Exon Sequence and Structure Requirements for tRNA Splicing in *Saccharomyces cerevisiae*†

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ABSTRACT: A survey of exon sequence and structure requirements for splicing was undertaken using labeled pre-tRNA substrates prepared by in vitro transcription of bacterial promoter–yeast $tRNA^{Tyr}$ gene fusions. Transcription templates were assembled from oligonucleotide cassettes allowing analysis of 22 derivatives affecting each of the potential secondary and certain tertiary interactions in the pre-tRNA. Effects on both excision of the intervening sequence by yeast endonuclease and joining of exons by ligase were examined. Replacements within the D- and T-stems and anticodon stems revealed that while the primary sequences of these segments were not essential for splicing, formation of base-paired structures was required. Replacements which altered the primary sequence while retaining the secondary structure of the aminoacyl stem allowed efficient excision by endonuclease but reduced joining by ligase. Potentially, the effects of changes within these stems may be indirect through effects on adjacent or overall structure. The presence of either structured or unstructured 5′ leader and/or 3′ trailer sequences had no effect on either splicing step. Alterations in the conserved Levitt tertiary pair (G15/C48), previously implicated in splicing of pre-$tRNA^{Phe}$, did not alter splicing of pre-$tRNA^{Tyr}$. A precursor in which the small (type I) extra arm in pre-$tRNA^{Tyr}$ was replaced with the large (type II) extra arm sequence from $tRNA^{Ser}$ was efficiently spliced. These and previous results suggest that only limited features of exon sequence or structure are recognized by the splicing enzymes.

Analyses of RNA splicing in a wide variety of systems have led to the identification of four general classes of splicing reactions (Sharp, 1985; Cech 1986). These classes, distinguished by differences in reaction mechanisms, substrate requirements, and trans-acting factors, are nuclear mRNA splicing, group I and group II self-splicing, and nuclear tRNA splicing. Significant similarities among the first three of these classes have been cited as evidence that all may have evolved from a common primitive reaction (Sharp, 1985; Cech, 1986). In contrast, nuclear tRNA splicing differs significantly from these other classes in the proposed role of protein factors, the role of exon elements in splice site identification, and the nature of reaction intermediates [see the review by Culbertson and Winey (1989) and references therein]. Thus tRNA splicing may have origins distinct from all other classes or, alternatively, may have diverged quite early from a common progenitor. Distinguishing between these two possibilities will require a greater understanding of the tRNA splicing reaction.

Among the distinguishing features of the tRNA splicing process are the nature of the substrate sequences and structures that are required for splicing. Both lowest free energy calculations (Ogden et al., 1984) and solution structure probing (Swerdlow & Guthrie, 1984; Lee & Knapp, 1985) reveal that pre-tRNA substrates include a mature tRNA-like domain which incorporates many of the secondary and tertiary interactions conserved among tRNAs and an intervening sequence (IVS)[1] domain which forms an extension of the anticodon arm. Constraints on IVS sequence or structure for splicing are few and may consist largely of a requirement for single-stranded structure at splice sites (Raymond & Johnson, 1983; Strobel & Abelson, 1986; Baldi et al., 1986; Szekely et al., 1988; Reyes & Abelson, 1988; Mattocia et al., 1988). In contrast, mutations throughout exon domains have been shown to affect splicing. These include mutations within the anticodon arm, the D-stem and loop, the variable extra arm, and the T-loop (Colby et al., 1981; Nishikura et al., 1982; Baldi et al., 1983; Willis et al., 1984; Pearson et al., 1985; Gandini-Attardi et al., 1985; Winey et al., 1986; Greer et al., 1987; Mattocia et al., 1988; Reyes & Abelson, 1988; Mathison et al., 1989). Additionally, the presence of a 5′ extension of the aminoacyl stem has been shown to affect splicing (Greer et al., 1987). Although, superficially, these results appear to implicate all of the exon structural elements in splicing, two mitigating factors should be considered. The first is that many of these mutations may exert their effects only indirectly through disrupting the formation of adjacent or distant structures. The second is that these studies have been carried out using a wide range of pre-tRNA substrates and splicing enzyme fractions. To define more precisely exon requirements for splicing, we have initiated a systematic survey of primary, secondary, and tertiary structures required for splicing of a single pre-tRNA substrate.

A λ promoter–yeast tRNA gene fusion was previously used to produce pre-tRNA transcripts by in vitro transcription with purified *Escherichia coli* RNA polymerase (Shapero & Greer, 1991). The resulting transcripts were efficiently spliced in vitro following a temperature- and magnesium-dependent reannealing step. This system served as the starting point for an analysis of requirements for splicing of the yeast tyrosyl-tRNA precursor (pre-$tRNA^{Tyr}$). A total of 22 templates were assembled from oligonucleotide cassettes, allowing analysis of the effects on IVS excision and exon joining of changes within

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[1] Abbreviations: amp, ampicillin; bp, base pairs; EDTA, ethylenediaminetetraacetic acid; IVS, intervening sequence; nt, nucleotide(s); Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; Tyr, tyrosine.

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each of the exon structural elements and the impact of 5′ leader and 3′ trailer sequences. The results suggest that only very limited features of exon sequence and/or structure are required for splicing.

Experimental Procedures

Enzymes and Chemicals. The restriction enzymes *Bam*HI, *Eco*RI, and *Pst*I were obtained from Boehringer Mannheim, and *Bst*NI, *Hin*dIII, and *Xma*I were from New England Biolabs. Ribonuclease T1 was from Calbiochem. Partially purified yeast tRNA endonuclease (23 units/mL) was equivalent to fraction VI of Peebles et al. (1983) while partially purified yeast tRNA ligase (180 units/mL) was equivalent to fraction IV of Greer et al. (1983b). Phage T4 polynucleotide kinase was from United States Biochemicals. Phage T4 RNA ligase, T4 DNA ligase, and *E. coli* RNA polymerase were from Pharmacia. Oligonucleotides used in directed mutagenesis were from the Protein/Nucleic Acid Analysis Laboratory of the Department of Biological Chemistry, University of California, Irvine, or from Genetic Designs, Inc., Houston, TX. Labeled nucleotides including [α-^{32}P]UTP (3000 Ci/mmol), [α-^{32}P]CTP (3000 Ci/mmol), [γ-^{32}P]ATP (5000 Ci/mmol), and [5′-^{32}P]cytidine 3′,5′-bisphosphate (3000 Ci/mmol) were from Amersham.

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. Stop mix contained 0.2% SDS, 10 mM EDTA, and 0.2 mg/mL proteinase K. Gel elution buffer contained 10 mM Tris-HCl (pH 7.4), 0.1% SDS, 1 mM EDTA, and 0.3 M NaCl.

Construction of Plasmid pBS(+)-Sup3. The strategy to be used for total tRNA gene synthesis required the introduction of a *Pst*I restriction site between the −10 and −35 segments of the λ P_R/C_{17} promoter in plasmid pSup3 (Shapero & Greer, 1991). For this purpose, single-strand M13 mp19 DNA containing the λ P_R/C_{17} hybrid promoter fused to the yeast SUP3 gene was used as the template for oligonucleotide-directed mutagenesis. The oligonucleotide used was a 24-mer of the sequence (5′-CAATTATCACCTGCAGAGGTAAAA-3′). The complete nucleotide sequence of this variant λ promoter–tRNA gene fusion, designated M13 mp19/MS-8, was confirmed by DNA sequencing (Sanger et al., 1977) using the M13 17-mer universal sequencing primer. The modified promoter was then subcloned into plasmid pBS(+) (Stratagene) as follows. M13 mp19/MS-8 RF DNA was digested with *Hin*dIII and *Eco*RI and an approximately 550 bp fragment was isolated and ligated into *Hin*dIII–*Eco*RI-digested pBS(+) plasmid DNA. The resulting plasmid is designated pBS(+)-Sup3.

Total tRNA Gene Synthesis. The sequences of all oligonucleotides used in this work are listed in Table I. Table II lists the subsets of oligonucleotides used in each of the gene syntheses. Oligonucleotides were purified by electrophoresis on 0.8-mm gels containing 12% acrylamide, 0.4% bisacrylamide, and 8 M urea. Visualization of the oligonucleotides was by shadowing with ultraviolet light at 254 nm. Oligonucleotides were eluted in 3 mL of gel elution buffer for 8 h with shaking at 37 °C. After precipitation with ethanol, oligonucleotides were resuspended in 20 μL of 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA and the concentration was determined by measuring absorbance at 260 nm. For synthesis of each tRNA gene, all oligonucleotides except the two 5′ end oligonucleotides (1A and 5B) were phosphorylated in 75-μL reactions containing 35 pmol of each oligonucleotide, 70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, and 0.3 unit/μL T4 polynucleotide kinase. Following incubation for 60 min at 37 °C, reactions were adjusted to 0.3 M NaOAc (pH 5.3) and extracted once with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). Oligonucleotides were recovered by precipitation with ethanol, and the pellet was washed with 70% ethanol and then dried. Oligonucleotides representing 1A and 5B (35 pmol each) were added to the pellet, and the volume was increased to 100 μL in buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl, and 1 mM DTT. The reaction was covered with paraffin oil, heated to 95 °C, and allowed to cool to room temperature over the course of several hours. The paraffin oil was removed, and 4 μL of 100 mM ATP and 800 units of T4 DNA ligase were added. The reactions were incubated for 4–8 h at room temperature. Ligated oligonucleotides were precipitated with ethanol, and the dried pellet was resuspended in 20 μL of DNA loading dye (6.7% sucrose, 0.0425% xylene cyanol, 0.0425% bromophenol blue) and iso-

Table I: Sequences of Deoxyoligonucleotides Used in Gene Synthesis

oligo[a]/ length[b]	sequence (5′ to 3′)
313/14	TTTATCACTACGAA
314/32	GGTGATAATTGTTATCTCTCGGTAGCCAAGTT
315/19	AATTCACCTGGTCTCCCGG
316/28	TACCGAGAGATAACAATTATCACCTGCA
317/22	CTCGCCCCCGGGAGACCAGGTG
318/22	GGTTTAAGGCGCAAGACTCTAA
319/28	CAAGATTTCGTAGTGATAAATTAGAGTC
320/22	TTGCGCCTTAAACCAACTTGGC
321/21	ATCTTGAGATCGGGCGTTCGA
322/22	GGGCGAGTCGAACGCCCGATCT
323/32	GGTGATAATTGTTATCTCTCGGTAGCCAAATT
324/21	ATCTTGAGATCCCCCCTTCGA
325/22	TTGCGCCTTAAACCAATTTGGC
326/21	ATCTTGAGATTGGGCGTTCGA
327/22	CTGGGGGCCGGGAGACCAGGTG
329/22	GGGCGAGTCGAACGCCCAATCT
330/22	GGGCGAGTCGAAGGGGGGATCT
331/22	CCCCCAGTCGAACGCCCGATCT
349/22	CCCCCAGTCGAAGGGGGGATCT
350/21	AAGAACAGATCGGGCGTTCGA
351/32	GGTGATAATTGTTATCTCTCGGTACGGAAGTT
352/32	GGTGATAATTGTTATCAGGGCCTAGCCAAGTT
353/28	CAAGATTTCGTAGTGATAAATTAGAGAG
354/22	AACCGCCTTAAACCAACTTGGC
355/28	TAGGCCCTGATAACAATTATCACCTGCA
356/28	GTTCTTTTCGTAGTGATAAATTAGAGTC
357/28	GTTCTTTTCGTAGTGATAAATTAGAGAG
359/19	AATTCACCTGGTCAGAGCC
362/22	GGTTTAAGGCGGTTCTCTCTAA
364/22	TTGCGCCTTAAACCAACTTCCG
366/22	TTGCCGGTTAAACCAACTTGGC
370/22	GGTTTAACCGGCAAGACTCTAA
371/22	TTGCCGGTTAAACCAACTTCCG
372/22	CTCGCCCGGCTCTGACCAGGTG
375/27	AATTCACCTGGAAAAAATATCTCCCGG
378/30	CTCGCCCCCGGGAGATATTTTTTCCAGGTG
379/39	TACCGAGAGTCGTTTTTGTGATAACAATTATC-ACCTGCA
380/29	CTCGCCCCCGGGAGACGTGATGCCAGGTG
382/43	GGTGATAATTGTTATCACAAAAACGACTCTCG-GTAGCCAAGTT
384/26	AATTCACCTGGCATCACGTCTCCCGG
385/39	TACCGAGAGACGTGATGCCGATAACAATTATC-ACCTGCA
386/43	GGTGATAATTGTTATCGGCATCACGTCTCTCG-GTAGCCAAGTT
400/30	CACAAGATTTCGTAGTGATAAATTAGAGTC
401/30	ATCTTGTGGGCTCTGCCCGCGGGCGTTCGA
402/29	GGGCGAGTCGAACGCCCGCGGGCAGAGCC

[a] Each oligonucleotide was assigned a unique number that is used in Table II and in the text. [b] Length in nucleotides.

Table II: Sets of Oligonucleotides Used in Assembling Transcription Templates

pre-tRNA[a]	position[b]									
	1A	1B	2A	2B	3A	3B	4A	4B	5A	5B
Sup 3	314[c]	316	318	320	313	319	321	322	317	315
G15A	323	316	318	325	313	319	321	322	317	315
C48U	314	316	318	320	313	319	326	329	317	315
G15A/C48U	323	316	318	325	313	319	326	329	317	315
5′ D-stem	351	316	318	364	313	319	321	322	317	315
3′ D-stem	314	316	370	366	313	319	321	322	317	315
D Dbl	351	316	370	371	313	319	321	322	317	315
5′ AC stem	314	316	362	354	313	353	321	322	317	315
3′ AC stem	314	316	318	320	313	356	350	322	317	315
AC Dbl	314	316	362	354	313	357	350	322	317	315
5′ T-stem	314	316	318	320	313	319	324	330	317	315
3′ T-stem	314	316	318	320	313	319	321	331	327	315
T Dbl	314	316	318	320	313	319	324	349	327	315
5′ AA stem	352	355	318	320	313	319	321	322	317	315
3′ AA stem	314	316	318	320	313	319	321	322	372	359
AA Dbl	352	355	318	320	313	319	321	322	372	359
5′1°	382	379	318	320	313	319	321	322	317	315
3′1°	314	316	318	320	313	319	321	322	378	375
1° Dbl	382	379	318	320	313	319	321	322	378	375
5′1° H	386	385	318	320	313	319	321	322	317	315
3′1° H	314	316	318	320	313	319	321	322	380	384
1° Dbl H	386	385	318	320	313	319	321	322	380	384
extra arm	314	316	318	320	313	400	401	402	317	315

[a] Transcription templates and corresponding pre-tRNA variants are described in Results. [b] The relative position of oligonucleotides within the assembled templates is indicated by the designations 1A through 5B using the scheme outlined in Figure 1. [c] Oligonucleotides are designated using the unique identifiers listed in Table I.

lated by electrophoresis on 0.8-mm gels containing 10% acrylamide and 0.33% bisacrylamide. After staining with ethidium bromide, the appropriately sized fragment was eluted from the gel in 3 mL of gel elution buffer. After precipitation with ethanol, the purified fragment was ligated with *Pst*I–*Eco*RI-digested pBS(+)-Sup3 plasmid DNA using T4 DNA ligase. Ampicillin-resistant bacterial colonies were screened for the presence of the restriction fragment by *Pst*I–*Eco*RI restriction digests. Single-strand DNA was prepared from individual candidates, and complete DNA sequence analysis was used to select correct constructs.

In Vitro Transcription Reactions. Transcription with *E. coli* RNA polymerase and purification of reaction products by gel electrophresis was carried out as described by Shapero and Greer (1991). Transcription reactions with yeast RNA polymerase III (Pol III) were carried out as described by Engelke et al. (1985) using plasmid pYleu2SUP6 as template (Johnson & Abelson, 1983). RNA polymerase III activity was provided by a yeast nuclear extract prepared as described by Engelke et al. (1985). Reactions (50 μL) were incubated at 30 °C for 30 min, and RNA was recovered as described above for bacterial polymerase reactions. The specific radioactivity of substrates prepared by transcription with RNA polymerase III or *E. coli* polymerase was 5000 dpm/fmol.

In Vitro Splicing Reactions. Splicing reactions were carried out as described by Greer et al. (1987) except that the reactions contained 25 mM NaCl and 5 mM $MgCl_2$. Also, pre-tRNA substrates were subjected to a renaturation cycle by heating to 68 °C in the presence of 25 mM $MgCl_2$ (Shapero & Greer, 1991) prior to use in splicing assays. Reactions were stopped by freezing the samples on crushed dry ice, and then an equal volume of electrophoresis sample buffer was added on ice. Reaction products were then separated by electrophoresis in 20 cm × 20 cm × 0.8 mm gels containing 12% acrylamide, 0.4% bisacrylamide, 8 M urea, and electrode buffer. Products visualized by autoradiography were then quantitated by measuring Cerenkov radiation in gel slices.

Analysis of Splicing Accuracy. One-dimensional RNase T1 oligonucleotide analysis was used to confirm accurate splicing of pre-tRNA substrates. This analysis was based on the following. Pre-tRNA^{Tyr} contains two RNase T1 oligonucleotides that are characteristic of the pre-tRNA [see Shapero and Greer (1991)]. These are a 19-mer that contains the 5′ splice site and the intron and an 8-mer that contains the 3′ splice site. In the correctly spliced tRNA product, these two RNase T1 oligomers are absent and a unique 13-mer, containing the splice junction, is generated.

For the RNase T1 oligonucleotide analyses, spliced tRNA was purified by electrophoresis, eluted from gel slices, and precipitated with ethanol. Following addition of 20 μg of RNA carrier (type VI; Sigma), samples were digested with 4 units of RNase T1 per 10 μg of RNA carrier in 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA for 30 min at 37 °C. After the addition of an equal volume of RNA loading dye, digestion products were resolved by electrophoresis on 25% acrylamide, 0.8% bisacrylamide and 8 M urea gels.

Although spliced tRNA fractions contained a small and variable amount of contaminating pre-tRNA, all contained high yields of the 13-mer characteristic of the spliced product, and in no case was evidence obtained for the use of alternate splice sites. We note that a slight mobility shift ($\sim^1/_2$ bandwidth) was observed for the 13-mer from the spliced product of AC stem Dbl reactions. This was most likely due to differences in sequence composition since the shift was less than that expected for a one-nucleotide increment and since the tRNA halves and spliced tRNA products were found to comigrate with those from pre-tRNA^{Tyr} reactions (data not shown). For other pre-tRNA substrates, the appearance of unique T1 oligonucleotides (other than that containing the spliced junction) provided independent confirmation of the sequences for these substrates.

Results

Assembly of tRNA Transcription Templates. Complete gene synthesis with overlapping oligonucleotide cassettes was used to facilitate the assembly of the extensive collection of tRNA^{Tyr} gene mutations required for this study. The starting plasmid, pSUP3, has been described previously (Shapero &

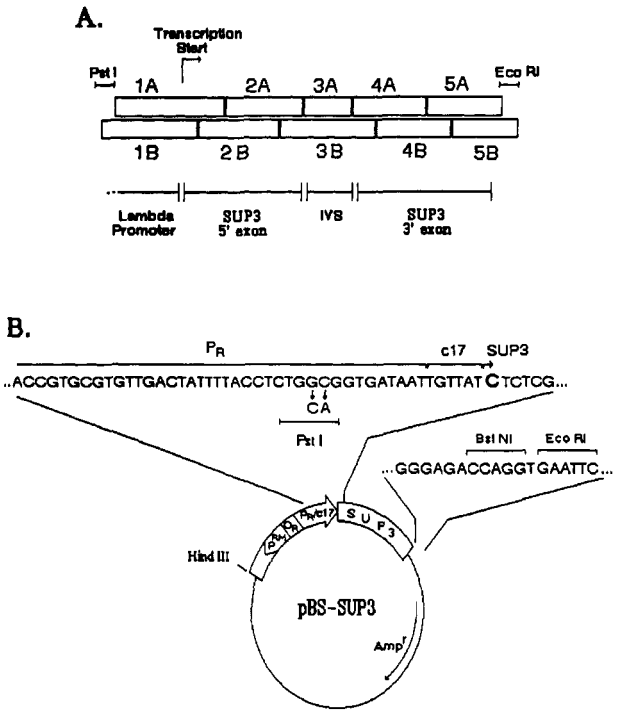

FIGURE 1: Transcription templates. Each variant tRNA gene was assembled from a unique set of 10 overlapping synthetic oligonucleotides as summarized in Table II. (A) A diagram of the relationship among overlapping oligonucleotides and the corresponding nomenclature is shown at the top of the panel. The approximate positions of the promoter, transcription initiation, and IVS-encoding sequences are indicated by brackets. (B) Assembled tRNA genes were used to replace the corresponding *Pst*I–*Eco*RI fragment in plasmid pBS-SUP3 (construction of this parental plasmid is described in Experimental Procedures). Templates contained a hybrid phage λ P_R/C_{17} promoter fused to the yeast pre-tRNA^{Tyr}[SUP3] coding sequence. The sequence of the noncoding DNA strand at the promoter is shown, including the two base changes introduced by directed mutagenesis to create a *Pst*I restriction site for tRNA gene insertion. Also shown is the sequence at the 3′ end of the tRNA gene including the *Bst*NI and *Eco*RI sites used for transcription runoff and cassette insertions, respectively.

Greer, 1991). Oligonucleotide-directed mutagenesis was used to introduce a *Pst*I restriction site within the λ promoter region in pSUP3. This allowed removal of the original tRNA gene from the plasmid as a *Pst*I–*Eco*RI fragment followed by its replacement with an altered gene assembled from oligonucleotides. The structure of the resulting plasmid, pBS/SUP3, is shown in Figure 1. This figure also shows schematically the relationship between overlapping oligonucleotides used in tRNA gene synthesis. Ten oligonucleotides were used in the assembly of each gene as described under Experimental Procedures.

Mutations Affecting Conserved Secondary Structures. Figure 2 shows the sequence of pre-tRNA^{Tyr} in a cloverleaf configuration to emphasize the secondary structures conserved among tRNAs. Requirements for sequence or structure within each of the four stems of the cloverleaf were examined as follows. The 5′ and 3′ portions of each double-stranded stem were altered in separate constructs to examine the effects of disrupting secondary structure. The sequence changes to each half of the stem were compensatory so that when introduced together in a third construct, the potential secondary structure of the stem was retained while the primary sequence was altered. These latter constructs with compensatory changes in both sides of the stem are designated "Dbl" (e.g., T-stem Dbl). The nomenclature and the sequence changes for this group of mutations are outlined in Figure 2, and the complete

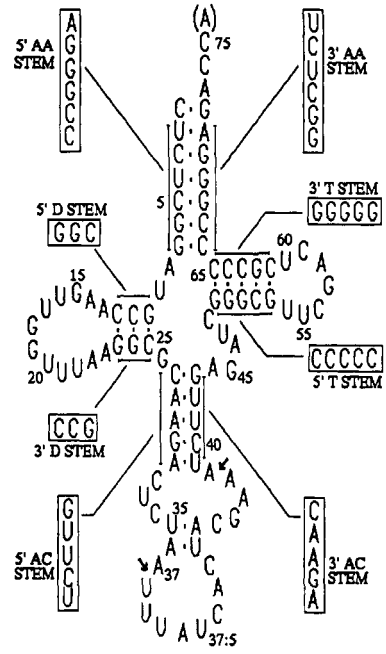

FIGURE 2: Summary of secondary structure replacements. The sequence of pre-tRNA^{Tyr} is shown with splice sites indicated by arrows and numbering according to the convention proposed for tRNA^{Phe} (Schimmel et al., 1979). The segments replaced in individual stem variants are indicated by brackets with the sequence introduced as a replacement shown boxed. The name assigned to the corresponding single replacement variant is shown beside each box. Double segment replacements (designated "Dbl") were also constructed for each stem in which both of the single segment replacements were introduced together.

sequence for each of the constructs is described under Experimental Procedures.

Labeled pre-tRNA substrates were prepared by in vitro transcription and purified by gel electrophoresis. Substrates were then incubated with yeast endonuclease to measure IVS excision or with both endonuclease and ligase to examine the complete splicing reaction. For measuring reaction time courses, products were resolved by electrophoresis and quantitated by measuring radioactivity in gel slices. To confirm accurate splicing, one-dimensional RNase T1 oligonucleotide analysis was carried out as described under Experimental Procedures. The results of reaction time courses are shown in Figure 3. Excision of the IVS by endonuclease was dramatically reduced for all of the mutations which disrupted secondary structure (Figure 3A). In contrast, endonuclease was fully active for all of the Dbl substrates in which the potential secondary structure of stems was preserved. Thus while no specific primary sequence requirements can be shown for any of the stems, the conserved secondary structures of these regions are required either directly or indirectly for cleavage by endonuclease. Joining by ligase in complete splicing reactions was measured for the Dbl variants only since the remaining variants were not cleaved by endonuclease. Joining of tRNA halves for all but one of the Dbl variants was comparable to that for pre-tRNA^{Tyr}, demonstrating that no specific sequences within the corresponding stems are required by yeast ligase. The exception was the aminoacyl stem Dbl (AA stem Dbl) variant, for which significantly reduced joining was observed (Figure 3B).

Basis for the Joining Defect in AA Stem Dbl Variant. One of the endonuclease cleavage products (identified below as the 3′ half) for the AA stem Dbl variant migrates ahead of its pre-tRNA^{Tyr} counterpart upon electrophoresis (see Figure 4). The possibility that failure to join these cleavage products was

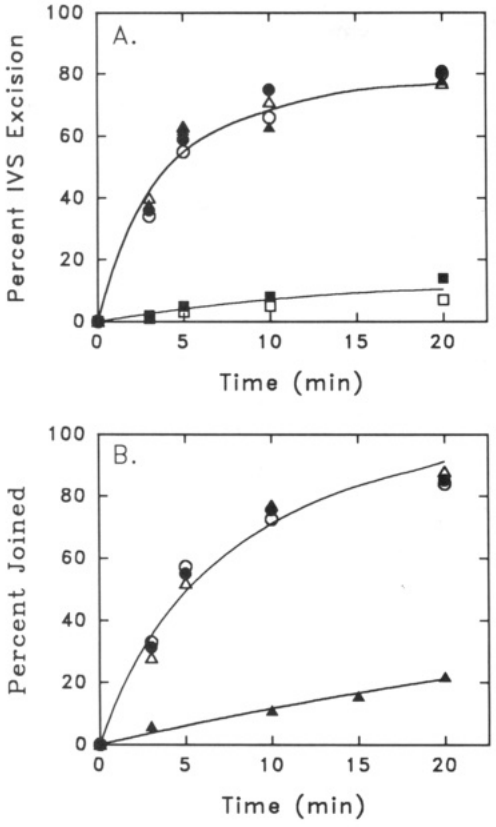

FIGURE 3: Time course for splicing of secondary structure variants. Labeled pre-tRNA substrates (~6 fmol) prepared by in vitro transcription were incubated at 30 °C in reactions (10 μL) containing endonuclease alone (panel A) or endonuclease plus ligase (panel B). Following incubation for the times indicated, reaction products were resolved by denaturing gel electrophoresis, visualized by autoradiography, and quantitated by measuring Cerenkov radiation in gel slices. Calculation of percent IVS excision by endonuclease and percent exon joining by ligase was as previously described (Greer et al., 1987). The nomenclature for the pre-tRNA variants is summarized in Figure 2 and corresponds to symbols as follows: D-stem Dbl (○), AC stem Dbl (●), T-stem Dbl (△), AA stem Dbl (▲), 5′ AA stem (□), and 5′ D stem (■). To save space, results for a representative set of variants are shown as follows. Endonuclease cleavage was significantly reduced among all of the single segment replacements, and only the 5′ AA stem and 5′ D stem variants are shown as examples of this group. The results of excision and joining assays with wild-type pre-tRNA^{Tyr} (see Figure 6C,D) were similar to that for the AC stem Dbl variant, and only the results for the Dbl variant are shown.

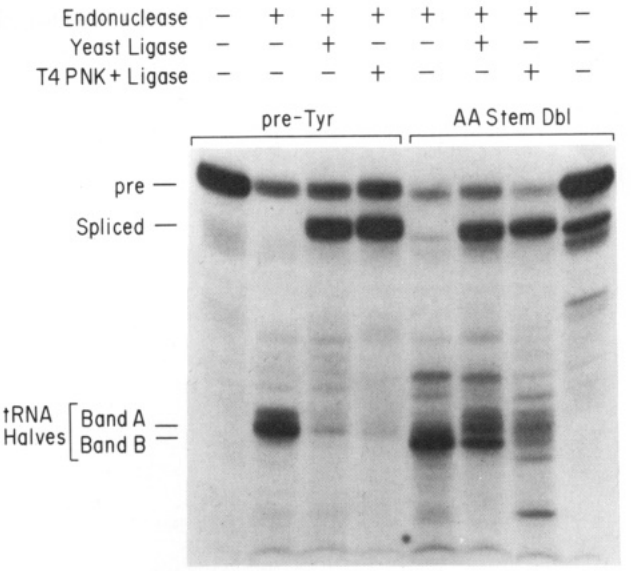

FIGURE 4: Splicing of the AA stem Dbl variant. A comparison of tRNA halves and spliced products is shown for pre-tRNA^{Tyr} and AA stem Dbl transcripts (variants are summarized in Figure 2). Labeled substrates (~6 fmol) were incubated in buffer alone or with varying combinations of endonuclease (0.92 unit/mL), yeast ligase (2.1 units/mL), or T4 kinase plus T4 ligase (500 units/mL each) as indicated at the top of the figure. Following incubation for 20 min at 30 °C, reaction products were resolved by denaturing gel electrophoresis and visualized by autoradiography. The identification of pre-tRNA substrates and reaction products is indicated at the left edge and summarized in the text.

due to inaccurate cleavage by endonuclease was examined by direct RNA sequence analysis (data not shown). Substrates labeled with [α-^{32}P]GTP or [α-^{32}P]UTP were prepared by in vitro transcription, and precursor and labeled endonuclease products were isolated by gel electrophoresis. RNase T1 fingerprint analysis of the isolated pre-tRNAs confirmed that the sequences of the substrates were as expected from the DNA sequences of the transcription templates. RNase T1 fingerprint analyses coupled with nuclease P1 secondary digests of T1 oligonucleotides were used to demonstrate that the bands which comigrate in pre-tRNA^{Tyr} and AA stem Dbl reactions (labeled band A in Figure 4) corresponded to 5′ halves. Nuclease P1 secondary analysis of appropriate RNase T1 oligonucleotides revealed that 5′ splice site selection was identical in these two substrates. RNase T1 fingerprinting [α-^{32}P]UTP-labeled substrates revealed that the more rapidly migrating AA stem Dbl cleavage product (Figure 4, band B) corresponded to the 3′ exon. RNase A secondary digestion analysis of splice site oligonucleotides revealed that cleavage at the 3′ splice site in the AA stem Dbl variant was also accurate. Thus the altered mobility of the 3′ half is not due to inaccurate cleavage and, instead, may reflect a change in its structure.

Potentially, a change in the structure of the 3′ half might account for poor joining by tRNA ligase. This effect might be specific through a change in a recognition site or might be more general through the formation of a structure which separates ends to be joined. This latter possibility was examined using phage T4 RNA ligase, a nonspecific enzyme (England & Uhlenbeck, 1978) capable of joining tRNA halves in conjunction with T4 polynucleotide kinase (Greer et al., 1983a). Joining of the AA stem Dbl halves by the T4 enzyme was substantially reduced relative to pre-tRNA^{Tyr} halves (Figure 4). Together, these results suggest the AA stem Dbl halves may adopt an alternate structure(s) in which ends to be joined are no longer juxtaposed. Circumstantial evidence consistent with this possibility includes the following. First, lowest free energy calculations suggest the AA stem Dbl 3′ half has the unique potential for forming an alternate, stable structure in which the aminoacyl and anticodon stem segments are paired (data not shown). This alternate structure might account for the anomalous electrophoretic mobility observed for these halves. Second, in many incubations the yield of 5′ exon did not appear proportional to that for the 3′ exon. Potentially, the two halves may dissociate, with the 3′ half forming a stable structure resistant to degradation.

Effects of 5′ Leader and 3′ Trailer Segments. Processing of tRNA^{Tyr} gene transcripts, both in yeast (O'Connor & Peebles, 1991) and in *Xenopus* (Melton et al., 1980), is thought to be ordered with removal of 5′ leader and 3′ trailer sequences preceding splicing. To test whether specificity on the part of the splicing enzymes might account for ordered processing of this precursor, the effects of leader and trailer segments on excision and joining reactions were examined. For this purpose, two different sets of variants were made (summarized in Figure 5). One set contained the flanking sequences from the primary transcript of the SUP6 gene [see Evans and En-

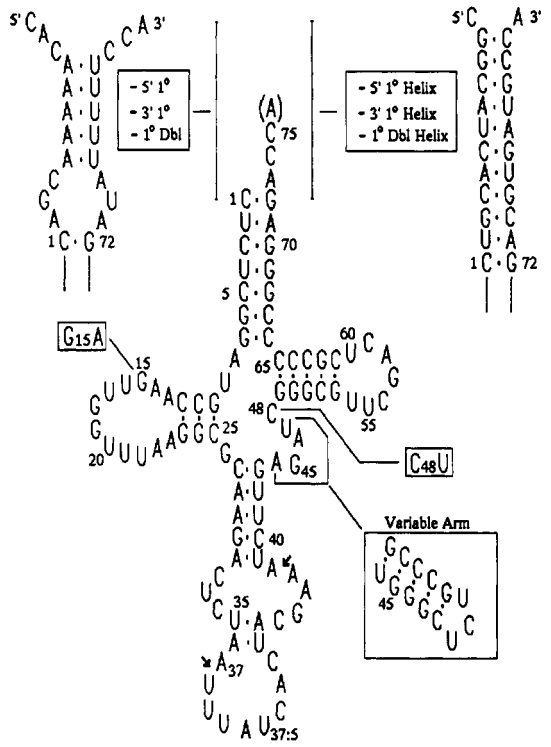

FIGURE 5: Summary of tertiary structure and flanking sequence replacements. The sequence of pre-tRNA^{Tyr} is shown with splice sites indicated by arrows and numbering according to the convention for tRNA^{Phe} (Schimmel et al., 1979). Flanking sequences inserted between the transcription start site and the mature 5′ end (indicated as position +1) and between the tRNA 3′ end (position 72) and the *Bst*NI transcription runoff site are shown at the top right and left. The names assigned to the corresponding single and double flanking sequence insertions are indicated in boxes. Also shown are replacements and assigned names for the Levitt tertiary pair (positions 15 and 48) variants and for replacement of the variable arm (positions 44–47).

gelke (1990)]. Variants were made that contained only the 5′ leader, only the 3′ trailer, or both the leader and trailer sequences (designated 5′1°, 3′1°, and 1° Dbl, respectively). The predicted helix formed by pairing of these leader and trailer segments is shown in Figure 5 and is composed entirely of A·U base pairs. To examine whether the potential to form a more stable structure might influence splicing, G/C-rich leader and trailer segments were also created. Constructs containing these 5′ leader and 3′ trailer segments separately and in combination were designated 5′1° helix, 3′1° helix, and 1° Dbl helix, respectively (Figure 5). In all of the templates designed to generate end-extended precursors, transcription initiation was with CTP and 3′ ends were formed by runoff at a *Bst*NI site.

Labeled precursors from transcription reactions were purified by gel electrophoresis and used as substrates in splicing assays. A time course for IVS excision by endonuclease is shown in Figure 6A and for splicing by both endonuclease and ligase in Figure 6B. In both reactions, processing of the end-extended variants was indistinguishable from that of the end-mature pre-tRNA. These results provide a clear demonstration that ordering of the end maturation and splicing steps for pre-tRNA^{Tyr} cannot be directly attributed to inherent specificity on the part of the yeast splicing enzymes.

Role of Tertiary Structural Elements. Hydrogen-bonding interactions between bases in the D-arm and variable arm are among the significant determinants of the L-shaped tertiary configuration characteristic of tRNA structure [reviewed by Kim (1976) and Rich and RajBhandary (1976)]. These

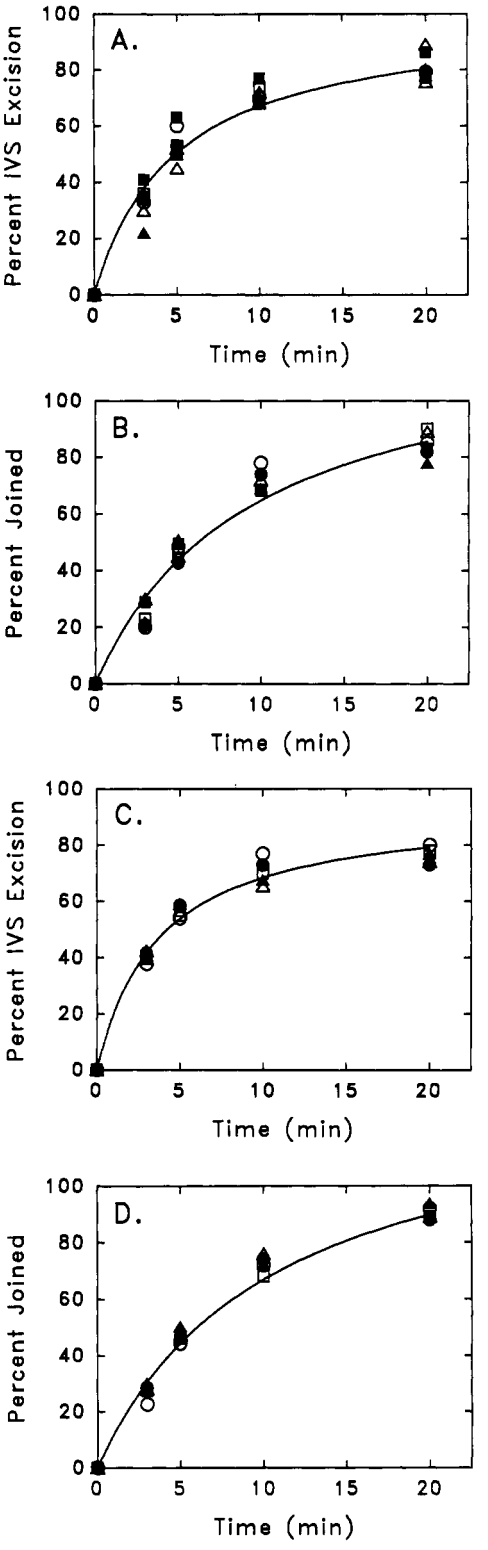

FIGURE 6: Time course for splicing of flanking sequence and tertiary structure variants. A comparison is shown for splicing of each of the pre-tRNA variants summarized in Figure 5. Labeled pre-tRNA substrates (~6 fmol) were incubated at 30 °C with endonuclease (panels A and C) or with endonuclease plus yeast ligase (panels B and D). Following incubation for the indicated times, reaction products were resolved by denaturing gel electrophoresis, visualized by autoradiography, and quantitated by measuring Cerenkov radiation in gel slices. Calculation of percent IVS excision and percent exon joining were as described previously (Greer et al., 1987). The nomenclature for pre-tRNA variants is summarized in Figure 5 and corresponds to symbols as follows. Panels A and B: 5′1° (○), 3′1° (●), 1° Dbl (△), 5′1° helix (▲), 3′1° helix (□), 1° Dbl helix (■). Panels C and D: pre-Tyr (○), G15A (●), C48U (△), G15A/C48U (▲), variable arm (□).

tertiary interactions occur at the corner of the "L" within a region previously implicated in pre-tRNA recognition by yeast endonuclease (Greer et al., 1987; Reyes & Abelson, 1988; Shapero & Greer, 1991). Disruption of the Levitt pair in pre-tRNA^{Phe} (type I variable arm) has been shown to reduce cleavage by endonuclease (Reyes & Abelson, 1988), making this a good candidate for a common recognition feature. To further test this possibility, single and compensatory double changes were made at these positions in pre-tRNA^{Tyr} (G15, C48) and their effects on splicing were examined. Also, a replacement of the pre-tRNA^{Tyr} variable arm was constructed to test whether additional tertiary interactions within this segment are required. For this purpose, the first 4 nt of the pre-tRNA^{Tyr} variable arm (positions 44–47) were replaced with a 13-nt sequence corresponding to the type II extra arm from pre-tRNA^{Ser}. Note that this replacement includes the potential for forming a stable stem–loop secondary structure characteristic of this class of variable arms and also changes the Pu/Pu pair (G26/A44) characteristic of type I tRNAs to a Pu/Py combination (G26/U44) common among type II sequences (Brennan & Sundaralingam, 1976).

The sequence changes and nomenclature for the variants affecting variable arm interactions are summarized in Figure 5. Labeled pre-tRNA substrates were prepared by in vitro transcription, purified by gel electrophoresis, and incubated in splicing reactions. The results for reactions with endonuclease alone are shown in Figure 6C and for both endonuclease and ligase in Figure 6D. The time courses for IVS excision and splicing among all of the variable arm derivatives were indistinguishable from those for pre-tRNA^{Tyr}. Thus neither retention of the conserved Levitt pair nor the presence per se of a type I extra arm in this precursor are required for efficient splicing.

Discussion

An essential role for exon sequence or structure in recognition by the pre-tRNA splicing enzymes has been suggested previously on the basis of four kinds of evidence. First, using gel shift assays, high-affinity binding dependent on tRNA-like structure has been demonstrated for *Xenopus* tRNA endonuclease (Baldi et al., 1986) and yeast tRNA ligase (Apostol & Greer, 1991). Second, a temperature- and magnesium-dependent conformational change within the tRNA-like portion of pre-tRNA^{Tyr} has been shown to affect cleavage by yeast endonuclease (Shapero & Greer, 1991). Third, exons are implicated indirectly by the apparent lack of conserved features within introns [cf. Szekely et al. (1988)] and the ability to tolerate extensive changes in IVS sequence and structure without affecting splicing accuracy [see Strobel and Abelson (1986) and Reyes and Abelson (1988) and references therein]. Finally, a large number of mutations within exon sequences have been shown to affect splicing [see the review by Culbertson and Winey (1989)]. Perhaps most telling among these exon mutations are those which affect the length of the anticodon stem (Greer et al., 1987; Mattoccia et al., 1988; Reyes & Abelson, 1988). Small insertions or deletions which change the length but preserve the structure of this stem result in a relocation of endonuclease cleavage sites so as to maintain a constant distance relative to the top of this stem. This result suggests that splice sites are defined by a measuring mechanism with the point of reference provided by a primary binding site within the tRNA-like portion of the substrate.

Collectively, these previous results provide good evidence that specific recognition of exons is required for pre-tRNA splicing. Conceptually, a site-specific RNA binding protein may recognize ligands through sequence-specific contacts or via structure-specific interactions [see the review by Steitz (1990)]. The conformation of exon segments in tRNA precursors is similar to that of their mature counterparts (Swerdlow & Guthrie, 1984; Lee & Knapp, 1985; Hall & Sampson, 1990) and is thought to include each of the 4 helical stems of the tRNA cloverleaf arranged in the characteristic L-shaped tertiary structure [reviewed by Kim (1976) and Rich and RajBhandary (1976)]. Additionally, conserved nucleotides common among all tRNAs can be found throughout the molecule [reviewed by Dirheimer et al. (1979) and Cedergren et al. (1981)]. Thus the exon segments of pre-tRNA substrates contain numerous conserved structural and sequence features that could serve as recognition elements.

Within the base-paired stems of the cloverleaf secondary structure, there may be no specific sequence requirements. Instead, any recognition of these regions by the splicing enzymes may be limited to structure-specific contacts. This conclusion is based on the experiments described here, previous results by others, and the results of a search of pre-tRNA sequences for conserved features (data not shown).

In searching for conserved features we used the same set of pre-tRNA sequences used previously for identifying intron and splice site sequences common among pre-tRNA substrates (Szekely et al., 1988). In comparing exon segments, we expected to find both sequences conserved among all tRNAs as well as features uniquely conserved among the available subset of intron-containing pre-tRNA sequences. Features in each of these two categories are described below along with a summary of the results of splicing assays.

Requirements within the Amino Acid Acceptor Arm. The acceptor arm consists of a 7 base pair stem, 2 single-strand nucleotides that link the 5′ half of the stem to the D-arm, and 4 nucleotides that extend from the 3′ half and include the conserved 3′-CCA sequence. The only common sequence feature within the stem is a bias (91%) for G or U at position 6 within pre-tRNAs. Efficient cleavage of the AA stem Dbl variant suggests this feature is not required for recognition by endonuclease. However, poor joining of this substrate by both yeast and T4 RNA ligases suggests the endonuclease cleavage products may be conformationally unstable. Failure to splice the 5′ or 3′ AA stem variants suggests the secondary structure of this stem is required either directly or indirectly for splicing. The conserved 3′-CCA sequence is added posttranscriptionally to nuclear-encoded tRNAs. Normal splicing of both the 3′1° and 3′1° helix substrates indicates the presence of a CCA sequence at positions 74–76 is not required for splicing.

Although cleavage of the AA stem Dbl substrate was normal, joining of the resulting halves by yeast ligase was reduced significantly. Two-dimensional RNase T1 fingerprints of the endonuclease products revealed that the effect on ligase could not be attributed to inaccurate cleavage. Instead, reduced joining by a nonspecific enzyme, phage T4-encoded RNA ligase, suggested halves produced from this substrate may adopt an inappropriate conformation in which the ends to be joined are inaccessible or held at a distance. Thus it is likely that the reduction of joining by yeast ligase of AA stem Dbl halves is not the result of changing a sequence-specific recognition element in the amino acid acceptor stem.

Requirements within the D-Arm. The D-arm consists of a 3 or 4 base pair stem completed by a loop of variable length. The loop contains several constant residues as well as the modified base dihydrouridine. Previously, one of these conserved bases, C19, and the tertiary pair in which it participates (C19/G56) have been shown to be dispensable for cleavage of pre-tRNA^{Phe} by yeast and *Xenopus* endonuclease (Reyes

et al., 1988; Mattocia et al., 1988). Our results with respect to the role of another conserved base, G15, differ from two previous observations. This base participates in a tertiary pair with C48 [first described by Levitt (1969)] which stabilizes stacking of the D- and T-stems. We found that single or compensatory double mutations affecting this pair had no effect on splicing. Previously, a point mutation (C48U) affecting this pair in pre-tRNA^{Ser} had been shown to reduce splicing in vivo (Willis et al., 1984; Pearson et al., 1985). Additionally, single mutations affecting this pair in pre-tRNA^{Phe} have a moderate effect on endonuclease cleavage in vitro which is restored by a compensatory second mutation (Reyes & Abelson, 1988). The differences in these observations may be due to the differences between in vivo and in vitro measurements and between the structures of these segments in pre-tRNA^{Tyr} and pre-tRNA^{Phe} (Swerdlow & Guthrie, 1984). Our results suggest this tertiary pair is not a general requirement for splicing and may contribute to the conformational stability of a subset of pre-tRNA substrates.

Within the D-stem, G10 and a pyrimidine at position 11 are common among tRNAs. Specific to pre-tRNAs is a pyrimidine bias at position 12. Despite these conserved sequence features, requirements within the D-stem appear limited. Disruption of the D-stem in the pre-tRNA^{Tyr} variants (5′ and 3′ D-stem) completely inhibited intron excision by tRNA endonuclease. Restoration of base-paired structure in the D-stem Dbl variant restored efficient splicing. These results are not consistent with any specific sequence requirement within the D-stem and suggest base-paired structure is required either directly or indirectly. Similar requirements within the D-stem have been demonstrated for splicing of yeast pre-tRNA$_3^{Leu}$ in *Xenopus* extracts (Mattoccia et al., 1988). For purified *Xenopus* endonuclease, a G/C- as opposed to an A/U-rich stem was essential, a requirement that was not observed in crude germinal vesicle extracts (Baldi et al., 1983; Gandini-Attardi et al., 1985). These results suggest that the stability of this stem may be crucial in the *Xenopus* system and that factor(s) in crude extracts may affect that stability.

Requirements within the Anticodon Arm. The anticodon arm in mature tRNAs consists of a 5 base pair stem completed by a loop of 7 bases. Within the loop, conserved sequences are found at three positions: a pyrimidine at position 32, U33, and a purine at position 37. Within pre-tRNA substrates, the intervening sequence interrupts this loop between positions 37 and 38. Changes in each of the three conserved loop positions have been shown to affect splicing either in yeast or in *Xenopus* (Colby et al., 1981; Nishikura et al., 1982; Greer et al., 1987; Reyes & Abelson, 1988). Thus exon sequence and/or structural elements near the 5′ splice site are important for splicing. Within the base-paired stem, conserved sequence features are limited to a slight bias (73%) among pre-tRNAs for an A/U pair at the base of the stem (A31/U39) and an adjacent G/C pair common among all tRNAs. These sequences are apparently not required for efficient splicing since changes at these positions in the AC Dbl substrate had no effect on splicing. However, disruption of the secondary structure of this stem in the 5′ and 3′ AC stem variants prevented splicing, indicating that this base-paired stem is required either directly or indirectly for splicing. These results are consistent with previous observations that single nucleotide changes within the anticodon stem affect splicing (Nishikura et al., 1982; Reyes & Abelson, 1988). Also, a systematic analysis of requirements at the 31/39 base pair was undertaken for splicing of pre-tRNA^{Pro} in yeast by Mathison et al. (1989). On the basis of the pattern of allowed substitutions, these workers concluded that structure rather than sequence was important at this position.

Requirements within the Variable Arm. Hydrogen-bonding interactions between bases in the variable arm and D-arm are among the significant determinants of the L-shaped tertiary configuration characteristic of tRNA structure [reviewed by Kim (1976) and Rich and RajBhandary (1976)]. The nature of these interactions varies, reflecting the variability in the sequence and structure of the variable arm region. Two general classes of tRNAs have been described with respect to variable arm structure [see Brennan and Sundaralingam (1976)]. The type I tRNAs are characterized by short variable arm segments (4–5 nt) and include extensive tertiary interactions between the variable and D-arms. These tertiary interactions occur at the corner of the "L" within a region previously implicated in pre-tRNA recognition by yeast endonuclease (Greer et al., 1987; Reyes & Abelson, 1988; Shapero & Greer, 1991). The type II tRNAs are characterized by long variable arm sequences which are capable of forming hairpin stem and loop secondary structures. Among the ten previously-identified yeast pre-tRNAs, four are typical of type II sequences whereas the remainder are type I (Ogden et al., 1984; Stucka & Feldman, 1988). Features thought to be common to both classes of variable arms are limited. In particular, only the Pu15/Py48 tertiary pair described by Levitt (1969) and the positions 26/44 non-Watson/Crick pair may be common to type I and type II tRNAs [see Brennan and Sundaralingam (1976)]. This variation in the sequence and structure of the variable arm among pre-tRNAs makes it an unlikely candidate for a common recognition element. Consistent with this is our observation that the type I variable arm in pre-tRNA^{Tyr} can be replaced with its type II counterpart from pre-tRNA^{Ser} without affecting splicing. Additionally, Mattocia et al. (1988) have shown that deletion of 8 nucleotides from the type II extra arm of tRNA$_3^{Leu}$ (producing a 5-nt arm) has no effect on cleavage by *Xenopus* endonuclease. Together, these results suggest the two types of variable arms are functionally interchangeable for splicing and suggest that variable arm mutations previously shown to affect splicing (Willis et al., 1984; Pearson et al., 1985; Greer et al., 1987) may act indirectly by affecting other structures.

Requirements within the T-Arm. The T-arm consists of 5 base pair stem closed by a 7 base pair loop. Within the loop, 6 of the 7 positions are conserved among tRNAs. Within the T-stem, only the G53/C61 pair is conserved among tRNAs and the pre-tRNAs show a slight purine bias (~80%) at positions 49, 51 and 52 in the 5′ half of the stem. Efficient splicing of the T-stem Dbl substrate indicates conserved sequence within this stem is not required for splicing. Instead, failure to splice the 5′ and 3′ T-stem variants suggests that the secondary structure of this stem is required, perhaps through contributing to the overall conformation.

Requirements for Mature 5′ and 3′ Ends. Processing of pre-tRNA^{Tyr} in yeast and in *Xenopus* is ordered, with maturation of 5′ and 3′ ends preceding splicing (Melton et al., 1980; O'Connor & Peebles, 1991). Here, the presence of 5′ leader or 3′ trailer sequences either alone or in combination had no effect on splicing in vitro. This was true for both authentic leader and trailer sequences derived from tRNA^{Tyr}[sup6] gene sequences and for a G/C-rich synthetic sequence.

Processing of a human pre-tRNA^{Tyr} gene transcript in HeLa extracts proceeds with splicing prior to end maturation (van Tol et al., 1987). However, when a plant (*Nicotiana*) pre-tRNA^{Tyr} gene is transcribed and processed by this same HeLa extract, splicing follows processing of the end-extended se-

quences. This previous observation is consistent with our results and suggests that, for both the yeast and HeLa enzymes, ordering of splicing and end processing events cannot be attributed to specificity on the part of the splicing enzymes themselves. Previously, cleavage by *Saccharomyces cerevisiae* endonuclease of *Schizosaccharomyces pombe* pre-tRNA^{Ser}-(sup3e) had been shown to be inhibited by the presence of an unpaired 5′ leader (Greer et al., 1987). The effect of the leader in this previous case may be indirect and may be unique to this particular substrate.

Substrate Recognition. The results of this and previous work reveal that sequence-specific requirements in pre-tRNA substrates may be limited to two largely single-stranded regions, the D- and T-loops, found together at the corner of the L-shaped tertiary structure and the anticodon loop sequences adjacent to splice sites. The anticodon stem appears to serve a central but purely structural function in linking these two regions. The remaining base-paired stems may also contribute indirectly by promoting the formation of the correct tertiary conformation. These results also demonstrate that the properties of the splicing enzymes alone cannot account for ordered end maturation and splicing of pre-tRNA^{Tyr} and, instead, kinetic constraints or functional compartmentation may determine ordering in vivo.

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