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A novel endoribonuclease cleaves at a priming site of mouse mitochondrial DNA replication

David D.Chang and David A.Clayton

Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305, USA

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Priming at the mouse mitochondrial origin of heavy-strand DNA replication is effected by transcripts from the light-strand promoter. The transition from RNA synthesis to DNA synthesis occurs at specific locations between 75 and 165 nucleotides downstream from the transcriptional initiation site. We have identified and partially purified an endonucleolytic activity that cleaves RNA accurately near one of these transition sites; this finding implies a role of specific RNA processing in DNA replication. Cleavage products possess 5′-phosphoryl and 3′-hydroxyl termini. Heterologous assays using mouse or human mitochondrial endoribonuclease with substrates containing the sequences of the human or mouse mitochondrial origins of heavy-strand DNA replication suggest that selection of the cleavage site is guided by sequences adjacent to the actual position of cleavage.

Key words: endoribonuclease/mitochondria/replication initiation/replication priming/RNA processing

Introduction

Mammalian mitochondrial DNA (mtDNA) replicates in a continuous fashion from two spatially and temporally distinct origins (Clayton, 1982). A round of replication begins with unidirectional synthesis of a daughter heavy strand (H strand) at the origin of H-strand replication (O_H). Synthesis of a daughter light strand (L strand) does not occur until the newly synthesized H strand displaces the parental H strand at the origin of L-strand synthesis (O_L), located two-thirds of the genomic distance away. The exposed single-stranded region of O_L then initiates synthesis of an L strand in the opposite direction. Subsequent completion of DNA synthesis and resolution of two daughter molecules mark the full cycle of replication.

Although both O_H and O_L promote unidirectional DNA synthesis, the structural features and modes of RNA priming at the two origins are quite different. O_L is characterized by a distinctive stem and loop structure of ~30 nucleotides and is functionally active only in a single-stranded template form (Martens and Clayton, 1979; Tapper and Clayton, 1982; Clayton, 1982). Recent *in vitro* studies have shown that RNA priming begins at the loop region of this dyadic structure and the transition from RNA synthesis to DNA synthesis occurs near the base of the stem (Wong and Clayton, 1985). DNA synthesis at O_H presents a different situation since at this origin replication must occur in a closed circular duplex DNA. A hallmark of O_H is the existence of three evolutionally conserved sequence blocks (CSBs I, II and III) at or near the 5′ end map positions of nascent H-strand DNAs (Gillum and Clayton, 1979; Walberg and Clayton, 1981; Chang *et al.*, 1985). Analyses of *in vivo* nucleic acids around O_H have indicated that for almost all 5′ termini of nascent H-strand DNAs, there exist RNA species with abutting 3′ termini (Chang and Clayton, 1985; Chang *et al.*, 1985). These RNA species all have 5′ ends mapping at the initiation sites of the L-strand promoter (LSP) (Chang and Clayton, 1986). Based on these findings we previously hypothesized that transcripts from the LSP have a direct role in H-strand DNA replication by serving as primer RNAs for DNA synthesis (Chang *et al.*, 1985). Furthermore, since most *in vivo* transcripts from the LSP extend beyond O_H, we speculated that specific 3′ ends of primer RNAs are generated by a post-transcriptional processing of primary transcripts from the LSP. Here we report the identification of an RNase activity from mouse mitochondria that can perform a specific endonucleolytic cleavage of L-strand transcripts at one of the transition sites located between CSB II and CSB III.

Results

Design of the in vitro cleavage assay

The transition from primer RNA synthesis to DNA synthesis occurs within a 90-nucleotide region overlapping the three CSBs

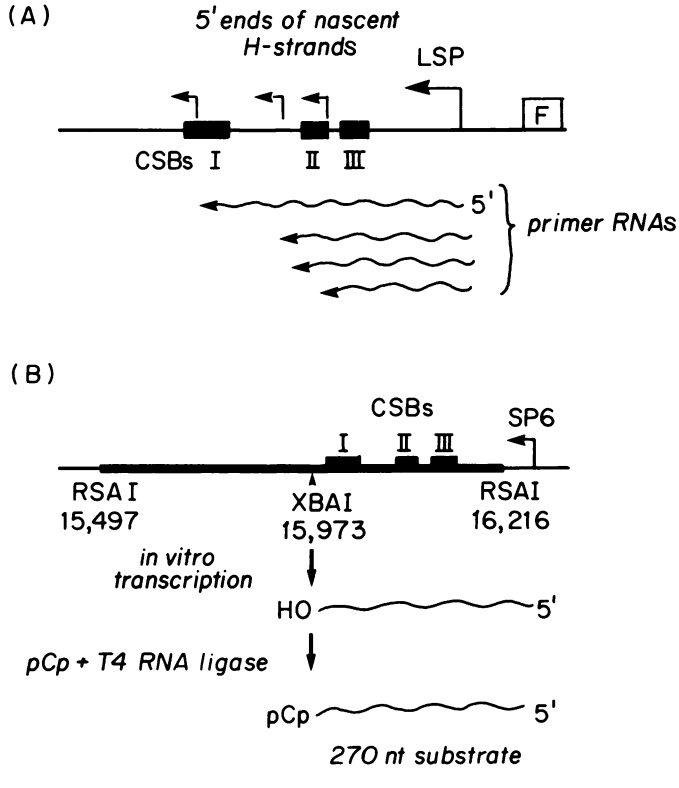

Fig. 1. O_H region of mouse mtDNA. **(A)** The structural organization of the O_H region, including the 5′ termini of nascent H-strand DNAs (three short, closed arrows) and the upstream transcriptional promoter (LSP), is shown. Also indicated are the positions of primer RNAs and the CSBs. F: the gene for $tRNA^{Phe}$. **(B)** Schematics of the 270-nucleotide substrate RNA synthesis. A physical map of pMR718B, used as the template for *in vitro* transcription by SP6 RNA polymerase, is shown. The locations of relevant restriction endonuclease cleavage sites and the SP6 promoter are also indicated.

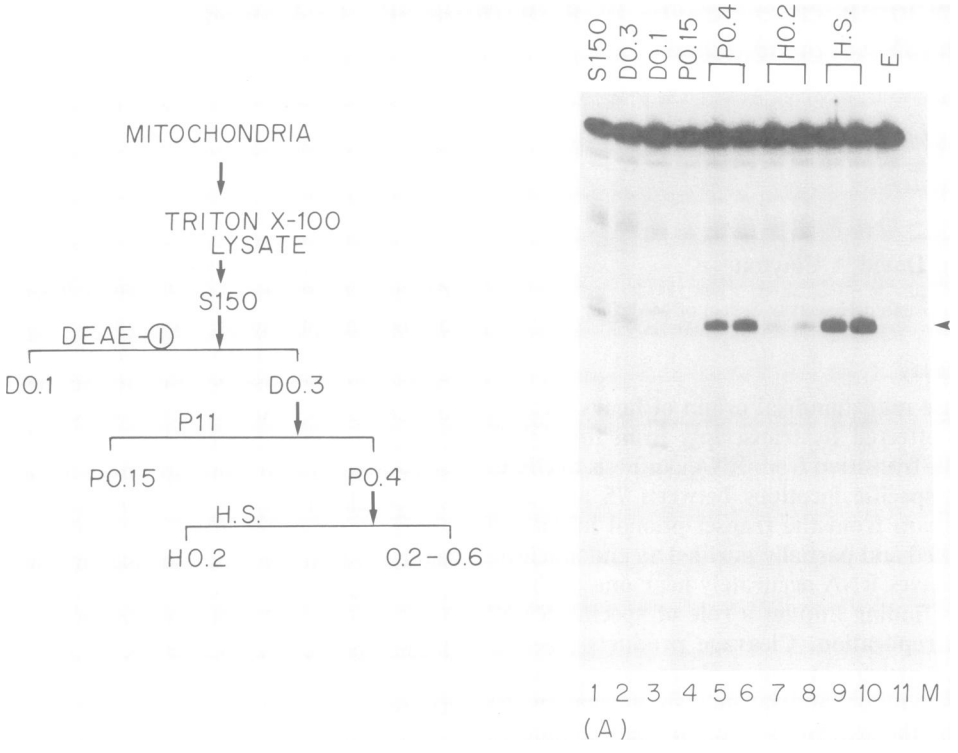

Fig. 2. Fractionation of mouse mitochondrial proteins. Mitochondrial proteins were fractionated as described under Materials and methods. The various chromatographic steps are schematically represented on the left side of the figure. H.S. denotes heparin–Sepharose. **(A)** *In vitro* processing of the 270-nucleotide pCp-labeled substrate RNA was assayed by using the indicated amounts of protein fractions. **Lane 1**, 8.5 μg of S150; **lane 2**, 1.3 μg of D0.3; **lane 3**, 2.3 μg of D0.1; **lane 4**, 0.4 μg of P0.15; **lanes 5** and **6**, 0.34 and 0.68 μg, respectively, of P0.4; **lanes 7** and **8**, 0.43 and 0.85 μg, respectively, of H0.2; **lanes 9** and **10**, 0.15 and 0.3 μg, respectively, of pooled heparin–Sepharose column fractions (see Figure 3); **lane 11**, no added mitochondrial proteins. *Hpa*II fragments of pBR322 are shown as size standards (**lane M**). Arrowhead: 130-nucleotide cleavage product.

(Figure 1A). Since primer RNAs without any deoxyribonucleotides could be identified (Chang *et al.*, 1985), it appeared that the generation of a primer RNA may not be obligatorily coupled to DNA synthesis. We therefore assayed for the formation of the 3′ ends of these RNAs by using, as a substrate, *in vitro* transcripts synthesized with SP6 RNA polymerase (Figure 1B). The template construct was pMR718B, which contains a 720-bp *Rsa*I fragment of mouse mtDNA cloned into the *Sma*I site of the pSP65 vector. Transcription of a *Xba*I-digested linear derivative of pMR718B by SP6 RNA polymerase generates 270-nucleotide transcripts that encompass all of the RNA to DNA transition sites at O_H. *In vitro* transcripts were subsequently labeled with [^{32}P]pCp by using T4 RNA ligase and the RNA of expected size was purified by gel electrophoresis. The use of end-labeled substrate was beneficial in that the position of processing sites relative to the 3′ end of the substrate (nucleotide 15973) could be determined directly from the size of the reaction products.

Detection and partial purification of a specific nuclease activity in mitochondrial lysates

A detergent–salt lysate of purified mitochondria from mouse LA9 cells was assayed for the presence of a specific nuclease activity capable of processing the pCp-labeled substrate described above. Following a 30-min incubation in a standard buffer containing Mg^{2+} and K^+, as well as human placental RNase inhibitor (RNasin) to reduce non-specific degradation of the substrate RNA, the reaction products were analyzed by gel electrophoresis (Figure 2). In the presence of a high-speed supernatant (S150) of the mitochondrial lysate, an RNA of 130 nucleotides, corresponding to a processing site around nucleotide 16103, was generated (Figure 2A, arrowhead). Because the substrate used was radiolabeled only at the 3′ end, it could not be determined from this experiment whether the 130-nucleotide RNA was generated by an exonucleolytic trimming from the 5′ end or by a specific endonucleolytic cleavage. However, when a 5′-end labeled substrate was used, an RNA of the different size expected for the 5′ half of an endonucleolytic cleavage event, was observed (data not shown). Furthermore, when an internally labeled substrate was used (see below, Figure 5), two products corresponding to the 5′ and 3′ halves of an endonucleolytic cleavage reaction were generated, thus demonstrating that the specific processing was due to a presence of an endonuclease in the mitochondrial lysate.

The cleavage reaction was not efficient and was dependent on the addition of RNasin to the reaction. Since non-specific nucleases and nucleic acids may interfere with the specific cleavage reaction, a preliminary attempt was made to remove these inhibitors by successively fractionating the S150 fraction on DEAE–Sephacel and phosphocellulose columns (Figure 2). The specific cleavage activity present in the S150 fraction was adsorbed on both columns at low salt and recovered at higher ionic strength. These simple chromatographic procedures increased the efficiency of the cleavage reaction and eliminated the requirement for RNasin in the reaction buffer by removing most of the non-specific nucleases present in the S150 fraction. The phosphocellulose fraction was subsequently chromatographed on a heparin–Sepharose column followed by a second DEAE–Sephacel column, each with a linear salt gradient for elution (Figure 3). The specific cleavage activity was eluted as a single peak from both the heparin–Sepharose and DEAE column. The

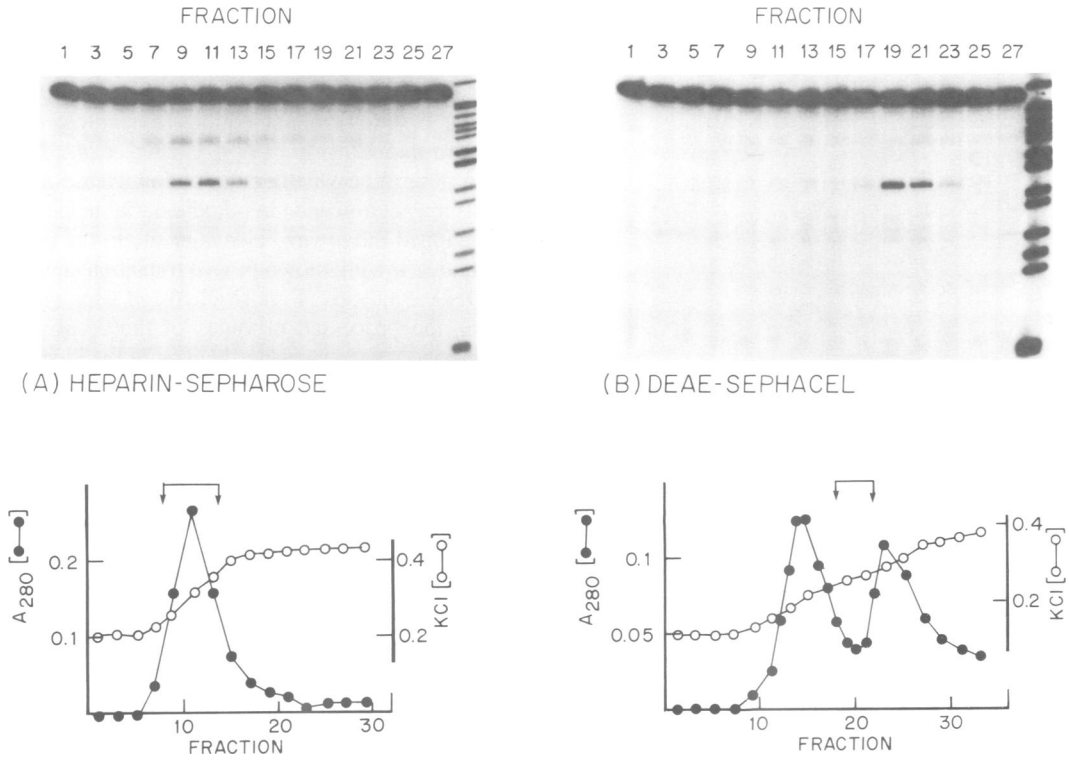

Fig. 3. Elution profiles of the processing activity from heparin–Sepharose and the second DEAE columns. The P0.4 protein fraction from the phosphocellulose column was further fractionated on a heparin–Sepharose column and then on a DEAE–Sephacel column as described under Materials and methods. Fractions from the heparin–Sepharose column **(A)** and DEAE–Sephacel column **(B)** were assayed for processing activity. Each assay contained 2 μl of column fraction in a 25-μl reaction volume. Protein profiles (A280; ●) and salt concentrations (KCl; ○) of column fractions are shown below. Fractions containing the processing activity are indicated by the horizontal bracket with arrowhead.

second DEAE fraction can be stored at −20°C for up to 1 year without a significant change in activity. Most of the subsequent experiments were carried out using this second DEAE fraction, which we refer to as Fraction V.

Requirements for the cleavage reaction

Various requirements for the cleavage reaction were investigated using Fraction V (Table I). The enzyme was active in the pH range 7.0–8.5 with an optimum of pH 8.0. The maximal cleavage reaction occurred between 37 and 42°C; above 50°C, the enzyme was rapidly inactivated. The enzymatic cleavage reaction had an absolute requirement for Mg^{2+} with an optimal concentration of 5–10 mM. This requirement for Mg^{2+} as the divalent cation could not be replaced by Mn^{2+} or Ca^{2+}. A 2-fold stimulation of the activity was observed when K^+ was added to 50 mM, but higher salt concentrations were inhibitory to the reaction.

Spermidine had a slightly stimulatory effect at a lower concentration, but inhibited the reaction by 60% at 5 mM. Single-stranded nucleic acids had a strong inhibitory effect on the cleavage reaction. At a concentration of 1 μg/ml, *Escherichia coli* tRNA reduced the reaction by 90%; single-stranded M13 DNA also inhibited the reaction, but required a 50-fold higher concentration than *E. coli* tRNA to achieve a 90% inhibition. Double-stranded plasmid DNA at concentrations up to 50 μg/ml had no significant effect on the cleavage reaction. The enzyme was also sensitive to *N*-ethylmaleimide, which inhibited the reaction by >90% at a concentration of 1 mM. DNA/RNA hybrids containing the CSB region are ineffective substrates for endonuclese cleavage *in vitro* (data not shown).

Table I. Requirements of the mitochondrial endoribonuclesae

Reaction conditions	Cleavage (%)
Complete system[a]	100
$-MgCl_2$	<1
$-MgCl_2$ + $MnCl_2$ (1–5 mM)	<1
$-MgCl_2$ + $CaCl_2$ (1–5 mM)	<1
−KCl	44
−KCl + 100 mM KCl	9
−KCl + 200 mM KCl	1.8
+Spermidine: 1 mM	121
2 mM	88
5 mM	40
+Nucleic acids: *E. coli* tRNA (1 μg/ml)	11.8
ssDNA[b] (10 μg/ml)	42
(50 μg/ml)	6.5
dsDNA[c] (10 μg/ml)	94
(50 μg/ml)	81.2
+*N*-ethylmaleimide: 1 mM	9.1
5 mM	2.8

[a]A complete system contains 20 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM DTT, 50 mM KCl, 15 U RNasin, 50 μg BSA/ml, and 0.1 μg of Fraction V in a 25-μl reaction volume.
[b]M13mp7-met: an M13mp7 derivative that contains a 210-bp *Eco*RI fragment (nucleotides 3803–4012) of mouse mtDNA.
[c]pBR322.

Precise mapping of the cleavage sites

The transition from primer RNA synthesis to DNA synthesis occurs *in vivo* at three distinct sites, all located within a 90-bp

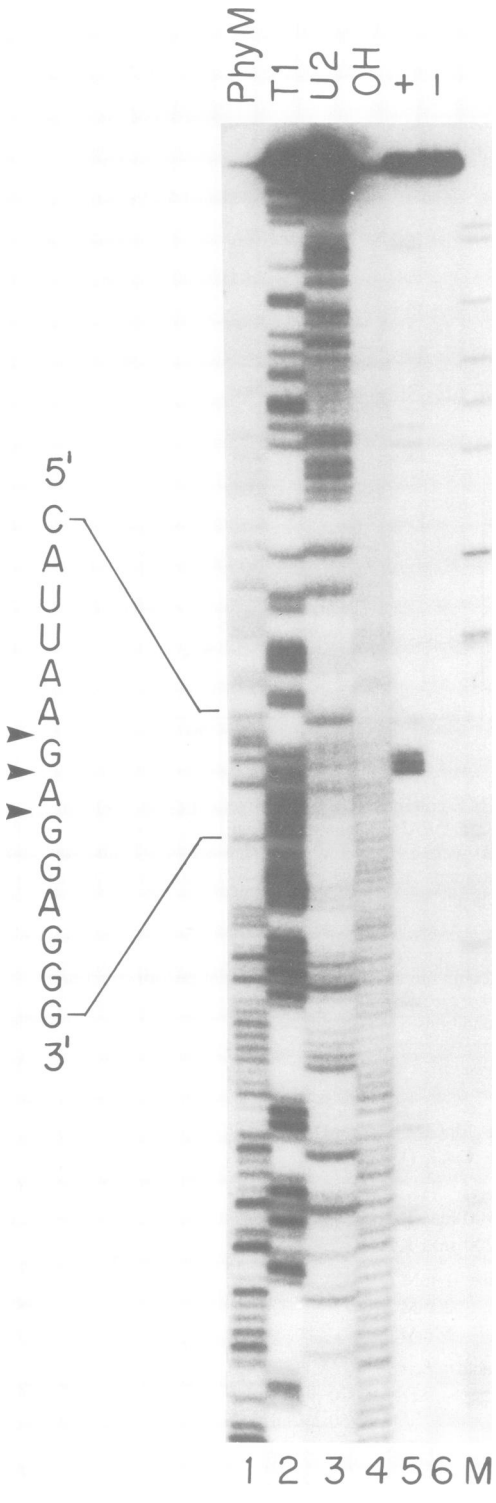

Fig. 4. Precise mapping of the endonucleolytic cleavage site. The products of an *in vitro* reaction were sized against the sequence ladders of the 270-nucleotide pCp-labeled substrate RNA to determine the nucleotide positions of endonucleolytic cleavage by the mitochondrial enzyme. The cleavage reaction was carried out by using, as a substrate, the 270-nucleotide pCp-labeled RNA in a 25-μl reaction volume containing 0.1 μg of Fraction V. The RNases used to generate sequence ladders are as follows: **lane 1**, PhyM (cleaves after A and U); **lane 2**, RNase T1 (cleaves after G); **lane 3**, RNase U2 (cleaves after A). **Lane 4**, partial alkaline hydrolysis ladder. **Lane 5**, products of the *in vitro* cleavage reaction. **Lane 6**, 270-nucleotide pCp-labeled RNA without any incubation. **Lane M**, *Hpa*II fragments of pBR322. The RNA sequence of the substrate around the nucleotide positions of cleavage sites (arrowheads) is shown next to the sequence ladders.

region. The majority of nascent H-strand DNA has 5′ ends mapping near nucleotide position 16030. The other two transition sites, which account for ~10% of the 5′ ends of nascent H strands, are located at positions 16059 and 16094; the latter group retains 5′ oligoribonucleotides extending to position 16104 (Gillum and Clayton, 1979; Chang *et al.*, 1985).

To determine whether the endonuclease activity in Fraction V has any significance in mitochondrial nucleic acid metabolism, we mapped the *in vitro* cleavage site to the nucleotide level and compared it with known *in vivo* transition sites of RNA synthesis to DNA synthesis. Precise cleavage sites were determined by sizing the processed products of the 3′ pCp-labeled substrate against enzymatic sequence ladders of the identical precursor substrate. The results shown in Figure 4 demonstrate that cleavage occurs at three adjacent nucleotides, 5′-GAG-3′, centered at position 16103. The three-nucleotide staggering of the cleavage sites appeared to be an intrinsic property of the endonuclease rather than the result of a contaminating exonuclease since an identical three nucleotide staggering at the abutting positions was also observed with a 5′-end labeled substrate (data not shown). Fraction V did not cleave substrate RNA at the other two transition sites, although specific cleavage around nucleotide 16059 was detected in phosphocellulose fractions eluted at higher ionic strength (unpublished observation).

The fate of the phosphate group at the cleavage site was determined by a nearest-neighbor analysis of the cleavage products. For this purpose the substrate RNA was internally labeled with [α -^{32}P]GTP. Following a standard *in vitro* reaction, the 5′ and 3′ halves of the cleaved products were individually isolated by gel electrophoresis and digested to completion with RNase T2 (Figure 5). Since RNase T2 hydrolyzes the phosphodiester bond between the 5′ ribose of an adjacent nucleotide, the end products of a complete RNase T2 digestion are nucleoside-3′-phosphate (Np) (Silberklang *et al.*, 1979). The nucleotide at the 5′ end of the RNA retains the 5′ phosphate, if present, and generates a nucleoside-3′,5′-diphosphate (pNp). Nucleotide composition analyses of the 3′ half of the cleavage products by two-dimensional t.l.c. showed the expected Np, as well as two additional spots corresponding to pGp and pAp. Thus the mitochondrial endonuclease cleaves RNA 5′ to phosphodiester bonds and generates products with 3′ hydroxyl and 5′ phosphoryl termini.

Selection of the cleavage site may be directed by the CSBs

A notable feature of the mammalian mitochondrial O_H is the presence of three evolutionally conserved sequence blocks (CSBs I, II and III) near the 5′ ends of nascent H-strand DNA (Walberg and Clayton, 1981; Chang and Clayton, 1985; Chang *et al.*, 1985). Because of this striking association between the CSB elements and the origin of DNA synthesis, we previously proposed that the CSBs may serve as control sequences involved in the transition from primer RNA synthesis to DNA synthesis. The *in vitro* cleavage site of this mitochondrial endonuclease is located between CSB II and CSB III. To investigate whether these adjacent CSB elements have a role in cleavage site selection, we assayed a heterologous substrate containing the O_H region of human mtDNA. Since the nucleotide sequences of the mouse O_H and human O_H are quite different, with the exception of the CSB elements (Anderson *et al.*, 1981; Bibb *et al.*, 1981), the overall effect of using a human RNA substrate is equivalent to introducing the three mouse CSB elements amongst foreign nucleotide sequences.

In the heterologous assay, a unique RNA of 190 nucleotides

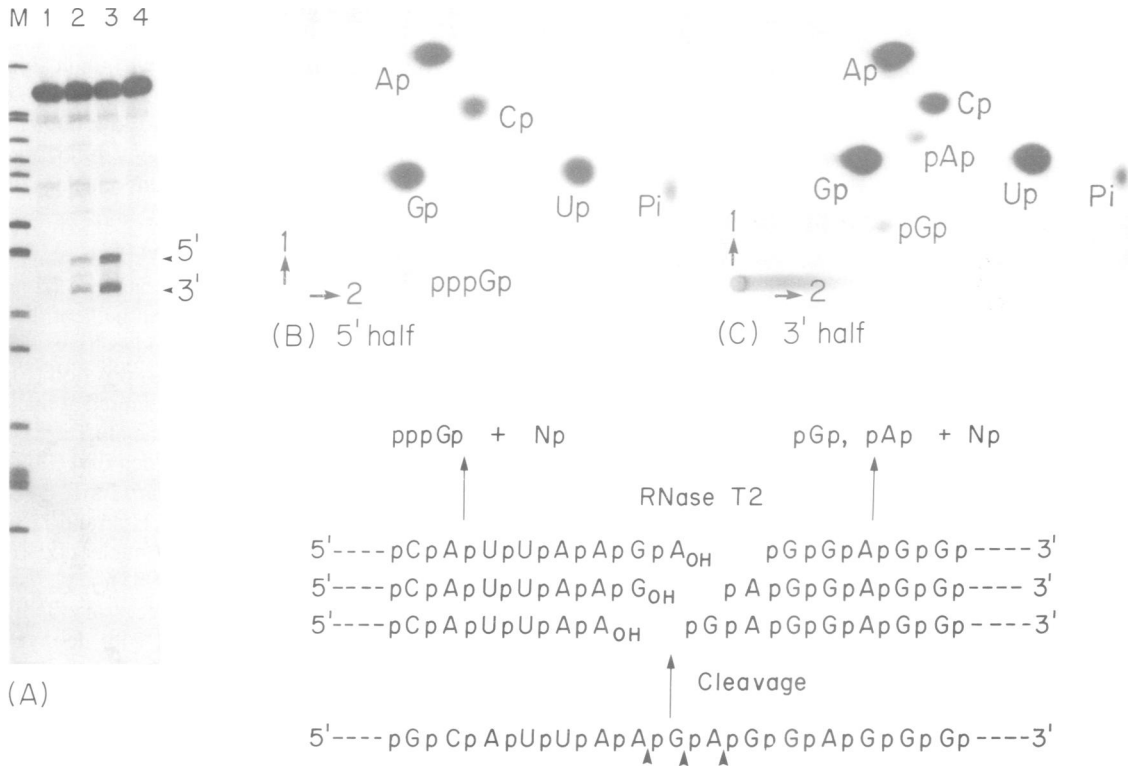

Fig. 5. Analysis of the phosphate groups at the cleavage site. **(A)** *In vitro* cleavage reactions were carried out by using the 270-nucleotide substrate RNA labeled internally with [α-^{32}P]GTP and 0.01, 0.04 and 0.28 μg of Fraction V (**lanes 1–3**). **Lane 4**, −enzyme. **Lane M**, *Hpa*II fragments of pBR322. Fragments corresponding to the 5′ and 3′ halves of the cleavage products are indicated on the right. **(B)** and **(C)** Isolated 5′ and 3′ halves of the cleavage reaction were digested with RNase T2 and the nucleotide compositions were determined as described under Materials and methods. The positions of individual nucleotides (Np, nucleoside 3′-phosphate; pNp, nucleoside 3′,5′-diphosphate; pppGp, guanosine 5′-triphosphate 3′-phosphate from the transcriptional initiation site of SP6 RNA polymerase) and free phosphorous (P_i) are indicated. The schematic of the nucleotide composition analysis along with the nucleotide sequence of substrate RNA around the cleavage sites is shown below.

was generated from the human RNA substrate; its presence was dependent on the added mouse Fraction III (Figure 6A). Although other distinct bands were present, their intensities remained the same independent of the amount of mouse Fraction III added; these species probably represent degradation products rather than specific processing by mitochondrial proteins. The cleavage site, as deduced from the size of the processed product, mapped at nucleotide position 320 of human mtDNA, again between CSB II and CSB III. Although a nucleotide sequence 5′-GAAG-3′, similar to the mouse cleavage sequence 5′-GAG-3′, was found at the cleavage site of the human DNA substrate, it is unlikely that this sequence alone is sufficient for a proper selection of the cleavage site since the sequences 5′-GAAG-3′ and 5′-GAG-3′, located elsewhere, were not cleaved by the mouse Fraction III.

If the CSB elements have a role in cleavage site selection, as the above experiment suggests, an analogous processing activity should exist in human mitochondria. To investigate this point, we prepared a human mitochondrial extract similar to the mouse Fraction III and assayed for its ability to cleave the 270-nucleotide mouse RNA substrate. The results showed a 130-nucleotide species when the mouse RNA substrate was incubated with the human mitochondrial extract (Figure 6B). This size of the RNA cleavage product was identical to the one generated by mouse Fraction III. The human mitochondrial extract, as expected, also cleaved the human RNA substrate at nucleotide position 320 (data not shown).

Discussion

The O_H of mammalian mtDNA is the only eukaryotic example where DNA synthesis is primed by RNA of significant size (75–165 nucleotides in the case of mouse mtDNA) (Chang *et al.*, 1985). Primer RNA synthesis begins at a major promoter that is also involved in gene expression and the transition from primer RNA synthesis to DNA synthesis occurs at three distinct sites. We have described here a site-specific endoribonuclease isolated from purified mitochondria that cleaves single-stranded RNA substrates at one of the previously identified transition sites, proximal to the transcriptional promoter; cleavage at the other two transition sites, including the major site at nucleotide 16030, has not been demonstrated with the Fraction V activity in *in vitro* assays. Currently we do not know whether processing at each of the three transition sites might be carried out by individual activities, but the presence of a second processing activity which cleaves the RNA substrate at a second minor transition site at nucleotide 16059 raises such a possibility.

Properties of the mitochondrial endonuclease

The endonuclease activity was easily solubilized by treating mitochondria with non-ionic detergent in the presence of 0.2 M KCl. Increasing the salt concentration up to 0.6 M had very little effect on the overall yield of activity in the high-speed supernatant fraction. Through several chromatographic procedures the endonuclease was recovered as a single entity, indicating both recogni-

tion and cleavage of the substrate are carried out by the same complex. Although the endonuclease was substantially purified, SDS gel analyses of polypeptide compositions of heparin–Sepharose and the second DEAE–Sephacel column fractions have failed to identify any polypeptide species that co-elutes with the activity. The endonuclease activity has a sedimentation coefficient of 15 S on a glycerol gradient and is excluded from a Sephacryl S-200 column (data not shown), indicating that the apparent mol. wt of the endonuclease is >250 kd. Since the coding capacity of mammalian mtDNA may now be accounted, with the exception of URF 6 (Chomyn *et al.*, 1985), it is likely that the entire coding information for the endonuclease resides in the nucleus.

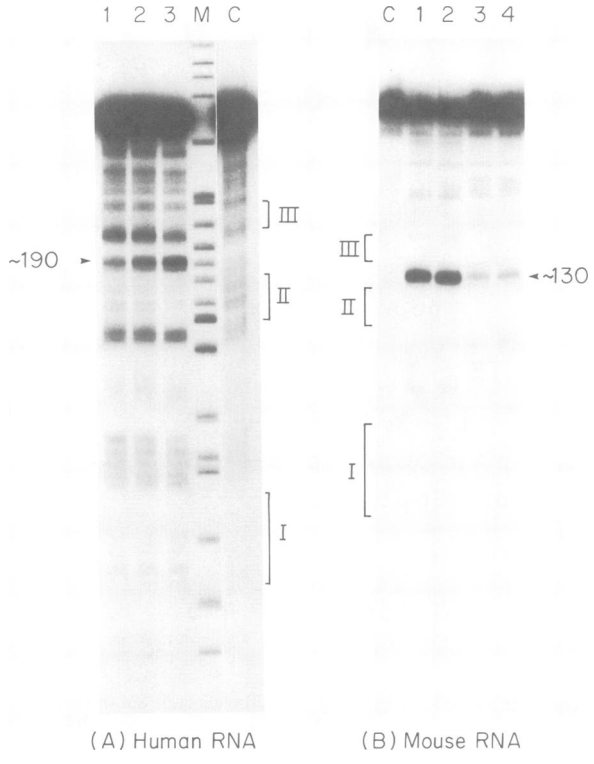

Fig. 6. *In vitro* cleavage assays using heterologous RNA substrates. **(A)** *In vitro* cleavage reactions were carried out by using, as a substrate, a 320-nucleotide pCp-labeled human mitochondrial RNA sequence and mouse Fraction III. **Lanes 1–3**, 0.34, 0.68 and 2 μg, respectively, of Fraction III. **Lane C**, –enzyme. **Lane M** shows *Hpa*II fragments of pKB741 which lack the 622-bp fragment of pBR322 and contain additional 635-, 463-, 164-, and 105-bp fragments. The position of the mouse Fraction III-dependent species is indicated with an arrowhead. **(B)** *In vitro* cleavage reactions were carried out by using, as a substrate, the 270-nucleotide pCp-labeled mouse mitochondrial RNA sequence. **Lane C**, –enzyme. **Lanes 1** and **2**, 1.4 μg of mouse Fraction III; **lanes 3** and **4**, 0.4 μg of human Fraction III. Lanes 2 and 4 were assayed in the absence of RNasin. CSBs I, II and III are indicated by brackets.

Relative location of the cleavage sites

By employing a 3′-end labeled substrate, we have mapped the exact cleavage site to three adjacent nucleotides centered at nucleotide position 16103. Previous studies have shown two types of *in vivo* nucleic acids mapping at this position (Figure 7). One is RNA with heterogeneous 3′ ends centered at nucleotide 16108 (extending from 16102 to 16114) and the other is nascent H-strand DNA with short 5′ oligoribonucleotides of 1–10 residues that extent to nucleotide 16104. The boundary for 5′ deoxyribonucleotide residues in this group of nascent H-strand DNAs, as determined by alkaline treatment, maps at nucleotide 16094 (Gillum and Clayton, 1979; Chang *et al.*, 1985).

The cleavage products have the necessary 3′ hydroxyl ends to serve as primers to be elongated with deoxyribonucleotide residues. However, based on the above mapping information, it is clear that there are an additional nine ribonucleotide residues between the *in vitro* cleavage site at 16103 and the *in vivo* RNA to DNA transition site at 16094. One plausible explanation for this nine-nucleotide discrepancy is that the fidelity of the endonuclease is somewhat altered during purification or under the *in vitro* reaction conditions employed. However, the presence of an RNA with a 3′ end at 16102, and the fact that some of the nascent H-strand DNA has 5′ oligoribonucleotides extending to 16104, argue for an *in vivo* processing event at nucleotides 16102, 16103 and 16104. These positions are the exact *in vitro* endonucleolytic cleavage sites; we therefore believe that the isolated endonuclease is faithfully recapitulating the *in vivo* processing event.

A role for RNA processing in DNA replication

What then is the functional role of the RNA processing event at this transition site? An interesting possibility is that the endonucleolytic processing centered at nucleotide 16103 is necessary for a required hybrid to form between the RNA and template

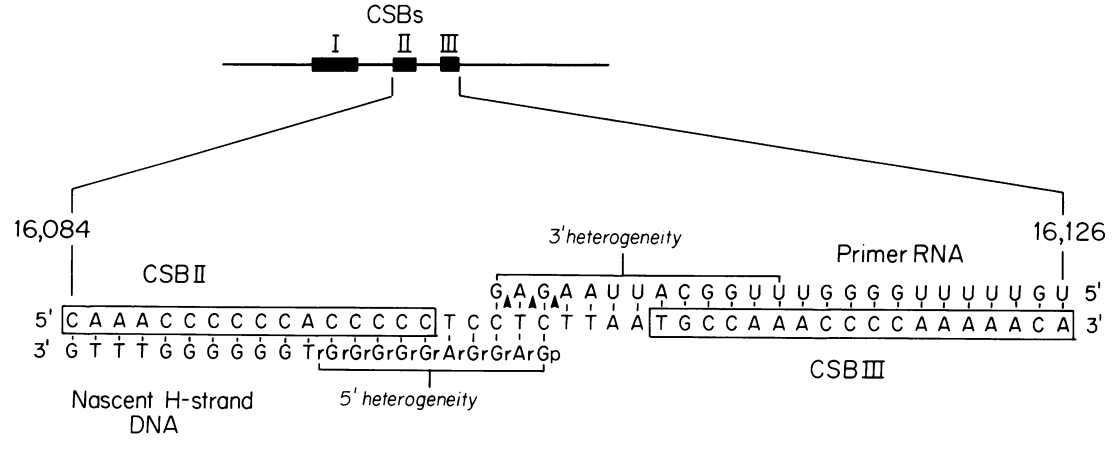

Fig. 7. Positions of *in vivo* nucleic acids at the endonucleolytic cleavage site. The L-strand sequence of the transition site of RNA synthesis to DNA synthesis between CSB II and CSB III is given. The sequences of CSB II and CSB III are boxed. The 3′ extent of primer RNA and the 5′ termini of nascent H-strand DNA, along with its heterogeneous 5′ oligoribonucleotides, are also shown. The *in vitro* endonucleolytic cleavage sites are indicated with arrowheads. The map positions of *in vivo* nucleic acids are as determined previously (Gillum and Clayton, 1979; Chang *et al.*, 1985).

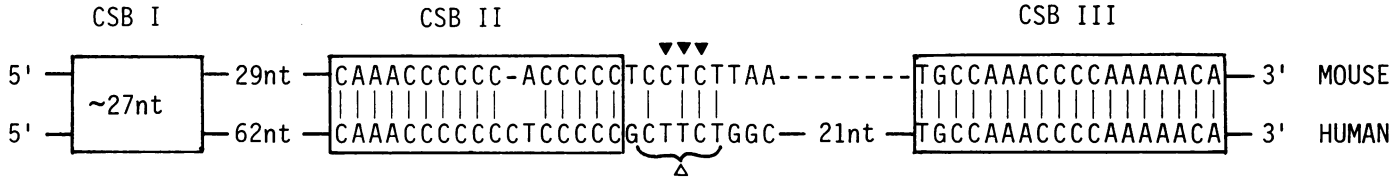

Fig. 8. Sequence alignment of the O_H regions of mouse and human mtDNA. The L-strand sequences are given. The endonucleolytic cleavage sites in mouse (▼) and human (Δ) are indicated. The cleavage sites in human mtDNA have not been determined to the individual nucleotide level. Sequence identities are indicated by vertical lines. The three CSB elements are boxed; the CSBs I in mouse and human consist of 26 and 27 nucleotides, respectively. A dash (−) indicates the absence of a nucleotide at that position.

DNA. As RNA polymerase transcribes through the replication origin, the free energy available from the superhelicity of a closed circular mtDNA would facilitate the formation of a hybrid. Such a hybrid should soon be displaced unless stabilized against branch migration. The region immediately downstream of the cleavage sites (see Figure 7), where the actual RNA to DNA transition occurs, consists of a polypyrimidine track that frequently adopts a non-B DNA structure in a superhelical DNA (Cantor and Efstratiadis, 1984; Pulleyblank *et al.*, 1985). This region of mtDNA, as expected, is hypersensitive to nuclease S1 digestion when torsionally strained by supertwisting (unpublished observation). It is attractive to speculate that this polypyrimidine tract promotes the formation of a stable hybrid between RNA and template DNA and that the cleavage event at the beginning of the polypyrimidine tract (at nucleotide 16103) further stabilizes the hybrid by removing a displaced RNA.

A second possibility, that we cannot presently rule out, is that the endonuclease is involved in removing primer RNA from a nascent DNA strand, rather than being principally active in generating primer RNA. The covalent joining of newly synthesized DNA at the completion of DNA replication requires the removal of RNA primers. Prokaryotic DNA polymerases have an associated 5′ to 3′ exonuclease activity that has been implicated in such a function (Kornberg, 1980). In contrast, DNA polymerase γ, found in mitochondria, may lack a 5′ to 3′ exonuclease activity (Kornberg, 1980). Furthermore, the O_H of mtDNA uses relatively long RNA primers that might branch migrate away from the template strand during unidirectional DNA synthesis. The endonuclease activity described here could then be involved in reducing the size of the displaced primer RNA from the nascent H-strand DNA to facilitate covalent joining of the daughter H-strand DNA at the completion of a replication cycle.

Although the relevance of the isolated endonuclease to mitochondrial nucleic acid metabolism is strongly supported by the precise correlation between the *in vitro* cleavage site and the known *in vivo* transition site, the specific function of this cleavage event in the overall mechanism of mtDNA replication remains to be determined. The development of an *in vitro* replication system capable of initiation at this origin should help our understanding of the exact role of the RNA processing event.

Selection of the cleavage site

Based on the mode of cleavage site selection, most site-specific endonucleases can be divided into two categories. The processing enzymes involved in tRNA maturation (RNase P and tRNA splicing endonucleases) cleave a broad spectrum of substrates and appear to recognize mainly common structural features of precursor tRNAs (Robertson *et al.*, 1972; Guerrier-Takada *et al.*, 1983; Peebles *et al.*, 1983). Although less well characterized, RNase III also seems to recognize a higher order structure in substrates (Gegenheimer and Apiron, 1981). A second class of endonuclease selects a cleavage site guided by primary sequences at or near the processing site. This class is exemplified by the self-catalyzing intron of *Tetrahymena* rRNA (Zaug and Cech, 1986; Waring *et al.*, 1986) and probably includes other Group I introns of fungal mitochondrial mRNAs (Garriga and Lambowitz, 1984; van der Horst and Tabak, 1985; Garriga *et al.*, 1986) as well as endonucleases involved in the 3′ maturation of mRNAs (Wickens and Stephenson, 1984; Birnstiel *et al.*, 1985; Moore and Sharp, 1985; Mason *et al.*, 1986).

We have tested, as substrates, SP6 RNA polymerase-generated transcripts of other regions of mtDNA to examine the specificity of the mitochondrial endonuclease. Thus far we find that the endoribonuclease cleaves only the substrate containing the O_H region and at a unique position located between CSB II and CSB III; other putative RNA processing sites at the boundaries of tRNAs (Van Etten *et al.*, 1980) are not cleaved. The overall nucleotide sequences of the O_H regions of mouse and human mtDNA are quite different. That a heterologous human RNA substrate was appropriately cleaved by the mouse endonuclease, and *vice versa*, indicate limited sequence information, common to both mouse and human RNA substrates, is sufficient for the proper recognition of the cleavage site. The sequence alignment of the O_H regions of mouse and human mtDNA, along with the positions of the endonucleolytic cleavage sites, is shown in Figure 8. Besides the three CSB elements, there are no conserved primary sequences of significant size nor any conserved potential secondary structures. Furthermore, the cleavage reaction is not affected when sequences downstream of CSB II, including the CSB I sequence, are removed (unpublished observation). Although the manner in which this mitochondrial endoribonuclease selects its cleavage site is unknown, the fixed distance between the cleavage sites and CSB II implicates this sequence element in the recognition process. Additional mutational analysis of CSB II should provide more definitive information regarding the mechanism of cleavage site selection.

Materials and methods

Reagents

Nucleoside triphosphates, nucleoside-3′-phosphates, nucleoside-3′,5′-diphosphates, T4 RNA ligase, RNase U2, RNase PhyM, DEAE−Sephacel and heparin−Sepharose were from Pharmacia, Inc. Radioisotopes, [α-^{32}P]GTP and [^{32}P]cytidine-3′,5′-bisphosphates, were from New England Nuclear. Phosphocellulose (P-11) and precoated cellulose t.l.c. plates were from Whatman, Inc. *N*-ethylmaleimide was from Sigma Chemical Co. SP6 RNA polymerase, RNasin, pSP64, and pSP65 were from Promega Biotech. Restriction endonucleases and RNase T2 were from New England Biolabs, Inc. and Bethesda Research Laboratories, Inc., respectively.

Recombinant plasmids

pMR718B is a derivative of pSP65 containing the O_H region of mouse mtDNA constructed by introducing a 720-bp *Rsa*I fragment (nucleotides 15497−16216) into the *Sma*I site of the vector. pK408 contains a nuclease *Bal*31-generted 408-bp fragment (nucleotides 1−408) of human mtDNA cloned into the *Bam*HI/*Eco*RI sites of pSP64. The orientations of these plasmids are such that the L strand of mtDNA inserts would be transcribed from the SP6 promoter in the vectors.

RNA substrate

RNA substrates for *in vitro* cleavage reactions were prepared using either a *Xba*I-digested pMR718B (mouse RNA substrate) or a *Fok*I-digested pK408 (human RNA substrate) as a template. *In vitro* transcription was carried out in a 50-μl reaction mixture containing 40 mM Tris−HCl (pH 7.5), 6 mM $MgCl_2$, 4 mM spermidine, 10 mM dithiothreitol (DTT), 500 μM each of four rNTPs, 2.5 μg of linearized template DNA, 25 U RNasin, and 10 U SP6 RNA polymerase (Melton *et al.*, 1984). The transcription mixture was incubated at 37°C for 1 h and RNA was recovered by precipitation with ethanol following Sephadex G-50 filtration to remove unincorporated rNTPs. Subsequent 3′-end labeling of RNA was carried out in a 20-μl reaction mixture containing 50 mM Hepes (pH 7.5), 15 mM $MgCl_2$, 3 mM DTT, 0.2 μg bovine serum albumin (BSA), 10% (v/v) dimethylsulfoxide, 30 μM ATP, 100 μCi [^{32}P]pCp, and 10 U T4 RNA ligase at 4°C for 12 h (England and Uhlenbeck, 1978). Incubation was terminated by extraction with phenol:chloroform (50:50, v/v). The reaction products were recovered by precipitation with ethanol and then electrophoresed in a 6% acrylamide−7 M urea gel. The 3′-end labeled RNA was eluted from the gel slices by soaking them in 0.4 ml of 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, and 0.1% SDS at 37°C for 4−6 h (Maxam and Gilbert, 1980). A typical yield of RNA was 2 μg at ~15 × 10^6 c.p.m. (Cherenkov)/μg.

Protein purification

Mouse LA9 fibroblast cells were grown in suspension cultures in Eagle's minimal essential medium supplemented with 10% calf serum. Mitochondria were prepared from cells harvested at mid-log phase (5−7 × 10^5 cells/ml) and further purified by centrifugation in sucrose density gradients as described previously (Tapper *et al.*, 1983). Unless otherwise stated, all the following procedures were performed at 4°C. Mitochondria (~3 g) were resuspended in 10 ml of buffer A (20 mM Tris−HCl, pH 8.0, 1 mM EDTA, 1 mM DTT and 10% glycerol) containing 1 mM phenylmethylsulfonyl fluoride; then Triton X-100 and KCl were added to 1% (v/v) and 0.2 M, respectively. The suspension was vortexed for 10 s every 5 min for 15 min. From these crude lysates, insoluble materials were pelleted by centrifugation at 45 000 r.p.m. for 1 h in a Beckman 75Ti rotor and the supernatant was recovered to yield Fraction I (87.2 mg). Fraction I was diluted with an equal volume of buffer A and applied at 20 ml/h to a 1.5 × 12-cm column of DEAE−Sephacel equilibrated in buffer A + 0.1 M KCl. The column was washed with the same buffer until the A_{280} of the effluent was <0.05. Bound proteins were then eluted from the column with buffer A + 0.3 M KCl to yield Fraction II (22.1 mg). Fraction II was diluted with an equal volume of buffer A and then immediately applied to a 1.5 × 12-cm column of phosphocellulose equilibrated in buffer A + 0.15 M KCl. The column was washed with two column volumes of the same buffer and then with buffer A + 0.4 M KCl. Fractions containing >0.1 mg/ml of protein were pooled and dialyzed against 1 l of buffer A + 0.1 M KCl for 2 h to obtain Fraction III (4.4 mg). Fraction III was applied at 5 ml/h to a 1 × 4-cm column of heparin−Sepharose that had been equilibrated in buffer A + 0.2 M KCl. The heparin−Sepharose column was washed with three column volumes of the same buffer and then developed with a 20-ml linear gradient of 0.2−0.6 M KCl in buffer A. Thirty 0.7-ml fractions were collected and 2 μl of every other fraction were assayed for endonuclease activity. Fractions 8−14 containing the activity were pooled (Fraction IV; 1.14 mg) and diluted with an appropriate volume of 20 mM Tris−HCl (pH 8.0), 1 mM DTT, and 10% glycerol to reduce the concentrations of KCl and EDTA to 0.1 M and 0.2 mM, respectively. The diluted sample was then applied at 3 ml/h to a 2.5-ml column of DEAE−Sephacel that had been equilibrated with buffer B (20 mM Tris−HCl, pH 8.0, 0.2 mM EDTA, 1 mM DTT, and 10% glycerol) + 0.1 M KCl. The column was washed with 10 ml of the same buffer and then developed with a 15-ml linear gradient of 0.1−0.4 M KCl in buffer B. Thirty-three 0.5-ml fractions were collected and 2 μl of every other fraction were assayed for the endonuclease activity. The active fractions (18−22) were pooled and dialyzed against 1 l of buffer that contained 20 mM Tris−HCl (pH 8.0), 0.2 mM EDTA, 1 mM DTT, 0.05 M KCl and 50% glycerol to yield Fraction V (0.07 mg). Fraction V was stored at −20°C. During the purification, when necessary, protein fractions were frozen in liquid nitrogen and stored at −70°C. Protein concentration was estimated by the modified dye-binding method of Read and Northcote (1981).

Assay for endonuclease

The cleavage reaction was assayed in a 25-μl reaction mixture containing 20 mM Tris−HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM DTT, 50 mM KCl, 15 U RNasin, 50 μg BSA/ml, and 2000 c.p.m. of substrate RNA at 37°C for 30 min. The reaction was terminated by adding 50 μl of 0.5 M sodium acetate (pH 5.0), extracted with an equal volume of phenol:chloroform (1:1, v/v) and then precipitated with ethanol in the presence of 5 μg of *E. coli* tRNA. Reaction products were pelleted and then analyzed by using 6% acrylamide−7 M urea gel electrophoresis.

Analysis of cleavage products

The enzymatic sequence ladders of the 270-nucleotide substrate RNA used to map the *in vitro* cleavage sites were generated by incubating 20 000 c.p.m. of the substrate RNA and 5 μg of carrier tRNA in 5 μl of reaction buffer (20 mM sodium citrate, pH 5.0, 1 mM EDTA, 7 M urea, and 0.025% each xylene cyanol and bromophenol blue) at 50°C. Following a 5-min incubation, 0.005 U RNase T1, 1 U RNase U2, and 0.2 U RNase PhyM were added to the respective tubes and then incubated for an additional 10 min (Donis-Keller *et al.*, 1977; Silberklang *et al.*, 1979). The reaction was terminated by transferring the tubes to an ice bath and the samples were immediately loaded onto an 8% acrylamide−7 M urea gel. Partial alkaline hydrolysis was carried out by heating 40 000 c.p.m. of the substrate RNA in 20 μl of 50 mM $NaHCO_3$ (pH 9.1) and 1 mM EDTA. Following a 10-min incubation at 90°C, the sample was lyophilized and then resuspended in 5 μl of the reaction buffer.

The nearest-neighbour analyses were carried out by incubating gel-purified 5′ and 3′ halves of the cleavage products in a 2-μl reaction mixture containing 50 mM sodium acetate (pH 4.6), 1 mM EDTA and 5 U RNase T2 at 37°C for 2 h (Silberklang *et al.*, 1979). The sample was then spotted onto a precoated cellulose thin-layer plate and the nucleotide composition was determined by two-dimensional chromatography using, as solvents, isobutyric acid:0.5 M ammonium hydroxide, 5:3 (v/v) for the first dimension and isopropanol:concentrated HCl:water, 70:15:15 (v/v/v) for the second dimension (Nishimura, 1979).

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