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Junction phosphate is derived from the precursor in the tRNA spliced by the archaeon *Haloferax volcanii* cell extract

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ABSTRACT

RNA splicing in archaea requires at least an endonuclease and a ligase, as is the case for the splicing of eukaryal nuclear tRNAs. Splicing endonucleases from archaea and eukarya are homologous, although they differ in subunit composition and substrate recognition properties. However, they all produce 2′,3′ cyclic phosphate and 5′-hydroxyl termini. An in vitro-transcribed, partial intron-deleted *Haloferax volcanii* elongator $tRNA^{Met}$ has been used to study splicing by *H. volcanii* cell extracts. Substrates and products were analyzed by nearest neighbor analyses using nuclease P1 and RNase T2, and fingerprinting analyses using acid-urea gels in the first dimension and gradient thin layer chromatography in the second dimension. The results suggest that 2′,3′ cyclic phosphate at the 3′ end of the 5′ exon is converted into the splice junction phosphate forming a 3′,5′-phosphodiester linkage. This resembles the animal cell type systems where the junction phosphate preexists in the transcript, and differs from yeast type systems, where GTP is the source of junction phosphate.

Keywords: archaea; cyclic phosphate; halophiles; RNA fingerprinting; RNA ligase; RNA splicing

INTRODUCTION

All three domains of life, Archaea, Bacteria, and Eukarya (Woese et al., 1990) contain intron-interrupted genes (Belfort et al., 1995). So far archaeal introns have been observed only in the stable RNA genes. Both the Euryarchaeota and Crenarchaeota kingdoms (Woese et al., 1990) of Archaea contain tRNA introns (Kaine et al., 1983; Daniels et al., 1985, 1986; Kaine, 1987; Wich et al., 1987; Datta et al., 1989; Kjems et al., 1989a, 1989b), whereas rRNA introns have been observed in some Crenarchaeota (Kjems & Garrett, 1985, 1991; Dalgaard & Garrett, 1992; Burggraf et al., 1993; Itoh et al., 1998; Nomura et al., 1998). Introns have also been reported in the tRNA genes of all published archaeal genomes (Bult et al., 1996; Klenk et al., 1997; Smith et al., 1997; Kawarabayasi et al., 1998, 1999), indicating probable occurrence of tRNA introns in all archaea. A structure, now called a bulge-helix-bulge (BHB) motif (see Fig. 1A) formed by pairing of the regions near the two intron–exon junctions, was originally proposed in the first report of the presence of introns in archaea (Kaine et al., 1983). This BHB motif can potentially be formed in nearly all archaeal tRNA and rRNA introns, although in some cases this BHB structure may be energetically less favorable.

At least two separate protein enzymes, an endonuclease and a ligase, seem to be involved in RNA splicing in archaea. The tRNA splicing endonuclease from *Haloferax volcanii* has been partially purified and characterized (Thompson & Daniels, 1988, 1990; Thompson et al., 1989). It appears to be 37 kDa in size and a homodimer under native conditions. A recombinant form of this enzyme has been expressed in *Escherichia coli* (Kleman-Leyer et al., 1997). The crystal structure of the endonuclease from *Methanococcus jannaschii* has been determined (Li et al., 1998). This enzyme is about 20 kDa in size and is a homotetramer in solution (Lykke-Andersen & Garrett, 1997). These archaeal enzymes recognize the above-mentioned BHB structure (Thompson et al., 1989; Kjems et al., 1989a; Thompson & Daniels, 1990; Kjems & Garrett, 1991; Lykke-Andersen & Garrett, 1994, 1997).

Eukaryal splicing endonucleases recognize three subsets of elements in their substrates (Di Nicola Negri et al., 1997). Both archaeal and eukaryal endonucleases produce 2′,3′ cyclic phosphate and 5′-hydroxyl termini (Filipowicz & Shatkin, 1983; Peebles et al., 1983;

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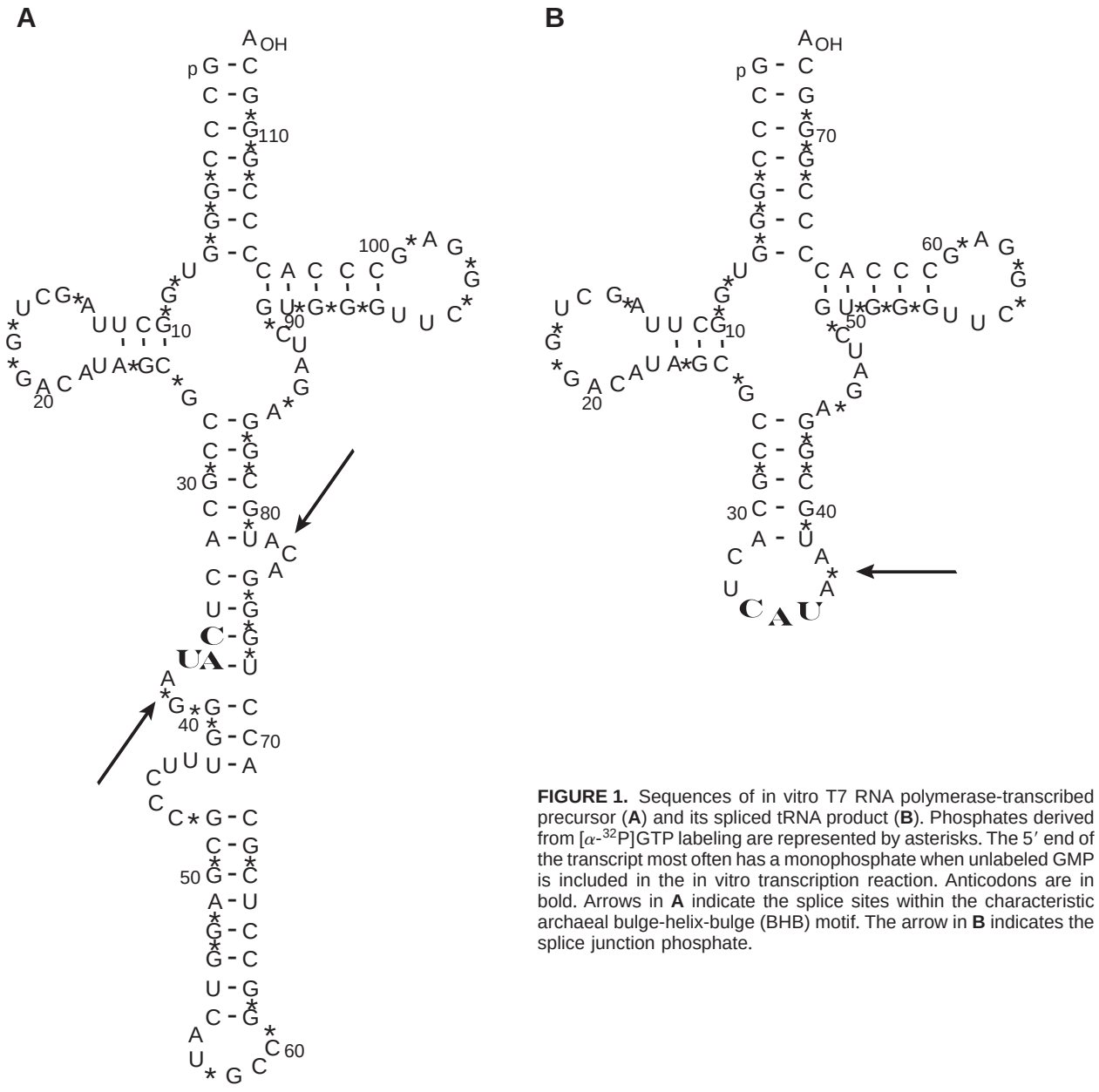

FIGURE 1. Sequences of in vitro T7 RNA polymerase-transcribed precursor (**A**) and its spliced tRNA product (**B**). Phosphates derived from [α-^{32}P]GTP labeling are represented by asterisks. The 5′ end of the transcript most often has a monophosphate when unlabeled GMP is included in the in vitro transcription reaction. Anticodons are in bold. Arrows in **A** indicate the splice sites within the characteristic archaeal bulge-helix-bulge (BHB) motif. The arrow in **B** indicates the splice junction phosphate.

Gandini Attardi et al., 1985; Stange et al., 1988; Thompson & Daniels, 1988). Archaeal and eukaryal splicing endonucleases are homologous (Kleman-Leyer et al., 1997; Lykke-Andersen & Garrett, 1997; Trotta et al., 1997; Abelson et al., 1998; Li et al., 1998; Trotta & Abelson 1999). However, they have evolved such that the mechanisms of substrate recognition in the two domains have become somewhat different. Nonetheless, eukaryal endonuclease can still recognize and cleave archaeal BHB motif containing synthetic tRNA substrates (Fabbri et al., 1998).

Very little is known about the ligation mechanism in archaeal RNA splicing (Kjems & Garrett, 1988; Gomes & Gupta, 1997). Two different types of ligation mechanisms have been observed in eukarya (Phizicky & Greer, 1993; Westaway & Abelson, 1995; Arn & Abelson, 1998; Trotta & Abelson, 1999). Yeast tRNA splicing ligase is the most extensively studied representative of the eukaryal tRNA ligases. It is a single polypeptide that has multiple functions (Greer et al., 1983b; Phizicky et al., 1986; Xu et al., 1990; Apostol et al., 1991; Belford et al., 1993; Westaway et al., 1993). It converts the 2′,3′ cyclic phosphate at the 3′ end of the 5′ exon to a 2′-phosphate and adds a phosphate at the 5′ end of the 3′ exon, using GTP as the source of this phosphate. The ligase is adenylated (derived from ATP) and the AMP is then transferred to newly added 5′-phosphate of the 3′ exon. Finally, ligase joins the two exons, releasing AMP and producing a 2′-phosphomonoester, 3′, 5′-phosphodiester linkage at the splice junction. The

γ-phosphate of GTP becomes the splice junction phosphate. A different 2′-phosphotransferase enzyme transfers the 2′-phosphate of the splice junction to NAD^+, yielding nicotinamide and ADP ribose 1″,2″ cyclic phosphate (Appr>p) (McCraith & Phizicky, 1991; Culver et al., 1993). Yeast tRNA ligase is also involved in splicing of a special nuclear pre-mRNA intron (Sidrauski et al., 1996). The mechanism of action of the tRNA ligase activity in wheat germ and *Chlamydomonas* extracts appears to be similar to the yeast system (Konarska et al., 1981, 1982; Kikuchi et al., 1982; Gegenheimer et al., 1983; Schwartz et al., 1983; Tyc et al., 1983). Activities similar to the yeast tRNA ligase and 2′-phosphotransferase producing Appr>p have also been identified in HeLa cells and *Xenopus*, suggesting that the yeast-like tRNA splicing pathway is conserved throughout eukarya (Zillmann et al., 1991, 1992). Animal cells also contain a second pathway where the phosphate at the splice junction is derived from the 2′,3′ cyclic phosphate at the 3′ end of 5′ exon that is produced during endonuclease reaction (Nishikura & De Robertis, 1981; Filipowicz & Shatkin, 1983; Filipowicz et al., 1983; Laski et al., 1983; Perkins et al., 1985). The roles for the two apparently redundant but different ligation pathways in animals are not yet clear. Recently it has been suggested that mammalian cells contain a default pathway that can join 2′,3′ cyclic phosphate and 5′-hydroxyl termini and can circularize ribozyme-processed RNAs (Reid & Lazinski, 2000).

Archaeal splicing ligases do not seem to be like the yeast tRNA ligase (Kjems & Garrett, 1988; Gomes & Gupta, 1997; Arn & Abelson, 1998; Trotta & Abelson, 1999). Probably the junction phosphate in archaeal splicing is derived from the precursor, as the phosphate of ATP or GTP apparently is not incorporated into the spliced RNA (Kjems & Garrett, 1988; Gomes & Gupta, 1997).

Here we describe the analyses of studies carried out to determine the origin of the splice junction phosphate in the tRNA spliced by the *H. volcanii* cell extracts. The results suggest that terminal 2′,3′ cyclic phosphate of the 5′ exon is converted into the junction phosphate by forming a 3′,5′-phosphodiester linkage.

RESULTS

Experimental design

Sequences of in vitro T7 RNA polymerase-transcribed precursor and its spliced tRNA product are shown in Figure 1. Specific reasons for using this construct are discussed below. The intron in this construct has a 36-base deletion when compared with the wild type (Gomes & Gupta, 1997). The sequences at the 5′ and 3′ splice sites are ApG and CpA, respectively (arrows in Fig. 1A). The phosphates on the 5′ side of the guanosine residues in the precursor are labeled when in vitro transcription reactions contain [α-^{32}P]GTP. Positions of these phosphates are marked as asterisks in Figure 1. One such labeled phosphate is present in the precursor at the 5′ splice site (Fig. 1A). *H. volcanii* intron endonuclease, like corresponding eukaryal endonucleases, produces 2′,3′ cyclic phosphate and 5′-hydroxyl termini (Thompson & Daniels, 1988). Therefore, endonuclease products of our precursor are expected to have a 5′ exon terminating in a labeled 2′,3′ cyclic phosphate-bearing adenosine residue (−A>p̆) (p̆ indicates ^{32}P-labeled phosphate), and a 3′ exon containing a hydroxyl bearing adenosine residue at its 5′ end ($_{OH}$A−). Splicing ligase reaction joins these two terminal adenosine residues of the two exons to produce the ligated tRNA, creating a new sequence, ApA, at the splice junction (arrow in Fig. 1B). Nearest-neighbor and fingerprinting analyses of the splicing substrates and products should be able to determine whether the labeled 2′,3′ cyclic phosphate of the 5′ exon is retained or removed during ligation.

Neighboring base of a phosphate of the precursor is changed during splicing

Ligated product was first separated from the precursor by polyacrylamide gel electrophoresis (Fig. 2B). Then gel-eluted precursor and ligated tRNA were digested with nuclease P1 and the digests were resolved by two-dimensional thin layer chromatography (Fig. 3). As expected, the precursor produced only pG as the labeled product (Fig. 3A), which was derived from the [α-^{32}P]GTP in the in vitro transcription reaction. However, digests of the ligated tRNA showed a small amount of labeled pA in addition to the labeled pG (Fig. 3B). This nearest neighbor analysis using nuclease P1 suggests that at least one adenosine residue in the ligated tRNA is preceded by a labeled phosphate. There was no [α-^{32}P]ATP in the in vitro transcription reaction. Therefore, this labeled phosphate in pA is derived from [α-^{32}P]GTP. Potentially this labeled pA can be derived from the newly created sequence ApA at the splice junction, if labeled 2′,3′ cyclic phosphate is converted to the splice junction phosphate.

RNA fingerprinting analyses of splicing substrates and products

RNA fingerprinting of the RNase T1 digests of precursor and its splicing products were done to confirm their predicted composition and to determine that the ligated product is correctly spliced tRNA. Splicing endonuclease and ligase reaction products were resolved by denaturing polyacrylamide gel electrophoresis to separate the precursor, ligated tRNA, two exons, and the intron from each other (Fig. 2). RNA from each band was eluted, digested with RNase T1 and analyzed by fingerprinting. The 3′ exon was always observed in two different bands (Fig. 2C); one of which also contained

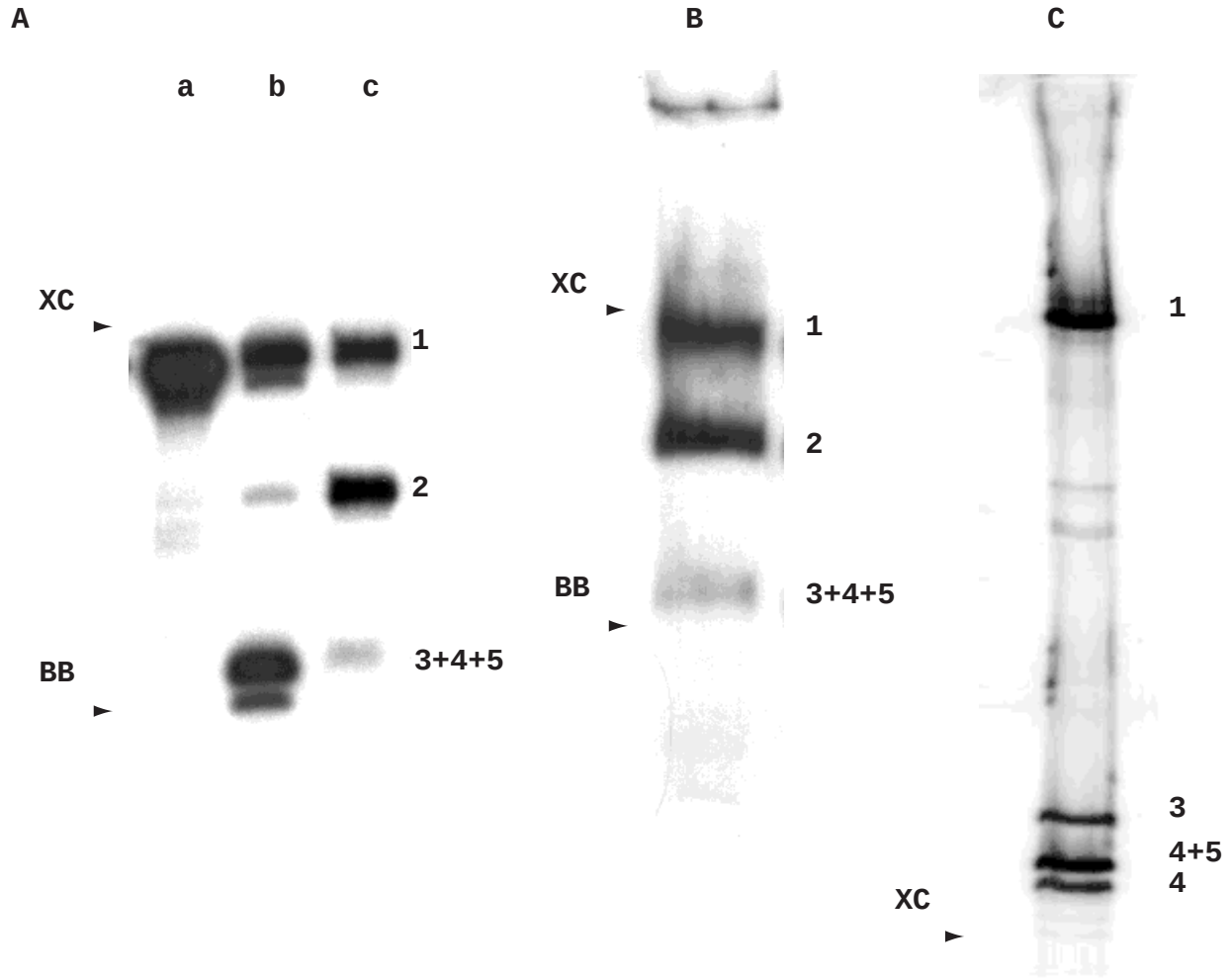

FIGURE 2. Labeled splicing reaction products resolved by denaturing polyacrylamide gel electrophoresis. **A**: A typical two-step reaction run on a 6% gel. [α-^{32}P]GTP-labeled transcripts (lane a) are used for the endonuclease reaction under low-salt concentrations (lane b), and then deproteinized endonuclease products are used for the ligase reaction under high-salt concentrations (lane c). **B**: Large scale ligation reaction products (as in **A**, lane c) resolved on 6% gel. **C**: Large scale endonuclease reaction products (as in **A**, lane b) separated on 15% gel. 1: precursor, 2: ligated product, 3: intron, 4: 3′ exon, and 5: 5′ exon. BB and XC are, respectively, the positions of bromophenol blue and xylene cyanol dyes.

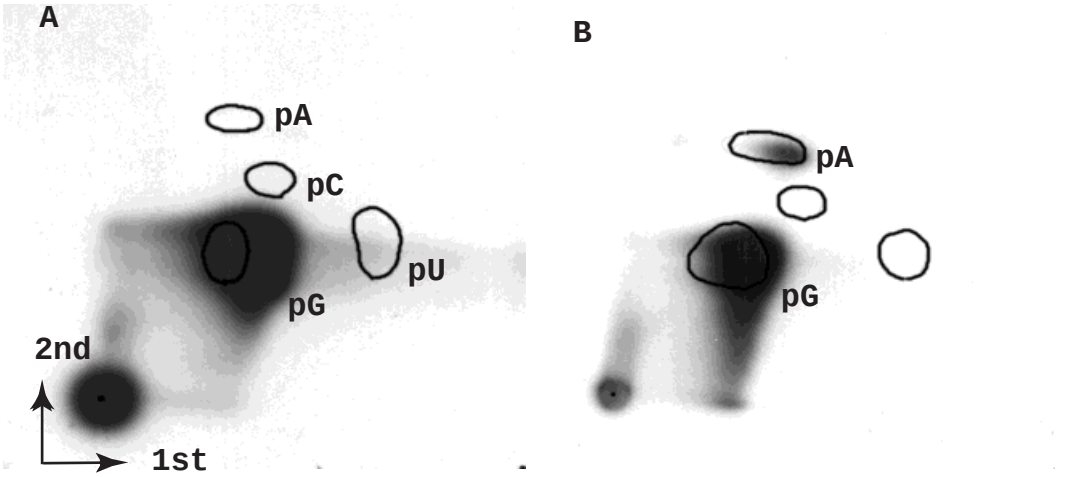

FIGURE 3. Autoradiograms of nuclease P1 digested [α-^{32}P]GTP labeled precursor (**A**) and its spliced tRNA (**B**) resolved by two-dimensional thin layer chromatography. Precursor RNA and spliced tRNA were digested independently with nuclease P1 and analyzed by chromatography on cellulose plates. Solvent a was used in the first dimension and solvent b in the second dimension. Outlined spots show the locations of unlabeled nucleoside-5′-monophosphate markers.

5′ exon (Fig. 2C, Band 4+5). No band exclusively containing nearly complete sized 5′ exon was observed. This was in spite of our attempts to separate endonuclease products on various different native and denaturing gels; varying urea, formamide, and polyacrylamide concentrations, and even using acid gels. In no case could the 5′ exon, whether generated by the cell extracts or by recombinant endonuclease, be separated from the 3′ exon.

RNA fingerprinting was done by using ultrathin acid-urea polyacrylamide gel electrophoresis (Branch et al., 1989) in the first dimension and ammonium formate gradient thin layer chromatography on DEAE-cellulose plates (Domdey & Gross, 1979) in the second dimension, as described in Materials and methods. These fingerprints are shown in Figure 4. Predicted sequences of the RNase T1 oligonucleotides of the precursor and its endonuclease and ligase products are shown in

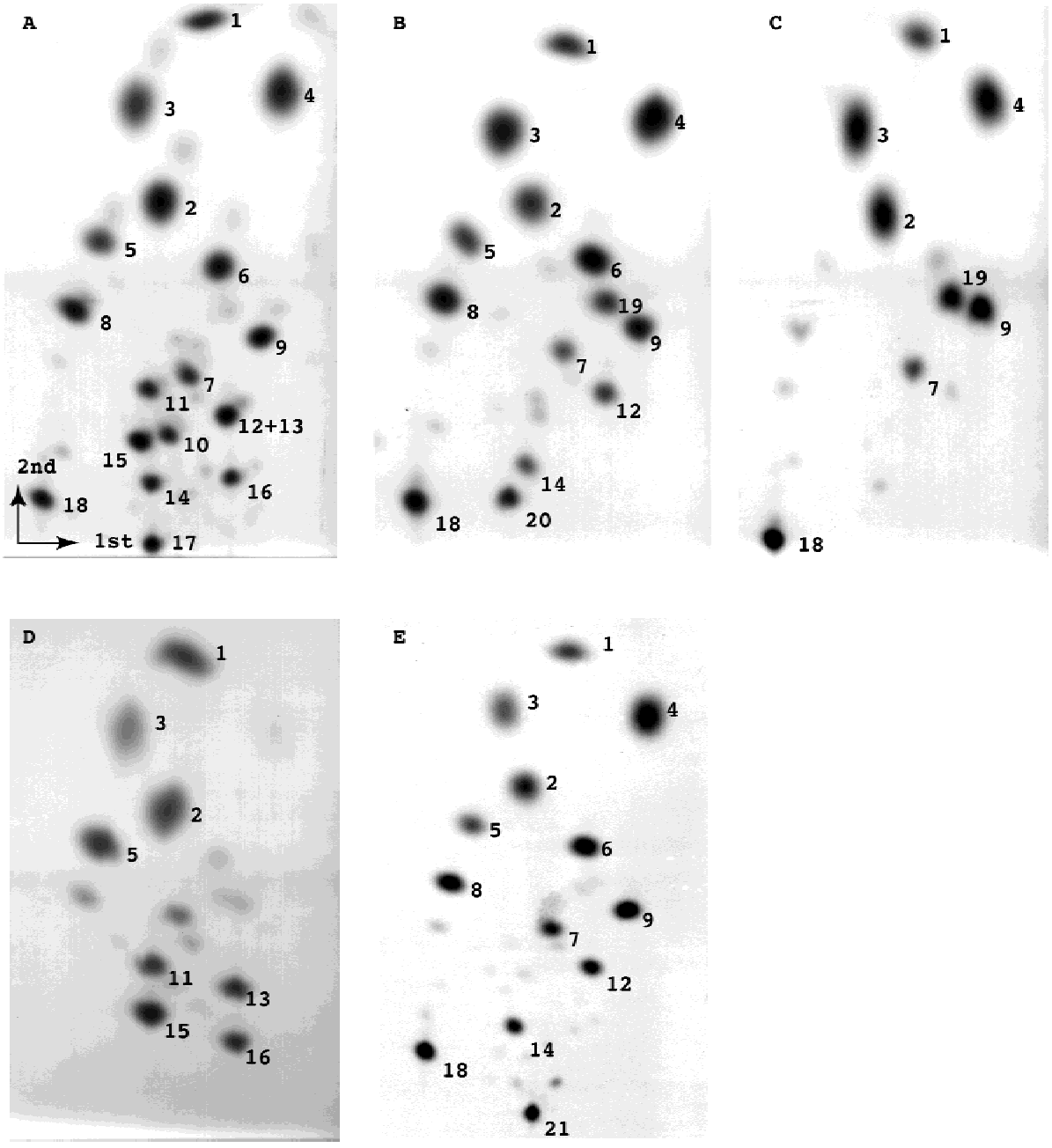

FIGURE 4. Autoradiograms of RNase T1 fingerprints of [α-^{32}P]GTP-labeled RNAs. RNAs eluted from the denaturing polyacrylamide gels are analyzed. Locations of these RNAs in the gels are marked in Figure 2. **A**: Precursor transcript; **B**: mixture of 3′ and 5′ exon; **C**: 3′ exon; **D**: intron; and **E**: the spliced tRNA. Arrows indicate the direction of the first and second dimensions. Numbering of the spots corresponds to the oligonucleotides listed in Table 1.

Table 1. Numbering in Table 1 corresponds to numbering on the fingerprints in Figure 4.

Initial identification of the oligonucleotides was done by comparison of their DNA sequences with their positions in the fingerprints (Domdey & Gross, 1979; Stackebrandt et al., 1985; Branch et al., 1989). The oligonucleotides were further characterized by nearest-neighbor analyses to confirm their identity.

There seems to be some degradation of the exons during endonuclease reactions. Recovery of the two exons was not equimolar. Figure 4C is the fingerprint of the 3′ exon (Fig. 2B, band 4), as it contains all of the predicted labeled oligonucleotides for the 3′ exon and no other (Table 1). For similar reasons Figure 4B must be a mixture of 5′ and 3′ exons (Fig. 2B, band 4+5). The 5′ exon is not significantly larger than the 3′ exon. Probably the base composition and end-group configurations increase the electrophoretic mobility of the 5′ exon or reduce 3′-exon mobility. Hydroxyl groups are present at both ends of the 3′ exon, whereas 5′ exon has a 5′-phosphate at one end and a 2′,3′ cyclic phosphate at the other end. Because all spots present in Figure 4C are also present in Figure 4B, it is assumed that the full-sized 3′ exon migrates with the 5′ exon (Fig. 2B, band 4+5) and the 3′ exon in the faster running band (Fig. 2B, band 4) has some small deletions at the 3′ end. These deletions would not change the fingerprints under our experimental conditions. Figure 4E is the fingerprint of the spliced tRNA. It only contains the spots that correspond to its oligonucleotides listed in Table 1.

Terminal 2′,3′ cyclic phosphate of the 5′ exon is converted into the splice junction phosphate

RNase T2 and nuclease P1 digestions followed by thin layer chromatography were used for further verification of the sequences of oligonucleotides in the fingerprints. This analysis for selected oligonucleotides is shown in Figure 5. RNase T2 digestion of the labeled oligonucleotides listed in Table 1 (except 1 and 21) can produce one of two patterns. When only one labeled nucleotide is observed, it can be Ap, Cp, or Up, but not Gp. When two labeled nucleotides are seen, one of them is a Gp. Similarly, nuclease P1 digestion of these oligonucleotides (except 1, 20, and 21) can also produce one of two patterns. If only one radioactive spot is observed, it is a pG. When two radioactive marks appear, one of them is pG and the other is Pi. These patterns have been observed in the analyses of oligonucleotides 2–19 (10, 17, and 19 are shown in Fig. 5).

Oligonucleotides 17 (CpApCpUpCpApUpAp̊/Gp̊) and 10 (ApCp/ApUp̊Gp) are present in the transcript at the 5′ and 3′ intron–exon junctions, respectively (see Fig. 1A; / denotes the position of the endonuclease

TABLE 1. Sequences of RNase T1 oligonucleotides of precursor and its endonuclease and ligase products.[a]

Number	Precursor transcript	Spliced product	5′ exon	3′ exon	Intron
	pGp[b]	pGp[b]	pGp[b]		
1.	Gp̊(5) + Gp (11)	Gp̊(3) + Gp (7)	Gp̊(1) + Gp (3)	Gp̊(2) + Gp (4)	Gp̊(3) + Gp (4)
2.	Ap̊Gp (2) + Ap̊Gp̊(1)	Ap̊Gp (2)		Ap̊Gp (2)	Ap̊Gp̊
3.	Cp̊Gp (2) + Cp̊Gp̊(1)	Cp̊Gp (1) + Cp̊Gp̊(1)	Cp̊Gp	Cp̊p̊	Cp̊Gp
4.	Up̊Gp̊(2)	Up̊Gp̊(2)	Up̊Gp̊	Up̊Gp̊	
5.	CpCp̊Gp (1) + CpCp̊Gp̊(1)	CpCp̊Gp	CpCp̊Gp		CpCp̊Gp̊
6.	CpUp̊Gp̊	CpUp̊p̊	CpUp̊Gp̊		
7.	ApUpCp̊Gp	ApUpCp̊Gp		ApUpCp̊Gp	
8.	CpCpCp̊Gp̊	CpCpCp̊Gp̊	CpCpCp̊Gp̊		
9.	UpUpCp̊Gp̊	UpUpCp̊Gp̊		UpUpCp̊Gp̊	
10.[c]	ApCpApUp̊Gp				
11.	CpCpUpCp̊Gp				CpCpUpCp̊Gp
12.	CpUpUpAp̊Gp	CpUpUpAp̊Gp	CpUpUpAp̊Gp		
13.	UpCpApUp̊Gp				UpCpApUp̊Gp
14.	ApCpApUpAp̊Gp	ApCpApUpAp̊Gp	ApCpApUpAp̊Gp		
15.	CpApCpCpUp̊G				CpApCpCpUp̊Gp̊
16.	UpUpUpCpCpCGpp̊				UpUpUpCpCpCp̊Gp
17.[c]	CpApCpUpCpApUpAp̊Gp̊				
18.	CpCpCpApCpCpCpCp̊Gp̊	CpCpCpApCpCpCpCp̊Gp̊		CpCpCpApCpCpCpCp̊Gp̊	
19.[c]				ApUp̊Gp	
20.[c]			CpApCpUpCpApUpA>p̊		
21.[c]		CpApCpUpCpApUpAp̊ApUp̊Gp[d]			
	CpA_{OH}			CpA_{OH}	
					ApC>p

[a] Numbered oligonucleotides contain ^{32}P (indicated as p̊) derived from [α-^{32}P]GTP (see Fig. 1). The numbering corresponds to the fingerprint spots in Figure 4. Number of copies of an oligonucleotide, when more than one in a spot, are indicated in the parentheses. >p and >p̊ indicate 2′,3′ cyclic phosphates.

[b] Labeled pp̊Gp is not detected in the fingerprints when unlabeled GMP is included in the reaction producing precursor.

[c] TLC analyses of the digests of oligonucleotides 10, 17, 19, 20, and 21 are shown in Figure 5.

[d] The p̊ between the two A (Ap̊A) in oligonucleotide 21 is the spliced junction phosphate.

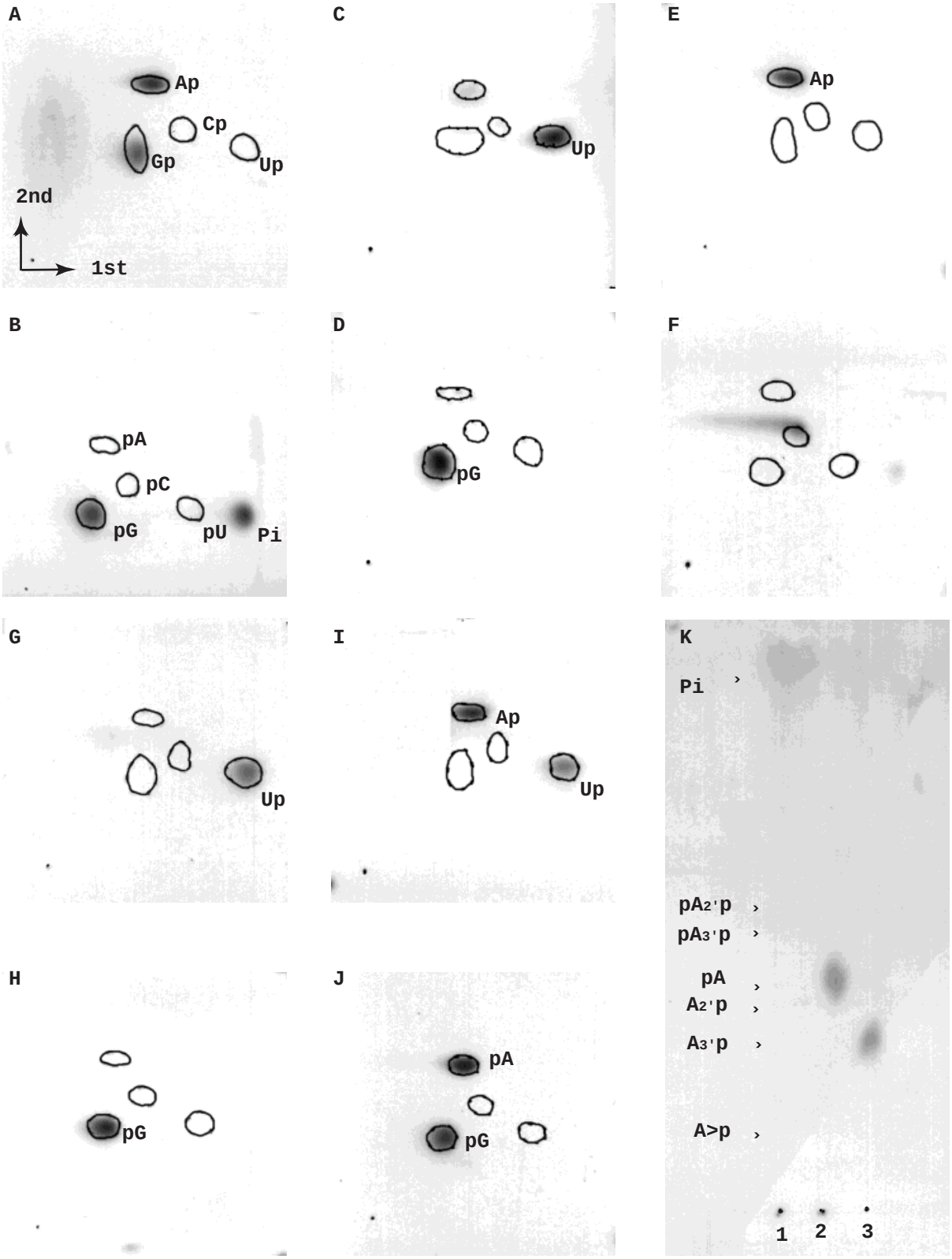

FIGURE 5. Autoradiograms of RNase T2 and nuclease P1 digests of selected oligonucleotides (Fig. 4) resolved by thin layer chromatography. Solvent a was used for the first dimension and solvent b for the second dimension in **A–J**. **A**,**C**,**E**,**G**,**I**: RNase T2 digests; **B**,**D**,**F**,**H**,**J**: nuclease P1 digests. **A**,**B**: oligonucleotide 17; **C**,**D**: oligonucleotide 10; **E**,**F**: oligonucleotide 20; **G**,**H**: oligonucleotide 19; **I**,**J**: oligonucleotide 21. Outlined spots in **A–J** show the locations of unlabeled nucleoside 3′- or 5′-monophosphate markers, as appropriate. **K**: One-dimensional separation of digests of oligonucleotide 20 using solvent c. Lane 1: Combined nuclease P1 and RNase T2 digestion (producing labeled Pi); lane 2: nuclease P1 digestion (producing pA>p̆); lane 3: RNase T2 digestion (producing Ap̆). Positions of various markers are indicated in **K**.

cleavage). Consequently these are present only in the precursor (Fig. 4A) and not in any of its endonuclease and ligase products (Table 1; Fig. 4). Further analyses of the corresponding spots in fingerprints (Fig. 4A) agrees with the location of the labels in these oligonucleotides (Fig. 5A–D).

Oligonucleotide 20 (CpApCpUpCpApUpA>p̂) is unique to the 5′ exon. This RNase T1 oligonucleotide forms the 3′ terminus of the exon. It should not contain Gp, but rather it should contain labeled 2′,3′ cyclic phosphate at its 3′ end, which would be produced by the endonuclease reaction. This oligonucleotide is included within oligonucleotide 17 of the precursor (Table 1). The labeled nucleotide produced by RNase T2 digestion of oligonucleotide 20 is $A_{3'}$p̂ (Fig. 5E,K, lane 3), where 2′,3′ cyclic phosphate is converted to a 3′-phosphate. Its mobility is different from $A_{2'}$p (see Fig. 5K, lane 3), which would be the expected product if oligonucleotide 20 had a 2′-phosphate at the terminus. Radioactive product of the nuclease P1 digestion of this oligonucleotide (Fig. 5F,K, lane 2) is an adenosine bisphosphate (can be either pA>p̂ or $pA_{2'}$p̂, but not $pA_{3'}$p̂), where 3′-phosphatase activity of the nuclease P1 cannot remove the terminal labeled phosphate. This radioactive product is not $pA_{2'}$p̂, as it runs slower than the $pA_{2'}$p marker (Fig. 5K, lane 2). Although we did not have an authentic pA>p marker, we consider the labeled nuclease P1 product (Fig. 5FK, lane 2) to be pA>p̂ by comparing its mobility with the other markers. As shown in Figure 5K, among the adenosine monophosphate markers, $A_{2'}$p runs faster than $A_{3'}$p and A>p runs slower than both. Similarly among the adenosine bisphosphates, $pA_{2'}$p runs faster than $pA_{3'}$p. The radioactive spot in lane 2 of Fig. 5K runs slower than both bisphosphates and can be pA>p̂. Furthermore, addition of a 5′-phosphate to $A_{2'}$p and $A_{3'}$p (making $pA_{2'}$p and $pA_{3'}$p, respectively) increases their mobilities in this system, that is, bisphosphates run faster than their corresponding monophosphates. Therefore, the radioactive spot in Fig. 5K, lane 2, running faster than the marker A>p is consistent with it being pA>p̂. The streaking of this radioactive product (pA>p̂) in Figure 5F seems to be due to the presence of 2′,3′ cyclic phosphate in this adenosine bisphosphate. Corresponding cyclic monophosphate (A>p) also streaks under these conditions, whereas none of the noncyclic adenosine monophosphates and bisphosphates streak in this chromatographic system (not shown). Figure 5K, lane 1, shows labeled Pi produced by digestion of oligonucleotide 20 with a combination of nuclease P1 and RNase T2. Here 3′-phosphatase activity of nuclease P1 removes the terminal phosphate after the 2′,3′ cyclic phosphate is converted to 3′-phosphate by RNase T2. Therefore, these RNase T2 and nuclease P1 digestions, independently and in combination, confirm that oligonucleotide 20 does contain a labeled 2′,3′ cyclic phosphate and terminates in −A>p̂.

Oligonucleotide 19 (ApUp̂Gp) is derived from the 5′ end of the 3′ exon. It is included within oligonucleotide 10 of the precursor (Table 1). The labeled products of its digestions are as expected (Fig. 5G,H).

Oligonucleotide 21 (CpApCpUpCpApUpAp̂ApUp̂Gp) is present in the spliced RNA (Fig. 4E; Table 1) and is created at the junction of the two exons (Fig. 1B). It is formed by the joining of oligonucleotides 20 and 19. Therefore, it is not present in the precursor or its endonuclease products. Digestion of this oligonucleotide with RNase T2 produces labeled Ap and Up (Fig. 5I) and, with nuclease P1, produces labeled pA and pG (Fig. 5J). This is possible only if the 2′,3′ cyclic phosphate of the 5′ exon, which is labeled in our system, is the source of the junction phosphate. No other oligonucleotide shown in Table 1 can produce this pattern of labeling. If the junction phosphate in the spliced RNA is derived from an external source, the labeled product of nuclease P1 digestion of this oligonucleotide would be only pG and that of RNase T2 digestion would be only Up. The amounts of radioactivity in Ap and Up (Fig. 5I) spots were about equal. This was also the case for pA and pG (Fig. 5J) spots. This is expected here, if oligonucleotide 21 included oligonucleotides 20 and 19 in 1:1 ratio.

Most of the spots are common to the fingerprints in Figure 4B (a mixture of the two unligated exons) and Figure 4E (ligated exons producing spliced tRNA). The only differences, as expected, are the presence of oligonucleotides 19 and 20 in Figure 4B and the presence of oligonucleotide 21 in Figure 4E. Oligonucleotides 19 and 20 present in the exon mixture are derived from the exon termini generated by the endonuclease reaction, whereas oligonucleotide 21 of the spliced tRNA is formed by the ligation of these two oligonucleotides.

DISCUSSION

As reported earlier (Gomes & Gupta, 1997), effects of cations on *H. volcanii* intron endonuclease and ligase activities are different. Endonuclease activity requires divalent cations and is inhibited by monovalent cations (Thompson & Daniels, 1988). Ligase activity does not need divalent cations and is abolished in monovalent cation-depleted extracts. Therefore, the ligase reaction is done in two steps (Gomes & Gupta, 1997). The transcript is first cleaved by the endonuclease, the products are purified, and then ligase reaction is done. Using these procedures our crude ligase preparations can ligate exons of both elongator $tRNA^{Met}$ (Gomes & Gupta, 1997; this work) and $tRNA^{Trp}$ (our unpubl. results). These are the only *H. volcanii* tRNA genes where introns have been found (Daniels et al., 1985; Datta et al., 1989). Therefore, it is expected that the mechanism of ligation for the two cases of *H. volcanii* tRNA exons would be the same.

A derivative of *H. volcanii* pre-$tRNA^{Met}$ was used in this study because of the presence of specific natural

sequences at the 5′ splice site (ApG) of the precursor, and at the spliced junction (ApA) of the ligated RNA. Corresponding natural sequences in $tRNA^{Trp}$ (both of which are GpA) were not useful in this study. Transcripts of a partial intron-deleted derivative of $tRNA^{Met}$ were used here, because full-sized intron-containing transcripts of this tRNA are not cleaved by the endonuclease (Gomes & Gupta, 1997). This seems to be due to improper folding of the in vitro-generated transcripts that contain complete intron.

In this report, we have shown that the splice junction phosphate in *H. volcanii* spliced tRNA is derived from the 2′,3′ cyclic phosphate present at the 3′ end of the 5′ exon. As is the case for the animal cell type systems, the junction phosphate preexists in the transcripts. This is different from the yeast-type systems, where this phosphate is derived from exogenous GTP. However, this does not rule out the possibility of existence of a second yeast-type ligase in *H. volcanii* cells, although it is not detectable in our system. Animal cells do contain two types of ligases.

Our RNA fingerprinting analyses of the oligonucleotides present at the 3′ end of the 5′ exon agrees with the previous report (Thompson & Daniels, 1988) that *H. volcanii* intron endonuclease produces a 2′,3′ cyclic phosphate terminus. In principle, no external source of energy is needed to convert this 2′,3′ cyclic phosphodiester linkage into a 3′,5′-phosphodiester linkage at the splice junction. This is consistent with the previous report (Gomes & Gupta, 1997) where ligase activity in *H. volcanii* cell extracts did not require any divalent cations or exogenously added source of energy. In this respect *H. volcanii* ligase differs from the animal pathway RNA ligase of HeLa cells that requires ATP or dATP and a divalent cation for activity (Perkins et al., 1985). Lack of the need for an added energy source for ligase reaction may suggest that the reaction observed in our system may really be a reversal of the cleavage by the splicing endonuclease present in the crude extracts. However, the possibility of this type of reaction is very low, because, as mentioned above, effects of monovalent and divalent cations on the stability and activity of the endonuclease and ligase are very different. Furthermore, *H. volcanii* endonuclease requires the presence of archaeal BHB structure in the precursor (Thompson & Daniels, 1990), whereas *H. volcanii* ligase can even join the gel-purified exons (Gomes & Gupta, 1997).

Kjems and Garrett (1988) have shown that the splicing endonuclease in the cell extracts of archaeon *Desulfurococcus mobilis* produces 3′-phosphate and 5′-hydroxyl termini, although 3′-phosphate derived from 2′,3′ cyclic phosphate by a cyclic phosphodiesterase present in the extract could not be excluded. In such a case, an RNA cyclase activity, like that found in animal cells (Filipowicz et al., 1983), might be needed to convert 3′-phosphate to 2′,3′ cyclic phosphate. Proteins similar to human RNA 3′-terminal phosphate cyclase are present in eukarya, bacteria, and archaea (Genschik et al., 1997).

In our experiments the phosphodiester bond formed at the splice junction can be cleaved by both RNase T2 and nuclease P1. This implies that the splice junction phosphate is in 3′,5′-phosphodiester linkage and not in 2′,5′-phosphodiester linkage. Furthermore, there is no 2′-phosphomonoester present along with 3′,5′-phosphodiester linkage, as is the case for yeast ligase. The RNA ligase activity in our *H. volcanii* cell extracts is different from the 2′,5′-RNA ligase found in several bacteria (Greer et al., 1983a; Arn & Abelson, 1996, 1998) that can join certain yeast tRNA exons by a 2′,5′-linkage. Several archaeal genomes contain homologs of bacterial 2′,5′-RNA ligase (Arn & Abelson, 1996, 1998). It is not known whether *H. volcanii* also has this 2′,5′-linking enzyme.

MATERIALS AND METHODS

Organism and growth

H. volcanii strain DS 2 was grown at 40–42 °C, aerobically, by vigorous shaking as described previously (Gupta, 1984).

RNA substrates

Plasmid pHVMΔi36 (Gomes & Gupta, 1997) was used to prepare the DNA for in vitro T7 RNA polymerase transcription. It produces a partial intron-deleted derivative of *H. volcanii* elongator pre-$tRNA^{Met}$. The plasmid was either digested with *Pst*I or PCR-amplified using primers TAATACGACTCAC TATAGCCCGGGTGGCTTAGC and TGCCCGGGGTGGGC TCCGAAC. 3′-end-labeled transcripts were prepared as described previously (Gomes & Gupta, 1997). Internally labeled transcripts using [α-^{32}P]GTP were prepared according to Milligan and Uhlenbeck (1989). A 100-μL reaction contained 10 μg of *Pst*I-digested pHVMΔi36 DNA (or varying amounts of PCR-amplified DNA), 5 μL of transcription buffer (200 mM Tris-Cl, pH 8.0, 40 mM $MgCl_2$, 150 mM DTT, 10 mM spermidine, 0.05% Triton X-100, 250 μg/mL bovine serum albumin), 0.75 mM each of ATP, UTP, and CTP, 0.3 mM GTP, 2 mM GMP, 100 μCi [α-^{32}P]GTP (ICN, sp. act. >3,000 Ci/mmol), and 0.12 μg T7 RNA polymerase (prepared as mentioned in Gomes & Gupta, 1997). Reactions were performed at 37 °C for 3 h. The transcripts were purified by polyacrylamide gel electrophoresis. Inclusion of excess unlabeled GMP in the reaction caused initiation of most of the transcripts with unlabeled GMP and increased the yield of full-length transcripts. The transcript of the PCR-amplified DNA (as shown in Fig. 1) has an extra A residue on the 3′ end when compared with the transcript of *Pst*I-digested pHVMΔi36 DNA. Labeled transcripts were purified by electrophoresis in denaturing (8.0 M urea, 89 mM Tris-borate, 2.5 mM EDTA) 6% polyacrylamide gels (20 × 20 × 0.08 cm), eluted and extracted with phenol/chloroform/isoamylalcohol (25/24/1). Approximate amounts of the labeled transcripts were estimated by Cerenkov counting.

Enzyme preparations

Cell extracts containing endonuclease and ligase activities were prepared according to Gomes & Gupta (1997) with some modifications. All of the following procedures were done at 4 °C unless stated otherwise. *H. volcanii* cells were grown to an A_{550} not exceeding 0.6 and harvested. For endonuclease preparations, the cell pellet was resuspended in 3 vol (w:v) of buffer A (40 mM Tris-Cl, pH 7.5, 20 mM $MgCl_2$, 0.5 mM EDTA, 10% glycerol) and passed twice through a French Pressure cell at 8,000 kPa. The cell extract was cleared by centrifugation at 10,000 × *g* for 10 min and then desalted by passing through a Sephadex G-25 column equilibrated with buffer A. Fractions containing endonuclease activity were pooled and stored at −70 °C. For ligase preparations, the cell pellet was resuspended in 2 vol (w:v) of buffer B (50 mM Na-HEPES, pH 7.5, 1.0 mM EDTA, 1.0 M KOAc, 0.5 mM DTT, 0.5 mM PMSF, 10% glycerol). The cells were lysed by passing twice through a French Pressure cell at 138,000 kPa and the lysate cleared by centrifugation at 12,000 × *g* for 15 min. Ligase enzyme preparation was stored at −70 °C. Recombinant *H. volcanii* endonuclease was prepared according to Kleman-Leyer et al. (1997).

Endonuclease and ligase reactions

Standard endonuclease and ligase reactions were done as described previously (Gomes & Gupta, 1997). These are separate reactions using two different enzyme preparations. First an endonuclease reaction is done in low-salt conditions and the products are phenol extracted and ethanol precipitated. These products are then used for ligase reaction under high-salt conditions. Endonuclease reactions contained 20,000 cpm (Cerenkov) end-labeled or 400,000 cpm internally labeled gel purified transcripts per 100-μL reaction. Ligase reactions contained 10,000 cpm end-labeled or 100,000 cpm internally labeled endonuclease reaction products per 100-μL reaction. For RNA fingerprinting analyses, the reaction products were separated by denaturing polyacrylamide gel electrophoresis either in 6% gel (20 × 20 × 0.08 cm) or in 15% gel (20 × 40 × 0.15 cm) and eluted as needed. Recombinant endonuclease was used, as described (Kleman-Leyer et al., 1997), in certain reactions to prepare exons and introns for RNA fingerprinting.

RNase T1 digestion of the substrates

Usually 2 μL of gel-eluted RNA samples in RNase T1 buffer (50 mM Tris-Cl, pH 7.5, 1 mM EDTA) were mixed with 1 μL of 1,000 U/μL RNase T1 (cloned; Ambion) in a glass capillary and incubated at 37 °C for 1 h. However, labeled ligated tRNA samples eluted from the gels include excess unlabeled tRNAs. These tRNAs are derived from the cell extracts in the reaction and they run with the labeled product in the gels. Therefore, in some cases, 2 μL of ligated tRNA samples were digested with 4 μL of RNase T1 for 1 h and then for another 30 min with an additional 1 μL of RNase T1. This reduced the appearance of incomplete digestion products in the fingerprints.

RNA fingerprinting

RNA fingerprinting was done by modifications of certain published procedures (Domdey & Gross, 1979; Stackebrandt et al., 1985; Branch et al., 1989).

The first dimension of fingerprinting was done in an ultrathin (20 × 40 × 0.04 cm) acid-urea (8.0 M urea, 0.025 M citric acid, pH 3.5) 10% polyacrylamide gel (Branch et al., 1989). RNase T1-digested RNA samples were mixed with two vol of 1.5× citrate dye (9 M urea, 0.0375 M citric acid, pH 3.5, 1% xylene cyanol FF, 0.5% acid fuchsin, 1% methyl orange), loaded onto the gel, and run at constant volts (1,300 V) until the yellow methyl orange and the blue xylene cyanol dyes were 16 cm apart. The gel was autoradiographed and the desired part of the gel (a 3- × 21-cm strip) was cut out. Oligonucleotides were transferred from the gel strip onto 20- × 40-cm flexible-backed DEAE-cellulose (Machery-Nagel, Polygram Cel 300 DEAE) thin layer plate essentially as described (Branch et al., 1989).

The second dimension of fingerprinting was done according to Domdey & Gross (1979), with some modifications. The DEAE-cellulose plate with transferred oligonucleotides was pre-run in 1 mM EDTA to just above the transfer line (usually about 15 cm) at room temperature and the remaining part was wetted using 1 mM EDTA-soaked Whatman 3MM paper. Such a pre-run and wetted DEAE-cellulose plate was developed in the second dimension in ammonium formate gradient, ranging from 0.3 M to 0.4 M, at 63 °C as described (Domdey & Gross, 1979). The plate was dried and autoradiographed.

Characterization of oligonucleotides and analyses by thin layer chromatography

A DEAE-cellulose plate was first aligned with the autoradiogram, and the regions corresponding to the fingerprint spots were marked on the plate. Then DEAE-cellulose material was scratched off the appropriate region of the plate, washed with 70% ethanol to remove urea, and dried. The oligonucleotide was then eluted from the DEAE-cellulose using 30% triethylammonium carbonate, pH 10.0 (Gupta, 1984), dried under vacuum, and digested with RNase T2 (an RNase T1/T2 preparation made according to Lichtler et al., 1992) or with nuclease P1 (Sigma) or a combination of the two enzymes. Gel-eluted RNA samples were also similarly digested. Typically the digests were resolved by two-dimensional thin layer chromatography on cellulose plates (Machery-Nagel, Polygram Cel 300) using solvent a (isobutyric acid/0.5 N NH_4OH, 5:3, v/v) in the first dimension and solvent b (isopropanol/HCl/H_2O, 70:15:15, v/v/v) in the second dimension (Nishimura, 1979; Gupta, 1984). RNase T2 or nuclease P1 digests of yeast total RNA were used as the source of unlabeled markers. In some cases, the digests were resolved by chromatography on cellulose plates using solvent c (saturated $(NH_4)_2SO_4$/3 M NaOAc/isopropanol, 80:6:2, v/v/v) in one dimension only (Genschik et al., 1997). Unlabeled markers (Sigma) were run in parallel. The plates were dried and positions of the markers were outlined under UV light, followed by autoradiography of the plates. In some cases the resolved spots were quantitated by a Packard Cyclone phosphorImager.

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