with dextrose, raffinose, or galactose was centrifuged and resuspended in 50 μl of the used media. We then treated this suspension with 3.5 μl of 0.35 M $KMnO_4$ for 1 min at 22°C. The reaction was stopped by dilution in sorbitol stop buffer [0.9 M sorbitol, 0.1 M tris-HCl (pH 8.0), 0.1 M EDTA, 40 mM β-mercaptoethanol]. Spheroplast preparation and DNA isolation was performed as described (*28*). Piperidine treatment and ligation-mediated polymerase chain reaction (LMPCR) analyses were performed as described (*15*). The primers used for LMPCR were the following: top strand, primer 1: GCGATTAGTTTTTTAGCCTTATTTCTGG; primer 2: TCTGGGGTAATTAATCAGCGAAGCGATG; and bottom strand, primer 1: CCAATGGTCTTGGTAATTCCTTTGC; primer 2: TTTGCGCTAGAATTGAACTCAGGTAC.

26. The primers used for LMPCR were the following: top strand, primer 1: ATTAGCTCTACCACAGTGTGTGAACC; primer 2: TGAACCAATGTATCCAGCACCACCTG; and bottom strand, primer 1: GGTTTTTTTAGGCTAAGATAATGGGG; primer 2: ATGGGGCTCTTTACATTTCCACAAC.
27. For the in vitro treatment of DNA, ~0.5 μg of plasmid DNA, either cut with Bam HI or at native superhelical density, was treated in 10 mM tris-Cl (pH 7.7) and 1 mM EDTA with 25 mM $KMnO_4$ for 30 s at the indicated temperatures.
28. D. A. Treco, in *Current Protocols in Molecular Biology*, F. M. Ausubel *et al.*, Eds. (Greene Publishing Associates and Wiley-Interscience, New York, 1989), pp. 13.11.1–13.11.5.
29. The *rpb1-1*–carrying yeast strain Y262 was the gift of R. Young. We also thank J. Roberts, T. O'Brien, and X. Hua for critically reading this manuscript.

1 April 1993; accepted 7 June 1993

Circularly Permuted tRNAs as Specific Photoaffinity Probes of Ribonuclease P RNA Structure

James M. Nolan, Donald H. Burke, Norman R. Pace*

Regions of *Escherichia coli* ribonuclease P (RNase P) RNA in proximity to a bound transfer RNA (tRNA) substrate were mapped by photoaffinity. A photoaffinity cross-linking reagent was introduced at specific sites in the interior of the native tRNA structure by modification of the 5′ ends of circularly permuted tRNAs (cptRNAs). The polymerase chain reaction was used for the production of cptRNA templates. After the amplification of a segment of a tandemly duplicated tRNA gene, the cptRNA gene was transcribed in vitro to produce cptRNA. Modified cptRNAs were cross-linked to RNase P RNA, and the conjugation sites in RNase P RNA were determined by primer extension. These sites occur in phylogenetically conserved structures and sequences and identify regions of the ribozyme that form part of the tRNA binding site. The use of circularly permuted molecules to position specific modifications is applicable to the study of many inter- and intramolecular interactions.

The ribonucleoprotein RNase P cleaves 5′ precursor sequences from precursor tRNAs (*1*). Bacterial RNase P RNA is catalytically active in vitro at high ionic strength in the absence of the protein component; it is a ribozyme (*2*). The model for the secondary structure of RNase P RNA is increasingly well refined, and the tertiary structure of the tRNA substrate is known (*3, 4*). To understand the structure of the catalytic complex, one must identify regions of contact between RNase P RNA and tRNA. Previously, nucleotides at or near the active site of RNase P RNA were identified with the use of a photoaffinity cross-linking reagent specifically attached at the 5′ end of mature tRNA, the site at which RNase P acts (*5*). The ability to introduce such an agent into other regions of tRNA would allow probing their interactions with the ribozyme.

Although the 5′ and 3′ termini of RNA molecules can be modified because of their unique chemical structures, specific modifications of internal sites in RNA are not easily achieved. Some internal modifications can be introduced by direct chemical RNA synthesis, but the efficiency of that process restricts its use to short molecules (*6*). A combination of chemical and enzymatic synthesis has been used to construct larger modified RNAs (*7*), but this approach is best suited for the synthesis of small amounts of material. We overcame these limitations by employing circularly permuted tRNA molecules. Circularly permuted tRNAs (cptRNAs) are tRNA structural analogs with native 5′ and 3′ ends linked by a synthetic loop and new termini in the interior of the native sequence. The choice of a particular nucleotide as the terminus of a cptRNA allows its specific modification by chemistry applicable to 5′ or 3′ ends. In general, cptRNAs can fold into biochemically active conformations (*8*).

We coupled photoagents to cptRNAs at 5′ termini that corresponded to native nucleotides 53 ($cptRNA_{53}$) or 64 ($cptRNA_{64}$) (*9*) because these nucleotides occur on the face of the tRNA molecule with which RNase P is thought to interact (*10*) (Fig. 1). To produce a template for transcription of a cptRNA, we amplified a selected portion of a tandemly duplicated gene encoding $tRNA^{Asp}$ (*11*) by polymerase chain reaction (PCR) (*12*) with primers that determined the 5′ and 3′ ends. The forward primer was a T7 RNA polymerase promoter plus the tRNA sequence selected to be the 5′ end of the cptRNA; the reverse primer determined the corresponding 3′ end. The PCR product was a cptRNA gene with a T7 promoter that could be transcribed to produce cptRNA. Transcription of PCR products in the presence of guanosine 5′-monophosphorothioate (GMPS) produced a cptRNA with a 5′-thiophosphate. This GMPS is incorporated as the first nucleotide of a transcript by T7 RNA polymerase but cannot be incorporated elsewhere in the transcript because the polymerase requires nucleoside triphosphates for chain elongation (*13*). The 5′-thiol can be specifically modified by conjugation with a photoaffinity agent for cross-linking to RNase P RNA.

We analyzed the kinetics of cleavage of cptRNAs by RNase P RNA to evaluate their suitabilities as model substrates. The ribozyme cleaves cptRNAs within the synthetic loop that connects the native 5′ and 3′ ends, so the effects of the presence of the loop and its size were tested. The cptRNAs bound well to RNase P RNA (Table 1); the Michaelis constant (K_m) of RNase P for each of the cptRNAs was no greater than threefold higher than that of the normal precursor. The catalytic rate constant (k_{cat}) for cleavage of each of the cptRNAs was five- to tenfold lower than the k_{cat} for the normal precursor. This was because the presence of the synthetic loop introduced nucleotides 3′ to the normally terminal CCA sequence. A native tRNA with nucleotides 3′ to the CCA and bipartite tRNAs with extra 3′ sequences also had a lower k_{cat} value (Table 1) (*14*). Bipartite tRNAs are formed by annealing appropriate in vitro transcripts of portions of tRNA genes (*15*). They have the same interior nick as cptRNAs but also have free termini, like normal precursor tRNAs (pre-tRNAs). The similarities of RNase P kinetics with bipartite and native tRNAs having the same CCA termini (Table 1) suggest that neither the presence of a nick in the interior of the tRNA structure nor the presence of a loop affects the binding of cptRNAs by the ribozyme. Because the differences in binding between the cptRNAs and normal pre-tRNAs to RNase P are relatively small, cptRNAs are suitable models for further study of the native reaction.

The 5′-thiolated cptRNAs produced by T7 transcription of PCR products in the presence of GMPS were coupled with azidophenacyl bromide (*5*). The ^{32}P-labeled azido-cptRNAs were incubated with RNase P RNA and irradiated at 300 nm of ultra-

J. M. Nolan and N. R. Pace, Department of Biology, Indiana University, Bloomington, IN 47405.
D. H. Burke, Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, CO 80309.

*To whom correspondence should be addressed.

violet light to induce cross-links, and the reaction products were resolved on a 4% polyacrylamide denaturing gel and autoradiographed (*15*). RNase P RNA–tRNA cross-links were evident as slowly migrating species for all three cptRNAs (Fig. 2A) and were identified as cross-linked conjugates by their dependence on the presence of RNase P RNA (Fig. 2A), azido-tRNA, and ultraviolet irradiation (*16*). The cross-links are >95% reversible by treatment with phenylmercuric acetate, which cleaves the thiophosphate linkage (*16*). The cross-link bands detected for $cptRNA_{53}$ and $cptRNA_{53Bst}$ were similar, regardless of loop size, and appeared as two or three major homogeneous species. The bands that corresponded to conjugates between

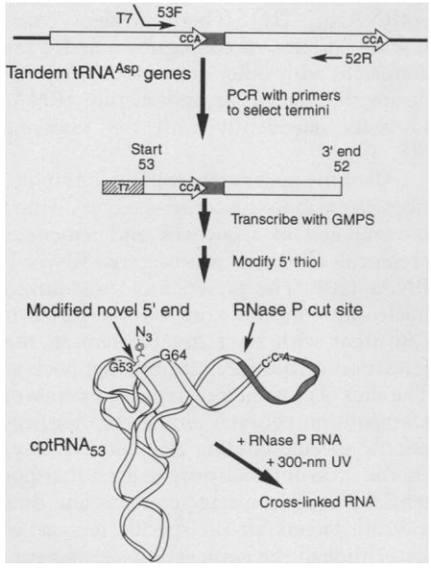

Fig. 1. Strategy for construction of cptRNAs. A 34-nucleotide forward primer, which consisted of a 17-nucleotide T7 promoter followed by *B. subtilis* $tRNA^{Asp}$ nucleotides 53 to 69, and a reverse primer of sequences complementary to nucleotides 52 to 36 were used to prime PCR amplification of a portion of a tandem repeat of $tRNA^{Asp}$. The product can be directly transcribed by T7 RNA polymerase in the presence of GMPS to produce 5′-thio-cptRNA. This 5′-thio-cptRNA was treated with azidophenacyl bromide to produce the 5′-azido-cptRNA shown. The size of the azido side chain is drawn to scale. Upon ultraviolet irradiation, the azido-tRNA cross-links to RNase P RNA and the cross-link sites were mapped by primer extension. The hatched box indicates T7 promoter sequences. Shaded regions indicate the synthetic linker sequence between the tRNA repeats in the tandem duplication and the cptRNA gene and in the cptRNA transcript. The 16-nucleotide synthetic loop of $cptRNA_{53}$ and $cptRNA_{64}$ is GCCAGGAUCCCAAAAU. For $cptRNA_{53Bst}$, the loop is GCCACCAAAAU. The first four nucleotides in these sequences correspond to the normal 3′ end of tRNA; RNase P cleaves 3′ to the last U of these loop sequences. The site of the 5′ end of $cptRNA_{64}$, G64, is also indicated on the tRNA backbone.

Table 1. Kinetic analysis of RNase P substrates (*15*). We incubated 10 to 100 nM tRNA with 1 nM *E. coli* RNase P RNA in P assay buffer [1 M NH_4Cl, 25 mM $MgCl_2$, 50 mM Hepes (pH 8.0), and 0.1% SDS] at 37°C for 10 to 75 min. The products were resolved by electrophoresis and quantitated as described (*18*). Bipartite tRNA, biptRNA. N_5 denotes GGAUC; nt, nucleotides.

tRNA	Structure	K_m (nM)	k_{cat} (min^{-1})
Pre-$tRNA^{Asp}$	Native	25	0.22
Pre-$tRNA^{Asp}N_5$	+ 5 nt at CCA end	14	0.086
$cptRNA_{64}$	Novel ends, 16-nt loop	68	0.041
$cptRNA_{53}$	Novel ends, 16-nt loop	44	0.047
$cptRNA_{53Bst}$	Novel ends, 11-nt loop	30	0.025
$biptRNA_{53}$	Novel ends, normal CCA	88	0.17
$biptRNA_{53}N_5$	Novel ends + 5 nt at CCA end	53	0.050

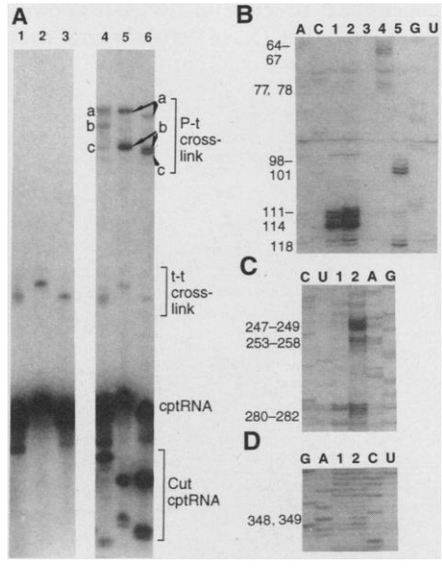

Fig. 2. Analysis of cptRNA cross-links. (**A**) Uniformly labeled with ^{32}P, 40 nM cptRNA was incubated in the presence (lanes 4 to 6) or absence (lanes 1 to 3) of 400 nM *E. coli* RNase P RNA in P assay buffer (Table 1; here, the $MgCl_2$ concentration was 100 mM) for 2 min at 37°C in 10 μl then irradiated with 300-nm ultraviolet light (*5*) for 15 min at room temperature. Samples were resolved on a 4% polyacrylamide denaturing gel and autoradiographed. Lanes 1 and 4, ^{32}P-labeled $cptRNA_{64}$; lanes 2 and 5, $cptRNA_{53}$; lanes 3 and 6, $cptRNA_{53Bst}$. Abbreviations: RNase P RNA–tRNA cross-links, P-t cross-links; tRNA-tRNA cross-links, t-t cross-links; and digested cptRNA products, cut cptRNA. The main cross-link bands a, b, and c are also indicated for each lane in which they are present. In (B) through (D), preparative cross-link reactions were performed in 100 μl of reaction buffer as in (A), except that the tRNA concentration was 375 to 500 nM. Cross-linked species were gel-purified, annealed to primer, and extended with AMV reverse transcriptase (Seikagaiku America, St. Petersburg, Florida) as described (*5*) with the pBluescript KS primer (Stratagene) or a primer complementary to nucleotides 196 to 174 of the *E. coli* RNase P RNA sequence (Fig. 3). Marker lanes are similar reactions performed on unmodified RNase P RNA in the presence of dideoxynucleotides; these lanes are designated with the sense-strand nucleotides that they determine. As controls for nonspecific reverse transcriptase terminations, control lanes are primer extensions on RNase P RNA that was irradiated but did not cross-link. Conjugates 53a, 53b, and 53c were purified from $cptRNA_{53Bst}$ cross-linking reactions; identical sites were mapped for $cptRNA_{53}$ conjugates (*16*). Nucleotide numbers indicate the position of the inferred cross-link; the nucleotide numbers of the actual termination products are one nucleotide 3′ to the position indicated. (**B**) Lane 1, cross-link 53c; lane 2, 53a; lane 3, control; lane 4, 64c; and lane 5, 64b. (**C**) Lane 1, control; and lane 2, 64a. (**D**) Lane 1, control; and lane 2, 53b.

Table 2. Efficiencies of cross-linking of cptRNAs to RNase P RNA. Samples were processed as in Fig. 2A and quantitated by Phosphorimager (Molecular Dynamics, Sunnyvale, California) analysis of the dried gel. Values shown are the percentage of total input of uniformly labeled cptRNA cross-linked to RNase P RNA from the gel in Fig. 2A. Total cross-link values include the entire region of the gel in which cross-links were detected and thus is greater than the sum of the individual species listed for each sample. The average percent cross-linked species for the three experiments from the total tRNA species ($cptRNA_{64}$, $cptRNA_{53}$, and $cptRNA_{53Bst}$, respectively) are 3.7, 7.1, and 4.3%, respectively; averages are not indicated for individual bands because $cptRNA_{53Bst}$ conjugates b and c did not resolve sufficiently in all experiments. The main cross-link sites in RNase P RNA determined for each band in Fig. 2, B through D, are indicated.

tRNA	Cross-linked species	Percent cross-linked	Main cross-link sites (nt)
$cptRNA_{64}$	Total	5.0	
	a	1.5	247–249
	b	1.2	98–101, 118
	c	0.8	64–67, 77, 78
$cptRNA_{53}$	Total	9.6	
	a	2.2	111–114
	b	5.6	348, 349
$cptRNA_{53Bst}$	Total	5.0	
	a	0.6	111–114
	b	1.9	348, 349
	c	1.6	111–114

cptRNA$_{64}$ and RNase P RNA were more diffuse, which suggests that there is more heterogeneity in cross-linked species. The cross-linking efficiencies for the bands designated in Fig. 2A are summarized in Table 2; the total cross-linked species for the experiment shown ranged from 5 to 9.6% of input tRNA. The high efficiencies of conjugation suggest that the cross-links reflect specific structural associations of cptRNAs with RNase P RNA.

The sites of cross-linking in gel-purified conjugates were analyzed by primer extension. Primers labeled with ^{32}P and complementary to RNase P RNA were annealed to the conjugates and extended with reverse transcriptase, which terminates DNA synthesis at the nucleotide preceding (that is, 3′ to) modified nucleotides (*17*), in this case the cross-link site. The primers allowed examination of the entire native RNase P RNA sequence (Fig. 2, B to D). The main sites of the cross-links are listed (Table 2) and indicated on the RNase P RNA secondary structure (Fig. 3). Additional, minor cross-link sites are not indicated—for example, those at nucleotides 253 to 258 or 280 to 282 (Fig. 2C; compare to main cross-links at nucleotides 247 to 249 in lane 2). The tRNA species with the 11-nucleotide synthetic loop, cptRNA$_{53Bst}$, yields a conjugate (Fig. 2A, lane 6, band c) not seen with cptRNA$_{53}$, which contains a 16-nucleotide loop. However, the sites of cross-linking in band c proved to be the same as for the conjugate represented by band a in Fig. 2A (compare Fig. 2B, lanes 1 and 2). Gel analysis of labeled conjugates after treatment with phenylmercuric acetate to break the cross-link showed that band c contains the enzymatically cleaved substrate, whereas the conjugate represented by the more slowly migrating band a contains uncleaved cptRNA$_{53Bst}$ (*16*). The equivalent cross-linking patterns of conjugates a and c are consistent with other observations that indicate that precursor and mature tRNAs interact equivalently with the ribozyme (*18, 19*).

All of the major cross-linking sites in the ribozyme map to the conserved core, which is composed of sequences and structures present in all the known bacterial RNase P RNAs (*20*). The presence of cross-linked nucleotides in the core of the RNA is consistent with their involvement in the formation of the substrate binding pocket. The sites of cross-linking represent points of juxtaposition between the RNAs, not necessarily specific binding elements. However, the cross-links identify regions that potentially contain specific contacts and thus indicate targets for site-specific mutagenesis. Although the conjugation sites for particular cptRNAs are separated in the model of the secondary structure of the ribozyme (Fig. 3), in the tertiary structure they must be fixed in the vicinity of the photoagent, which is 9 Å in length. Because the three-dimensional structure of tRNA is known, these and other (*5, 14*) cross-linking data spatially orient the cross-linked regions within RNase P RNA. Because RNase P is not known to interact with its substrate by base-pairing interactions (*10*), it is difficult to predict local interactions between tRNA and structures of RNase P RNA that cross-link to it. Thus, only when a coherent three-dimensional structure of RNase P RNA is available can a more detailed mechanism for this interaction be proposed. The use of photoagents with different geometries and at different positions in cptRNAs will refine our understanding of this structure.

Amplification by PCR of segments of tandemly duplicated genes is a convenient way to produce cptRNAs. Although the structures of different circularly permuted genes are determined by the choice of PCR primers, there are constraints that can limit the applicability of this strategy. One constraint is that the native 5′ and 3′ ends of the RNA need to be adjacent to each other to minimize the possible effects of the synthetic loop on the global structure. A constraint on the efficient production of cptRNAs by T7 RNA polymerase is the preference of that enzyme for a G nucleotide as the site of transcription initiation (*21*). This limitation, however, can be circumvented by the use of di- or trinucleotide primers to initiate T7 transcription (*13*) or by the use of sequence variants of homologous genes from different organisms with a G nucleotide at the transcription start site. PCR of tandemly duplicated sequences could also be used for the production of circularly permuted DNAs to study DNA bending. The strategy could be adapted to the expression of circularly permuted proteins for the study of protein folding (*22*) or to introduce specific modifications at the new termini, as done here with RNA.

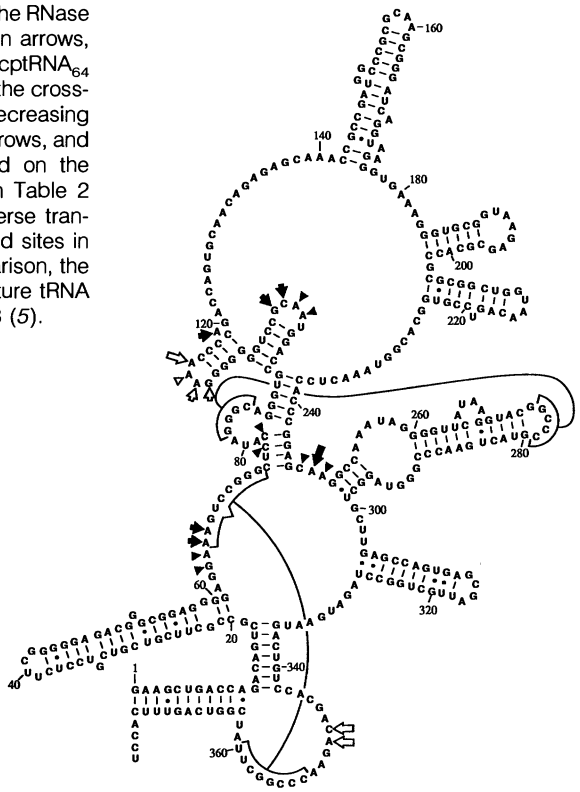

Fig. 3. Location of cross-link sites on the RNase P RNA secondary structure (*4*). Open arrows, cptRNA$_{53}$ cross-links; closed arrows, cptRNA$_{64}$ cross-links. The relative intensities of the cross-links detected are represented, in decreasing order, by the long arrows, the short arrows, and the arrowheads. Hierarchy is based on the percentage cross-linked values from Table 2 and the estimated proportion of reverse transcriptase terminations at the indicated sites in primer extension analysis. For comparison, the main cross-link sites for 5′-azido mature tRNA were nucleotides 248 and 330 to 333 (*5*).

REFERENCES AND NOTES

1. S. Altman, *Adv. Enzymol. Relat. Areas Mol. Biol.* **62**, 1 (1989); N. R. Pace and D. Smith, *J. Biol. Chem.* **265**, 3587 (1990).
2. C. Guerrier-Takada *et al.*, *Cell* **35**, 849 (1983).
3. G. J. Quigley and A. Rich, *Science* **194**, 796 (1976).
4. J. W. Brown, E. S. Haas, N. R. Pace, *Nucleic Acids Res.* **21**, 671 (1993).
5. A. B. Burgin and N. R. Pace, *EMBO J.* **9**, 4111 (1990).
6. N. Usman and R. Cedergren, *Trends Biochem. Sci.* **17**, 334 (1992).
7. M. J. Moore and P. A. Sharp, *Science* **256**, 992 (1992).
8. T. Pan *et al.*, *ibid.* **254**, 1361 (1991).
9. D. H. Gauss, F. Gruter, M. Sprinzl, in *Transfer RNA: Structure, Properties, and Recognition*, P. R. Schimmel, D. Söll, J. N. Abelson, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1979), pp. 518–519.
10. C. J. Green and B. S. Vold, *J. Biol. Chem.* **263**, 652 (1988); D. Kahle, U. Wehmeyer, G. Krupp, *EMBO J.* **9**, 1929 (1990); W. H. McClain, C. Guerrier-Takada, S. Altman, *Science* **238**, 527 (1987); D. L. Thurlow, D. Shilowski, T. L. Marsh, *Nucleic Acids Res.* **19**, 885 (1991).

11. DNA manipulations and gel electrophoresis were performed by standard methods (*23*). The *Bacillus subtilis* tRNA^{Asp} gene was excised from pT7-1 pre-tRNA^{Asp} (*25*) and cloned into pUC19 to yield pUC153. Subsequently, pT7-1 pre-tRNA^{Asp} was digested with either Bst NI or Bam HI, its ends were filled in by treatment with the Klenow fragment of DNA polymerase, and it was digested with Eco RI and gel-purified. These fragments were ligated to pUC153 DNA that had been digested with Kpn I, treated with Klenow as above, digested with Eco RI, and gel-purified. The DNA fragments from pT7-1 pre-tRNA^{Asp} were ligated to pUC153 and used to transform a *rec*$^-$ *E. coli* host (SURE, Stratagene, La Jolla, CA); the resulting tandem duplications were named p153Bamtan and p153Bsttan depending on the enzyme used in the construction. The constructions were confirmed by DNA sequencing. The p153Bamtan duplication was used as a template for amplification of the genes for cptRNA$_{53}$ and cptRNA$_{64}$; the p153Bsttan duplication was used for amplification of cptRNA$_{53Bst}$. Amplifications were performed in 50 to 200 μl of standard buffer (*12*) that contained 6 mM $MgCl_2$ and DNA template (approximately 10 ng/ml); the amplification program was 30 cycles at 92°C for 1.5 min, 50°C for 2 min, and 72°C for 1 min. Primer pairs used for cptRNA$_{53}$ were forward (53F) (5′-TAATACGACTCACTATAGTTCGAGTCCCGTCC-3′) and reverse (52R) (5′-CCGCGACCTCCTGCGTG-3′); for cptRNA$_{64}$ the primers were forward (64F) (5′-TAATACGACTCACTATAGTCCGGACCGCCAGGAT-3′) and reverse (63R) (5′-GGGACTCGAACCCGCGA-3′). The PCR products were purified by extraction with phenol and ethanol precipitation. Product ends were made blunt with Klenow, phenol extracted, and purified by Sephadex G-50 (Pharmacia, Piscataway, NJ) spin column chromatography. The eluate was precipitated and resuspended in TE [10 mM tris-HCl (pH 8.0) and 1 mM EDTA] at one-tenth of the original PCR reaction volume.
12. R. K. Saiki *et al.*, *Science* **239**, 487 (1988).
13. J. F. Milligan and O. C. Uhlenbeck, *Methods Enzymol.* **164**, 51 (1989).
14. C. Guerrier-Takada, N. Lumelsky, S. Altman, *Science* **246**, 1578 (1989).
15. *Escherichia coli* RNase P RNA was transcribed from a derivative of pDW27 (*25*) and purified by gel electrophoresis as described (*5, 13*). Native tRNA was transcribed from pT7-1 pre-tRNA^{Asp} that had been cleaved with either Bst NI or Bam HI. Transcriptions were performed as above but with 4 mM guanosine monophosphate (GMP), 0.2 mM guanosine triphosphate (GTP), [α-^{32}P]GTP (0.3 mCi/ml), plus the other nucleoside triphosphates (1 mM each). PCR products were transcribed as for tRNA, except that polyethylene glycol 8000 (Sigma) was added to a final concentration of 8%. One microliter (~0.1 μg) of PCR template was added per 20 μl of reaction buffer. Transcripts were purified as above. The CCA half of the bipartite tRNAs was transcribed from cptDNA$_{53Bam}$ that had been digested with either Bam HI or Bst NI. The template for the precursor half of the bipartite tRNAs was amplified from a derivative of pT7-1 pre-tRNA^{Asp} that has a pre-tRNA^{Asp} gene under control of a T7 promoter but lacks most of the polylinker sequences present in pT7-1 pre-tRNA^{Asp}. The precursor half of this tRNA gene was amplified with the use of 52R (above) as the reverse primer and an M13 universal primer (5′-CCCAGTCACGACGTTGT-3′) as the forward primer. Amplifications were performed as described for cptRNA genes (*11*) with a 45°C annealing temperature. Both halves of the bipartite tRNAs were transcribed and purified as for cptRNAs. For analytical cross-linking, GMPS-primed transcripts were prepared as above, with GMPS substituted for GMP and modified with azidophenacyl bromide (Fluka, Ronkonkoma, NY) as described (*5*). The RNase P RNA used for preparative cross-links was pEcKS RNA, which is *E. coli* RNase P with an additional 26 nucleotides at the 3′ end, complementary to the pBluescript KS primer (Stratagene). The pEcKS plasmid is an Nco I–Sna BI fragment containing the T7 promoter and *E. coli* RNase P gene from pDW27 (*25*) that was treated with Klenow and ligated to pBluescript KS+ that was digested with Cla I and Nae I and treated with Klenow. Then pEcKS was digested with Xho I and transcribed with T7 RNA polymerase to produce pEcKS RNA. Similar results were seen with normal-length RNase P RNA transcripts and a primer complementary to the 3′ terminal 14 nucleotides of RNase P RNA (*16*). Unlabeled GMPS transcripts were prepared and modified with azidophenacyl bromide as described (*5*). Kinetic parameters were determined by linear regression analysis with the Enzyme Kinetics computer program (dogStar Software, Bloomington, IN).
16. J. M. Nolan, unpublished data.
17. T. Inoue and T. R. Cech, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 648 (1985).
18. D. Smith, A. B. Burgin, E. S. Haas, N. R. Pace, *J. Biol. Chem.* **267**, 2429 (1992).
19. C. Reich *et al.*, *Science* **239**, 178 (1988).
20. J. W. Brown and N. R. Pace, *Nucleic Acids Res.* **20**, 1451 (1992).
21. J. J. Dunn and F. W. Studier, *J. Mol. Biol.* **166**, 477 (1983).
22. A. Buchwalder, H. Szadkowski, K. Kirschner, *Biochemistry* **31**, 1621 (1992).
23. F. M. Ausubel *et al.*, *Current Protocols in Molecular Biology* (Wiley, New York, 1989), vols. 1 and 2.
24. C. I. Reich, thesis, Universidad de Buenos Aires (1988).
25. D. S. Waugh, C. J. Green, N. R. Pace, *Science* **244**, 1569 (1989).
26. We thank members of the Pace lab for critical review of this manuscript and for many helpful discussions. Dedicated to the memory of T. F. Nolan. Supported by Public Health Service grant GM34527 from the National Institute of General Medical Sciences and Department of Energy grant FG02-92ER2088.

17 February 1993; accepted 27 May 1993

Primers for Mitochondrial DNA Replication Generated by Endonuclease G

Jacques Côté and Adolf Ruiz-Carrillo*

Endonuclease G (Endo G) is widely distributed among animals and cleaves DNA at double-stranded $(dG)_n \cdot (dC)_n$ and at single-stranded $(dC)_n$ tracts. Endo G is synthesized as a propeptide with an amino-terminal presequence that targets the nuclease to mitochondria. Endo G can also be detected in extranucleolar chromatin. In addition to deoxyribonuclease activities, Endo G also has ribonuclease (RNase) and RNase H activities and specifically cleaves mouse mitochondrial RNA and DNA-RNA substrates containing the origin of heavy-strand DNA replication (O_H). The cleavage sites match those found in vivo, indicating that Endo G is capable of generating the RNA primers required by DNA polymerase γ to initiate replication of mitochondrial DNA.

Endo G is a low-abundance endonuclease of vertebrates (*1*) that has been purified from calf thymus as a dimer of two identical subunits of 26 to 28 kD (*2*). The term Endo G was originally coined (*1*) to reflect the preference of the partially purified nuclease for the G strand of $(dG)_n \cdot (dC)_n$ DNA tracts. However, the highly purified nuclease nicks either strand of the homopolymer with similar kinetics (*2*), producing 5′-phosphoryl and 3′-hydroxyl ends (*1*). Although the occurrence of $(dG)_n \cdot (dC)_n$ tracts next to regions of deletions, translocations, and DNA hypervariability suggested a causal relation (*1*), the function of Endo G remained undetermined.

To clone the Endo G cDNA, the NH_2-terminal sequence of the purified nuclease from calf thymus and liver (*3*) was determined (Fig. 1) (*4, 5*). A short Endo G cDNA probe was obtained by polymerase chain reaction (PCR) of total calf liver cDNA with degenerate oligonucleotide primers derived from the NH_2-terminal (AGLPAVP) and COOH-terminal (ELAKYGL) Endo G sequence (*6*) (Fig. 1). This DNA probe hybridized with RNA of ~1000 nucleotides in length in blots of polyadenylated RNA from bovine and other mammalian species, including human (HeLa cells).

An end-labeled 53-nucleotide fragment of the Endo G cDNA hybridized with 24 clones in a partial calf liver cDNA expression library (*7*). All 24 clones contained essentially the same sequence, and none was in the proper reading frame to produce a β-galactosidase fusion protein, suggesting that expression of Endo G was deleterious in *Escherichia coli*. From the initial cDNA enrichment and the frequency of positive clones, we estimate that Endo G mRNA represents ≈1/60,000 of all bovine liver mRNA. This low concentration is in accord with the low abundance of the protein (*2*).

The sequence (*8*) of the longest cDNA clone (pbEG4) contained an open reading frame (ORF) predicting a protein of molecular weight 32,261 (Fig. 1), larger than the Endo G monomer (*2*). The NH_2-terminal sequence of the purified Endo G protein did not correspond to that predicted by the cDNA but rather coincided with amino acids 49 to 75 (Fig. 1). Hybridization of

Cancer Research Center and Department of Biochemistry, Medical School of Laval University, L'Hôtel-Dieu de Québec, Québec, Canada G1R 2J6.

*To whom correspondence should be addressed.