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Conserved Mechanism of tRNA Splicing in Eukaryotes

MARTIN ZILLMANN,[1]* MARTIN A. GOROVSKY,[1] AND ERIC M. PHIZICKY[2]

Department of Biology, University of Rochester, Rochester, New York 14627,[1] and Department of Biochemistry, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642[2]

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The ligation steps of tRNA splicing in yeast and vertebrate cells have been thought to proceed by fundamentally different mechanisms. Ligation in yeast cells occurs by incorporation of an exogenous phosphate from ATP into the splice junction, with concomitant formation of a 2′ phosphate at the 5′ junction nucleotide. This phosphate is removed in a subsequent step which, in vitro, is catalyzed by an NAD-dependent dephosphorylating activity. In contrast, tRNA ligation in vertebrates has been reported to occur without incorporation of exogenous phosphate or formation of a 2′ phosphate. We demonstrate in this study the existence of a yeast tRNA ligase-like activity in HeLa cells. Furthermore, in extracts from these cells, the entire yeastlike tRNA splicing machinery is intact, including that for cleavage, ligation, and removal of the 2′ phosphate in an NAD-dependent fashion to give mature tRNA. These results argue that the mechanism of tRNA splicing is conserved among eukaryotes.

Splicing of intron-containing pre-tRNAs has been studied extensively in *Saccharomyces cerevisiae*, *Xenopus laevis*, wheat germ, and humans. Splicing always involves endonucleolytic excision of the intron, located one base 3′ of the anticodon, by a specific endonuclease and subsequent ligation of the resultant half molecules.

Splicing is initiated by an endonuclease that is similar in yeast and vertebrate cells. In yeast cells, this reaction is catalyzed by a membrane-associated enzyme that recognizes and binds a specific structure within the mature domain of the pre-tRNA and cleaves at a fixed distance from the point of binding (25). The nature of the intervening sequence is relatively unimportant, as long as the mature domain is unaltered and the 3′ splice site is exposed in a single-stranded region (9, 14, 29, 30). The tRNA endonuclease of *X. laevis* is also known to recognize the mature domain of pre-tRNA and cleave a fixed distance away from where it binds (16). Endonuclease cleavage in yeast (20), wheat germ (28), and vertebrate (2, 5, 6) cells generates a 5′ half molecule terminating with a 2′,3′ cyclic phosphodiester and a 3′ half molecule beginning with a 5′ hydroxyl, suggesting conservation of the endonuclease reaction among eukaryotes.

Ligation has been characterized most extensively in yeast cells, in which the half molecules are joined in an ATP-dependent reaction by tRNA ligase, a 95-kDa enzyme with several different activities (1, 8, 22). The cyclic phosphate is opened by a 2′,3′ cyclic phosphodiesterase activity to create a 2′ phosphate, leaving a free 3′ OH terminus on the 5′ half molecule. A phosphate derived from ATP is added to the 5′ OH of the 3′ half molecule by the kinase activity; this added phosphate will form the splice junction in the mature tRNA (Fig. 1A, step 1). The halves are then joined in a reaction that uses another molecule of ATP (Fig. 1A, step 2), resulting in a mature-size tRNA bearing a 2′ phosphate at the splice junction. This ligase is likely responsible for tRNA splicing in vivo, because lack of the protein is correlated with accumulation of unligated half molecules (21a). A ligase with similar activities also has been described in wheat germ (11, 12, 23, 24) and in *Chlamydomonas* spp. (10, 31), suggesting that this ligation mechanism is in widespread use. In *Saccharomyces cerevisiae*, the 2′ phosphate remaining at the splice junction is efficiently removed in vitro by an NAD-dependent, 2′ phosphate-specific dephosphorylating activity (Fig. 1A, step 3) that has recently been partially purified (17, 18).

The ligation reaction in vertebrates appears to be different from that in *S. cerevisiae* (Fig. 1A). Yeast pre-$tRNA^{Tyr}$ is accurately transcribed and processed after microinjection into *Xenopus* oocyte nuclei. In most of the spliced transcripts, the junction phosphate was shown to be derived from within the phosphate backbone (19), suggesting a ligation mechanism different from that of *S. cerevisiae*. Experiments with HeLa cell extracts yielded similar results (5, 13). When yeast pre-$tRNA^{Ser}$ and *Xenopus* pre-$tRNA^{Tyr}$ were transcribed and subsequently processed in these extracts, the spliced tRNAs that were detected contained junction phosphates derived from the precursor. Although in one study 50% of the nonligated 3′ half molecules were 5′ phosphorylated in the presence of ATP (5), indicative of the first step of splicing by a yeastlike ligase, it was concluded that these molecules were "dead-end" intermediates that arose from a kinase activity in the extract. The ligase implicated in these in vivo and in vitro studies of vertebrate splicing was first characterized in HeLa cell extracts, although it is known to exist in extracts from *Xenopus* oocyte nuclei as well. This ligase has an estimated molecular weight of 160 kDa and is capable of ligating various RNAs bearing 5′ hydroxyl and 2′,3′ cyclic phosphate termini in an ATP-requiring reaction (4, 21). Little is known about the mechanism of action of vertebrate ligase, its intracellular location, or its in vivo substrate(s), since a variety of RNAs in addition to tRNA halves have been found to be acceptable substrates in vitro.

Because no yeast tRNA ligase-like activity has been observed in vertebrates and the end requirements (2′,3′ cyclic phosphate and 5′ hydroxyl) of the HeLa cell ligase are met by cleavage of tRNA precursors into halves by HeLa endonuclease (5), it has been suggested (5, 13) and generally accepted that this HeLa cell type of ligase joins tRNA halves in vivo in vertebrates. However, it is also possible that a yeast tRNA ligase-like enzyme operates in vivo in vertebrates but has escaped detection. Data obtained from experiments done with isolated mouse cell nuclei suggest that a

* Corresponding author.

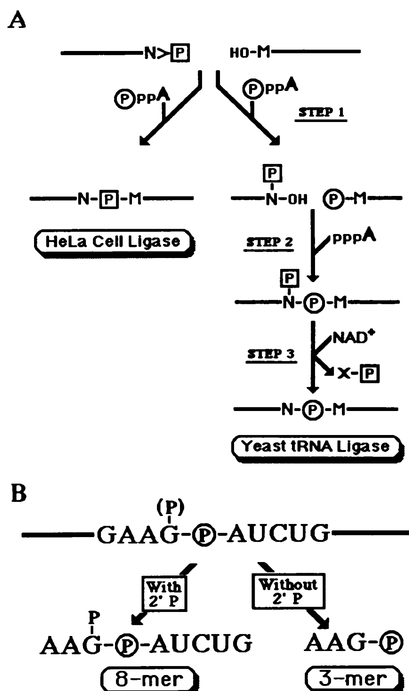

FIG. 1. Detection of yeastlike activities during splicing. (A) Analysis of ligation. tRNA endonuclease in HeLa cells or in yeast cells generates half molecules bearing a 3' 2',3' cyclic phosphodiester and a 5' hydroxyl terminus, as shown at the top. HeLa ligase conserves the phosphate originally present at the 3' terminus of the 5' half molecule (squared phosphate), whereas the yeast tRNA ligase adds a phosphate from ATP (circled phosphate) to form the splice junction while opening the 3' terminal cyclic phosphate (squared) to the 2' position. Removal of the 2' phosphate by an NAD-dependent activity completes splicing in yeast extracts. The product of each splicing pathway can then be distinguished only by the source of the junction phosphate. (B) Assay for the splice junction 2' phosphate. $tRNA^{Phe}$ spliced in the presence of [γ-^{32}P] ATP produces a mature tRNA bearing a radioactive (circled) splice junction phosphate and, in the absence of dephosphorylation, a 2' phosphate. The sequence surrounding the junction is shown, but only relevant phosphates are indicated. When tRNA bearing this phosphate is digested with RNase T_1, a radiolabelled 8-mer containing an uncleaved G residue would be expected. With prior removal of the 2' phosphate from the internal G residue, a radiolabelled 3-mer would be expected.

yeast tRNA ligase-like activity may function in vertebrates. Mouse L-cell nuclei incubated with [γ-^{32}P]ATP incorporate radiolabelled phosphates not only at the 5' termini (phosphatase sensitive) of many RNAs but also internally (phosphatase insensitive) in RNAs comigrating with mature tRNA (33). This observation is consistent with a splicing mechanism similar to that of yeast cells, although other explanations are also possible.

The apparent conservation of the endonuclease reaction between organisms as diverse as yeasts and vertebrates seems at odds with the apparent divergence of the ligase reaction. In this report, we demonstrate that extracts from HeLa cells can in fact ligate tRNA halves, utilizing the same overall reaction as that of yeast tRNA ligase. Furthermore, we demonstrate that these extracts, like those from yeast cells, contain an NAD-dependent activity capable of efficiently removing the 2' phosphate created at the splice junction during ligation, producing mature tRNA. These data lead to the satisfying conclusion that the tRNA ligase reaction is as evolutionarily conserved as the endonuclease reaction, making it likely that the yeast-type tRNA splicing pathway is universally retained in eukaryotes.

MATERIALS AND METHODS

Reagents and buffers. Calf intestinal phosphatase, RNase T_1, cobra venom phosphodiesterase, RNase T_2, and nuclease P1 were from Boehringer Mannheim. Isotopes (specific activity, 3,000 Ci/mmol) were obtained from Dupont/NEN. Roeder buffer D contains 20 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.5 mM EDTA, 1 mM dithiothreitol (DTT), and 20% glycerol. tRNA splicing buffer contains 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2.5 mM spermidine-HCl, 0.1 mM DTT, 0.4% Triton X-100, 10% glycerol, and 2 mM ATP. Urea buffer contains 5 mM Tris-HCl (pH 8.0), 175 mM NaCl, 5 mM EDTA, 0.5% sodium dodecyl sulfate, and 7 M urea.

Preparation of extracts. HeLa nuclear extract was prepared from log-phase spinner cultures of HeLa cells essentially as described by Dignam et al. (3). Aliquots of extract were frozen in liquid nitrogen and stored at −80°C. *Xenopus* oocyte S150 extract was prepared as previously described (27) and was the kind gift of Alison Bertuch.

Heparin agarose fractionation of HeLa extract. One milliliter of extract (approximately 5 mg of protein) was loaded onto a 5-ml column of heparin agarose (Bio-Rad) equilibrated in Roeder buffer D without KCl. The column was washed with 5 ml of the same buffer, and proteins were eluted with 5 ml of Roeder buffer D containing 0.5 M KCl. The extract was dialyzed against two 250-ml changes of Roeder buffer D and stored at −80°C.

In vitro incorporation of γ-^{32}P into yeast $tRNA^{Phe}$. $tRNA^{Phe}$ transcripts were synthesized from a construction made by Reyes and Abelson (25), using T7 polymerase. Approximately 5 μg of pre-$tRNA^{Phe}$ was produced from the transcription of 2 μg of plasmid with 200 U of T7 polymerase.

Cleavage and ligation of tRNA were carried out in tRNA splicing buffer (without unlabelled ATP) for 1 h at 30°C in a 100-μl volume containing 50 μl of extract, 600 μCi of [γ-^{32}P]ATP (1 μM, final concentration), and about 200 ng of unlabelled pre-$tRNA^{Phe}$. To stop the reaction, 400 μl of urea buffer was added, and the reaction mixtures were phenol-$CHCl_3$ extracted, $CHCl_3$-isoamyl alcohol (24:1) extracted, and ethanol precipitated after the addition of 1 μg of glycogen carrier. RNA pellets were resuspended in 15 μl of formamide loading dye, boiled, and chilled on ice prior to being loaded onto a 10% polyacrylamide-bisacrylamide (30:1) sequencing-length denaturing preparative gel. After electrophoresis, RNA was visualized by brief autoradiography, and the region of the gel containing mature tRNA-size RNA was excised. RNAs were eluted, ethanol precipitated, and washed in 70% ethanol.

Partially purified yeast endonuclease (a generous gift of Chris Greer) and ligase (22) were used to incorporate the γ phosphate of ATP into spliced $tRNA^{Phe}$ as described previously (17).

Removal of the 2' phosphate from the splice junction. Half of the radioactive RNA generated from the incorporation of radioactive phosphate was resuspended in 100 μl of CIP buffer (15) containing 100 U of calf intestinal phosphatase and incubated at 37°C for 1 h. Then 400 μl of urea buffer was added, and the reaction mixtures were extracted, precipitated, and gel purified as described above. Alternatively, the 2' phosphate was removed by using partially purified 2'

dephosphorylating activity from yeast cells (17). RNA was incubated in a 10-μl reaction mixture in tRNA splicing buffer (without ATP) containing 1 mM NAD and 1 μl of 2′ dephosphorylating activity at 30°C for 20 min. Assays of the dephosphorylating activity present in HeLa cell and *Xenopus* oocyte extracts were conducted in the same manner, in either the presence or absence of NAD.

RNase T_1 digestion of tRNA and electrophoresis of RNA fragments. RNase T_1 digestion of gel-purified, labelled RNA was carried out in 15 μl of TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) containing 5 U of RNase T_1 at 37°C for 1 h. Reaction mixtures were saturated with urea, and RNA fragments were resolved on a 20% polyacrylamide-bisacrylamide (20:1) denaturing gel. RNAs were detected by autoradiography.

Nuclease digestion and PEI-cellulose thin-layer chromatography. Radioactive RNA was digested in a 10-μl volume containing 1 μg of carrier tRNA and either 1 μg of nuclease P1, 1 U of RNase T_2, or 100 μg of cobra venom phosphodiesterase per ml. Digestions were carried out at 37°C for 2 h. For RNase T_2-nuclease P1 double digestion, the nuclease P1 was added after 1 h of RNase T_2 digestion at 37°C. Digestion was continued for an additional hour. The reaction mixtures were then extracted with 10 μl of phenol-$CHCl_3$, and 4 μl of the aqueous phase was spotted onto a polyethyleneimine (PEI)-cellulose plate (Sigma). The chromatogram was washed in methanol for 5 min and developed in 1 M LiCl or 1.2 M sodium formate (pH 3.5) until the solvent front reached the top of the thin-layer plate. Nonradioactive standards, prepared by the digestion of 10 μg of tRNA with either nuclease P1 or RNase T_2, were visualized by short-wave UV illumination.

RESULTS

Assay for yeast tRNA ligase-like activity in HeLa cell extracts. The pathways for tRNA ligation described to date in *S. cerevisiae* (8) and higher eukaryotes (5, 13) are fundamentally different (Fig. 1A). Both pathways begin with endonuclease-generated tRNA half molecules having 2′,3′ cyclic phosphate (squared in Fig. 1A) and 5′ hydroxyl termini. HeLa cell ligase joins the half molecules as shown, with conservation of the squared phosphate at the junction. Yeast tRNA ligase joins the half molecules by a more circuitous path. The γ phosphate from ATP (circled) is used to phosphorylate the 3′ half molecule, while the cyclic phosphodiester on the 5′ half molecule is hydrolyzed to the 2′ position (Fig. 1A, step 1). Ligation then proceeds by using another molecule of ATP (Fig. 1A, step 2). The net result of yeast ligase catalysis is the incorporation of the ATP-derived phosphate (circled) into the splice junction, with conversion of the original 2′,3′ cyclic phosphate to a 2′ phosphate (squared). This 2′ phosphate is then removed in an NAD-dependent fashion (18), leaving only the phosphate (circled) originating from ATP at the splice junction (Fig. 1A, step 3).

We have taken advantage of the difference in the products produced by the two types of ligases to assay HeLa cell extracts specifically for a yeast tRNA ligase-like activity, even in a large background of HeLa cell ligase activity. If only the circled phosphate is made radioactive by using [γ-^{32}P]ATP in the reaction, only the product of the yeast tRNA ligase-like activity will be detected. The presence of the expected 2′ phosphate can be assayed by digestion of the ligated tRNA with RNase T_1 (Fig. 1B). A radioactive 8-mer is formed in the presence of the 2′ phosphate, because the internal G residue bearing this phosphate is insensitive to cleavage. If this phosphate is removed, a radioactive 3-mer is produced.

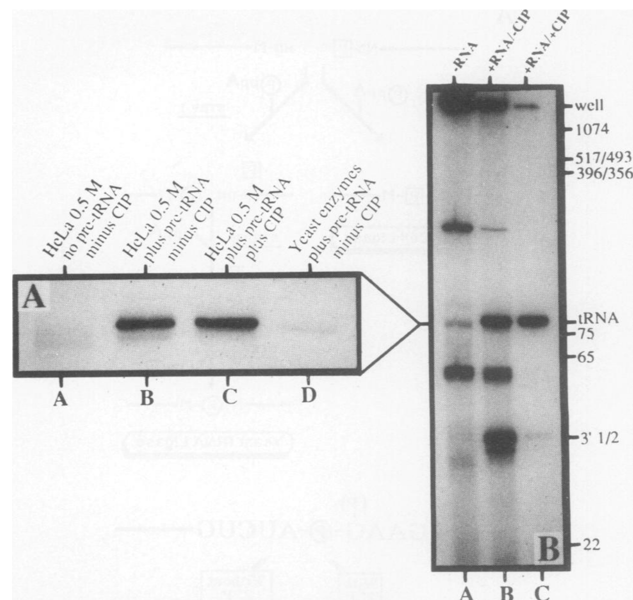

FIG. 2. RNA-dependent incorporation of [γ-^{32}P]phosphate from ATP into tRNA^{Phe}. (A) High-resolution analysis of radiolabel incorporated into tRNA-sized RNAs in HeLa cell extract. Fractionated HeLa extracts were incubated with [γ-^{32}P]ATP in the presence or absence of tRNA^{Phe} precursor as indicated. RNA was analyzed on a sequencing-length 10% denaturing polyacrylamide gel, a portion of which is shown. Lanes: A, no added RNA; B, unlabelled pre-tRNA^{Phe}; C, as lane B but treated with an excess of calf intestinal phosphatase (CIP); D, tRNA^{Phe} ligated by yeast enzymes. Exposure was for 1 h at −80°C with an intensifying screen. (B) Low-resolution analysis. A portion of the samples shown in panel A was also analyzed on a short 10% polyacrylamide denaturing gel. Relevant RNA species are indicated at right along with the mobilities of radiolabelled DNA markers (nucleotide lengths are given at right). Exposure was overnight at −80°C with an intensifying screen.

HeLa cell extracts ligate pre-tRNA^{Phe} with incorporation of the γ phosphate from ATP. When nonradioactive pre-tRNA^{Phe} was incubated in heparin agarose-fractionated HeLa cell nuclear extract in the presence of [γ-^{32}P]ATP, radiolabelled RNA having the mobility of tRNA^{Phe} was produced (Fig. 2). Appearance of this RNA was dependent on the addition of nonradioactive pre-tRNA^{Phe} (Fig. 2A, lanes A and B). Since the incorporated radioactivity was not removed by treatment with a large excess of calf intestinal phosphatase (Fig. 2A, lanes B and C), the label was likely at an internal location. Figure 2B shows a low-resolution gel of the samples in Fig. 2A. Several endogenous extract RNAs were radiolabelled in fractionated HeLa extract (lane A). Two bands were specifically produced by addition of unlabelled pre-tRNA^{Phe} (compare lanes A and B), one migrating at the position of mature tRNA^{Phe} and the other migrating at the position of 5′ phosphorylated 3′ half molecules. Upon treatment with calf intestinal phosphatase, radioactivity was lost from all RNA species other than that migrating as mature tRNA (compare lanes B and C). This finding argues that the endogenous extract RNAs and 3′ tRNA halves are labelled primarily at the termini, whereas ligated tRNA is labelled internally.

The splice junction formed in HeLa extracts is sensitive to RNase T_1 cleavage only after removal of the 2′ phosphate. To

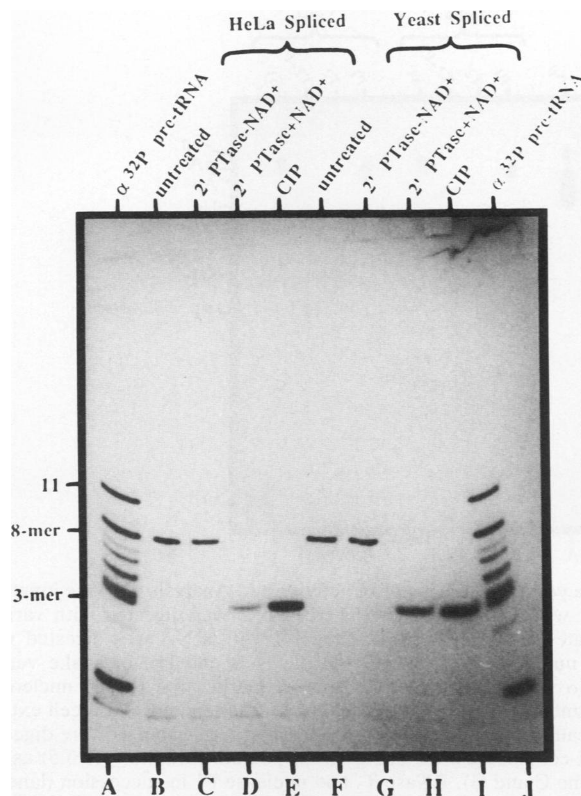

FIG. 3. Evidence that the internal G residue at the splice junction is sensitive to RNase T_1 digestion after dephosphorylation. $tRNA^{Phe}$ spliced in the presence of [γ-^{32}P]ATP either in fractionated HeLa cell extract (lanes B to E) or with yeast enzymes (lanes F to I) was digested with RNase T_1 and analyzed by electrophoresis on a 20% polyacrylamide denaturing gel. Lanes: B and F, untreated tRNA as in Fig. 2A, lane B; C, D, G, and H, tRNA treated with yeast dephosphorylating activity in the absence of NAD (C and G) or in the presence of 1 mM NAD (D and H); E and I, tRNA treated before isolation with excess calf intestinal phosphatase (CIP) as in Fig. 2A, lane C; A and J, uniformly [α-^{32}P]ATP-labelled pre-$tRNA^{Phe}$ digested with RNase T_1. PTase, phosphotransferase.

test whether the radioactivity incorporated into tRNA was the result of a yeastlike splicing mechanism, RNA obtained from the gel shown in Fig. 2A was analyzed by RNase T_1 digestion. The possible outcomes are shown in Fig. 1B. Modification at the junction by a 2′ phosphate was expected to yield an 8-mer, whereas pretreatment with phosphatases, either with a large excess of calf intestinal phosphatase or with the NAD-dependent 2′ dephosphorylating activity isolated from yeast cells, should result in a 3-mer. Both treatments are known to remove the 2′ phosphate from the splice junction (17). Digestion of tRNA cleaved and ligated in fractionated HeLa extract yielded the expected 8-mer (lane B). Treatment with 2′ dephosphorylating activity from yeast cells rendered the internal G residue in the 8-mer sensitive to RNase T_1 cleavage, resulting in the production of 3-mer; as expected, this occurred only in the presence of NAD (Fig. 3, lanes C and D). Since this activity has been shown to be highly selective for 2′ phosphates (17), it seems likely that the junction bears a 2′ phosphate. Similarly, treatment of the ligated tRNA with excess calf intestinal phosphatase and subsequent RNase T_1 cleavage also produced a 3-mer (Fig. 3, lane E). Analogous treatment of tRNA cleaved and ligated with purified yeast enzymes produced products identical in mobility to those produced from tRNA ligated in HeLa cell extract (Fig. 3, lanes F to I). All of these results are consistent with the presence of a 2′ phosphate at the splice junction of tRNA cleaved and ligated in fractionated HeLa cell extracts.

The structure of the ligation junction is identical for tRNA ligated in fractionated HeLa cell extract or with yeast enzymes. To determine the structure of the splice junction, tRNA ligated in the presence of [γ-^{32}P]ATP either in fractionated HeLa nuclear extract or by yeast enzymes was compared after digestion with various nucleases prior to or after treatment with excess calf intestinal phosphatase (Fig. 4A and B). This approach has been used previously to determine the nature of the splice junctions produced by yeast tRNA ligase (8) and wheat germ ligase (11, 26).

Ligation by a mechanism identical to that of yeast tRNA ligase is expected to yield the splice junction depicted in Fig. 1B, containing a 2′ phosphate (...pG^{P}_{p}Ap...). In the absence of phosphatase treatment, the splice junction should be resistant to cleavage by nuclease P1 or RNase T_2, producing either pG^{P}_{p}A or G^{P}_{p}Ap, respectively. Successive digestion with RNase T_2 and nuclease P1 would be expected to produce G^{P}_{p}A. Snake venom phosphodiesterase is capable of cleaving 3′ of the G residue, producing radiolabelled pA even in the presence of a 2′ phosphate. Because standards for these dinucleotides are not readily available, we compared the digestion products from tRNA cleaved and ligated in fractionated HeLa extract with those from tRNA cleaved and ligated with yeast enzymes.

Thin-layer analysis of $tRNA^{Phe}$ ligated without subsequent phosphatase treatment is shown in Fig. 4A. For each enzyme digestion, the mono- or dinucleotides produced from tRNA cleaved and ligated in fractionated HeLa cell extract (lanes B to E) comigrated with the corresponding digestion products obtained from tRNA spliced with yeast enzymes (lanes F to I).

Digestion of calf intestinal phosphatase-treated, radiolabelled $tRNA^{Phe}$, cleaved and ligated either in fractionated HeLa extract or with yeast enzymes, also gave the expected products upon thin-layer analysis (Fig. 4B). For both of the ligated tRNAs, digestion with either nuclease P1 or snake venom phosphodiesterase gave a radiolabelled species migrating at the position of pA (compare lanes B and E with lanes F and I). Similarly, treatment of either tRNA with RNase T_2 produced a radiolabelled species migrating at the position of Gp (lanes C and G), and successive digestion with RNase T_2 and nuclease P1 released a radioactive species migrating at the position of free phosphate (lanes D and H). These analyses unambiguously identify the junction created in fractionated HeLa cell extracts as -G^{P}_{p}A- and show that HeLa cells contain an activity capable of ligating intron containing tRNAs by the mechanism demonstrated for tRNA ligase from yeast cells.

Extracts from HeLa cells contain an NAD-dependent activity capable of removing the 2′ phosphate from the splice junction. If tRNA splicing in HeLa cells can proceed by the yeast pathway, there must be a mechanism for removing the 2′ phosphate that is generated at the junction during ligation. It is evident from the data in Fig. 3 and 4 that the vast majority of the ligated tRNA bearing a phosphate incorporated from ATP also bears a 2′ phosphate. Thus, there is no obvious 2′ dephosphorylating activity operating in these extracts during cleavage and ligation of this RNA. By analogy to the yeast system (17, 18), a possible reason for the failure of extracts to remove the 2′ phosphate from the splice

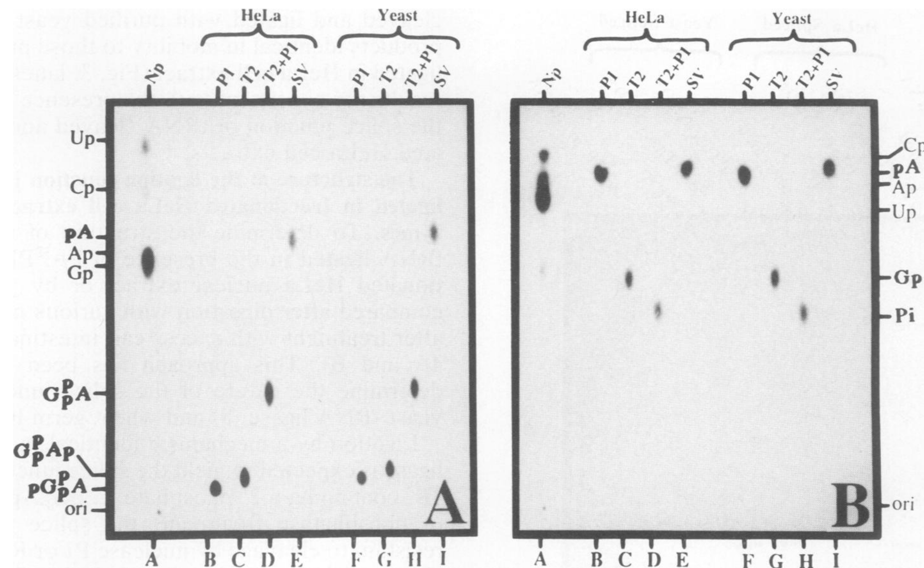

FIG. 4. Evidence that the splice junction has the structure expected from a yeast tRNA ligase-like activity. (A) Analysis of splice junction. $tRNA^{Phe}$ spliced either in fractionated HeLa cell extract (lanes B to E) or with yeast enzymes (lanes F to I) was digested with various nucleases and subjected to thin-layer chromatography on PEI-cellulose plates, using 1 M LiCl as the solvent. tRNA was digested with nuclease P1 (lanes B and F), RNase T_2 (lanes C and G), RNase T_2 and nuclease P1 in succession (lanes D and H), or snake venom phosphodiesterase (SV; lanes E and I). Lane A, RNase T_2 digest of [α-^{32}P]ATP-labelled RNA as a marker for the 3' nucleotide monophosphates. (B) Analysis of the splice junction after phosphatase treatment. $tRNA^{Phe}$ spliced either in fractionated HeLa cell extract (lanes B to E) or with yeast enzymes (lanes F to I) was treated with excess calf intestinal phosphatase and analyzed. Samples were digested with various nucleases and subjected to thin layer-chromatography on PEI-cellulose plates, using 1.2 M sodium formate (pH 3.5) as the solvent. tRNA was digested with nuclease P1 (lanes B and F), RNase T_2 (lane C and G), RNase T_2 and nuclease P1 in succession (lanes D and H), or snake venom phosphodiesterase (lanes E and I). Lane A, RNase T_2 digest of [α-^{32}P]ATP-labelled RNA as a marker for the 3' nucleotide monophosphates.

junction is the absence of NAD from splicing reactions. We therefore tested whether the addition of NAD could stimulate removal of the 2' phosphate from ligated tRNA in HeLa extracts.

To assay extracts for their ability to remove the 2' phosphate from the splice junction, tRNA spliced with yeast enzymes in the presence of [γ-^{32}P]ATP was incubated in HeLa cell nuclear extracts in the presence or absence of NAD. Dephosphorylating activity was measured by subsequent analysis of the RNase T_1 digestion products (17). Retention of the 2' phosphate is expected to produce a radiolabelled 8-mer, while its removal is expected to produce a 3-mer (Fig. 1B and 3). As shown in Fig. 5 (lanes F to I), both of the fractionated HeLa extracts that were used during the course of our experiments could efficiently remove the splice junction 2' phosphate, but only in the presence of NAD. Furthermore, as shown in lanes B and C, the original (unfractionated) nuclear extract could also efficiently remove the 2' phosphate only in the presence of NAD, indicating that this activity is the principal 2' dephosphorylating activity in this extract. Lanes J and K demonstrate that a similar NAD-dependent dephosphorylating activity is present in *Xenopus* oocyte S150 whole cell extracts. Thus, HeLa cell extracts contain enzymes that catalyze all three steps of tRNA splicing (cleavage, ligation, and 2' phosphate removal) as they occur in *S. cerevisiae*.

DISCUSSION

Using the incorporation of exogenous phosphate from [γ-^{32}P]ATP into the phosphodiester backbone of ligated tRNA as an assay, we have demonstrated the conservation of all of the activities implicated in yeast tRNA splicing in extracts from HeLa cells. These extracts were shown to be capable of ligating tRNA halves created by endogenous endonuclease, using the γ phosphate of ATP to form the phosphodiester bond at the splice junction, with concomitant formation of a 2' phosphate. The splice junction so produced was shown by extensive comparison of nuclease digestion products to be indistinguishable from that described for purified yeast tRNA ligase (Fig. 3 and 4). Consistent with these results, we have found that HeLa cell nuclear extracts contain a protein that cross-reacts with a polyclonal serum raised against purified yeast tRNA ligase upon Western immunoblot analysis; this protein has approximately the same molecular weight as yeast tRNA ligase (data not shown). Demonstration of yeast tRNA ligase-like activities in organisms as diverse as humans (this report), wheat germ (10–12), and *Chlamydomonas* sp. (10, 31) argues strongly that the yeastlike tRNA ligation mechanism is highly conserved in all eukaryotes.

Extracts from both HeLa cells and *Xenopus* oocytes, like those from yeast cells (17, 18), contain an NAD-dependent activity capable of removing the 2' phosphate formed at the splice junction (Fig. 5). The yeast enzyme has been implicated in tRNA splicing because of its high degree of specificity for substrates bearing a 2' phosphate and by the absence of other activities that can efficiently catalyze this dephosphorylation. The presence of an NAD-dependent dephosphorylating activity in both HeLa and *Xenopus* oocyte extracts argues that this activity is the same as that in yeast cells. The specificity and catalytic properties of the dephosphorylating enzyme from HeLa cells are currently under investigation.

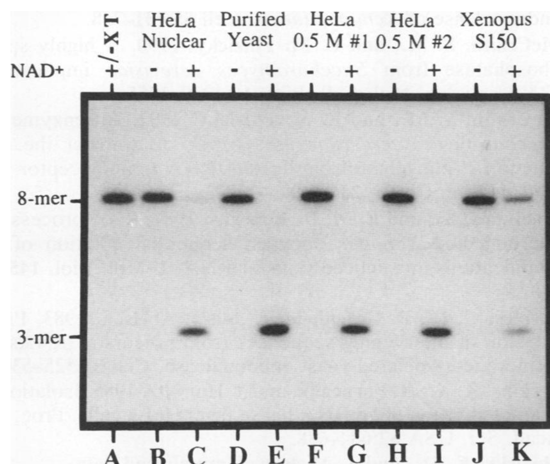

FIG. 5. Evidence that extracts from HeLa cells and *Xenopus* oocytes contain an NAD-dependent 2′ dephosphorylating activity. $tRNA^{Phe}$ labelled exclusively at the splice junction by yeast enzymes (see Materials and Methods) was incubated with various extracts in the presence or absence of 1 mM NAD as indicated. tRNA was then cleaved with RNase T_1, and the resulting oligonucleotides were resolved on a 20% polyacrylamide denaturing gel. Lanes: A, no added protein; B and C, extensively dialyzed HeLa cell nuclear extract; D and E, partially purified yeast 2′ dephosphorylating activity; F to I, the 0.5 M KCl heparin agarose fractions used throughout; J and K, extensively dialyzed *Xenopus* oocyte whole cell extract.

The presence in HeLa cells of both HeLa cell RNA ligase (5, 13) and a yeastlike ligation/dephosphorylation pathway, as demonstrated here, raises the question of what the yeastlike enzymes are doing in HeLa cells. The vast majority of spliced tRNA generated in HeLa extracts comes from the HeLa pathway (5, 13), and our comparison of the ratio of activities of the two ligation pathways in unfractionated nuclear extracts confirm this result (data not shown). Furthermore, microinjection experiments in *X. laevis* oocytes demonstrate that a majority of tRNA splicing occurs by the HeLa pathway (19). However, the conservation of the yeastlike splicing machinery in HeLa cells argues on evolutionary grounds that at least some tRNA splicing in vivo must occur by the yeast pathway. Several points, described below, attempt to justify this hypothesis.

First, experiments in HeLa extracts demonstrating that most ligation of tRNAs occurs by using the HeLa cell ligase can be biased in several ways against ligation by the yeastlike ligase. For example, the activity of the HeLa cell RNA ligase could be overrepresented in such extracts because it is normally in a subcellular compartment different from that in which tRNA splicing occurs. Alternatively, the activity of the yeastlike ligase may be underrepresented in such extracts because it is particularly sensitive to degradation or is inefficiently extracted.

Second, such in vitro experiments have measured only the flux of tRNA splicing through a particular pathway at a given concentration of precursor; at different concentrations, depending on the amounts and kinetic parameters of the two ligases, the ligases might participate in ligation to different extents.

Third, the $tRNA^{Tyr}$ gene microinjection experiments of Nishikura and De Robertis (19) could allow for a significant amount of splicing by the yeast pathway. As shown in Fig. 5, *Xenopus* oocytes also have an NAD-dependent 2′ dephosphorylating activity which would presumably remove the splice junction 2′ phosphate generated by a yeastlike ligase in *Xenopus* oocytes. Any yeastlike splicing of [α-^{32}P]UTP-labelled transcripts would then generate, after dephosphorylation, a mature tRNA with an unlabelled splice junction phosphodiester bond. This would lead to an underrepresentation of i^6A as quantitated by [α-^{32}P]UTP labelling and RNase T_2 digestion relative to that measured by [α-^{32}P]ATP labelling and nuclease P1 digestion. Although the amounts of i^6A generated in both cases were found to be comparable (19), a small difference in the two numbers, indicative of as much as 10% splicing utilizing the yeastlike ligase, would have been within the bounds of experimental error.

There are several possible roles of the two tRNA ligation pathways in vertebrate cells. First, different tRNA precursors may, for reasons that are presently unclear, be spliced by one or the other ligase in vertebrate cells. At least two different tRNA gene families contain intervening sequences in mammalian cells (7, 32), and more may be found. Although the tRNA ligase in *S. cerevisiae* is required for both cell viability and the ligation step of most, if not all, tRNA genes that are spliced (21a), the corresponding ligase in vertebrate cells may not work with certain tRNAs. The tRNA endonuclease from wheat germ, for example, excises intervening sequences efficiently only from homologous tRNAs (28). Second, under different growth conditions or during different stages of development or the cell cycle, the different ligases may be expressed at different levels and the tRNA precursor concentration may change. To ensure efficient production of tRNAs at all times, there may be a requirement for two ligation systems with different kinetic parameters. Third, the retention of the yeastlike ligation/dephosphorylation system may be required to ensure production of the small, as yet unidentified molecule (labelled X-p in Fig. 1A), which is a product of the NAD-dependent dephosphorylation reaction (18). This small molecule, which is not a common cellular nucleotide, may have additional metabolic and regulatory roles in the cell. Fourth, the yeastlike ligase may be required for an as yet unrecognized RNA ligation event in both vertebrate and yeast cells, in addition to its role in tRNA splicing in yeast cells. Fifth, the conservation of the yeast tRNA ligation system may indicate that it plays a major (or even an exclusive) role in tRNA splicing, while the HeLa cyclase/ligase system plays another, as yet undetermined role in RNA metabolism.

Understanding the role of the yeastlike ligation system in vertebrate cells will clearly require more work. The conservation of the entire yeastlike ligation pathway in vertebrate cells suggests that it is essential for the splicing of at least some tRNAs. Other eukaryotes, more suited to genetic analysis, may also possess two different RNA ligases as do human cells. Further experimentation will allow a full understanding of the role of each ligase.

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