Minimum Intron Requirements for tRNA Splicing and Nuclear Transport in *Xenopus* Oocytes†

Rosalind C. Haselbeck‡ and Chris L. Greer*

Department of Biological Chemistry, College of Medicine, University of California, Irvine, California 92717

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ABSTRACT: The presence or absence of an intron defines two classes of eukaryotic nuclear tRNA genes whose transcripts differ in a requirement for splicing. Using quantitative nuclear microinjection, we have previously found that nucleocytoplasmic transport of these two classes of tRNAs involves pathways which differ in one or more limiting components. To examine substrate features which distinguish these two pathways, a series of variants of a *Xenopus* $tRNA^{Tyr}$ gene were constructed in which the intron size was altered. The splicing and transport properties of the resulting transcripts were examined in oocyte microinjection and *in vitro* processing assays. The addition of one or two nucleotides at the splice site equivalent in an intronless gene produced transcripts which could be transported without splicing. However, transport was reduced relative to the mature-sequence tRNA, suggesting the anticodon loop (interrupted in pre-tRNAs) may be recognized by the intronless tRNA transport apparatus. Transcripts with four- or six-nucleotide intervening sequences were incompletely spliced with cleavage at only the 3′ splice site. Neither unspliced precursor nor partially processed intermediates were efficiently transported. The results of coinjection experiments using tRNA and pre-tRNA competitors suggest that simple retention by the splicing apparatus may not account for failure to export these RNAs. Finally, a requirement for splicing is not unique to transport of pre-$tRNA^{Tyr}$ since a pre-$tRNA_3^{Leu}$ variant which was not spliced was also not exported.

Among eukaryotic nuclear tRNA genes, only a fraction contain an intron while the majority are uninterrupted. This is best characterized in yeast where an estimated 20% of tRNA genes, encoding 10 different tRNAs, have an intron (Abelson, 1990). Thus, splicing is required for the synthesis of a distinct subset of cytoplasmic tRNAs. Features of this unique requirement and its impact on a subsequent essential step, nucleocytoplasmic transport, are the subjects of this research.

For clarity, the term IVS^{-1} will be used throughout the text to refer to the transcripts of intronless tRNA genes. Transcripts of intron-containing genes will be designated IVS^+. Introns where they occur within nuclear tRNA genes are characteristically small, ranging from 8 to 60 bp in length [see the compilation by Sprinzl et al. (1991)], and all interrupt exons at the same relative position, one nucleotide 3′ to the anticodon sequence. These common intron features and the high degree of conservation among tRNAs result in a common general structure among intron-containing pre-tRNA transcripts. All include a mature tRNA-like domain, formed by folding of exon segments, with the IVS extending from the anticodon arm (Swerdlow & Guthrie, 1984; Lee & Knapp, 1985). Conserved elements of tRNA structure within exons provide recognition sites for the splicing apparatus, and splice sites are defined, in part, by their position relative to these exon elements [reviewed by Culbertson and Winey (1989)]. Two distinct activities mediate the splicing reaction. These are a site-specific endonuclease, which cuts at each splice site to excise the linear IVS, and an NTP-dependent RNA ligase which joins exons. Both of these enzymes are thought to be restricted to the nucleus, and thus splicing must precede nuclear transport.

Previously, Zasloff and co-workers have used nuclear microinjection in *Xenopus* oocytes to study transport of an IVS^- tRNA (Zasloff et al., 1982; Zasloff, 1983; Tobian et al., 1985). Transport is specific, saturable, and temperature-dependent, consistent with a carrier-mediated mechanism. Additionally, the transport process is dependent on nuclear pore function (Neuman de Vegvar & Dahlberg, 1990; Dworetsky & Feldherr, 1988). Thus, tRNA transport may involve interaction of the RNA first with a specific carrier or adapter followed by directed transit through the pore.

We have used a similar microinjection approach to study pre-tRNA splicing and transport in *Xenopus* oocytes (Haselbeck and Greer, unpublished work). Both splicing and transport were found to be saturable with transport selective for the mature-sequence products of intron-containing and intronless genes. The effects of pre-tRNA and tRNA competitors on splicing and transport of radiolabeled substrates were also examined. The unique competition properties of IVS^+ and IVS^- tRNAs suggest that transport of these two classes involves functionally distinct pathways and that introns in tRNA transcripts may function in specifying a transport pathway. To examine the role of substrate features in specifying transport pathways, both splicing and transport were examined for a series of intron variants of *Xenopus* $tRNA^{Tyr}$ and yeast $tRNA_3^{Leu}$. The results suggest the IVS^- transport pathway is sensitive to the structure of the anticodon loop in tRNA substrates. Splicing is required for the IVS^+ transport pathway, and coinjection experiments suggest that

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* To whom correspondence should be addressed. Telephone: (714) 856-4565. FAX: (714) 725-2688.

‡ Present address: Department of Biology 0322, 9500 Gilman Dr., University of California at San Diego, La Jolla, CA 92093.

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; IVS, intervening sequence; Leu, leucine; Lys, lysine; Met, methionine; nt, nucleotide(s); [α-^{32}P]pCp, cytidine 3′,5′-[5′-^{32}P]bisphosphate; PEI, poly(ethylenimine); Phe, phenylalanine; *S. cerevisiae*, *Saccharomyces cerevisiae*; *S. pombe*, *Schizosaccharomyces pombe*; Tris, tris(hydroxymethyl)aminomethane; Tyr, tyrosine.

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simple retention by the splicing apparatus may not account for failure to export unspliced or partially spliced intermediates.

EXPERIMENTAL PROCEDURES

Suppliers. Sexually mature female frogs were obtained from Nasco (Ft. Atkinson, WI). Collagenase A and calf intestinal phosphatase were from Boehringer Manheim. Ethyl *p*-aminobenzoate (benzocaine), pancreatic ribonuclease, proteinase K, and RNA carrier (type VI) were from Sigma Chemical Co. RNases T1 and T2 were from Calbiochem. *Escherichia coli* RNA polymerase and poly(A) RNA were from Pharmacia. [α-^{32}P]UTP (3000 Ci/mmol) and cytidine 3′,5′-[5′-^{32}P]bisphosphate (3000 Ci/mmol) were from Amersham. Oligonucleotides were obtained from Operon Technologies, Alameda, CA. Yeast endonuclease fraction 71 was a shoulder fraction from a Sepharose CL-6B column with a protein concentration of approximately 5 μg/mL kindly provided by Reinhart Rauhut (Rauhut et al., 1990). The restriction enzyme *Bst*NI was from New England Biolabs.

Buffers. TE buffer contained 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA. Electrophoresis sample buffer contained 20% sucrose, 8 M urea, 0.1% xylene cyanol, and 0.1% bromophenol blue. Electrode buffer contained 90 mM Tris–borate (pH 8.3) and 2.4 mM EDTA. Gel elution buffer contained 50 mM KOAc, 200 mM KCl, and 20 mM EDTA. Splicing reaction buffer contained 20 mM Hepes (pH 7.5), 2.5 mM spermidine, 5 mM $MgCl_2$, 0.1 mM DTT, 0.4% Triton X-100, and 25 mM NaCl. Stop mix contained 2 mg/mL proteinase K, 2% SDS, and 0.1 M EDTA. Diluent contained 20 mM Hepes (pH 8.0), 0.5 mM EDTA, 1 mM DTT, 25 mM NaCl, 0.5% Triton X-100, and 20% glycerol. Injection sample buffer contained 20 mM Tris-HCl (pH 7.5), 88 mM NaCl, and 0.4% trypan blue.

Preparation of Radiolabeled RNA Substrates. Transcription reactions using λ promoter templates linearized by *Bst*NI were performed with *E. coli* RNA polymerase, and products were purified by gel electrophoresis as described previously (Shapero & Greer, 1992). CTP was included in a preincubation step with template and polymerase. The standard specific radioactivity of substrates was 5000 dpm/fmol. Low specific radioactivity transcripts were also prepared with the following modifications. In 100-μL reactions, unlabeled nucleotide concentrations of 100 μM ATP, GTP, and UTP and 1.0 mM CTP were used with 10 μCi of [α-^{32}P]UTP (3000 Ci/mmol) to give a final specific activity of 50 dpm/fmol.

Construction of Pre-tRNA^{Tyr} Intron Variants. Intron variants of *Xenopus* pre-tRNA^{Tyr} were constructed by total gene synthesis as described by Shapero and Greer (1992) using the same oligonucleotides listed in Haselbeck (1992) except that the oligonucleotide encoding the intron sequence (3A) was omitted and the oligonucleotide encoding the opposite strand (3B) was replaced with one of the following oligonucleotides:

I1 (1-nt intron) = 5′-AAGGATTCTACAG-3′

I2 (2-nt intron) = 5′-AAGGATTCCTACAG-3′

I4 (4-nt intron) = 5′-AAGGATTGCCCTACAG-3′

I6 (6-nt intron) = 5′-AAGGATTGCCACCTACAG-3′

The 1-nt and intronless constructs were amplified in *E. coli* strain BSJ72 (obtained from Tom St. John, Fred Hutchinson Cancer Research Center). All other constructs were amplified in strain DH5a (Hanrahan, 1983).

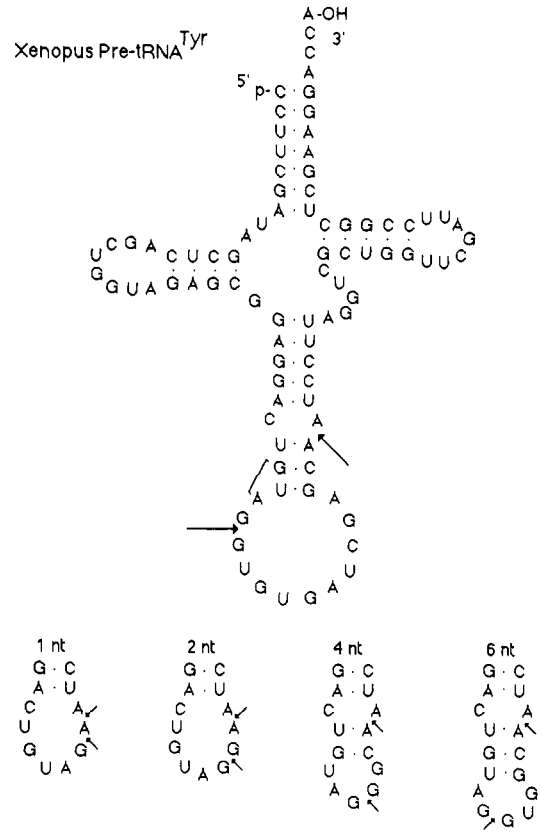

FIGURE 1: Sequence and secondary structure of *Xenopus* pre-tRNA^{Tyr} and intron variants. The sequence and predicted secondary structure of *Xenopus* pre-tRNA^{Tyr} (Muller & Clarkson, 1980) are shown with the positions of the two splice sites indicated by arrows and the anticodon by a bracket. The sequence and potential secondary structure of the anticodon stem/loop region of constructed intron variants (described under Experimental Procedures) with intron lengths of 1, 2, 4, and 6 nt are shown below.

Microinjection Experiments. Preparation of oocytes, injection of radiolabeled RNA substrates, fixation, and dissection conditions were all performed as described previously (Haselbeck, 1991). Unless noted otherwise, time courses were carried out with time points at 2, 20, 40, 60, and 120 min. The calculations used for graphs were as follows. The value for percent splicing was calculated as [(cpm nuclear + cytoplasmic spliced tRNA)/(cpm pre-tRNA + mature tRNA in both fractions)] × 100. The value for percent transport of spliced product was calculated as [(cpm cytoplasmic mature tRNA)/(cpm pre-tRNA + mature tRNA in both fractions)] × 100. The value for percent transport of unspliced pre-tRNA was calculated as [(cpm cytoplasmic pre-tRNA)/(cpm nuclear + cytoplasmic pre-tRNA)] × 100. The percent cytoplasmic tRNA was calculated as [(cpm cytoplasmic tRNA)/(cpm nuclear + cytoplasmic tRNA)] × 100. For cleavage of pre-tRNA substrates by the splicing endonuclease, the percent cutting was calculated as [(cpm nuclear cleavage products)/(cpm nuclear cleavage products + cpm pre-tRNA in both fractions)] × 100. Curves for graphs were generated by nonlinear regression analysis.

In Vitro Endonuclease Digests. Endonuclease reactions (10 μL) with radiolabeled substrates (5000 dpm/fmol) were carried out in splicing reaction buffer. Reactions containing 12 fmol of RNA substrates and 3 μL of endonuclease were incubated at 30 °C for 20 min. Reactions were stopped with the addition of 0.1 volume of stop mix and then incubated at

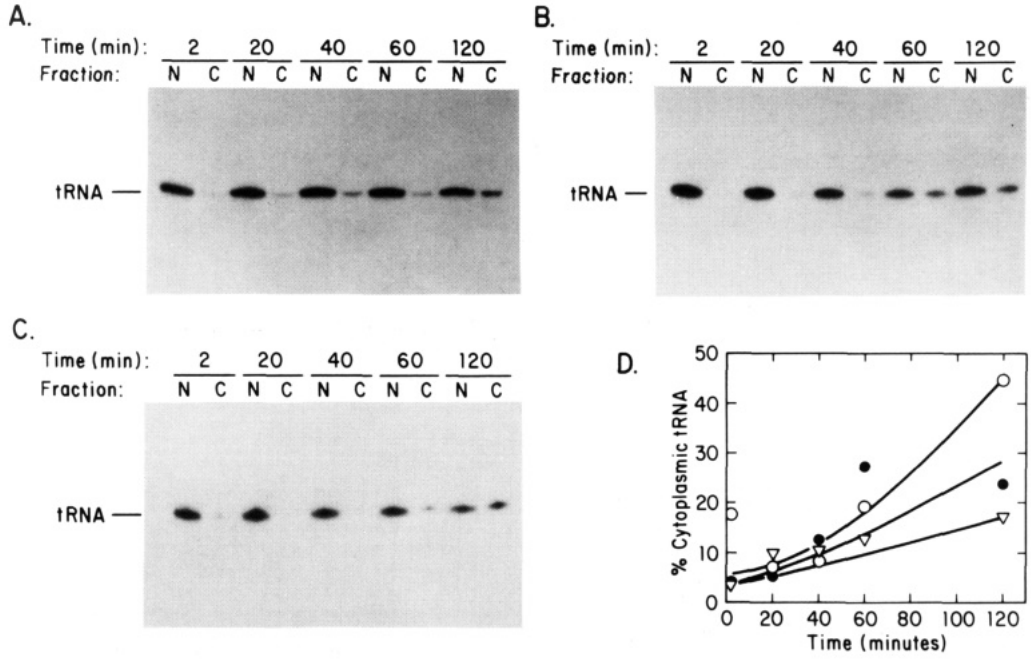

FIGURE 2: Transport of 0-, 1-, and 2-nt IVS variants. Radiolabeled pre-tRNA^{Tyr} (2-nt IVS), pre-tRNA^{Tyr} (1-nt IVS), and tRNA^{Tyr} (ΔIVS) were prepared by *in vitro* transcription, oocytes were microinjected, and values for the percent cytoplasmic tRNA were calculated as described under Experimental Procedures. Fractions from five oocytes were pooled for each time point, and extracted RNA products were resolved by electrophoresis and visualized by autoradiography. (A) Injection of labeled pre-tRNA^{Tyr} (2-nt IVS) (0.004 ng). (B) Injection of labeled pre-tRNA^{Tyr} (1-nt IVS) (0.008 ng). (C) Injection of labeled tRNA^{Tyr} (ΔIVS) (0.01 ng). For (A), (B), and (C), the identities of labeled products are indicated at panel edges. For (D), products resolved by electrophoresis were quantitated by measuring Cerenkov radiation in gel slices. Symbols correspond to injection of labeled pre-tRNA^{Tyr} (ΔIVS) (○), pre-tRNA^{Tyr} (1-nt IVS) (●), and tRNA^{Tyr} (2-nt IVS) (▽).

50 °C for 15 min. Reaction products were precipitated with ethanol and purified by electrophoresis on 12% polycrylamide/8 M urea gels.

One-Dimensional Analysis of RNase T1 Digestion Products. Endonuclease cleavage products were characterized by RNAse T1 oligonucleotide analysis as follows. Endonuclease reactions were performed as described above. For analysis of each substrate, reaction products were pooled from 10 separate 10-μL incubations. RNase T1 digestions were carried out on purified cleavage products in TE buffer with 10 μg of RNA carrier and 7 units of RNase T1 for 30 min at 37 °C. An equal volume of sample buffer was added, and digestion products were resolved on polyacrylamide gels containing 18% acrylamide, 0.95% bisacrylamide, and 8 M urea.

For preparation of 5′-dephosphorylated pre-tRNA^{Tyr} substrates, transcripts were incubated at 50 °C for 60 min in 10-μL reactions containing calf intestinal phosphatase (CIP; 2 units), 50 mM Tris-HCl (pH 8.5), and 0.1 mM EDTA. Reactions were stopped by the addition of 1 μL of 200 mM EGTA followed by heating to 65 °C for 10 min.

Structure of Endonuclease Cleavage Products. Analysis of the structure of 3′ ends of endonuclease cleavage products was carried out by postlabeling of purified cleavage products as follows. Endonuclease digests were performed with low specific radioactivity transcripts (50 dpm/fmol) as described above for transcripts with high specific radioactivity. Products were pooled from 10 separate reactions for each analysis. Labeling of RNA 3′ ends using [α-^{32}P]pCp and T4 RNA ligase was performed essentially as described by Szekely et al. (1988) except that 3′ end-labeled tRNA halves were purified by electrophoresis on 12% polyacrylamide/8 M urea gels, and 5′ exons were pretreated with T4 polynucleotide kinase (12 units) for 30 min at 37 °C to dephosphorylate cyclic 2′,3′-phosphate termini.

Analysis of nucleotides at labeled 3′ ends was done by one-dimensional chromatography of RNase T2 digests or by two-dimensional chromatography of double digests with pancreatic ribonuclease and RNAse T1 as follows. RNase T2 digests were carried out for 2 h at 37 °C in 5-μL reactions containing 20 mM NaOAc (pH 5.0), 30 μg of RNA carrier, and 0.5 units of RNase T2. Products were analyzed by thin-layer chromatography as described by Volckaert and Fiers (1976). Double digests with pancreatic ribonuclease and RNase T1 were carried out essentially as described by Volckaert and Fiers (1977) with the following modifications. Digests were carried out on purified 3′ end-labeled exons in a 5-μL volume containing 0.5 mg/mL pancreatic ribonuclease, 12.5 units of RNase T1, and 20 μg of RNA carrier in 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA for 2 h at 37 °C, and then applied to PEI plates. Plates were then developed and analyzed as described by Volckaert and Fiers (1977).

RESULTS

The experimental strategy used here can be summarized as follows. Radiolabeled pre-tRNA substrates for measuring splicing and transport were prepared by in vitro transcription. Labeled transcription products were then purified by gel electrophoresis and injected into oocyte nuclei. Following incubation, oocytes were fixed and dissected into nuclear and cytoplasmic fractions. Substrates and processed products in each fraction were then separated by gel electrophoresis and quantitiated, allowing simultaneous measurement of both splicing and transport.

Using this experimental strategy, we had previously shown that IVS$^+$ and IVS$^-$ tRNAs fail to compete with one another for a saturable transport step (Haselbeck, 1992). To examine the role of the IVS in both splicing and transport, 0-, 1-, 2-, 4-, and 6-nt variants of the authentic 13-nt IVS found in *Xenopus* pre-tRNA^{Tyr} were constructed. This mini-intron series, summarized in Figure 1, was constructed by omitting central IVS segments so as to maintain splice site sequence where possible.

Role of Anticodon Loop Structure. Previously, we had found that unspliced precursor accumulates within the nucleus and is not transported even at saturating pre-tRNA concentrations (Haselbeck, 1992). Thus, the presence of an IVS effectively prevents transport. Potentially, discrimination between tRNA and pre-tRNA substrates by the IVS$^-$ pathway may involve recognition of the anticodon loop segment since other regions of these molecules are thought to share a common structure. To test this possibility, transport of the 0-, 1-, and 2-nt IVS variants was compared. This size range was chosen since 1-nt loop insertions are found among functional frameshift suppressors in yeast [see Culbertson et al. (1977)] and thus must support at least minimal nuclear transport. Additionally, within this range splicing might not be expected as was observed for a 2-nt IVS variant of pre-tRNA$_3^{Leu}$ (Strobel & Abelson, 1986a).

To measure transport rates, labeled transcripts (0.1 ng/oocyte) were injected along with poly(A) RNA carrier (0.1–0.5 ng/oocyte), and cells were incubated at 25 °C for varying times. Fixed oocytes were then dissected into nuclear and cytoplasmic fractions, and RNA in each fraction was extracted and analyzed by gel electrophoresis. Examples of autoradiograms for time courses with the 0-, 1-, and 2-nt IVS variants are shown in Figure 2A–C. The results are quantitated in Figure 2D. All three of these substrates accumulated in cytoplasmic fractions in a time-dependent manner, albeit at different rates. Transport of the 1-nt IVS variant in this experiment was only slightly reduced relative to the mature-sequence tRNA (0-nt variant). In other experiments, no significant reduction was observed. Transport of the 2-nt IVS variant was significantly reduced, with only 17% of the labeled tRNA found in the cytoplasmic fraction after a 120-min incubation. Thus, the IVS$^-$ tRNA transport system appears to be highly selective for anticodon loop sequence or structure, effectively discriminating between 1- and 2-nt insertions within this segment.

Mini-Introns and Splicing. Previously, we had found that a 10-nt IVS variant of yeast tRNA^{Tyr} (SUP3) (with a wild-type IVS of 14 nt) was efficiently spliced in yeast extracts (data not shown). In addition, a naturally-occurring *S. pombe* tRNA^{Lys} (CUU) gene with an 8 bp intron is functional in both *S. pombe* and *S. cerevisiae* (Gamulin et al., 1983). In contrast, 2-nt IVS variants of pre-tRNA^{Tyr} (see above) or tRNA$_3^{Leu}$ (Strobel & Abelson 1986a) are not substrates for tRNA endonuclease. Thus, we anticipated the minimum IVS size for splicing was between 2 and 8 nt. To test this possibility and to examine transport of unspliced precursor or partially-processed intermediates, the properties of 4- and 6-nt IVS variants were compared to the pre-tRNA with the authentic 13-nt IVS.

Figure 3 shows autoradiographs of labeled RNA extracted from nuclear and cytoplasmic fractions and resolved by electrophoresis for the 4-, 6-, and 13-nt constructs. The 13-nt variant was efficiently spliced, with transient appearance of tRNA halves at intermediate time points and subsequent accumulation of spliced tRNA. Only spliced product was observed in cytoplasmic fractions with both precursor and halves restricted to the nucleus. Thus, either the presence of an IVS or the failure to close the anticodon loop in the second step in splicing prevents transport.

No spliced product was observed in either nuclear or cytoplasmic fractions for the 4- or 6-nt IVS substrates. Instead, nuclear fractions contained primarily pre-tRNA and two smaller species whose sizes were consistent with specific endonuclease cleavage products. These results suggest the minimum intron size for complete splicing is greater than 6 nt for this substrate. Within cytoplasmic fractions, only a small amount of pre-tRNA accumulated over the 120-min time course (13% and 12% for the 4- and 6-nt variants, respectively), consistent with rates for nonspecific redistribution as measured in previous experiments (Haselbeck, 1992). Thus, neither unspliced nor incompletely or inaccurately spliced products are efficient substrates for transport.

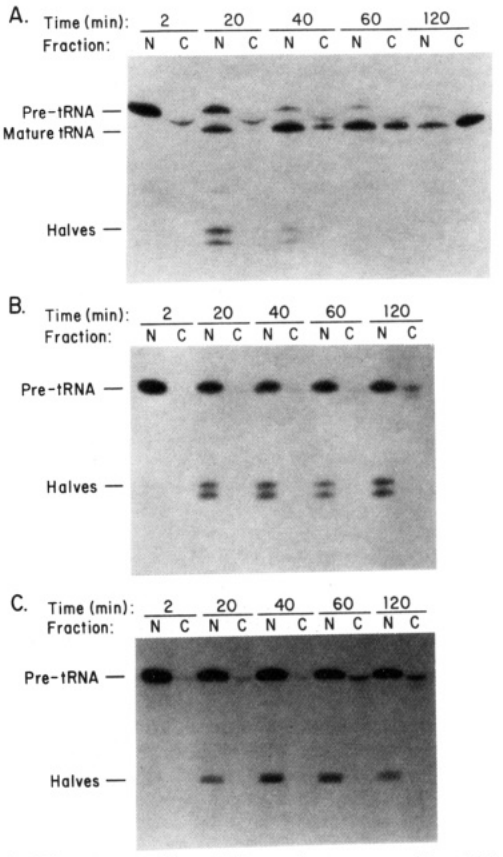

FIGURE 3: Time courses for splicing and transport of 4- and 6-nt IVS variants. Radiolabeled pre-tRNA^{Tyr}, pre-tRNA^{Tyr} (6-nt IVS), and pre-tRNA^{Tyr} (4-nt IVS) were prepared by *in vitro* transcription, and oocytes were microinjected as described under Experimental Procedures. Fractions from five oocytes were pooled for each time point, and extracted RNA products were resolved by electrophoresis and visualized by autoradiography. Identities of labeled products are indicated at panel edges. (A) Injection of labeled pre-tRNA^{Tyr} (0.01 ng). (B) Injection of labeled pre-tRNA^{Tyr} (6-nt IVS) (0.01 ng). (C) Injection of labeled pre-tRNA^{Tyr} (4-nt IVS) (0.007 ng).

Structure of Cleavage Products. Potentially, the cleavage products observed for 4- and 6-nt IVS variants might result from specific cleavage by tRNA endonuclease or through nonspecific breakdown. To differentiate between these possibilities, the effects of coinjected pre-tRNA and tRNA competitors on the yield of cleavage products were measured (the competitor concentrations chosen were based on those shown to affect splicing in previous coinjection experiments; Haselbeck, 1992). The results are shown in Figure 4A,B. Coinjection of unlabeled pre-tRNA^{Phe} competitor reduced the yield of labeled cleavage products for both the 4- and 6-nt variants. In contrast, coinjection of tRNA^{Phe} competitor, previously shown to stimulate pre-tRNA splicing (Haselbeck, 1992), increased the yield of cleavage products for both variants. Thus, the effects of both competitors suggest the cleavage products observed for the 4- and 6-nt IVS substrates are the result of specific cleavage by tRNA endonuclease.

The distinct electrophoretic patterns among the cleavage products for these variants suggested they might be derived

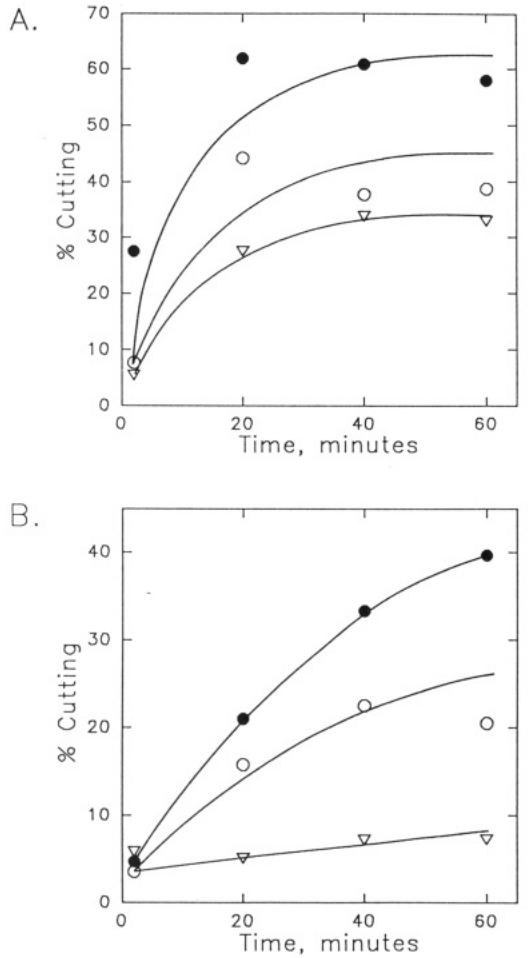

FIGURE 4: Effect of competitors on cleavage of 4- and 6-nt IVS variants. Microinjection of labeled pre-tRNA^{Tyr} (6-nt IVS) and pre-tRNA^{Tyr} (4-nt IVS) was carried out as described under Experimental Procedures. Products resolved by electrophoresis were quantitated by measuring Cerenkov radiation in gel slices. The percent cutting was calculated as described under Experimental Procedures. (A) Cleavage of labeled pre-tRNA^{Tyr} (6-nt IVS). Symbols correspond to injection of labeled pre-tRNA only (0.012 ng) (○), co-injection of labeled pre-tRNA (0.012 ng) and unlabeled tRNA^{Phe} (21.6 ng) (●), and co-injection of labeled pre-tRNA (0.011 ng) and unlabeled pre-tRNA^{Phe} (18.6 ng) (▽). (B) Cleavage of labeled pre-tRNA^{Tyr} (4-nt IVS). Injection of labeled pre-tRNA only (0.007 ng) (○), co-injection of labeled pre-tRNA (0.006 ng) and unlabeled tRNA^{Phe} (26.5 ng) (●), and co-injection of labeled pre-tRNA (0.009 ng) and unlabeled pre-tRNA^{Phe} (32.3 ng) (▽).

by inaccurate cleavage by endonuclease at an inappropriate site(s), or by accurate cutting at one splice site only. Note that either possibility might account for the failure to observe spliced tRNA products. To discriminate between these possibilities, an attempt was made to determine the sequence at ends among cleavage products. Because of the small amount of labeled RNA that could be injected and the large quantity of bulk cellular RNA obtained in oocyte fractions, it was not readily feasible to sequence oocyte cleavage products directly. We observed, however, that an identical pattern of cleavage products was obtained for cleavage of these same substrates in vitro by a yeast endonuclease fraction (Figure 5). Furthermore, the products obtained from oocytes and those from in vitro reactions comigrate precisely upon electrophoresis (data not shown). Thus, the sequence of the *in vitro* cleavage products was examined to determine whether the mobilities of the oocyte products were consistent with incomplete as opposed to inaccurate cleavage.

The sequence composition of each of the *in vitro* cleavage products was examined by one-dimensional electrophoretic

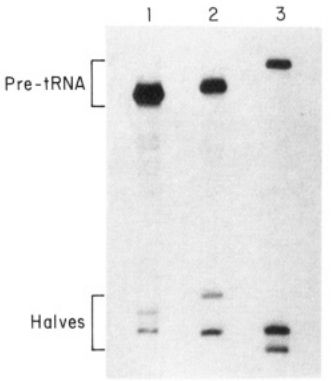

FIGURE 5: Comparison of endonuclease products for the 4- and 6-nt IVS variants. Endonuclease digests were carried out using labeled pre-tRNA^{Tyr} (4-nt IVS), pre-tRNA^{Tyr} (6-nt IVS), or pre-tRNA^{Tyr} for 20 min at 30 °C as described under Experimental Procedures, and products were resolved by electrophoresis in 12% polyacrylamide/8 M urea gels. Identities of labeled products are indicated at the panel edge. Lane 1 contains products from pre-tRNA^{Tyr} (4-nt IVS), lane 2 contains products from pre-tRNA^{Tyr} (6-nt IVS), and lane 3 contains products from wild-type pre-tRNA^{Tyr}.

analysis of RNase T1 digests (data not shown). The results can be summarized as follows. The 4-, 6-, and 13-nt IVS substrates all yielded a band of identical mobility which included sequences characteristic of the 3′ half. Each substrate yielded a second band with unique mobility, and, in each case, this species included sequences characteristic of the 5′ half. Thus, the products of the 4- and 6-nt IVS substrate are consistent with cleavage at the 3′ splice site only.

To confirm precise cleavage at the 3′ splice site, postlabeling of cleavage products purified from reactions with low specific activity substrates was carried out (data not shown). Postlabeling was carried out with T4 polynucleotide kinase and [γ-^{32}P]ATP to examine 5′ ends and with T4 ligase and [α-^{32}P]pCp for 3′ ends as described under Experimental Procedures. The products of subsequent digestion with RNase T2 and also a mixture of RNase A and RNase T1 were examined by thin-layer chromatography. The results confirm that cleavage occurred at 3′ splice sites for both the 4- and 6-nt IVS substrates. Thus, the mobility of the oocyte cleavage products and the effects of coinjected competitors are consistent with precise cleavage of these substrates by the *Xenopus* endonuclease at one splice site only.

Pre-tRNA$_3^{Leu}$ Variants. To determine whether the link between splicing and transport defects observed for pre-tRNA^{Tyr} was unique to this precursor or more general, the properties of two pre-tRNA$_3^{Leu}$ IVS variants were examined. Both variants contain hexanucleotide inserts within the 32-nt IVS found in this yeast precursor (Szekely et al., 1988). In the DSI variant, this insert is complementary to the 3′ splice site and forms a double-stranded structure which prevents splicing in yeast. The insert in the SSI variant does not interact with the 3′ splice site and has no effect on splicing in yeast. Labeled SSI and DSI substrates were injected into oocytes, and, after varying incubation time, extracted RNA was resolved by electrophoresis and visualized by autoradiography as shown in Figure 6A,B. The results are shown quantitatively in Figure 6C and can be summarized as follows. No apparent splicing of the DSI precursor was observed at any of the time points. In contrast, splicing of the SSI precursor was comparable to that observed for the *Xenopus* pre-tRNA^{Tyr}. Thus, a requirement for single-stranded structure at the 3′ splice site is common to the yeast and *Xenopus* splicing systems. During the time course, only a small amount of the unspliced DSI precursor appeared in the cytoplasm, consistent with

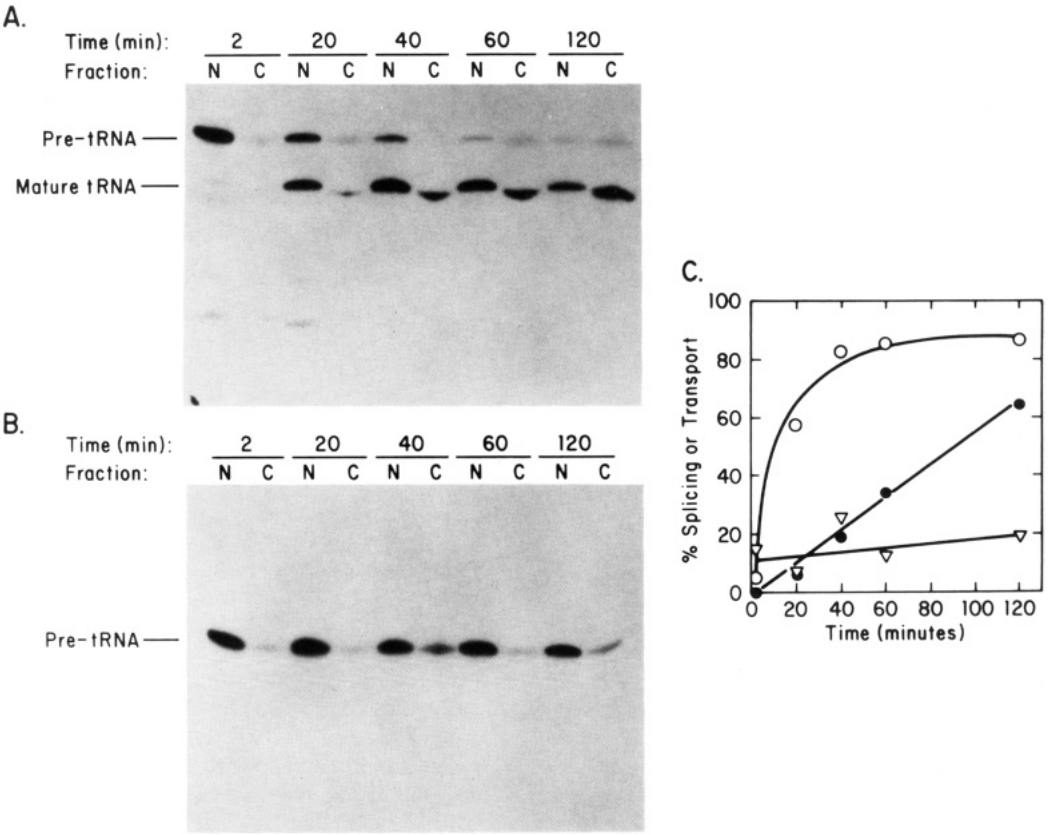

FIGURE 6: Splicing and transport of SSI and DSI substrates. Radiolabeled SSI and DSI substrates were prepared by *in vitro* transcription, oocytes were microinjected with 0.01 and 0.005 ng, respectively, and the values for percent splicing, percent transport SSI and percent transport DSI were calculated as described under Experimental Procedures. (A) Injection of labeled SSI. (B) Injection of labeled DSI. Fractions from five oocytes were pooled for each time point, and extracted RNA products were resolved by electrophoresis and visualized by autoradiography. Identities of labeled products are indicated at the edges of each panel. (C) Splicing (○) and transport of labeled SSI (●); transport of DSI (▽) substrates. Products resolved by electrophoresis were quantitated by measuring Cerenkov radiation in gel slices.

nonspecific relocation rates as measured previously (Haselbeck, 1992). The spliced product for the SSI substrate was transported at a rate similar to that observed for $tRNA^{Tyr}$-spliced product. Thus, a linkage between splicing and transport defects is not limited to a single precursor nor to a particular splicing defect.

DISCUSSION

The experiments described here were designed to identify tRNA intron features which affect splicing and transport. With respect to splicing, several conclusions can be drawn. The first is based on the observation that the 2-nt IVS variant was not a substrate for endonuclease while the 4-nt precursor was partially processed. Thus, the minimum intron size for cleavage by endonuclease is either 3 or 4 nt.

Although apparently recognized by endonuclease, the 4-nt IVS variant was cut at the 3′ splice site only. This result is consistent with previous work demonstrating distinct requirements for cleavage at the two splice sites. Other examples of cleavage of a tRNA precursor at a single splice site include the effects of certain tRNA gene mutations (Strobel & Abelson, 1986b; Greer et al., 1987; Mattocia et al., 1988; Reyes & Abelson, 1988; Mathison et al., 1989) as well as a mutation in the SEN2 gene thought to encode a subunit of yeast tRNA endonuclease (Ho et al., 1989). Thus, the 5′ and 3′ splice sites may be differentially recognized by a single endonuclease active site, or, potentially, the enzyme may have distinct active sites which function independently in the two cleavage steps [see Ho et al. (1989) and Miao and Abelson (1992)].

Previously it had been shown that single-stranded structure at the 3′ splice site is required for endonuclease cleavage in yeast. On the basis of a comparison of splicing of the SSI and DSI precursors described here, we conclude that a similar requirement applies to the *Xenopus* system. We note that when alternate structures are considered for the mutations described by Baldi et al. (1986), single-stranded structure may also be required for 5′ splice site cleavage in *Xenopus*. For at least one pre-tRNA substrate, adjacent exon sequences may also influence 3′ splice site selection (Baldi et al., 1992). Thus, requirements in addition to single-stranded character may account for functional differentiation between the 5′ and 3′ splice sites by endonuclease.

The effects of each of the IVS changes on nuclear transport were also examined, and a number of conclusions can be drawn from the results. First, in all cases, splicing was required for efficient transport of pre-tRNAs with an IVS length ≥4 nt. Thus, pre-tRNAs accumulated either as the result of a mutation preventing splicing or by injection of high concentrations of a functional substrate were not efficiently transported. Similarly, partially-spliced intermediates accumulated for the 4- and 6-nt IVS variants or appearing transiently for a wild-type substrate were retained within the nucleus.

Potentially, nuclear retention of pre-tRNAs and splicing intermediates might result from active discrimination among RNAs for export, reflecting the specificity of the transport system. Alternatively, binding of substrates to the splicing apparatus could serve indirectly to prevent transport. These alternatives could be tested in this experimental system, in the latter case, saturation of the splicing apparatus should

result in pre-tRNA transport. In experiments reported here and conducted previously (Haselbeck, 1992), we found no evidence for efficient transport of IVS-containing transcripts either at high substrate concentrations or in the presence of splicing competitors. Thus, our results are consistent with active discrimination among export candidates by the tRNA transport system.

Previously, Zasloff and co-workers have examined the effects of a series of point mutations on transport of an IVS$^-$ tRNA ($tRNA_i^{Met}$; Tobian et al., 1985). Their results implicated seven conserved and semiconserved bases within the D and T stem/loop regions as critical for transport. Recognition of these specific elements by the transport apparatus may not be sufficient to account for pre-tRNA discrimination since the structure within these regions is thought to be similar in precursors and spliced products. Comparison of the transport properties of the 1- and 2-nt IVS variants described here suggests that pre-tRNA discrimination may be based on sequence or structure within the anticodon loop region. Potentially, the anticodon loop might serve as a primary recognition site or might act indirectly by affecting structures at a distance [see Leontis et al. (1988)]. These possibilities will be tested in future experiments.

Although the tRNA and mRNA transport systems share certain general features in common, these two systems apparently differ in at least two respects. First, regulated transport of IVS-containing transcripts is essential for certain mRNA expression patterns. Well-characterized examples include expression patterns in tissue-specific transposition of the *Drosophila* P element (Laski et al., 1986) and late mRNA and genomic RNA expression among retroviruses [cf. Chang and Sharp (1990)]. Equivalent examples for pre-tRNA transport have not been described.

A second difference may be specific recognition of IVS-containing precursors for tRNA transport in contrast to competition between splicing and transport for mRNA export. This latter model for mRNA transport is based primarily on the observation that mutations which prevent the initial steps in spliceosome assembly allow transport of unspliced mRNA precursors (Legrain & Rosbash, 1989). This difference may reflect both the regulatory complexity unique to mRNAs and the location of *cis* elements required for recognition by the corresponding splicing and transport systems. Pre-mRNAs are characterized by common elements at splice sites and within introns for recognition by the splicing apparatus. Exon features in this class are necessarily divergent. Thus, controlling pre-mRNA transport through splicing competition includes no inherent conflict with requirements for export of IVS$^-$ mRNAs. In contrast, pre-tRNA splicing involves recognition of conserved exon features common to precursors, spliced products and IVS$^-$ tRNAs. Thus, both pre-tRNAs and IVS$^-$ tRNAs would be subject to competition in a splicing versus transport competition model. Active discrimination of the presence of an IVS by the tRNA transport apparatus may effectively avoid this conflict.

In summary, the presence and size of introns in tRNA transcripts may be the basis for direction into a splicing pathway or a pathway for direct transport. Both the splicing and transport steps appear to involve specific recognition of appropriate substrates, and this recognition, rather than a simple competition process, may account for the observed tRNA transport patterns. The oocyte microinjection system provides a unique opportunity to test this possibility and to examine essential features of the transport system.

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