Conformational Transition Required for Efficient Splicing of Transcripts from Hybrid λ Promoter Yeast tRNA Gene Fusion†

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ABSTRACT: Fusion of a prokaryotic promoter to a yeast tRNA gene provides a means for uncoupling analyses of mutations affecting splicing from requirements for transcription and other processing steps. For this purpose, a phage λ promoter was fused to the *Saccharomyces cerevisiae* $tRNA^{Tyr}$(SUP3a) coding sequence. This fusion allows the synthesis of an end-mature precursor by in vitro transcription with *Escherichia coli* RNA polymerase. This precursor was accurately spliced by purified yeast endonuclease and ligase fractions. However, both the initial rate and the extent of the endonuclease cleavage reaction were reduced in comparison to those for substrates produced by yeast RNA polymerase III. Efficient splicing could be restored in a magnesium- and temperature-dependent renaturation step, suggesting a conformational transition was required. Enzymatic solution structure probing of transcripts from wild-type and intron-variant templates revealed that the essential conformational transition involved a segment of the tRNA-like portion of the precursor. These results (1) suggest that the primary sequence of the precursor alone may not be sufficient to ensure formation of the active conformer during synthesis, (2) provide direct evidence that endonuclease recognizes mature tRNA-like structure in the precursor, and (3) suggest a general caution for the use of semisynthetic transcripts in RNA processing reactions. Potentially, transcription and processing of $tRNA^{Tyr}$ in yeast may provide a useful paradigm for examining active control of conformation in RNA biosynthesis.

Splicing of intron-containing tRNA precursors (pre-tRNAs) in the yeast *Saccharomyces cerevisiae* involves at least two distinct enzymes: a tRNA endonuclease and an ATP-dependent RNA ligase (Peebles et al., 1979, 1983). The endonuclease cleaves precisely at the two splice sites to yield paired tRNA halves and the linear intervening sequence (IVS)[1] (Knapp et al., 1979; Peebles et al., 1983). The ligase joins the halves to form the mature sequence tRNA (Knapp et al., 1978; Greer et al., 1983). Evidence for single endonuclease and ligase activities in yeast includes extensive copurification of cleavage activity measured for a variety of pre-tRNA substrates (Peebles et al., 1983; P. Green, R. Rauhaut, and J. Abelson, personal communication) and the pleiotropic character both of conditional ligase gene mutations (E. Phizicky, personal communication) and of mutations affecting endonuclease activity (Winey & Culbertson, 1988; Ho et al., 1990). The ability of a single set of splicing enzymes to process an apparently diverse set of pre-tRNA substrates is the subject of this research.

The yeast haploid genome contains at least 10 distinct tRNA gene families that contain introns (Ogden et al., 1984; Stucka & Feldman, 1988). A comparison of tRNA gene sequences and analysis of pre-tRNA transcripts reveal certain common features which might be recognized by the splicing enzymes. While the primary sequences of these introns show little in common (Ogden et al., 1984; Szekely et al., 1988), their conserved position (all interrupt the anticodon loop sequence at the same site) and small size (14–60 bp) suggest the overall structures of the pre-tRNA substrates may be similar (Lee & Knapp, 1985). Both lowest free energy calculations (Guthrie & Abelson, 1982; Ogden et al., 1984) and the results of solution structure probing (Swerdlow & Guthrie, 1984; Lee & Knapp, 1985) suggest these precursors all form a structure consisting of a tRNA-like domain with the conserved secondary and tertiary interactions characteristic of their mature tRNA counterparts and an IVS-containing domain extending from the anticodon stem. The primary sequence of exons includes numerous conserved features common to all tRNA genes [see the compilation by Sprinzl et al. (1987)]. Thus, the tRNA-like structure of the precursor, including conserved primary, secondary, and tertiary structure within exons, may provide common features for recognition by the splicing enzymes.

These considerations have focused our attention on specific elements of exon sequence and structure required for splicing. However, analysis of these elements via mutagenesis is constrained in many expression systems by requirements for exon sequences in transcription by RNA polymerase III [reviewed by Geiduschek and Tocchini-Valentini (1988)] and in processing steps in addition to splicing [cf. Deutscher (1984)]. The construction of artificial promoter–tRNA gene fusions provides an effective strategy for eliminating these constraints cf. Leontis et al. (1988), Sampson and Uhlenbeck, (1988). Reyes and Abelson (1988), and Samuelsson et al. (1988)]. In this study, we report the characterization of a splicing substrate produced from such a fusion template by in vitro transcription. Efficient splicing of the resulting pre-$tRNA^{Tyr}$ substrate required a temperature-dependent conformational transition within the mature domain of the molecule. This result provides independent evidence for the role of the mature domain in

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[1] Abbreviations: amp, ampicillin; bp, base pairs; CpU, cytidylyl-(3′,5′)uridine dinucleotide; ECpTyr, pre-$tRNA^{Tyr}$ produced by transcription of plasmid pSUP3 with *E. coli* RNA polymerase; EC:IVSpTyr, pre-$tRNA^{Tyr}$ transcripts of plasmid pSUP3[IVS(Pvu)] with *E. coli* RNA polymerase; EDTA, ethylenediaminetetraacetic acid; IVS, intervening sequence; nt, nucleotide(s); Pol III, RNA polymerase III; Tris, tris(hydroxymethyl)aminomethane; Tyr, tyrosine; YpTyr, pre-$tRNA^{Tyr}$ produced by transcription with RNA polymerase III.

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splicing, suggests alternative folding of transcripts may be an important factor in vivo, and provides a general caution for the use of semisynthetic substrates in RNA processing.

Experimental Procedures

Enzymes and Chemicals. The restriction enzymes *Bam*HI and *Pst*I were obtained from Boehringer Mannheim. *Xma*I and *Bst*NI were from New England Biolabs. Oligonucleotides used in directed mutagenesis were from the Agouron Institute, La Jolla, CA, or from the Protein/Nucleic Acid Analysis Laboratory of the Department of Biological Chemistry, University of California, Irvine. 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. (1983). Phage T4 polynucleotide kinase was from United States Biochemicals. Cobra venom ribonuclease V1, DNase I, phage T4 RNA ligase, and *Escherichia coli* RNA polymerase were from Pharmacia. Nuclease P1 and RNases T1 and T2 were obtained from Calbiochem. Pancreatic ribonuclease was from Sigma Chemical Co. Nuclease S1 was from BRL. RNA carrier was obtained from Sigma (type X-S). 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 cytidine 3',5'-[5'-^{32}P]bisphosphate (3000 Ci/mmol) were from Amersham.

Buffers. TE buffer contained 10 mM Tris-HCl (pH 7.4)/1 mM EDTA. Electrophoresis sample buffer contained 20% sucrose, 8 M urea, 0.1% xylene cyanol, and 0.1% bromophenol blue, and electrode buffer contained 90 mM Tris–borate (pH 8.3)/2.4 mM EDTA.

Construction of Plasmid pSUP3 and IVS(Pvu) Mutant. (A) SUP3a Gene Construct. Plasmid pWJ39 containing the yeast SUP3a gene (Weeks-Levy, 1985) was provided by Dr. Rodney Rothstein. A 137 bp *Bam*HI fragment (including the SUP3a gene) derived from pWJ39 was inserted in the *Bam*HI site of plasmid pSP64 (Melton et al., 1984). A portion of the 5'-flanking region in pSP64(SUP3) was removed by digestion with *Xba*I and *Hin*dIII, treatment with nuclease S1, and ligation with DNA ligase to create plasmid pMS1. Digestion of plasmid pMS1 with *Xma*I followed by religation was used to delete a 49 bp fragment containing the last 3 bp of tRNA coding sequence and the 3'-flanking segment (including the downstream *Bam*HI site) remaining from the SUP3a gene. The resulting plasmid was designated pMS2.

(B) E. coli Expression Vector. Plasmid pPM1 (Ahlquist & Janda, 1984), containing a temperature-sensitive allele of the λ cI repressor gene and the λ P_M promoter (a derivative of the P_R promoter), was provided by Dr. Paul Ahlquist and Agrigenetics Research Associates. For insertion of the SUP3a gene, plasmid pPM1 was digested sequentially with *Sma*I and *Eco*RI, producing a linear form with blunt and *Eco*RI-compatible ends. A 110 bp SUP3a fragment, excised from plasmid pMS2 by sequential digestion with *Bam*HI, nuclease S1, and *Eco*RI, was then joined to these ends to produce plasmid pMS3.

(C) Modification of Transcription Initiation and Runoff Sites. The initial fusion construct from pMS3 was modified by using oligonucleotide-directed mutagenesis to allow transcription initiation at the first nucleotide of the mature tRNA sequence and to introduce the 3'-CCA sequence (not encoded in yeast nuclear tRNA genes) within an appropriate restriction site for transcription runoff. For this purpose, a 900 bp *Pst*I–*Eco*RI fragment including the λ promoter–SUP3a segment from plasmid pMS3 was subcloned into the corresponding sites in the polylinker of M13mp19 replicative-form (RF) DNA. A series of oligonucleotides was used to carry out site-directed mutagenesis essentially as described by Zoller and Smith (1982). The first oligonucleotide changed the −1 to −10 region of the λ P_M promoter to that of the λ c17 promoter (Rosenberg et al., 1979) and also deleted 10 base pairs of 5'-flanking sequence from the SUP3a gene. A second oligonucleotide was used to introduce the 3'-CCA sequence as either a *Bst*NI or an *Nde*I restriction site at the 3' end of the tRNA gene. The complete sequences of the λ promoter–tRNA gene fusions and the IVS(Pvu) variant (described under Results and generated by direct mutagenesis as described above) were confirmed by using the chain termination method of Sanger et al. (1977) and a 17 bp universal M13 sequencing primer obtained from United States Biochemicals. Following directed mutagenesis, a 900 bp *Pst*I–*Eco*RI fragment containing the λ cI gene and the λ promoter–tRNA gene fusion from each of the M13 derivatives was subcloned into the corresponding sites in the polylinker of plasmid pUC19 (Yanisch-Perron et al., 1985). The resulting plasmids are designated pSUP3 (containing the SUP3a gene with a *Bst*NI runoff site), pSUP3n (containing the SUP3a gene with an *Nde*I runoff site), and pSUP3[IVS(Pvu)] (containing the SUP3a gene with a modified intron as described in the text and a *Bst*NI runoff site). For producing pre-tRNA transcripts at high levels in bacterial cells, two distinct terminator segments were inserted downstream of the yeast SUP3a gene. The resulting plasmids were pSUP3T'$_{ilv}$ containing a terminator derived from the *E. coli ilv*GMEDA locus (Lawther et al., 1987) and pSUP3T$_{trpa}$ containing a synthetic terminator based on the sequence of the *trpA* attenuator (Christie et al., 1981). These templates produce pre-tRNA transcripts with approximately 120 and 30 nucleotides of 3'-trailer sequence, respectively.

In Vitro Transcription Reactions. Transcription with *E. coli RNA* polymerase was carried out at 37 °C for 30 min in 50-μL reactions containing 40 mM Tris-HCl (pH 7.9), 150 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 10 μM each of ATP, GTP, and UTP, 150 μM CTP, [α-^{32}P]UTP (50 μCi), 12 units of *E. coli* RNA polymerase, and 10 μg of linearized plasmid template. At the end of the reaction, 2.3 Kunitz units of DNase I were added, and samples were further incubated for 10 min at 37 °C. The reactions were then adjusted to 0.3 M sodium acetate (pH 5.3) and extracted once with phenol/chloroform/isoamyl alcohol (25:24:1), and RNA was then recovered by precipitation with 2.5 volumes of ethanol.

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 RNA pellets from both yeast Pol III and *E. coli* polymerase transcription reactions were resuspended in 10 μL of water on ice, and an equal volume of electrophoresis sample buffer was added. Purification of pre-tRNA substrates was carried out by electrophoresis in 16.5 cm × 26 cm × 0.4 mm gels containing 10% polyacrylamide [30:1 acrylamide:bis-(acrylamide) ratio] and 4 M urea. Following electrophoresis, pre–tRNA substrates were eluted by soaking 4 h at 30 °C in 3 mL of buffer containing 10 mM Tris-HCl (pH 7.4), 0.1% SDS, 1 mM EDTA, and 0.3 M NaCl. Samples were precipitated with the addition of 2.5 volumes of ethanol and resuspended in TE buffer to a final concentration of 0.6 nM.

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 $MgCl_2$. Reactions were stopped by freezing the samples on crushed dry ice, and then an equal volume of RNA dye mix (described above) was added on ice. Reaction products were then separated by electrophoresis in 20 cm × 20 cm × 0.8 mm gels containing 12% polyacrylamide [30:1 acrylamide:bis(acrylamide) ratio], 8 M urea, and electrophoresis buffer (described above). Products visualized by autoradiography were then quantitated by measuring Cerenkov radiation in gel slices.

Two Dimensional Oligonucleotide Mapping. Labeled pre-tRNA substrates for sequence analysis were prepared by in vitro transcription as described above for *E. coli* RNA polymerase except that reactions (50 μL) contained 10 μM each of ATP, GTP, CTP, and UTP, [α-^{32}P]UTP (50 μCi), [α-^{32}P]CTP (50 μCi), 150 μM cytidylyl(3′,5′)uridine dinucleotide, and 10 μg of linearized plasmid pSUP3n template. Digestions with RNase T1 and two-dimensional oligonucleotide mapping were as described by Gegenheimer et al. (1983). The identity of T1-generated oligonucleotides was confirmed by secondary digestion with pancreatic ribonuclease as described by Volkaert and Fiers (1977). Nucleotide composition was analyzed by digestion with RNase T2 or nuclease P1 as described by Gegenheimer et al. (1983). Digestion products were then resolved by two-dimensional chromatography on cellulose thin-layer plates according to the method of Nishimura (1972).

Labeling of 5′ and 3′ Ends. Substrates for labeling of 5′ and 3′ ends were prepared as described above for in vitro transcription with *E. coli* RNA polymerase except that the labeled nucleotide was omitted or was present at low concentration (2 nCi/μL). Reaction products were purified by gel electrophoresis [10% polyacrylamide [30:1 acrylamide:bis(acrylamide) ratio] and 4 M urea] using a high specific radioactivity pre-tRNA substrate loaded in adjacent lanes as a mobility marker. Labeling of 5′ ends with polynucleotide kinase and [γ-^{32}P]ATP and of 3′ ends with phage T4 RNA ligase and cytidine 3′,5′-[5′-^{32}P]bisphosphate was carried out as described by Szekely et al. (1988). Labeled pre-tRNA was reisolated by two-dimensional gel electrophoresis. The first-dimension gel contained 8% polyacrylamide [19:1 acrylamide:bis(acrylamide) ratio] and 8 M urea, and the second-dimension gel contained 20% polyacrylamide [30:1 acrylamide:bis(acrylamide) ratio] and 4 M urea. Products were eluted from gel slices as described above, precipitated with the addition of 2.5 volumes of ethanol, and resuspended in TE buffer at a final concentration of 0.6 nM. The specific radioactivity of both 5′ and 3′ end-labeled substrates was 1800 dpm/fmol.

Solution Structure Probing. Limited RNase digestions for solution structure probing were carried out in 10-μL reactions containing 10 mM Tris-HCl (pH 7.4), 25 mM NaCl, 2.5 mM spermidine, 5 mM $MgCl_2$, 1 μg of RNA carrier, ~30000 dpm of 3′ end-labeled pre-tRNA substrate, and either RNase VI (0.02 unit/μg of RNA carrier) or RNase T1 (0.047 unit/μg of RNA carrier). Reactions were incubated 5 min at 30 °C and stopped by freezing on crushed dry ice, and then an equal volume of electrophoresis sample buffer was added to the samples on ice. Products were then analyzed by electrophoresis in 40 cm × 40 cm × 0.4 mm gels containing 8% polyacrylamide [19:1 acrylamide:bis(acrylamide) ratio]. Cleavage sites were determined by comparing the mobilities of RNase T1 and V1 fragments to those for complete size ladders produced by limited alkaline hydrolysis of end-labeled substrates as described by Donis-Keller et al. (1977). The presence of a 5′-phosphate on fragments produced by RNase V1 caused these fragments to migrate faster than their counterparts produced by RNase T1 or partial alkaline hydrolysis. This increased mobility, corresponding to an apparent size difference of 1.5 nucleotides, was taken into account in determining the sites of cleavage by RNase VI.

Results

Pre-tRNA Expression System. A hybrid λ promoter and the yeast SUP3a(tRNA^{Tyr}) gene were selected for the construction of an expression template based on several considerations. Three factors were considered in choosing among the available phage and bacterial promoters. The first was the ability to produce transcripts of high specific radioactivity, a feature essential to our enzymatic assays. The low K_s for ribonucleoside triphosphates of *E. coli* polymerase (5–20 μM; Rhodes & Chamberlin, 1975) as compared to phage T7 or SP6 polymerases [40–100 μM; cf. Chamberlin and Ryan (1982)] weighed in favor of the bacterial enzyme. The second was the ability to initiate transcription with CTP corresponding to the mature 5′ end of yeast pre-tRNA^{Tyr}. A conflict with sequence requirements at initiation sites in phage promoters (Chapman & Burgess, 1987; Milligan et al., 1987; Chapman et al., 1988) in contrast to examples of bacterial promoters which direct efficient CTP starts [see the review by Rosenberg and Court (1979)] also weighed in favor of promoters for bacterial polymerase. A third factor was a need for producing transcripts at high levels in an inducible bacterial expression system for future use in physical chemical studies. A plasmid containing a derivative of the λ P_R promoter and operator and encoding a temperature-sensitive λ cI repressor was available for this purpose (plasmid pPM1; Ahlquist & Janda, 1984).

The yeast SUP3a gene was chosen for this expression system since the secondary and tertiary structures of the precursor and mature tRNA have been analyzed (Swerdlow & Guthrie, 1984) and since numerous mutations affecting splicing have been studied for this gene family (Colby et al., 1981; Nishikura et al., 1982). The SUP3a gene contains a 14 bp intron and encodes an amber suppressor derivative of tyrosyl-tRNA (Rothstein, 1977; Weeks-Levy, 1985).

The resulting plasmid, pSUP3, is shown in Figure 1A. The λ promoter in the final construct is a hybrid consisting of the −35 region of the λ P_R promoter and the −10 region of the λ c17 promoter (selected because of its ability to direct initiation with CTP both in vitro and in vivo; Rosenberg & Court, 1979). Additionally, a *Bst*NI restriction site was created at the 3′ end of the SUP3a gene, allowing the production of runoff transcripts ending in the 3′-CCA(OH) sequence characteristic of the mature tRNA.

In Vitro Synthesis of Pre-tRNA Substrates. A limitation inherent in the choice of *E. coli* polymerase for expression systems is the presence of multiple promoters on most plasmid templates. To overcome this limitation, reaction conditions were adjusted to provide preferential initiation at the c17 promoter. In particular, transcription reactions included either an appropriate dinucleotide (CpU) for initiation or high concentrations of CTP with the remaining nucleoside triphosphates at concentrations below those required for efficient initiation at other promoters (see Experimental Procedures).

Labeled pre-tRNA substrate for use in control incubations was prepared in reactions with a yeast nuclear extract fraction and plasmid pYLeu2SUP6 (Johnson & Abelson, 1983). The

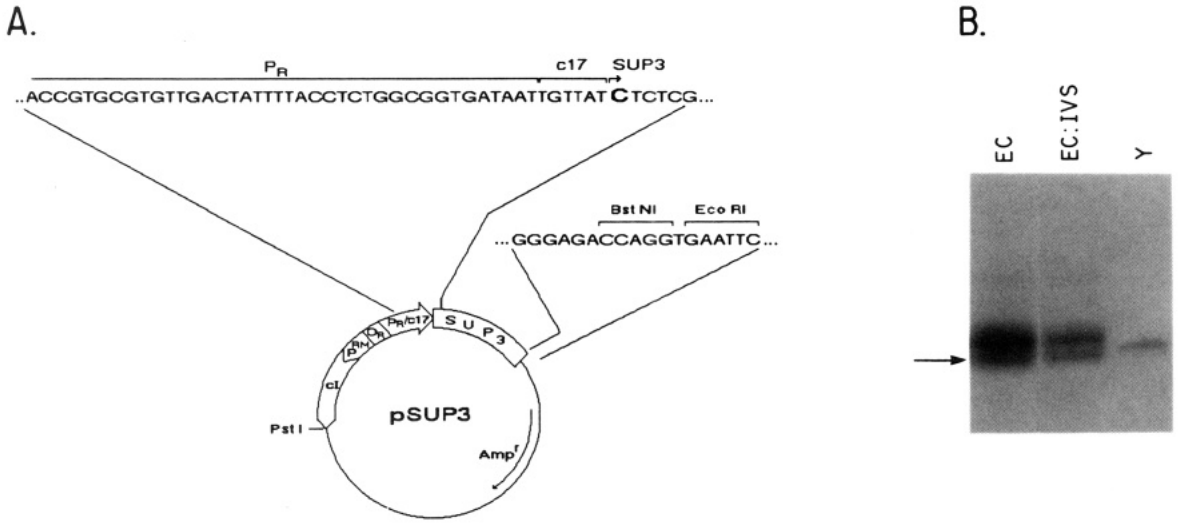

FIGURE 1: Yeast tRNA gene transcription system. (A) A schematic representation is shown of plasmid pSUP3, constructed for transcription with *E. coli* RNA polymerase. The open arrow and open box correspond to segments derived from phage λ and the yeast $tRNA^{Tyr}$(SUP3) gene, respectively. Sequences derived from the P_R and c17 promoters are shown in brackets at the top with the transcription initiation site marked by an arrow and a boldface letter. The sequence at the 3′ end of the SUP3 gene, including the *Bst*NI site for linearizing transcription templates, is also shown. (B) An autoradiograph of labeled pre-$tRNA^{Tyr}$ transcripts, produced by in vitro transcription and resolved by electrophoresis, is shown. Lanes marked EC and EC:IVS were samples containing *E. coli* RNA polymerase and plasmids pSUP3 and pSUP3[IVS(Pvu)], respectively. The sample marked Y included yeast nuclear extract (containing RNA polymerase III) and plasmid pYSUP6. The arrow denotes the position of transcripts which were isolated and used for end analysis and in vitro splicing reactions. Plasmid construction procedures and transcription reaction conditions are described under Experimental Procedures.

SUP6o gene contained on this plasmid encodes a tyrosine-inserting ochre suppressor which differs from the SUP3 gene product only in the first position of the anticodon and at a position of natural heterogeneity within the intron [cf. Ogden et al. (1984)]. The yeast nuclear extract fraction contains RNA polymerase III (Pol III), end maturation activities including nucleotidyl transferase, certain base modification enzymes, and splicing endonuclease and ligase (Engelke et al., 1985; our unpublished results). The predominant product in nuclear extract incubations was the end-mature, IVS-containing precursor (see below) since splicing is inhibited under transcription reaction conditions while other processing steps are not. Splicing of substrates produced by nuclear extract fractions in vitro previously has been shown to be equivalent to that for precursors produced in vivo (Greer et al., 1983a). For brevity, transcripts of the SUP6 gene produced by yeast Pol III are designated YpTyr. Transcripts of the yeast SUP3 gene made by *E. coli* RNA polymerase are designated ECpTyr.

Figure 1B shows a comparison of the products of *E. coli* polymerase and yeast Pol III transcription reactions. The *E. coli* polymerase reactions contained two major transcripts, one of which comigrates with YpTyr in this partially denaturing polyacrylamide gel system and another which migrates slightly more slowly. The identities of the major ECpTyr transcripts were examined by primary sequence analysis. For this purpose, the two major *E. coli* polymerase transcripts and the Pol III product were purified by electrophoresis and analyzed by oligonucleotide mapping. Examples of two-dimensional RNase T1 fingerprints of transcripts labeled with both [α-^{32}P]CTP and [α-^{32}P]UTP are shown in Figure 2. Panel A shows the pattern obtained for the yeast Pol III transcript as a reference while panel B shows that obtained for the faster migrating of the *E. coli* polymerase transcripts. Table I provides a summary of the results of sequence analyses including a listing of RNase T1 oligonucleotides, secondary digestion products, and deduced sequences. The pattern of labeled internal RNase T1 oligonucleotides was essentially identical for digests of YpTyr and ECpTyr. The deduced oligonucleotide sequences confirm the identification of the ECpTyr transcripts as SUP3 gene transcripts. The differences between the *E. coli* polymerase and Pol III transcripts detected in this analysis were limited to (1) the presence of the modified nucleotides ribothymidine and pseudouridine in YpTyr and (2) the presence of 5′-triphosphate (elevated CTP transcriptions) or 5′-hydroxyl (dinucleotide transcriptions) termini in ECpTyr transcripts in place of a 5′-monophosphate in the end-processed YpTyr transcript.

The sequences at the 5′ and 3′ ends of ECpTyr transcripts were further analyzed by postlabeling of purified transcripts.

Table I: RNase T1 Oligonucleotides from Labeled Pre-tRNA Substrates[a]

oligonucleotide no.[b]	RNase A digest	RNase T2 digest	deduced sequence
1	G'	G	G
2	G	G	CG
4	G,U,AC	A,C,G,U	ACUCG
6	C,AAG	C,G	CCAAG
7	C	C	CCCCCG
8	AG	G	UAG
9	AU	A,U	AUCG
11	U	U	UUG
12	U	U,ψ,T[c]	TψCG
12a	U	U	UUCG
13	U	U	UUUAAG
14	C,U,pCp	C,U,pCP	pCUCUCG
14a,b	C,U	C,U	CUCUCG[d]
15	C,U,AAAU	A,C,U,ψ	AAAψCUUG
15a	C,U,AAAU	A,C,U	AAAUCUUG
19	U,AC,AAU	A,C,U	ACUUUAAUU-UACCACUACG
20	C,U,AC,AU,AAU	A,C,U	ACUCUAAU-UUAUCA-CUACG

[a] Methods for oligonucleotide analyses are described under Experimental Procedures. [b] The numbering scheme is that of O'Farrell et al. (1978). Unique oligonucleotides characteristic of the amber and ochre suppressors have been numbered 19 and 20, respectively. [c] The symbols ψ and T refer to pseudouridine and ribothymidine, respectively. [d] Labeled precursor for fingerprint analysis was prepared in *E. coli* transcription reactions using CpU as the initiating dinucleotide as described under Experimental Procedures.

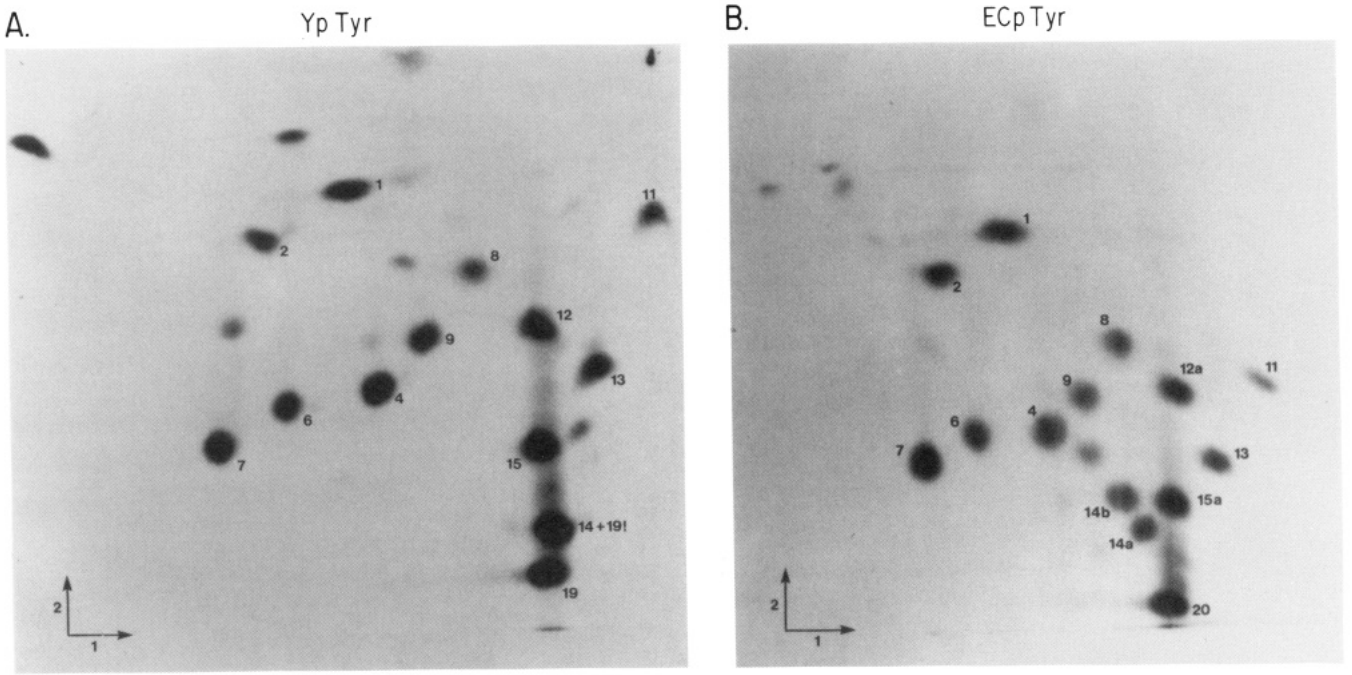

FIGURE 2: Two-dimensional RNA fingerprints of YpTyr and ECpTyr. Two-dimensional RNase T1 fingerprints are shown of pre-tRNA^{Tyr} substrates produced by transcription with yeast RNA polymerase III (YpTyr; panel A) and *E. coli* RNA polymerase (ECpTyr; panel B). Transcription products, labeled to a high specific activity with both [α-^{32}P]CTP and [α-^{32}P]UTP, were isolated and digested with ribonuclease T1. Oligonucleotides were then separated by electrophoresis in the first dimension and homochromatography in the second dimension as described under Experimental Procedures. Labeled oligonucleotides were visualized by autoradiography and subsequently eluted for ribonuclease redigestion and nucleotide composition analyses. Deduced sequence assignments for the numbered oligonucleotides are summarized in Table I.

For 5′-end analysis, both ECpTyr and YpTyr transcripts were treated with phosphatase and then labeled with polynucleotide kinase and [γ-^{32}P]ATP. For 3′-end analysis, purified transcripts were labeled with T4 RNA ligase and cytidine [α-^{32}P]bisphosphate. Postlabeled transcripts were then analyzed by two-dimensional RNase T1 oligonucleotide mapping followed by secondary digestion (data not shown). Oligonucleotides corresponding to 5′ ends were identical for all of the transcripts and corresponded to the 5′ end of the mature tRNA. Oligonucleotides derived from the 3′ end of YpTyr and the faster migrating of the ECpTyr transcripts were identical, and both corresponded to transcripts ending in the sequence 3′-CC(OH). Thus, both lack the terminal adenosine found in the mature tRNA (the terminal adenosine is apparently not required for splicing since the Pol III transcript has previously been shown to be an efficient substrate; Greer et al., 1983a). The slower migrating of the ECpTyr transcripts produced a series of distinct oligonucleotides after 3′ end-labeling. Analysis of these oligonucleotides indicates these transcripts end in 3′-CCAX(OH), where X indicates a mixed yield of A, C, and G. The additional 3′-nucleotide is presumably added by *E. coli* polymerase in a template-independent reaction similar to that described previously for phage T7 polymerase (Milligan et al., 1987; Reyes & Abelson, 1987).

Thus, primary sequence analysis reveals that the two ECpTyr transcripts and the pre-tRNA produced in a yeast Pol III transcription and processing extract differ only in the phosphate content at 5′ ends, the presence of certain base modifications in YpTyr, and the presence of two additional nucleotides in the larger of the ECpTyr products. In the experiments described below, splicing of the faster migrating ECpTyr transcripts is compared to that for YpTyr. However, similar results were obtained with the larger ECpTyr transcript in experiments not shown.

Accurate Splicing of Semisynthetic Substrates. Partially purified pre-tRNA endonuclease and ligase fractions were used to compare splicing of the ECpTyr and Pol III transcripts. Figure 3 shows an electrophoretic analysis of the products obtained in such a comparison. In the absence of added en-

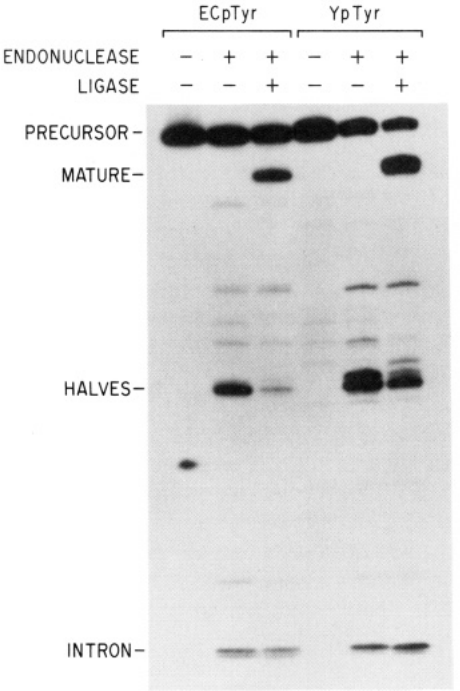

FIGURE 3: Splicing of in vitro transcription products. Pre-tRNA substrates were prepared in transcription reactions with [α-^{32}P]UTP and *E. coli* RNA polymerase (ECpTyr) or yeast RNA polymerase III (YpTyr) and isolated by gel electrophoresis. Splicing reactions contained constant amounts of pre-tRNA incubated with buffer alone, with partially purified endonuclease, or with partially purified endonuclease and ligase as indicated at the top of the panel. Following incubation for 10 min at 30 °C, products were separated by polyacrylamide gel electrophoresis and visualized by autoradiography. The identification of reaction products, shown at the left edge, was based on sequence analyses (data not shown).

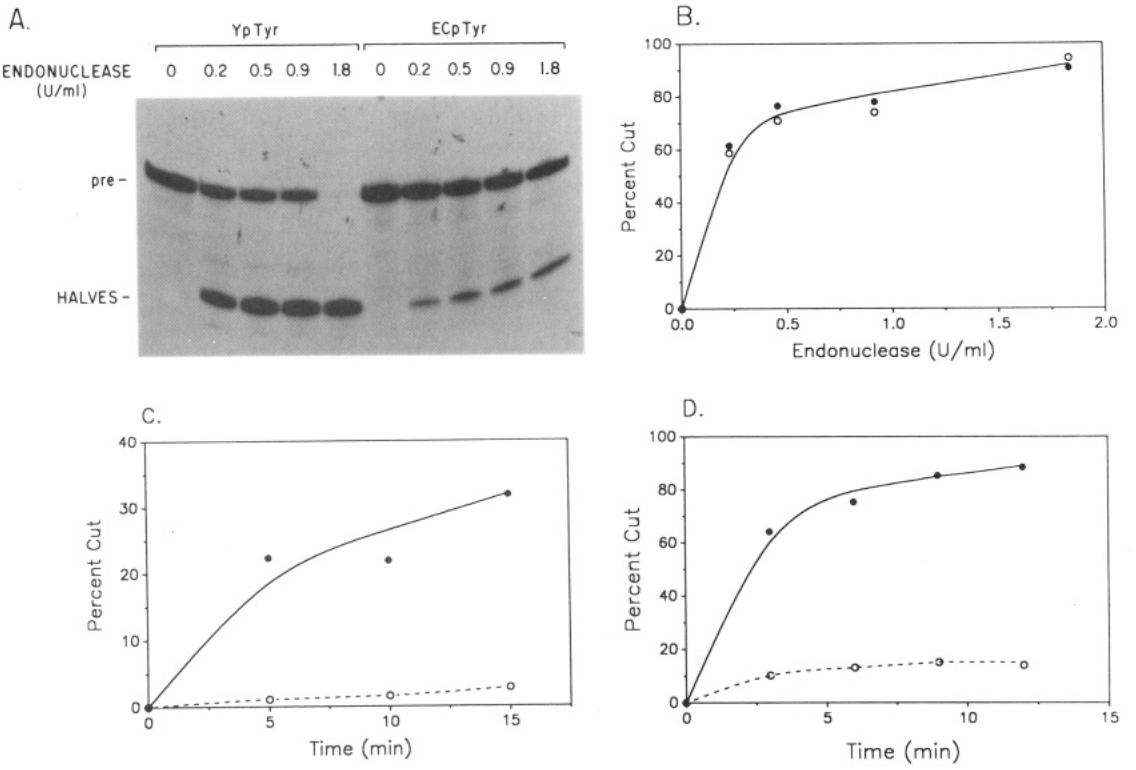

FIGURE 4: Time course and concentration dependence for cleavage by endonuclease. Labeled pre-tRNA substrates, prepared by transcription with yeast (YpTyr; indicated by closed circles in panels B–D) or *E. coli* (ECpTyr; indicated by open circles in panels B–D) RNA polymerase, were incubated with yeast endonuclease as indicated. Products were resolved by electrophoresis and quantitated by Cerenkov counting. The yield of endonuclease cleavage products ("percent cut") was calculated as described previously (Greer et al., 1987). (A) An autoradiograph is shown of reaction products for YpTyr and ECpTyr substrates incubated with increasing amounts of endonuclease as indicated at the top. The migration position of pre-tRNA (pre) and exon cleavage products (halves) is shown at the left. (B) ECpTyr and YpTyr substrates were preincubated at 68 °C for 4 min in buffer containing 20 mM $MgCl_2$ and then cooled to room temperature. The reannealed substrates were then incubated for 10 min at 30 °C with increasing amounts of endonuclease as indicated. Time courses are also shown for cleavage of pre-tRNA substrates (without prior reannealing) at low (0.077 unit/mL, panel C) and high (0.23 unit/mL, panel D) endonuclease concentrations.

zyme fractions, ECpTyr and YpTyr migrate identically as expected from the results of sequence analysis. The addition of a yeast endonuclease fraction resulted in the appearance of a similar set of cleavage products for the two substrates. The major products in each case migrate as expected for the tRNA halves (each 39 nucleotide in length) and the excised IVS (14 nucleotides)(Knapp et al., 1979). In the presence of both endonuclease and ligase, an additional product is formed whose mobility corresponds to that expected for the spliced tRNA product. The identification of this species as spliced tRNA was confirmed by RNase T1 oligonucleotide mapping (data not shown). In particular, oligonucleotides containing IVS sequences found in the precursor were absent, and a new oligonucleotide corresponding to the spliced junction was present (secondary ribonuclease redigestion and nucleotide composition analyses were used to confirm the identity of spliced junction oligonucleotides). Thus, the semisynthetic ECpTyr substrate was correctly spliced by the yeast enzymes in vitro.

Nucleotide composition analysis of RNase T1 oligonucleotides from spliced ECpTyr revealed the presence of pseudouridine (data not shown). Apparently, the partially purified ligase fraction contained pseudouridine synthase activity since this modification was not found after incubation with endonuclease alone. This modification was detected only at position 55 at the base of the anticodon stem (see Figure 7C for numbering). The yield of pseudouridine in spliced products was found to vary significantly (15–80%), suggesting that the introduction of pseudouridine was not a prerequisite for joining. This and similar results obtained previously for pre-$tRNA^{Phe}$ (Reyes & Abelson, 1988) suggest that, in general, splicing by the yeast enzymes may include no prerequisite for one or more specific modifications.

Inefficient Splicing of Semisynthetic Substrates. Among tRNA gene mutations which affect splicing, we have previously identified two categories of effects: changes in the accuracy of splicing and changes in the efficiency of the reaction (Greer et al., 1987). This latter parameter, efficiency, is based on our observation that mutations may affect the initial rate, the maximum extent, or both of splicing in vitro. To determine whether the ECpTyr and YpTyr substrates were spliced with similar efficiencies, the extent of splicing was compared in enzyme titrations, and splicing rates were compared in time courses at constant enzyme concentrations. An electrophoretic analysis of an endonuclease titration is shown in Figure 4A. At all of the endonuclease concentrations examined, the yield of tRNA halves for YpTyr was significantly greater than that for ECpTyr. This differential yield was not affected by extended incubation or by the introduction of additional aliquots of endonuclease at late time points in ECpTyr incubations (data not shown). A comparison of the time course for splicing of these two substrates at a low endonuclease concentration (0.077 unit/mL) is shown in Figure 4C. Again, the yield of YpTyr halves was significantly greater at all time points than that for ECpTyr. A comparison of the ECpTyr and YpTyr time courses at low and intermediate endonuclease concentrations (Figure 4C,D; see also Figure 5) suggests both the rate and the extent of splicing of the ECpTyr substrate were significantly reduced. A characteristic feature of the ECpTyr substrate was that the maximum extent of splicing was low but variable among substrate preparations, ranging from 15 to 50% of the input substrate.

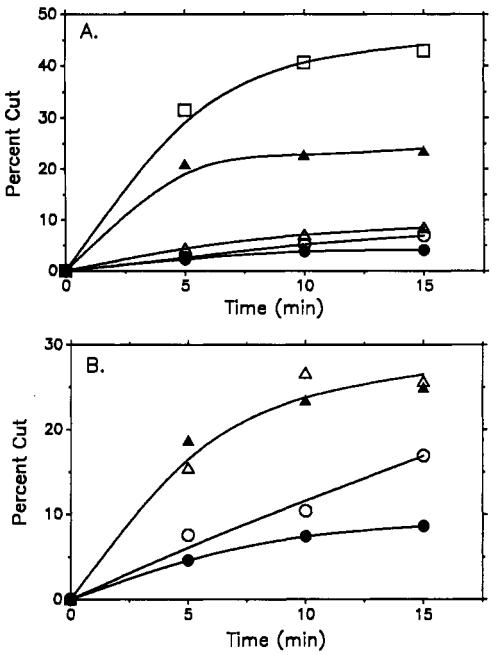

FIGURE 5: Temperature- and Mg^{2+}-dependent activation of the ECpTyr substrate. Labeled ECpTyr substrates were preincubated for 4 min under the conditions described below prior to measurement of cleavage by endonuclease. (A) Substrates were preincubated as follows: 4 °C in TE buffer (see Experimental Procedures) plus 25 mM $MgCl_2$ (●); 30 °C in TE buffer (○); 30 °C in TE buffer plus 25 mM $MgCl_2$ (▲); 37 °C in TE buffer (△); or 37 °C in 25 mM $MgCl_2$ (□). (B) Labeled ECpTyr substrates were preincubated 4 min at 30 °C in TE buffer with the following additions: no addition (●); 5 mM $MgCl_2$ (○); 15 mM $MgCl_2$ (▲); or 25 mM $MgCl_2$ (△). Cleavage of preincubated substrates was then measured by incubation with endonuclease (0.077 unit/mL) at 30 °C for the times indicated. Reaction products were resolved by electrophoresis and quantitated as described in the legend to Figure 4.

The low yield of halves for the ECpTyr substrate prevented a systematic comparison of joining of the two substrates by yeast tRNA ligase. However, essentially all of the halves produced in ECpTyr incubations could be joined by ligase at concentrations which gave complete joining of YpTyr halves (data not shown). Thus, the defect in splicing of the ECpTyr substrate appeared to be primarily at the level of IVS excision by endonuclease.

Activation of the ECpTyr Substrate. The resistance of a portion of the ECpTyr substrate to endonuclease cleavage raised the possibility that a fraction of the substrate might exist in one or more inactive conformations. In this instance, the observed variation among substrate preparations might reflect differences in the ratio of active to inactive conformers resulting from slight variations in preparation procedures. To test possible interconversion among alternate structural forms, the effect of a thermal denaturation/renaturation cycle on subsequent cleavage of the ECpTyr substrate was examined. Both the ECpTyr and YpTyr substrates were heated to 68 °C in buffer containing 20 mM $MgCl_2$ for 4 min and then allowed to cool slowly to room temperature. As shown in Figure 4B, the extent of cleavage of these two reannealed substrates was similar at all of the endonuclease concentrations tested and was nearly complete at the highest concentrations. At low endonuclease concentrations, both the rate and the extent of the reaction are affected by prior reannealing (Figure 4). Thus, while YpTyr was efficiently spliced with or without pretreatment, the ECpTyr substrate was efficiently cleaved only after renaturation. For clarity, this renaturation-dependent increase in endonuclease cleavage of the ECpTyr substrate will be referred to here as substrate activation.

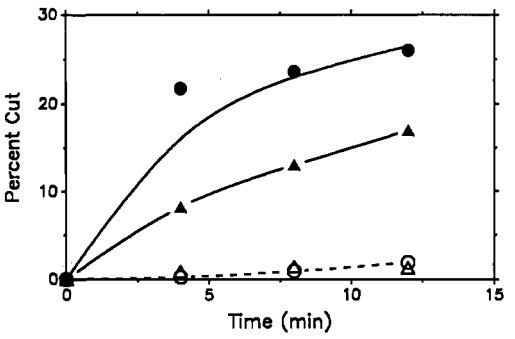

FIGURE 6: Endonuclease cleavage of the EC:IVSpTyr substrate. A comparison of the time course for cleavage of ECpTyr and EC:IVSpTyr substrates is shown. Both substrates were labeled at 3′ ends (see Experimental Procedures) and preincubated (4 min) as follows prior to measurement of endonuclease cleavage: ECpTyr preincubated at 4 °C in TE buffer (○); ECpTyr, 68 °C in TE buffer plus 10 mM $MgCl_2$ (●); EC:IVSpTyr, 4 °C in TE buffer (△); EC:IVSpTyr, 68 °C in TE buffer plus 10 mM $MgCl_2$ (▲). Following preincubation, cleavage was measured in reactions with endonuclease (0.077 unit/mL) at 30 °C for the times indicated. Reaction products were resolved by gel electrophoresis and quantitated as described in the legend to Figure 4.

The requirements for activation of the ECpTyr substrate were examined by varying the temperature and $MgCl_2$ concentrations in preincubations. The results are shown in Figure 5. Preincubation of the substrate at intermediate temperatures (30–37 °C) and $MgCl_2$ concentrations (5–15 mM) results in partial conversion to an active form. Preincubation at higher temperatures (>65 °C) resulted in maximal activation with or without added Mg^{2+} (data not shown). Note that very little activation is observed under the conditions used in splicing assays and in the structure-probing experiments described below (30 °C, 5 mM $MgCl_2$).

Structure of the ECpTyr Substrate. Activation of the ECpTyr substrate in a temperature- and Mg^{2+}-dependent fashion provided indirect evidence for interconversion among alternate structural forms. Enzymatic solution structure probing using limited ribonuclease digestion was used to examine this possibility directly. Purified ECpTyr substrate was labeled at 3′ ends by using T4 RNA ligase and cytidine [α-^{32}P]bisphosphate. As shown in Figure 6, the substrate subjected to the end-labeling procedure retained the same activation requirement as the original precursor. Two enzymes were used as structure probes. These were RNase T1, which cleaves after guanosine residues and preferentially in single-stranded regions (Takahashi, 1961), and cobra venom ribonuclease V1, which shows little sequence specificity but cleaves preferentially in double-stranded regions (Lockard & Kumar, 1981). Labeled ECpTyr was preincubated at 4 or 68 °C, or at 68 °C in the presence of 10 mM $MgCl_2$. Limited ribonuclease digestions were then carried out for 5 min at 30 °C in buffer identical with that used for splicing reactions except that the nonionic detergent Triton X-100 was omitted. The cleavage products were then resolved by electrophoresis and visualized by autoradiography. An example of the results obtained for partial RNase V1 cleavage is shown in Figure 7A. As can be seen in this figure, changes are observed in many regions of the precursor when comparing substrates preincubated at 4 °C and at 68 °C with Mg^{2+}. However, only a few of these changes are also observed in the 68 °C preincubations without Mg^{2+} (see Figure 7A). Only those changes in cleavage patterns observed at 68 °C both in the presence and in the absence of Mg^{2+} are correlated with splicing since Mg^{2+} was not required for complete conversion at high temperatures. When this criterion was used, changes in cleavage

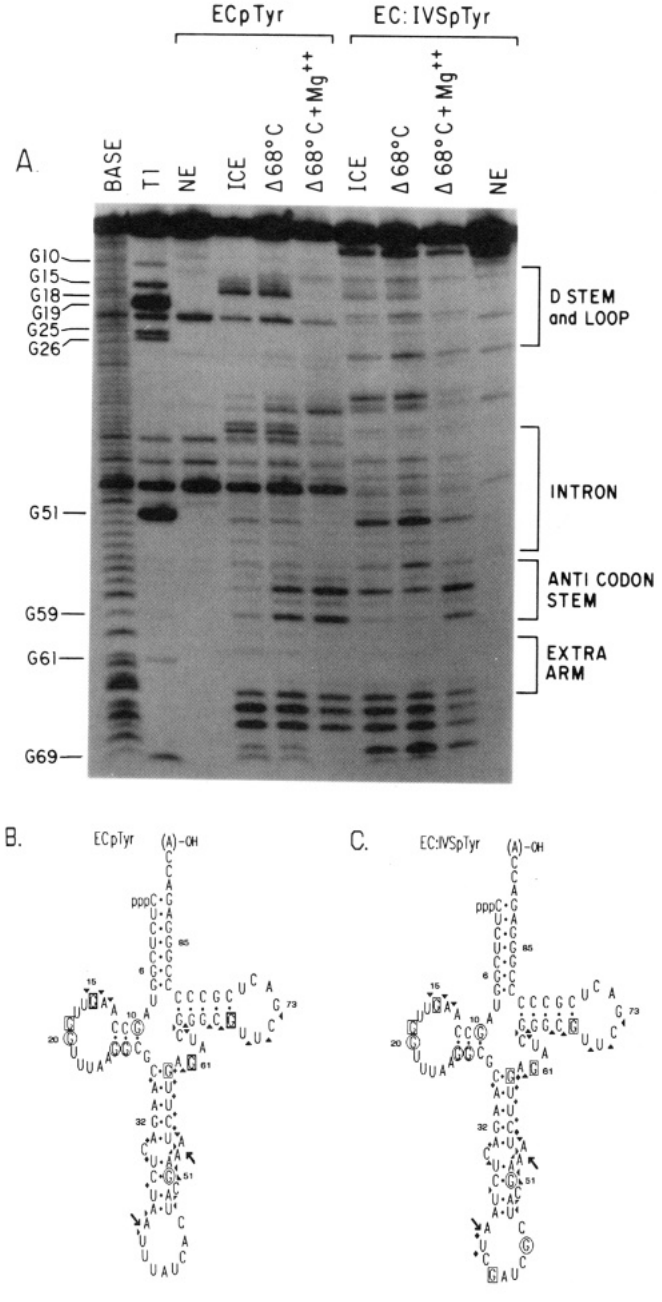

FIGURE 7: Enzymatic solution structure probing of pre-tRNA substrates. The structure of end-labeled pre-tRNA substrates was examined by partial digestion with ribonucleaes T1 and nuclease V1 as described under Experimental Procedures. Panel A shows an autoradiograph of nuclease V1 cleavage products for 3′ end-labeled ECpTyr and EC:IVSpTyr substrates (indicated in brackets at the top). Nuclease V1 digests were carried out at 30 °C for 4 min with substrates preincubated as indicated for each lane and summarized as follows: 4 °C in TE buffer with 10 mM $MgCl_2$ (ice); 68 °C in TE buffer (Δ68 °C); or 68 °C in TE buffer with 10 mM $MgCl_2$ (Δ68 °C + Mg^{2+}). Partial base hydrolysis and partial ribonuclease T1 digests of untreated 3′ ECpTyr were included as markers and are shown in the lanes labeled BASE and T1, respectively. The lanes labeled NE refer to no enzyme added. The deduced positions of ribonuclease T1 cleavage sites are indicated at the left edge with corresponding segments of the pre-tRNA indicated at the right. (B, C) A summary of differences observed in the intensity of cleavage by ribonuclease T1 and nuclease V1 for substrates reannealed by preincubation at 68 °C in TE buffer plus 10 mM $MgCl_2$ as compared to a mock incubation (4 °C in TE buffer) is shown for ECpTyr in panel B and for EC:IVSpTyr in panel C. Bold squares mark sites of elevated T1 cleavage upon reannealing, decreases by a circle, and no change by a square. Sites of elevated V1 cleavage upon reannealing are marked by a plus, decreases by a triangle, and no change by a diamond.

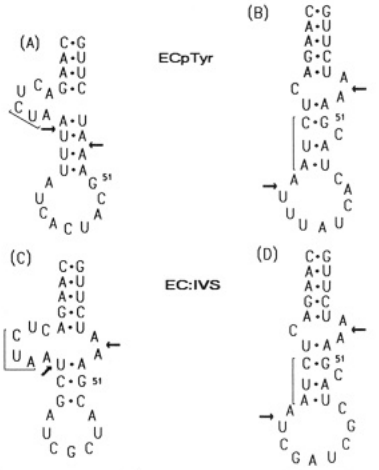

FIGURE 8: Alternate secondary structures. Potential alternate conformations of the IVS domains of ECpTyr and EC:IVSpTyr were compared. Predicted lowest free energy structures were derived by using the PCFOLD program (Zucker & Steigler, 1981) and the calculated free energy values described by Turner et al. (1988). Potential alternate secondary structures for the anticodon stem/loop and IVS regions are shown for ECpTyr in panels A and B and for EC:IVSpTyr in panels C and D.

rates correlated with splicing were limited to segments near splice sites and within exons at the anticodon and D stems as follows. In the regions around splice sites, these were (1) increased V1 cleavage at position 36 within the anticodon and (2) decreased T1 cleavage at position 51 near the 3′-splice site. Within exons, these were (1) increased V1 cleavage within the anticodon stem and (2) decreased T1 cleavage at position 10 within the D stem.

Previously, 3′-splice site structure had been shown to be important in splicing (Szekely et al., 1988). Thus, the ECpTyr structural changes observed near splice sites merited careful consideration. Potential alternate conformations of the IVS domain based on lowest free energy calculations and consistent with the results of structure probing are shown in Figure 8. The structure shown in panel A is consistent with the cleavage data prior to renaturation whereas that in panel B is consistent with cleavage patterns following activation in the presence of Mg^{2+}. Two lines of evidence suggest that a transition between these two structures was not responsible for activation. The first was the observation that V1 cleavage patterns at splice sites were unchanged after activation in the absence of Mg^{2+} (Figure 7A). The second was obtained through the use of directed mutagenesis. Sequence changes designed to disrupt the predicted structure of the IVS domain without disrupting features important in splicing were made in the SUP3 template. Since exon sequences adjacent to splice sites are conserved and, for 5′-splice sites, have been implicated in splicing (Colby et al., 1981; Reyes & Abelson, 1988), these sequence changes were restricted to 5′ IVS positions which are not conserved among yeast precursors [cf. Szekely et al. (1988)]. Precursor derived from the resulting template was designated EC:IVS and differs from ECpTyr at three positions; the second, third, and seventh nucleotides of the IVS. Potential structures for the IVS domain of the EC:IVS precursor based on lowest free energy calculations are shown in Figure 8C,D.

Endonuclease assays with the ECpTyr and EC:IVS substrates are shown in Figure 6. When preincubated at 4 °C, both transcripts are poor endonuclease substrates. Preincubation at 68 °C in the presence of 10 mM $MgCl_2$ produced a substantial increase in cleavage of both substrates. However, the magnitude of the increase was reduced for the EC:IVS precursor. This quantitative difference in activation levels

suggests the sequence changes in the EC:IVS substrate may not be benign with respect to splicing. Nonetheless, the EC:IVS precursor retains the same qualitative activation properties in the presence of Mg^{2+} as the ECpTyr substrate.

The results of solution structure probing of the EC:IVS precursor are shown in Figure 7. Panel A shows an example of RNase V1 cleavage products for this transcript, and panel C provides a summary of the cleavage sites. The only changes in cleavage patterns consistently correlated with activation were increased V1 cleavage in the 3′-anticodon stem and decreased cleavage by both V1 and T1 in the 5′ D-stem and loop region. This latter result suggests the formation of a more compact and less accessible structure within the D-arm region. Cleavage at splice sites by V1 was low under all conditions while cleavage at nearby sites by T1 remained high. This result suggests EC:IVS splice sites were single stranded both before and after activation. Together, these results suggest changes in exon structural elements rather than at splice sites are responsible for activation of both the ECpTyr and EC:IVS substrates. These exon structures include double-stranded character within the anticodon stem and the structure of the D-loop and stem regions.

Surprisingly, we found that preincubation of the EC:IVS transcript at 68 °C in the absence of Mg^{2+} completely prevented cleavage of this precursor by endonuclease (data not shown). This result suggests that in the absence of Mg^{2+} the EC:IVS mutations may allow the formation of a structure which is completely resistant to endonuclease. Changes in cleavage patterns unique to preincubation of EC:IVS at 68 °C without Mg^{2+} included increases in V1 cleavage at C50 within the IVS and at A54 between the 3′-splice site and the base of the anticodon stem. Thus, resistance to endonuclease in this substrate may be associated with a structural change which includes the IVS domain and which is distinct from that required for activation.

Discussion

Although the pre-tRNA substrate produced by in vitro transcription with *E. coli* RNA polymerase was accurately spliced by the yeast enzymes, both the rate and the extent of cleavage by endonuclease were low. We attribute this inefficient cleavage to an inappropriate substrate conformation based on the following lines of evidence. First, the extent of cleavage could not be enhanced by extended incubation or by further addition of fresh endonuclease aliquots. Second, efficient splicing was restored by reannealing substrate fractions prior to incubation with endonuclease. Third, the effect of the reannealing step on subsequent cleavage was dependent both on the temperature and on the Mg^{2+} concentration. Fourth, solution structure probing revealed significant changes in pre-tRNA structure upon reannealing including a consistent correlation with tRNA-like structural elements in exon domains. These results suggest the initial substrate preparation contained a mixture of active and inactive conformers with the active form stabilized by the presence of Mg^{2+}.

Several examples exist of alternate structural forms for tRNAs from yeast (Lindahl et al., 1966; Adams et al., 1967; Reeves et al., 1970; Streeck & Zachau, 1971; Kearns et al., 1974; Hall et al., 1989) and *E. coli* (Gartland & Sueoka, 1966; Eisinger & Gross, 1975; Hyde & Reid, 1985; Hyde, 1986). In many of these examples, the alternate forms have been shown to have distinct functional properties with the active form stabilized by Mg^{2+}. This effect of bound Mg^{2+} has been attributed to stabilization of secondary and tertiary interactons critical to the native L-shaped conformation (Schrier & Schimmel, 1974; Stein & Crothers, 1976; Quigley et al., 1978). Thus, the requirement we have observed for Mg^{2+}-dependent reannealing in splicing may reflect requirements for formation of native tRNA-like structure within the precursor.

The results of solution structure probing (Swerdlow & Guthrie, 1984; Lee & Knapp, 1985) and, more recently, NMR spectroscopy (K. Hall, personal communication) suggest a common structural motif among pre-tRNA substrates. This motif consists of a mature tRNA-like domain formed by exons and an IVS domain extending from the anticodon stem. An essential role for exon domains in splicing has been suggested by previous analyses of tRNA gene mutations affecting splicing (Colby et al., 1981; Nishikura et al., 1982; Willis et al., 1984; Pearson et al., 1985; Greer et al., 1987; Reyes & Abelson, 1988; Szekely et al., 1988; Mathison et al., 1989). In contrast to exons, a largely passive role for IVS feature in splicing is suggested by the rare occurrence of intron mutations affecting splicing (Kurjan et al., 1980; Willis et al., 1984), the lack of intron sequence conservation [cf. Szekely et al. (1988)], and the ability to create large IVS insertions, deletions, and rearrangements without affecting splicing (Johnson et al., 1980; Raymond & Johnson, 1983; Strobel & Abelson, 1986a,b; Szekely et al., 1988; Reyes & Abelson, 1988). Moreover, analyses of mutations affecting splice site or anticodon stem structure support a measuring model for splice site identification (Greer et al., 1987; Szekely et al., 1988; Reyes & Abelson, 1988). In such a model, splice sites are identified by their presence within single-stranded loops located at a fixed distance and orientation relative to a primary exon recognition site. The results presented here provide additional evidence in support of this model. Both solution structure probing and mutational analysis suggested activation of the ECpTyr substrate required tRNA-like structure within the anticodon stem and D-stem regions of the precursor. These structural studies provide direct evidence that tRNA-like structure within these regions, previously indirectly implicated by mutational analysis, is required for splicing.

Processing of yeast pre-$tRNA^{Tyr}$ in *Xenopus* oocytes is ordered with maturation of 5′ and 3′ ends and the introduction of most base modifications preceding splicing (Melton et al., 1980; Nishikura & DeRobertis, 1981). Evidence that similar, albeit perhaps more flexible, ordering occurs in yeast includes (1) the detection of distinct sets of intermediates among whole cell RNA from wild-type yeast strains and of end-mature IVS-containing precursors accumulated in strains with splicing defects (Hopper & Kurjan, 1981; Willis et al., 1984; Wang & Hopper, 1988; O'Connor & Peebles, 1991), (2) analysis of the time course for appearance of intermediates in yeast transcription and processing extracts (Pearson et al., 1985; Engelke et al., 1985; our unpublished observations), and (3) the demonstration that certain base modifications may take place only prior to splicing (Johnson & Abelson, 1983; Strobel & Abelson, 1986). Thus, pre-tRNA substrates for the splicing enzymes in vivo may normally include base modifications. Both the results presented here for an unmodified pre-$tRNA^{Tyr}$ substrate (activated ECpTyr) and the results presented previously for pre-$tRNA^{Phe}$ (Reyes & Abelson, 1988) demonstrate that modifications are not required for splicing. Thus, the properties of the splicing enzymes could not account for ordering of modification and splicing steps in yeast, and, instead, kinetic constraints or functional compartmentation may determine ordering.

Factors which influence folding of $tRNA^{Tyr}$ gene transcripts in vivo might include (1) the transcribing polymerase, (2) the nuclear environment (including ionic strength, Mg^{2+} concentration, etc.), (3) the introduction of base modifications which

stabilize the native tertiary structure [cf. Bjork (1984)], and (4) protein factors which may function to direct conformation. A candidate for this latter category of factors is the STP1 gene product identified recently by Wang and Hopper (1988). Mutations at this locus were initially identified by their ability to suppress a $tRNA^{Tyr}$ gene mutation affecting splicing. Subsequently, strains bearing a null allele at the STP1 locus were found to be viable and exhibited normal splicing activity in extracts. Thus, this gene does not encode an essential splicing function. These strains do show the accumulation of a specific subset of IVS-containing tRNA precursors (including pre-$tRNA^{Tyr}$). Thus, the STP1 gene affects splicing in a precursor-specific manner. One model which accounts for these results is that the STP1 product may affect the efficiency of splicing by influencing the conformation of the affected tRNA precursors. This possibility could be tested by examining activation of ECpTyr substrates by wild-type and *stp1* mutant cell extracts.

The use of prokaryotic promoter fusions for the production of semisynthetic substrates has been widely applied in the field of RNA processing. The results presented here serve to emphasize that factors influencing folding in vivo may be absent from such expression systems. Thus, a means for correlating results obtained with semisynthetic substrates to those for authentic substrates is certainly desirable where possible. The use of *E. coli* polymerase for the production of processing substrates has advantages in two specific applications. The first is in the production of transcripts where nucleotides at the 5′ end are important but do not correspond to a phage promoter consensus. For example, our construct would have required initiation by T7 polymerase with CTP, a reaction which is 10-fold reduced relative to initiation with GTP (Mulligan & Uhlenbeck, 1989). A second advantage is the ability to synthesize in good yield transcripts of high specific radioactivity. This advantage derives from the relatively low K_s for ribonucleotide triphosphates characteristic of the bacterial enzyme (Rhodes & Chamberlin, 1975). In this regard, the use of *E. coli* polymerase may provide an important alternative in a variety of RNA synthesis applications.

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