in the DEAE fraction [fraction V as described in (*13*)]. The nucleic acids present in fraction V were labeled with [^{32}P]pCp (*15*) and then separated by gel electrophoresis (Fig. 2). Since the 3′ termini of RNA species, present as ribonucleoproteins, may not be accessible in native form to T4 RNA ligase, an equal amount of fraction V was digested with PK to release any complexed RNA species, and the RNA was subsequently 3′ end labeled. Three prominent bands of 260, 135, and 65 nt were seen in the proteinase K–treated fraction V (*16*). Other species were present in low abundance (for example, the band above the 260-nt species) or were labeled with equal efficiency, especially the species of transfer RNA (tRNA) size, in both untreated and proteinase K–digested fraction V.

We next examined the elution profile of these RNA species from a Mono Q FPLC column and compared it with the endonucleolytic activity profile from the same column (Fig. 3). The input fraction was similar to the fraction III previously described (*13*), except that the endonuclease activity was further purified by a 10 to 30 percent glycerol gradient sedimentation before it was placed on the Mono Q column. The maximal cleavage activity was eluted with column fraction 54 (Fig. 3B). Analysis of the RNA species in the Mono Q column fractions, as examined by pCp 3′ end labeling, showed that the 135-nt RNA species has an elution profile that correlates precisely with the activity profile (Fig. 3A). The two other RNA species, of 260 and 65 nt, were eluted in subsequent fractions. Furthermore, the amounts of these two RNA species varied considerably in different enzyme preparations, suggesting that they are not related to the endonuclease activity itself.

Sequence of the 135-nt RNA. The 135-nt RNA was characterized by determining its sequence by partial enzymatic digestions. For this purpose, the 5′ end of the 135-nt RNA was first dephosphorylated with calf intestinal phosphatase and then labeled with [γ-^{32}P]ATP with the use of T4 polynucleotide kinase. The 5′ end labeled 135-nt RNA was subsequently purified by gel electrophoresis and digested with RNase T1 (cleaves after G) (*17*), RNase U2 (cleaves after A) (*17*), RNase PhyM (cleaves after A and U) (*18*), RNase from *Bacillus cereus* (cleaves after C and U) (*17*), or chicken liver RNase (cleaves after C) (*19*). Because of the limited availability of the 135-nt RNA and ambiguities inherent with direct RNA sequencing (especially with pyrimidines and possibly modified nucleotides), a complete sequence could not be determined. However, the sequences of two small regions of 15 to 30 nt, near the 5′ end and in the middle of the RNA, were obtained (Fig. 4). These sequences are not present in the genomic sequence of mouse mtDNA and, when compared with the sequences of human and bovine mtDNA's, failed to show any homology (*20*).

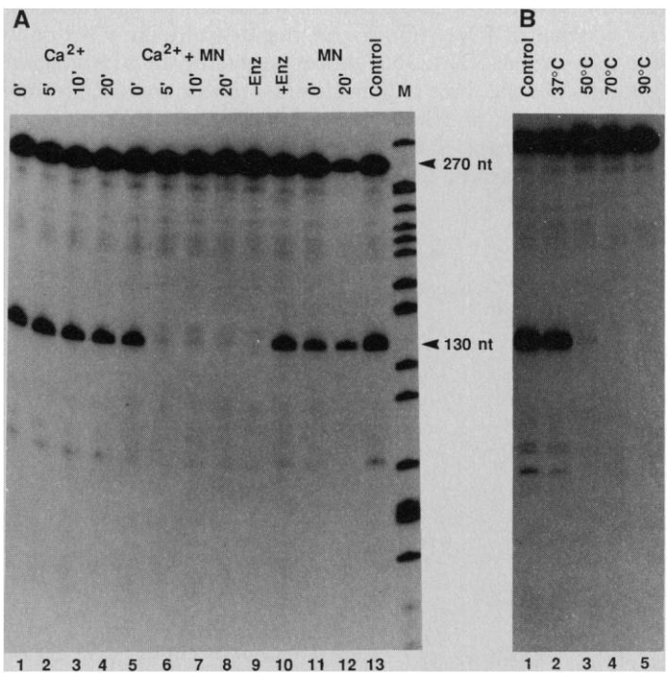

Fig. 1. (**A**) Inactivation of RNase MRP by nuclease digestion. RNase MRP was first incubated with $CaCl_2$ alone (lanes 1 to 4), with $CaCl_2$ and MN (lanes 5 to 8), or with MN alone (lanes 11 and 12) for various time intervals. Nuclease digestion was terminated by addition of EGTA, and the activity of RNase MRP was assayed. A 20-minute incubation alone (lane 10) had no significant effect on the activity of RNase MRP (compare with lane 13, no incubation). Lane M contains the Hpa II fragments of pBR322. (**B**) Heat-inactivation of RNase MRP. RNase MRP was first incubated at the designated temperature for 15 minutes and the remaining activity was assayed. RNase MRP (fraction V) and a [^{32}P]pCp-labeled 270-nt RNA substrate were prepared as described previously. In (A) 0.1 μg of fraction V was incubated at 37°C in a 20-μl volume containing 2 μg of bovine serum albumin (BSA) and either $CaCl_2$ (2 m*M*) alone, $CaCl_2$ (2 m*M*), and MN (0.8 unit), or MN (0.8 unit) alone. At the indicated time, 1 μl of 0.1*M* EGTA (*p*H 8.0) and then 5 μl of 5× reaction buffer—0.1*M* tris-HCl, *p*H 8.0, 50 m*M* $MgCl_2$, 5 m*M* dithiothreitol (DTT), 0.25*M* KCl—containing 15 units of RNasin and 0.2 ng of the substrate RNA (10×10^6 to 15×10^6 cpm, μg^{-1}) were added. The reaction was incubated for 30 minutes more at 37°C, and terminated; the cleavage products were then analyzed (*13*). In (B) incubation was carried out in a 20-μl volume containing 2 μg of BSA.

We also obtained sequence information for the 260-nt and 135-nt RNA's from their respective 3′ termini after labeling with [^{32}P]pCp. The nucleotide sequences of these two RNA's from the 3′ ends were identical. Furthermore, indirect RNA sequence derived from a partial complementary DNA sequence of the 260-nt RNA is completely homologous with the available sequence of the 135-nt RNA, suggesting a possible precursor-product relation between the 260-nt and the 135-nt RNA's.

Complementary oligonucleotide directed inactivation of the endonuclease. Agents that interact noncovalently with an enzyme can interfere with the function of the protein (*21*). In most cases, such agents are competitive inhibitors or neutralizing antibodies. In the case of ribonucleoprotein-mediated reactions, oligonucleotides complementary to the RNA moiety of the complex should function as noncovalent inhibitors by hybridizing to the RNA. Similar approaches have been used to demonstrate the involvement of U1 and U2 small nuclear ribonucleoprotein particles in splicing reactions, although in both cases the use of RNase H resulted in irreversible inactivation of the ribonucleoprotein functions (*22–24*).

To demonstrate that the 135-nt RNA is indeed an essential component of the endonuclease, we synthesized three oligonucleotides that are complementary to different regions of the RNA as indicated on Fig. 4. These complementary oligonucleotides were initially incubated with the isolated 5′end labeled 135-nt RNA in the presence of RNase H; this enzyme degrades the RNA only in the region of an RNA:DNA duplex created by the added oligonucleotides. Of the three oligonucleotides tested, one (2C), complementary to the middle region of the 135-nt RNA, induced a significant cleavage of the RNA (Fig. 5). The other two complementary oligonucleotides (2A and 2B), and an unrelated oligonucleotide made complementary to 5.8*S* ribosomal RNA (rRNA), had no noticeable effect on the 135-nt RNA. Since the RNase H cleavage reaction absolutely requires RNA:DNA duplex formation, the lack of cleavage with oligonucleotides 2A and 2B might be attributable to inaccessibility of the corresponding regions of the 135-nt RNA to these oligonucleotides. In addition, oligonucleotide 2B was derived from a region of the 135-nt RNA with sequence ambiguities and may not be perfectly complementary to the RNA.

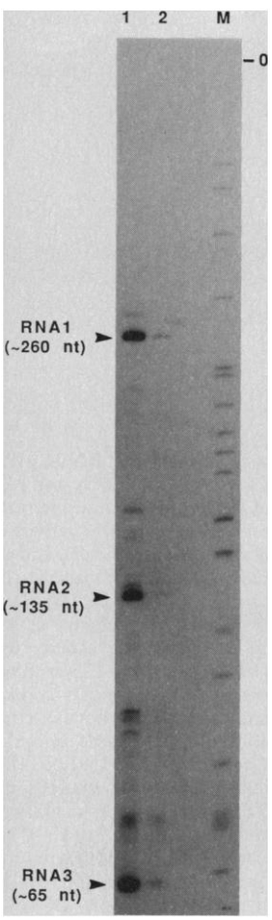

Fig. 2. Complexity of the RNA species present in RNase MRP fraction V. Nucleic acids in fraction V (10 μg) were labeled at the 3′ end either directly (lane 2) or after proteinase K digestion (lane 1). (Lane M) Hpa II fragments of pBR322. Proteinase K treatment was carried out in 100 μl containing 0.2 percent (w/v) SDS and 20 μg of proteinase K at 37°C. The sample was incubated for 30 minutes and then extracted twice with an equal volume of phenol and $CHCl_3$ (1:1, v/v) and then precipitated with ethanol at −20°C for 24 hours. The 3′ end labeling was carried out (overnight at 4°C) in a 40-μl volume containing 50 m*M* Hepes (*p*H 7.5), 15 m*M* $MgCl_2$, 3 m*M* DTT, 0.4 μg of BSA, 10 percent (v/v) dimethyl sulfoxide (DMSO), 30 μ*M* adenosine triphosphate (ATP), 60 μCi of [^{32}P]pCp (specific activity, 2000 Ci/mmol), and 20 units of T4 RNA ligase (*13*). The end-labeled products were recovered by precipitation with ethanol and then analyzed by gel electrophoresis on a 6 percent acrylamide–7*M* urea gel.

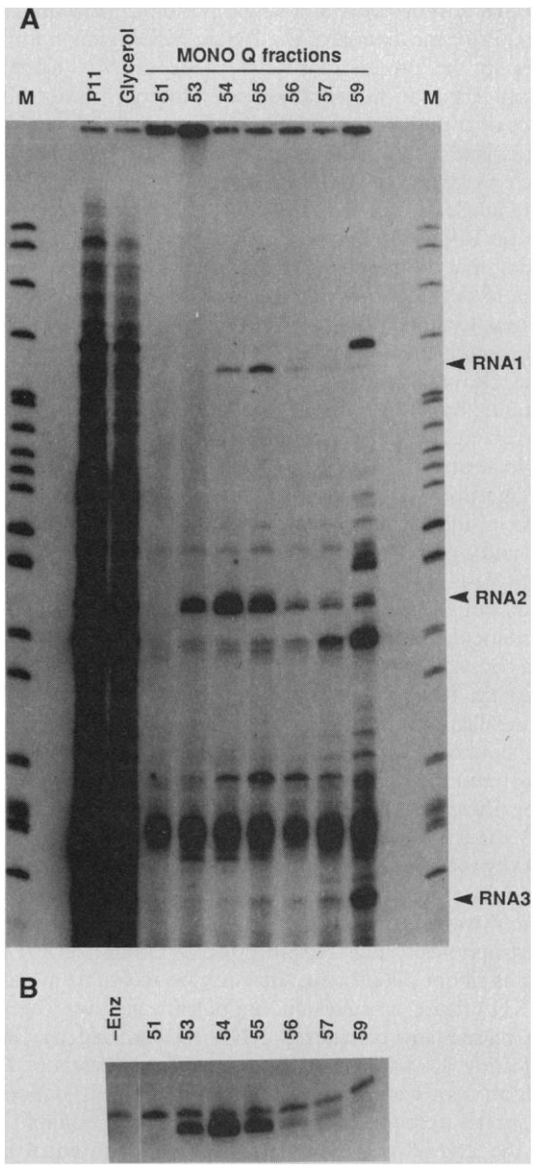

Fig. 3. Coelution of the 135-nt RNA and the RNase MRP activity. The elution profiles of RNA species (**A**) and the RNase MRP activity (**B**) from a Mono Q FPLC column are shown. Mitochondrial protein fraction III (1.6 mg) was loaded onto two tubes containing a 4-ml linear gradient of 10 to 30 percent glycerol in buffer C (20 m*M* tris-HCl, *p*H 8.0, 0.1*M* KCl, 0.5 m*M* EDTA, and 1 m*M* DTT) and sedimented at 60,000 rpm for 5 hours (SW 60 Ti rotor). Active fractions (60 μg) were pooled and loaded onto a Pharmacia Mono Q HR 5/5 column. The column was washed with 6 ml of 0.15*M* KCl in buffer D (buffer C + 10 percent glycerol) and then developed with a 25-ml linear gradient of 0.15*M* to 0.6*M* KCl in buffer D at a flow rate of 1 ml min^{-1}. Twenty-five 1-ml fractions (fractions 41 to 65) were collected and assayed for RNase MRP (B). Fractions 51 to 59 encompassing the RNase MRP activity were labeled at the 3′ end with [^{32}P]pCp after proteinase K digestion and analyzed by gel electrophoresis as described in the legend to Fig. 2A. Lanes M contain the Hpa III fragments of pBR322.

The effects of these oligonucleotides on the function of RNase MRP were subsequently tested by incubating the enzyme fraction with varying amounts of oligonucleotides. We found that the oligonucleotide 2C, which induced a selective degradation of the 135-nt RNA, also significantly reduced the efficiency of RNase MRP in a concentration-dependent manner (Fig. 6). The other two complementary oligonucleotides, as well as the oligonucleotide complementary to 5.8*S* rRNA (anti-5.8*S*), had no effect on the cleavage reaction even at the highest concentration (50 μM) tested.

The inhibition of RNase MRP by oligonucleotide 2C occurred in the absence of RNase H, suggesting that RNA:DNA duplex formation between oligonucleotide 2C and the 135-nt RNA, rather than an irreversible degradation of the RNA by RNase H, was sufficient to render the RNase MRP inactive. Consistent with this interpretation, the selective inactivation by oligonucleotide 2C showed temperature dependence; under identical conditions, simply raising the cleavage reaction temperature from 30° to 42°C resulted in reversal of the inhibition. The melting temperature of the expected hybrid between oligonucleotide 2C and the RNA under the salt conditions used is 37.3°C (*25*). At a reaction temperature of 42°C, 5°C above the expected melting temperature, this hybrid should not be stable. We therefore believe that the oligonucleotide-mediated inhibition is due to a hybrid formation between the RNA component of the endonuclease and the added complementary oligonucleotide.

Subcellular distribution of RNase MRP and the 135-nt RNA. This assignment of RNase MRP as a mitochondrial enzyme is essentially on a functional basis; the enzyme cleaves an RNA substrate containing the sequence of mouse mtDNA at a previously recognized processing site. To provide additional support for RNase MRP being a mitochondrial enzyme, we separated total cellular proteins into three distinct fractions representing nuclear, mitochondrial, and cytosolic compartments. Each fraction was subsequently assayed for RNase MRP activity with the 270-nt RNA as substrate (Fig. 7A). In this and cognate experiments (Fig. 7, B, C, and D) the amounts of protein were adjusted to reflect equivalent cell numbers rather than protein amount.

Of the three compartments tested, most of the RNase MRP activity was isolated in the mitochondrial fraction (Fig. 7A). The activity in the cytosolic fraction accounted for less than 5 percent of that present in mitochondria. In addition, a significant portion of the activity fractionated with the nuclear compartment. In this experiment, the level of RNase MRP in the nuclear compartment was falsely underrepresented because of the presence of nonspecific nucleases in the crude nuclear extract as evidenced by the extensive degradation of the 270-nt substrate RNA (Fig. 7A, lane 3). When the RNase MRP activity in the nuclear extract was partially purified to remove these interfering nonspecific nucleases, the yield of RNase MRP approximated that from the mitochondrial fraction.

We next examined the distribution of 135-nt RNA by Northern analysis with an oligonucleotide probe made complementary to the 135-nt RNA. The RNA in each of the three subcellular fractions was isolated after the polypeptides were removed by proteinase K digestion. The recovered RNA species were separated by gel electrophoresis, immobilized onto a nitrocellulose filter (*26*), and subsequently hybridized with ^{32}P-labeled oligonucleotide. In the mitochondrial fraction, the labeled probe hybridized to the expected 135-nt RNA (RNA2) as well as to the 260-nt RNA (RNA1), which shares an identical 3′ end sequence. The probe also hybridized to an additional, previously unidentified RNA species of 230-nt (denoted as RNA1*). This species was not present in the isolated enzyme fraction (Fig. 2), and its relation to enzymatic activity is not known although its size is suggestive of a processing intermediate between the 260-nt and 135-nt RNA.

The same three RNA species were also seen in the nuclear and cytosolic fractions. However, the abundances of these RNA species were both quantitatively and qualitatively different in each compartment. First, RNA2, which coeluted with the RNase MRP activity from the Mono Q column, was detected at a significant level only in nuclear and mitochondrial fractions, correlating with the subcellular distribution of the enzymatic activity. Second, in the nuclear fraction, the larger RNA1 was present as the major species, amounting to a 100-fold excess (or more) over the smaller RNA2. In the mitochondrial fraction, this ratio was reversed and the smaller RNA2 was seen as the major species. Although each species was present in much less amount, the relative amounts of the RNA species in the cytosolic fraction were similar to that in the nuclear

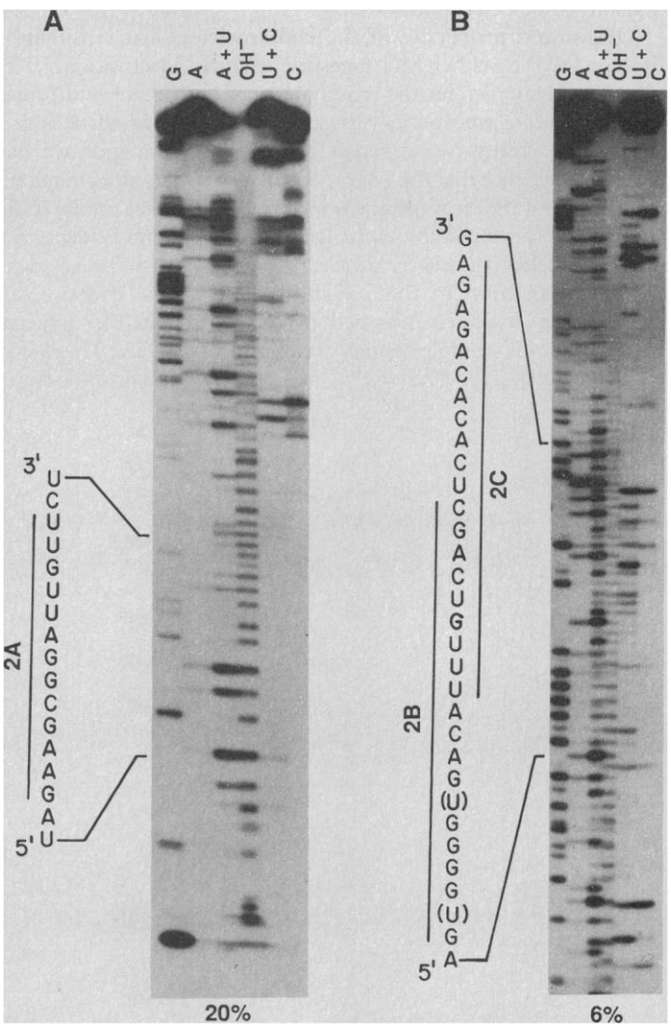

Fig. 4. Sequence analysis of the 135-nt RNA. The 135-nt RNA labeled at the 5′ end was subjected to partial enzymatic sequence analysis with RNase T1 (G), RNase U2 (A), RNase PhyM (A+U), RNase *Bacillus cereus* (U+C), and chicken liver RNase (C). OH^- indicates partial alkaline hydrolysis ladder. The reaction products were separated by electrophoresis on a 20 percent (**A**) or 6 percent (**B**) polyacrylamide gel containing 7*M* urea. The sequence of RNA used to generate complementary oligonucleotides 2A, 2B, and 2C is shown next to the sequence ladders. Some of the ambiguous nucleotides are indicated by parentheses. RNase MRP fraction V (50 μg or pooled Mono Q fractions) was digested with proteinase K, and RNA was recovered by precipitation with ethanol (legend to Fig. 2). The RNA sample, after being treated with calf intestinal alkaline phosphatase, [γ-^{32}P]ATP-labeled with the use of T4 polynucleotide kinase, and the 135-nt RNA was isolated from a 6 percent acrylamide–7*M* urea gel. A typical yield of the 135-nt RNA labeled at the 5′ end was 2000 to 4000 cpm. The digestions with sequence-specific RNases were carried out as described (*13*).

fraction and may reflect nuclear contamination during the isolation procedure (see below, Fig. 7D).

To determine whether the RNase MRP activity and the corresponding 135-nt RNA moiety of this enzyme found in the nuclear fraction represent a presence of a separate nuclear pool or a contamination by mitochondria, we conducted additional Northern analyses using nucleic acid probes specific for mitochondrial methionyl–transfer RNA (tRNA) (Fig. 7C) or the small nuclear RNA U1 (Fig. 7D). In each case, the extents of cross-contamination of these nucleic acids between the nuclear and mitochondrial preparations were less than 1 percent and cannot account for the amount of RNase MRP found in the nuclear fraction. Whether the RNase MRP activity in our nuclear fraction reflects a functionally significant RNA processing activity or simply a maturation process remains to be determined.

Origin and properties of the endonuclease. The sensitivity of RNase MRP to either MN digestion, thermal inactivation, or PK digestion demonstrates the requirement of both RNA and protein components for site-specific cleavage by this mitochondrial endoribonuclease. Using two different experimental strategies, we have obtained evidence that the 135-nt RNA species present in fraction V is involved in the cleavage reaction. First, the elution profile of this 135-nt RNA from an ion-exchange Mono Q column is identical to the endonucleolytic activity profile, thus providing physical evidence for the association of the 135-nt RNA with the endonuclease. Second, the endonucleolytic activity can be inhibited by a specific oligonucleotide complementary to the 135-nt RNA. These data, along with the large sedimentation coefficient of the endonuclease in a glycerol density gradient centrifugation, suggest that RNase MRP is a ribonucleoprotein with at least one RNA component.

Repeated attempts to identify the protein component of RNase MRP have not yet resulted in the correlation of any specific polypeptide with the activity. Nevertheless, on the basis of current knowledge of the protein coding capacity of mammalian mtDNA (*27–29*), it is highly unlikely that a protein component of RNase MRP is encoded in mtDNA. In regard to the origin of the 135-nt RNA of the endonuclease, there are persuasive arguments for its being a nuclear gene product. The available RNA sequence information clearly indicates that this RNA is not encoded in mtDNA. Although the presence of mitochondrial plasmids has been documented in some fungi (*30*) and plants (*31*), no evidence for any such extragenomic genetic element exists for mammalian mitochondria despite numerous and extensive analyses of DNA isolates from mammalian sources. Thus it is difficult to argue that the 135-nt RNA is mitochondrially encoded. A second line of evidence is that, of the three major RNA species present in fraction V (see Fig. 2), two, the 260- and 135-nt RNA's, are related in sequence. The fact that a large amount of the 260-nt RNA was detected in the nuclear RNA preparation supports a nuclear origin of this RNA, as well as for that of the 135-nt RNA.

Besides the postulated precursor-product relation between the 260- and the 135-nt RNA species, little is known about the larger RNA. Whether the accumulation of the 260-nt RNA in the nucleus is simply a reflection of a precursor-product relation or an indication of a separate functional role of this RNA in the nucleus remains to

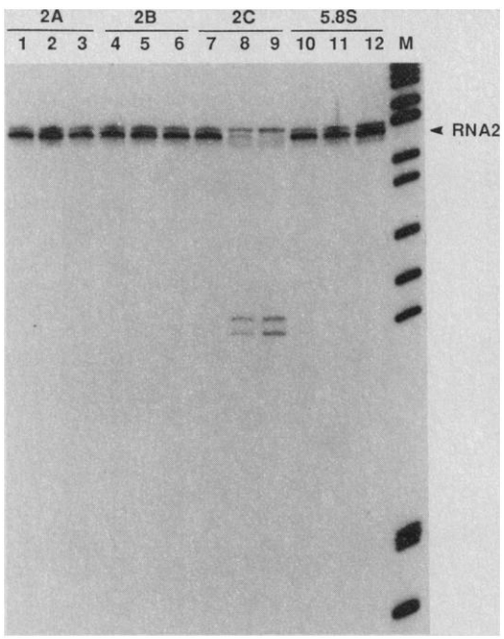

Fig. 5. Selective cleavage of the 135-nt RNA by complementary oligonucleotides. Oligonucleotides were synthesized by means of an automated (Applied Biosystems, model 380A) solid-phase phosphite triester method and purified by acrylamide gel electrophoresis. The sequences of oligonucleotides are as follows: 2A, 5′-AACAATCCGCTTC-3′ (13 nt); 2B, 5′-GCTGACAAATGTCACCCCAC-3′ (20 nt); 2C, 5′-TCTCTGTGTG AGCTGACAAA-3′ (20 nt); 5.8*S*, 5′-CCACCGCTAAGAGTC-3′ (15 nt). The oligonucleotide 5.8*S* is complementary to nucleotides 2 to 16 of human 5.8*S* rRNA (*53*). 5′ end labeled 135-nt RNA was incubated with 8 μ*M* (lanes 2, 5, 8, and 11) or 40 μ*M* (lanes 1, 3, 4, 6, 7, 9, 10, and 12) of oligonucleotide 2A, 2B, 2C, or 5.8*S* in a 25-μl reaction buffer containing 0.8 unit of *Escherichia coli* RNase H at 37°C for 30 minutes and then analyzed by gel electrophoresis. Lanes 1, 4, 7, and 10 did not contain *E. coli* RNase H. The position of the 135-nt RNA (RNA2) is indicated. The slow-migrating RNA species above RNA2 is a contaminant and is not related to RNA2. Lane M contains the Hpa III fragments of pBR322.

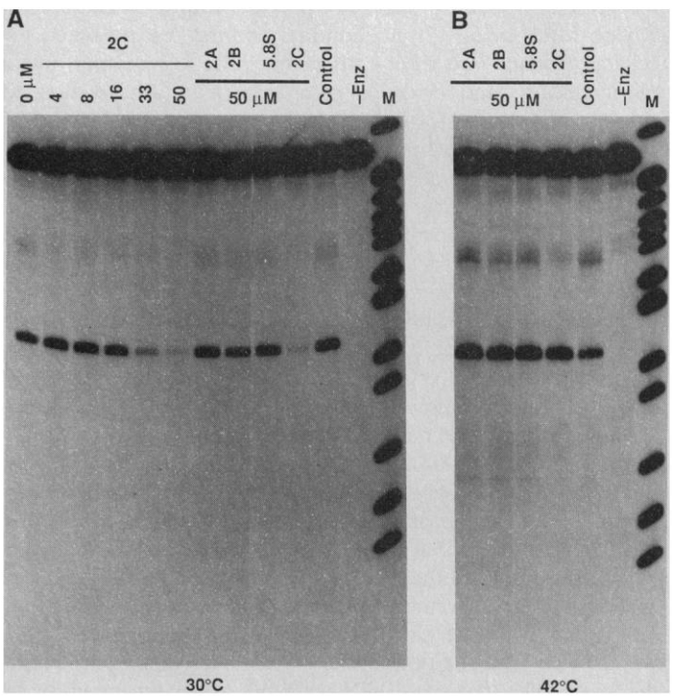

Fig. 6. Effect of complementary oligonucleotides on the RNase MRP activity. RNase MRP was incubated with varying amounts of oligonucleotide 2C or 50 μ*M* of oligonucleotide 2A, 2B, or 5.8*S*, and then assayed for endonuclease activity at 30°C (**A**) or at 42°C (**B**). Individual oligonucleotides were added to a 6-μl volume containing 0.2 μg of RNase MRP fraction V in 20 m*M* tris-HCl (*p*H 8.0), 50 m*M* KCl, 10 m*M* $MgCl_2$, 1 m*M* DTT, and 6 units of RNasin to the specified concentration and incubated at 4°C. Samples (3 μl) of each mixture were assayed for the endonuclease activity with the 270-nt substrate RNA at the indicated temperature; the reaction products were analyzed as described previously (*13*). In control lanes, the cleavage reaction was carried out in the absence of oligonucleotide. Lane M contains the Hpa III fragments of pBR322.

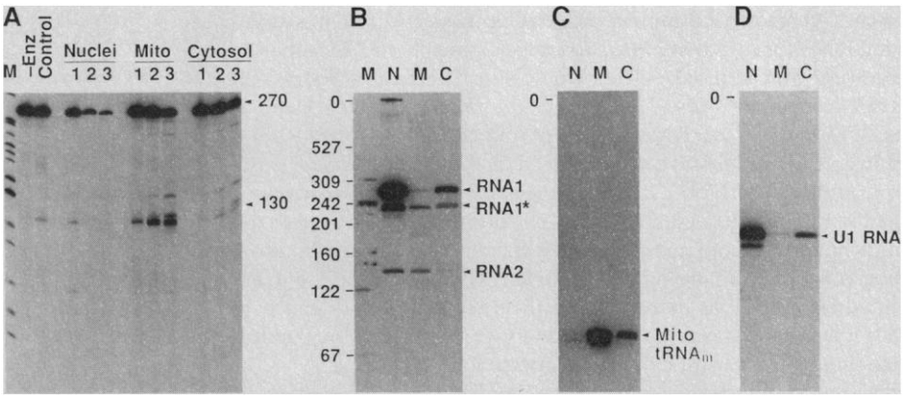

Fig. 7. Subcellular localization of RNase MRP and the 135-nt RNA. (**A**) the RNase MRP activity from a nuclear, mitochondrial, or cytosolic extract was assayed under standard in vitro cleavage reaction conditions (*13*). The amounts of each extract used were equivalent to $\sim 5 \times 10^3$ cells (lanes 1), $\sim 2.5 \times 10^4$ cells (lanes 2), and $\sim 1.25 \times 10^5$ cells (lanes 3). The control reaction was carried out with 0.05 μg of RNase MRP fraction V. The positions of the 270-nt substrate RNA and the 130-nt product RNA are indicated. (**B** to **D**) Nucleic acid was obtained from an equivalent ($\sim 7 \times 10^6$ cells) amount of nuclear (N), mitochondrial (M), or cytosolic (C) extract and analyzed by the Northern blot technique with probes complementary to the 135-nt RNA (B), mitochondrial $tRNA^{Met}$ (C), and nuclear U1 RNA (D). The positions of respective RNA species are indicated. Nuclear and cytosolic extracts were prepared as described (*54*) with slight modification. The mitochondria were removed from the cytoplasm by centrifugation at 20,000*g* for 10 minutes, and the resulting supernatant was used to prepare a cytosolic extract. The pellet obtained was lysed by a detergent-salt procedure (*13*) to obtain a mitochondrial extract equivalent to fraction I of (*13*). A typical yield from 3 liters of exponentially growing mouse L cells (5×10^5 cells per milliliter) was 24 mg of nuclear, 39 mg of cytosolic, and 13 mg of mitochondrial proteins. For Northern analysis, each extract was digested with proteinase K (200 μg/ml) at 37°C for 60 minutes, and nucleic acid was recovered by precipitation with ethanol. Contaminating DNA was removed by DNase I (Miles, RNase-free) digestion, and the remaining RNA was separated by gel electrophoresis on a 6 percent urea-acrylamide gel. RNA was then transferred to a nitrocellulose filter membrane (*26*) and hybridized with probes overnight at 42°C in 5× SSPE (0.9*M* NaCl, 5 m*M* EDTA, 50 m*M* NaH_2PO_4, *p*H 7.5) containing 5× Denhardt's solution (0.1 percent Ficoll, 0.1 percent polyvinylpyrrolidone, 0.1 percent BSA), 0.5 percent SDS, and *E. coli* tRNA (0.2 mg/ml). (**C** and **D**) Formamide and dextran sulfate (40 and 5 percent, respectively) were added to the hybridization solution. The filter membrane was successively washed in 5× SSPE plus 0.2 percent SDS, in 1× SSPE plus 0.2 percent SDS, and then in 0.2× SSPE + 0.2 percent SDS at 24°C for 10 minutes with each solution. In (C) and (D) the filter membranes were additionally washed in 0.2× SSPE and 0.2 percent SDS at 55°C for 10 minutes. The probe used in (B) was generated from a complementary oligonucleotide by labeling the 5′ termini with [γ-^{32}P]ATP to a specific activity of 0.5×10^9 cpm/μg. A probe complementary to mitochondrial methionyl tRNA was generated from M13mp7-met (*13*) by extending a hybridization primer (P-L Biochemicals). The U1 RNA-specific probe was synthesized with the use of SP6 RNA polymerase and plasmid SP64/Ulc$^-$ (*55*), linearized with Eco RI digestion, as the template. M in the left-hand column of (A) and (B) represent the Hpa III fragments of pBR322; otherwise, in (B) to (C), the M signifies mitochondrial.

be investigated. The available sequence of the 260-nt RNA is not homologous to any of the known U-series RNA's or other small nuclear or cytoplasmic RNA sequences (*20*).

Since oligonucleotide 2C is complementary to both of these RNA species, we cannot exclude the possibility that the 260-nt RNA, as well as the 135-nt RNA, is required for endonuclease function based on the complementary oligonucleotide-mediated inhibition assays. It is possible that the 135-nt RNA is a degradation product of the larger RNA, generated during enzyme purification procedures. However, the 5′ end of the 135-nt RNA is homogeneous, and the relative abundance of the 135-nt RNA, not that of the 260-nt RNA, correlates better with the endonucleolytic activity in different enzyme preparations. Furthermore, the relative abundances of these two RNA species vary significantly among different intracellular compartments. In both nuclei and cytosol the amount of the 260-nt RNA exceeds that of the 135-nt RNA by more than 100-fold. In mitochondria, this ratio between the two RNA species is reversed and the 135-nt RNA becomes the major species; this suggests that the smaller 135-nt RNA is not a simple degradation product of the larger RNA. These findings, along with the elution profile of the 135-nt RNA from the Mono Q column, argue that the 135-nt RNA is the RNA component of the endonuclease. It will be of interest to identify the gene encoding the 260-nt RNA and to study its function in the nucleus as well as to determine the precise relationship between the 260-nt RNA and the 135-nt RNA component of RNase MRP.

A role for RNA import in organelle biogenesis. All known nuclear gene products involved in mitochondrial biogenesis are proteins (*6, 32*). Translocation of such proteins synthesized in the cytosol, into mitochondria, occurs posttranslationally (*5, 33, 34*). The RNase MRP was isolated from purified mitochondria and cleaves a mitochondrial RNA substrate specifically at a known in vivo processing site (*13*). That this mitochondrial enzyme requires a nucleus-encoded RNA component for its activity mandates a requirement for transporting nucleic acid into mitochondria.

How does a highly charged nucleic acid traverse the hydrophobic lipid membrane of mitochondria? Little is known about translocation of nucleic acids across any biological membrane. Ribosomal RNA's exit through nuclear pores after assembly of ribosomal particles in the nucleus (*35*). Conversely, the nuclear segregation of the U series of RNA's involves the assembly of ribonucleoprotein particles, which occurs in the cytoplasm (*36–39*). This interaction between the RNA and Sm antigens appears to activate the necessary karyophilic signals for nuclear homing (*38*). The transport of mRNA's out of the nucleus is also a multistep process requiring RNA processing and, probably, interaction with poly(A) binding proteins as well (*40*). Such RNA-protein interactions in nucleic acid transport across the nuclear envelope may be an indication that the RNase MRP is transported into mitochondria as a ribonucleoprotein where the protein components of the complex may serve to shield the charged groups of the RNA. However, the mitochondrial membrane has no known structural equivalent of the nuclear pore. Furthermore, it is believed that proteins partially unfold while being translocated across mitochondrial membranes (*41, 42*) and it is doubtful that the endonuclease activity, particularly given its size, is transported as a complexed structure. The importing of this 135-nt RNA into mitochondria, nevertheless, is likely to be an active process and represents a potential regulatory site of mitochondrial biogenesis—especially since the 135-nt RNA is required for site-specific RNA processing at the leading-strand origin of mtDNA replication.

The transport of nucleic acid into or, perhaps, out of mitochondria may be general. The presence of nucleus-encoded tRNA's in mitochondrial RNA preparations has been reported for yeast (*43*) and *Tetrahymena* (*44*), although any clear demonstration of a functional role for these species remains to be established. The

recently characterized human mtDNA primase also requires structural RNA for its activity (*45*), and it is possible that RNA import is involved. Furthermore, all known RNase P activities require an RNA component (*46–51*). In yeast, the tRNA synthesis locus of mtDNA encodes the RNA moiety of mitochondrial RNase P (*52*). However, no candidate gene for the RNA moiety of RNase P exists in mammalian mtDNA (*27–29*), suggesting that the RNA component is nucleus-encoded. Therefore, translocation of RNA across the mitochondrial membrane could be a general aspect of mitochondrial biogenesis. Identifying nucleus-encoded RNA's essential for mitochondrial biogenesis and studying their regulation and transport into mitochondria may provide substantive insight into our understanding of nuclear-mitochondrial interactions.

REFERENCES AND NOTES

1. G. Schatz, E. Halsbrunner, H. Tuppy, *Biochem. Biophys. Res. Commun.* **15**, 127 (1964).
2. M. M. K. Nass, S. Nass, B. A. Afzelius, *Exp. Cell Res.* **37**, 516 (1965).
3. P. Borst and L. A. Grivell, *Cell* **15**, 705 (1978).
4. G. Schatz and T. L. Mason, *Annu. Rev. Biochem.* **43**, 51 (1974).
5. R. Hay, P. Böhni, S. Gasser, *Biochim. Biophys. Acta* **779**, 65 (1984).
6. A. Tzagoloff and A. M. Myers, *Annu. Rev. Biochem.* **55**, 249 (1986).
7. J. W. Chase and I. B. Dawid, *Dev. Biol.* **27**, 504 (1972).
8. J. Heasman, J. Quarmby, C. C. Wylie, *ibid.* **105**, 458 (1984).
9. L. S. Dillon, *Ultrastructure, Macromolecules, and Evolution* (Plenum, New York, 1981), pp. 377–386.
10. K. J. Newton and V. Walbot, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6879 (1985).
11. D. A. Clayton, *Cell* **28**, 693 (1982).
12. ———, *Annu. Rev. Biochem.* **53**, 573 (1984).
13. D. D. Chang and D. A. Clayton, *EMBO J.* **6**, 409 (1987).
14. T. R. Cech and B. L. Bass, *Annu. Rev. Biochem.* **55**, 559 (1986).
15. T. E. England and O. C. Uhlenbeck, *Nature (London)* **275**, 560 (1978).
16. Although there is a periodicity in the sizes of these fragments, the two larger species are not artifactual multimers of the smaller 65-nt RNA generated during the in vitro labeling with T4 RNA ligase since the three RNA species can be separated by subsequent fractionation (see Fig. 3).
17. R. E. Lockard *et al.*, *Nucleic Acids Res.* **5**, 37 (1978).
18. H. Donis-Keller, *ibid.* **8**, 3133 (1980).
19. M. S. Boguski, P. A. Hieter, C. C. Levy, *J. Biol. Chem.* **255**, 2160 (1980).
20. The computer analyses were carried out with the MicroGenie Program (SciSoft, Inc.).
21. A. Fersht, *Enzyme Structure and Mechanism* (Freeman, San Francisco, 1977), pp. 93–96.
22. A. Kramer, W. Keller, B. Appel, R. Lührman, *Cell* **38**, 299 (1984).
23. A. R. Krainer and T. Maniatis, *ibid.* **42**, 725 (1985).
24. D. L. Black, B. Chabot, J. A. Steitz, *ibid.*, p. 737.
25. The melting temperature was determined from the following equation: $T_m(°C) = 16.6 \log[Na^+] + 0.41[\%(G + C)] + 81.5 - (820/L)$, where L is the length of the oligonucleotide.
26. J. C. Alwine *et al.*, *Methods Enzymol.* **68**, 220 (1979).
27. S. Anderson *et al.*, *Nature (London)* **290**, 457 (1981).
28. M. J. Bibb, R. A. Van Etten, C. T. Wright, M. W. Walberg, D. A. Clayton, *Cell* **26**, 167 (1981).
29. S. Anderson *et al.*, *J. Mol. Biol.* **156**, 683 (1982).
30. R. A. Collins, L. L. Stohl, M. D. Cole, A. M. Lambowitz, *Cell* **24**, 443 (1981).
31. D. R. Pring, C. S. Levings, III, W. W. L. Hu, D. H. Timothy, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2904 (1977).
32. P. Borst, *Annu. Rev. Biochem.* **41**, 333 (1972).
33. G. Schatz, *FEBS Lett.* **103**, 203 (1979).
34. G. Schatz and R. A. Butow, *Cell* **32**, 316 (1983).
35. F. Wunderlich, in *The Cell Nucleus: Nuclear Particles*, H. Busch, Ed. (Academic Press, New York, 1981), pp. 249–281.
36. G. Zieve and S. Penman, *Cell* **8**, 19 (1976).
37. S. J. Madore, E. D. Wieben, T. Pederson, *J. Cell Biol.* **98**, 188 (1984).
38. I. W. Mattaj and E. M. DeRobertis, *Cell* **40**, 111 (1985).
39. D. E. Fisher, G. E. Conner, W. H. Reeves, R. Wisniewolski, G. Blobel, *ibid.* **42**, 751 (1985).
40. B. Setyono and J. R. Greenberg, *ibid.* **24**, 775 (1981).
41. M. Schleyer and W. Neupert, *ibid.* **43**, 339 (1985).
42. M. Eilers and G. Schatz, *Nature (London)* **322**, 228 (1986).
43. R. P. Martin, J. M. Schneller, A. J. C. Stahl, G. Dirheimer, *Biochemistry* **18**, 4600 (1979).
44. N. Chiu, A. Chiu, Y. Suyama, *J. Mol. Biol.* **99**, 37 (1975).
45. T. W. Wong and D. A. Clayton, *Cell* **45**, 817 (1986).
46. B. C. Stark, R. Kole, E. J. Bowman, S. Altman, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3717 (1978).
47. K. Gardiner and N. R. Pace, *J. Biol. Chem.* **255**, 7507 (1980).
48. E. Akaboshi, C. Guerrier-Takada, S. Altman, *Biochem. Biophys. Res. Commun.* **96**, 831 (1980).
49. L. Kline, S. Nishikawa, D. Söll, *J. Biol. Chem.* **256**, 5058 (1981).
50. C. J. Doerson, C. Guerrier-Takada, S. Altman, G. Attardi, *ibid.* **260**, 5942 (1985).
51. M. Hollingsworth and N. C. Martin, *Mol. Cell. Biol.* **6**, 1058 (1986).
52. D. L. Miller and N. C. Martin, *Cell* **34**, 911 (1983).
53. V. A. Erdmann and J. Walters, *Nucleic Acids Res.* **14**, r1 (1986).
54. J. D. Dignam, R. M. Lebovitz, R. G. Roeder, *ibid.* **11**, 1475 (1983).
55. E. T. Schenborn and R. C. Mierendorf, Jr., *ibid.* **13**, 6223 (1985).

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Chemistry of Antibody Binding to a Protein

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The chemistry of antibody recognition was studied by mapping the antigenicity of the protein myohemerythrin with peptide homologs of the protein sequence. The results suggest that the entire protein surface is antigenic, but the probability of there being antibodies to a given site is influenced by local stereochemistry. Although accessible to an antibody binding domain, the least reactive positions cluster in the most tightly packed and least mobile regions and are closely associated with narrow, concave grooves in the molecular surface containing bound water molecules. The most frequently recognized sites form three-dimensional superassemblies characterized by high local mobility, convex surface shape, and often by negative electrostatic potential.

ANTIGENIC SITES IN PROTEINS ARE OFTEN COMPLEX CONformations dependent on the tertiary folding of the protein chain. Unless sequenced mutational or evolutionary variants of the protein are available, the exact amino acid residues to which an antibody binds are difficult to determine. Moreover, the proteins that are best defined structurally and antigenically are often very conserved among species, thus producing limited immunological responses experimentally. In this article we describe our study of the immune response to the protein myohemerythrin (MHr), in which a large number of synthetic peptides were used to map antigenic sites on the three-dimensional crystallographic structure.

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