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Substrate Recognition and Identification of Splice Sites by the tRNA-Splicing Endonuclease and Ligase from *Saccharomyces cerevisiae*

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We have examined the substrate requirements for efficient and accurate splicing of tRNA precursors in *Saccharomyces cerevisiae*. The effects of *Schizosaccharomyces pombe* tRNA^{Ser} gene mutations on the two steps in splicing, intron excision and joining of tRNA halves, were determined independently by using partially purified splicing endonuclease and tRNA ligase from *S. cerevisiae*. Two mutations (G14 and A46) reduced the efficiency of excision and joining in parallel, whereas two others (U47:7 and C33) produced differential effects on these two steps; U47:7 affected primarily the excision reaction, and C33 had a greater impact on ligation. These data indicate that endonuclease and ligase recognize both common and unique features of their substrates. Another two mutations (Ai26 and A37:13) induced miscutting, although with converse effects on the two splice sites. Thus, the two cutting events appear to be independent. Finally, we suggest that splice sites may be determined largely through their position relative to sites within the tRNA-like domain of the precursors. Several of these important sites were identified, and others are proposed based on the data described here.

The *sup3-e* and *sup9-e* loci in *Schizosaccharomyces pombe* encode efficient serine-inserting UGA suppressors (11, 12, 33). The dimeric transcripts of these two loci contain tRNA^{Ser} and tRNA$_i^{Met}$ sequences joined by a seven-nucleotide spacer. The tRNA$_i^{Met}$ and spacer sequences are identical, while the tRNA^{Ser} sequences differ at one position (Fig. 1). Production of mature tRNAs from the dimeric transcripts requires the action of a number of site-specific processing enzymes (32). These processing enzymes are required for the synthesis of a wide range of tRNAs and thus must recognize elements of sequence or structure common to all of their substrates (4). The nature of these common recognition elements can be probed by examining the effects of tRNA gene mutations on the processing of their corresponding transcripts. We have taken this approach in analyzing the interactions of the tRNA-splicing enzymes with their substrates.

A number of years ago, Hofer et al. (11) isolated *S. pombe* strains in which nonsense suppression by *sup3-e* or *sup9-e* tRNA was abolished as a result of mutations at the suppressor locus. Subsequently, the genes encoding the nonsense suppressors and a number of the suppressor-inactive derivatives were isolated and characterized (12, 21, 33). As was observed in an analysis of the tRNA^{Tyr} *SUP4* gene in *Saccharomyces cerevisiae* (15), nearly all of the inactivating mutations occur within the tRNA-coding sequence. The two exceptions found among the *sup3-e* and *sup9-e* mutations are located within the 15-nucleotide intervening sequence (IVS) which interrupts the tRNA^{Ser} coding sequence. Such a distribution of mutations suggests that sequences essential for tRNA biosynthesis are contained primarily within the mature tRNA. The *S. pombe sup3-e* and *sup9-e* genes have been shown to function in vivo in *Saccharomyces cerevisiae* (12, 33) and can also be transcribed and processed in vitro by *Saccharomyces cerevisiae* extracts (21, 33). Transcription and processing in this in vitro system has been used to examine the biosynthetic effects of the various suppressor-inactive mutants. Several of these were found to produce defective splicing substrates (21, 33).

The mechanism of tRNA splicing in extracts of *Saccharomyces cerevisiae* has been well defined. The reaction proceeds in two stages (14, 23). First, the IVS is excised through the action of a site-specific endonuclease yielding paired tRNA "halves." Second, the halves are joined in an ATP-dependent reaction to produce mature-sized tRNA. The endonuclease and ligase which carry out these two steps can be resolved in crude extracts (22) and have been purified to various degrees (8, 22, 24). These purified fractions were used to examine the splicing of several *sup3-e* and *sup9-e* mutants. Certain mutations reduced the efficiency of the excision and joining steps in parallel, whereas others had differential effects on these two steps. Another two mutations were found to alter the accuracy of IVS excision by endonuclease. The results suggest that both endonuclease and ligase recognize specific elements of their respective substrates and provide the outlines of a general model for the interaction of the tRNA-splicing enzymes with their substrates.

MATERIALS AND METHODS

Enzymes and chemicals. *Saccharomyces cerevisiae* endonuclease (23 U/ml) was equivalent to fraction VI of Peebles et al. (22). *Saccharomyces cerevisiae* ligase (210 U/ml) was equivalent to fraction IV of Greer et al. (8). Units of endonuclease and ligase activity are defined in these two references. RNases T_1 and T_2 were obtained from Calbiochem, and pancreatic RNase was from Sigma Chemical Co. [α-^{32}P]GTP, [α-^{32}P]UTP (410 Ci/mmol), and [5,6-^{3}H]UTP (40 Ci/mmol) were obtained from Amersham.

tRNA gene mutations. The isolation and characterization of suppressor-inactive mutants of *sup3-e* and *sup9-e* has been described previously (11, 21, 33). Mutations are desig-

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FIG. 1. *S. pombe* pre-*sup3-e*. The sequence of the intron-containing precursor is depicted in the traditional cloverleaf configuration. The 5'-flanking sequence present in the *sup3-e* primary transcript is shown in italics, and the site at which RNase P cleaves to generate a 5'-end-matured precursor is indicated by an arrow pointing downwards. Normal splicing endonuclease cleavage sites are indicated by thin arrows pointing inwards. The suppressor anticodon is boxed, and the sequence of its wild-type counterpart (*sup3*$^{+}$) is indicated. Dashed lines identify potential base-pairing interactions. The single base change at the tip of the extra arm which distinguishes the *sup3-e* (U) and *sup9-e* (C) tRNAs is also shown. The location and base change of each mutation described in the text are indicated. Sites of miscutting by endonuclease for Ai26 and A37:13 precursors are indicated by arrowheads and are numbered 1 to 4 for reference to the text. The solid and open arrowheads indicate the aberrant cleavage sites in the Ai26 and A37:13 precursors, respectively.

nated by the position and nature of the base change found in the resulting pre-tRNA (the 5'- and 3'-end-matured, intron-containing tRNA precursor). For example, mutation G14 produces an A to G change at position 14 in the D loop of pre-tRNA^{Ser}. The mutations G14, C33, and Ai26 are present in a *sup3-e* background (10, 21), while A37:13, A46, and U47:7 are derived from *sup9-e* (33).

Preparation of pre-tRNA substrates. Labeled *S. pombe* pre-tRNA substrates were prepared by in vitro transcription with a soluble cell extract from *Saccharomyces cerevisiae* prepared by the method of Klekamp and Weil (13). Transcription reactions (100 μl) contained 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid, pH 7.9), 10 mM $MgCl_2$, 130 mM NaCl, 10% (vol/vol) glycerol, 0.5 mM dithiothreitol, 2 μg of plasmid DNA, 10 μl of cell extract, 1.2 mM ATP, 0.6 mM each CTP and UTP, and 10 μM [α-^{32}P]GTP (100 Ci/mmol). Where noted in the text, transcription was carried out with [α-^{32}P]UTP (100 Ci/mmol) or [5,6-^{3}H]UTP (10 Ci/mmol), and the concentrations of UTP and GTP were adjusted to 10 μM and 0.6 mM, respectively. Tritiated and ^{32}P-labeled pre-tRNA (approximately 5.6×10^{6} dpm/pmol) was purified by gel electrophoresis as described previously (8).

Endonuclease and ligase assays. Endonuclease reactions (10 μl) contained 20 mM Tris hydrochloride (pH 8.0), 2.5 mM spermidine, 0.1 mM dithiothreitol, 40 mM NaCl, 10 mM $MgCl_2$, 0.5% Triton X-100, and 3.3×10^{4} dpm of pre-tRNA substrate. Ligase reactions were similar, with the addition of 2 mM ATP and constant amounts of endonuclease as noted in the text. Reactions were incubated for 10 min at 30°C, stopped with the addition of 90 μl of 0.4 M ammonium acetate (pH 7.0), and immediately extracted with phenol-chloroform-isoamyl alcohol (25:24:1). Samples were then extracted once with ether and precipitated with the addition of 3 volumes of ethanol. Reaction products were analyzed by electrophoresis in thin (0.4-mm) gels containing 12% acrylamide, 0.4% bisacrylamide, and 8 M urea essentially as described by Peebles et al. (22). Following autoradiography, substrates and reaction products were quantitated by measuring Cerenkov radiation in gel slices.

Endonuclease competition experiments were carried out as described above in the presence of 0.12 U of endonuclease per ml and 3.3×10^{4} dpm of ^{32}P-labeled Ai26 pre-tRNA. This amount of endonuclease results in half-maximal cutting of Ai26. Tritiated pre-*sup3-e* was added as the competitor over a concentration range corresponding to molar ratios from 0.25 to 8.0 with respect to Ai26.

RNA structure analysis. Two-dimensional mapping of RNase T_1-generated oligonucleotides and subsequent secondary analysis of eluted oligonucleotides with RNase A and RNase T_2 were carried out as described by Willis et al. (32).

RESULTS

Efficient splicing of pre-*sup3* in *Saccharomyces cerevisiae* extracts. The relative efficiency of splicing of homologous and heterologous substrates was examined with pre-tRNA substrates transcribed from the wild-type *S. pombe sup3*$^{+}$ gene and the *Saccharomyces cerevisiae SUP6* gene (21, 31). The amount of endonuclease required for optimal IVS excision was measured by incubating constant amounts of labeled pre-tRNA substrates with increasing amounts of endonuclease as described in Materials and Methods. The substrate and reaction products were resolved by electrophoresis, visualized by autoradiography, and quantitated by measuring the radioactivity in gel slices (Fig. 2A). Notably, *S. pombe* pre-*sup3*$^{+}$ was more efficiently cleaved by the *Saccharomyces cerevisiae* endonuclease than was the homologous substrate (pre-*SUP6*). The efficiency of cleavage and of ligation (see below) as defined here refers to both the initial reaction velocity (~0 to 0.1 U of endonuclease per ml or ~0 to 1.5 U of ligase per ml) and the maximal extent of the reaction.

The efficiency of joining of tRNA halves was examined by incubating pre-tRNA substrates with a constant amount of endonuclease and increasing amounts of ligase. The *S. pombe* precursor was joined more efficiently than was pre-*SUP6* (Fig. 2B). Together these results demonstrate that *S. pombe* pre-*sup3*$^{+}$ is spliced by the *Saccharomyces cerevisiae* enzymes with an efficiency greater than that of homologous substrate.

The UGA suppressor derivatives *sup3-e* and *sup9-e* differ from their corresponding wild-type genes by a single anticodon base change (Fig. 1). We therefore examined the

effect of this base change on the excision and ligation reactions. The results (Fig. 2) demonstrate that no significant effect could be detected.

A number of mutations in the *sup3-e* and *sup9-e* genes have been shown to reduce significantly the efficiency of 5′-end maturation by RNase P (21, 32, 33). The effects of these mutations on splicing could only be examined in this in vitro system by measuring the splicing of a 5′-end-extended precursor. For this reason, the effect of the 5′-flanking sequence on splicing was examined. At early time points *sup3-e* in vitro transcription products include small amounts of a $tRNA^{Ser}$ species which has been processed at its 3′ end but retains both the IVS and the 7-nucleotide 5′ flank of the primary transcript (21). This 5′-end-extended precursor was isolated from a transcription reaction and incubated with endonuclease (Fig. 2A). No significant endonuclease products were detected in any of the incubations with the end-extended precursor. Reduced splicing was not unique to the 5′-flanking sequence in *sup3-e* since the end-extended precursor to *sup9-e*, whose flanking sequence differs from that of *sup3-e* (see reference 32), was also a poor endonuclease substrate (data not shown). This results suggests that sequences at the 5′ end of a tRNA precursor, presumably far removed from the splice sites, can have a dramatic effect on splicing. Consequently, this in vitro transcription and splicing system cannot be used to examine the splicing of *sup3* and *sup9* precursors which substantially reduce the rate of 5′-end maturation.

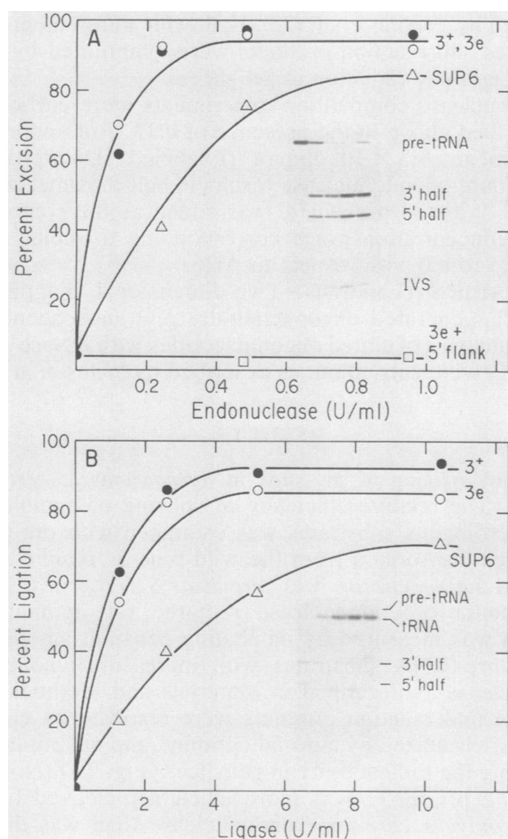

FIG. 2. Efficient in vitro splicing of pre-*sup3*. (A) The efficiency of IVS excision was measured in reactions containing constant amounts of labeled pre-tRNA substrates (3.3×10^4 dpm) and various amounts of endonuclease. (B) Joining of tRNA halves was measured in similar reactions with addition of ATP (2 mM), constant amounts of endonuclease (0.96 U/mL), and various amounts of ligase. Substrates and reaction products were resolved by polyacrylamide gel electrophoresis and visualized by autoradiography. The insets in each panel show autoradiographs obtained in the analysis of pre-*sup3-e*. The relevant species are identified. The amount of each of these species was then determined by measuring Cerenkov radiation in gel slices. The percent excision was calculated as [(cpm 5′ and 3′ halves + cpm IVS)/(cpm pre-tRNA + cpm 5′ and 3′ halves and IVS)] × 100. Percent joining was calculated as [(cpm tRNA)/(cpm tRNA + cpm 5′ and 3′ halves)] × 100. Symbols: ●, pre-*sup3*⁺; ○, pre-*sup3-e*; △, pre-*SUP6*; □, pre-*sup3-e* containing the 7-nucleotide 5′ leader sequence of the primary transcript (3e + 5′ flank).

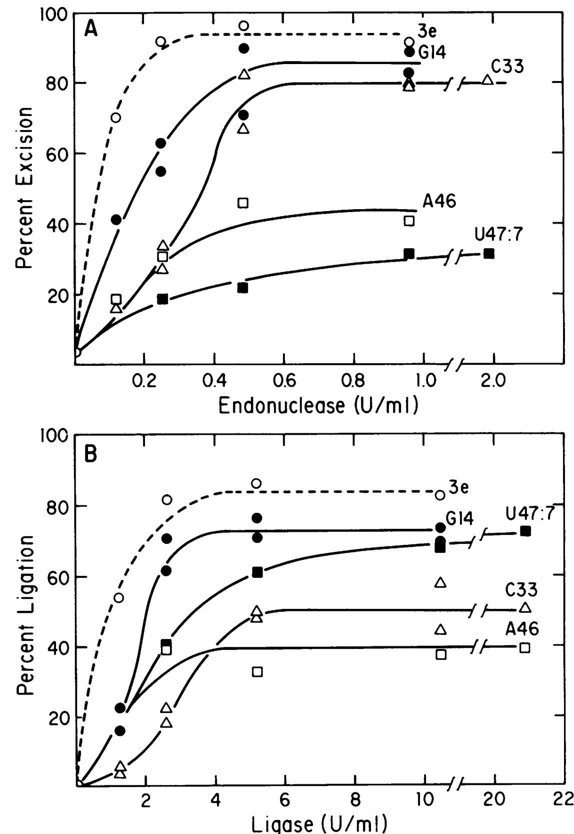

FIG. 3. Splicing of mutant transcripts. The efficiency of IVS excision (A) and joining of halves (B) was measured as described in the legend to Fig. 2. Results for pre-*sup3-e* are shown for comparison (○---○). Symbols: ●, G14; △, C33; □, A46; ■, and U47:7.

Mutations which reduce the efficiency of splicing. Six mutations in the *sup3-e* and *sup9-e* genes were chosen for this study (Fig. 1). These had previously been shown to affect splicing in a yeast whole-cell extract without substantially reducing either transcription or end trimming (21, 33). To determine whether the effects of these mutations were unique to one of the steps in splicing, the efficiency of the excision and joining reactions were examined independently.

Among the six mutations that were studied, four were found to affect the efficiency but not the accuracy of splicing. These were mutations G14 and C33 in *sup3-e* and mutations A46 and U47:7 in *sup9-e* (Fig. 3). Two of these mutations, G14 within the D loop and A46 within the extra arm, affected both the excision and joining reactions in a similar way. For both reactions the G14 mutation produced a slight decrease in the maximum extent of the reaction and an increase in the

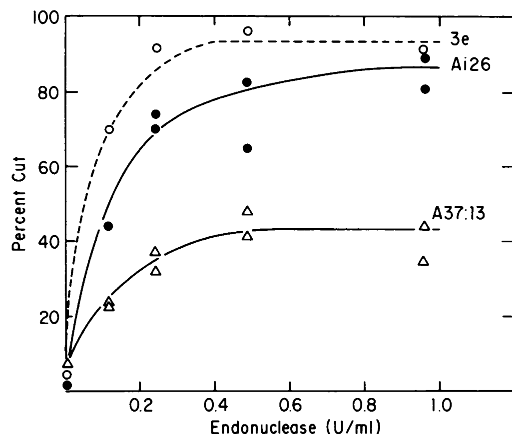

FIG. 4. Efficiency of endonuclease cutting of Ai26 and A37:13 precursors. The efficiency of cleavage of Ai26 (●) and A37:13 (△) pre-tRNAs into smaller products by endonuclease is compared with that of pre-*sup3-e* (○). All of the products identified in Fig. 5 were taken into account in determining the percent excision with these substrates.

amount of enzyme required for half-maximal conversion. The A46 mutation produced a more dramatic decrease in the maximum extent of both excision and joining, less than 50% of that measured for the *sup3-e* transcript.

Mutations C33, in the anticodon loop, and U47:7, in the extra arm, had differential effects on the excision and joining reactions. The U47:7 mutation had a greater effect on IVS excision than on subsequent joining of halves. At the highest concentration of endonuclease tested, only about 30% of the U47:7 pre-tRNA was converted to halves plus IVS. Of the halves that were produced by endonuclease, more than 70% could be converted to tRNA at high ligase concentrations. Note that the reduced joining observed for U47:7 may be due, in part, to reduced concentrations of halves as a result of poor endonuclease cutting. In contrast to U47:7, the C33 mutation had greater impact on joining than on excision. The maximum extent of joining was reduced from 84% for the *sup3-e* transcript to 50% for C33, even at very high ligase concentrations. The maximum extent of IVS excision was reduced only slightly, however (from 94% for *sup3-e* to 80% for C33). Additionally, there was a reproducible change in the shape of the C33 endonuclease titration curve. The significance of this is not known. Comparison of C33 and U47:7 (a poor endonuclease substrate) suggests that reduced joining of C33 may not be attributed solely to a reduction in substrate concentrations. Additionally, the observed reduction in the extent of the reaction for C33 was not the result of incorrect cleavage by endonuclease since C33 and *sup3-e* halves were identical, as judged by comigration on thin denaturing gels, and exhibited the expected pattern of oligonucleotides after RNase T_1 fingerprint analysis (data not shown). Furthermore, fingerprint analysis revealed the presence of 2′, 3′-cyclic phosphate and 5′-hydroxyl termini, which are appropriate for joining by ligase (8).

The basis for the effect of C33 and other mutations on the extent of the excision and joining reactions has not been defined but should be considered in light of the following. Splicing is not a single reaction but rather a pathway of reactions involving at least four distinct enzymatic activities (8, 22) associated with two or more polypeptides which are capable of acting in a concerted fashion (7). Second, while the ligase has been well characterized (8, 24), information about the structure and mechanism of action of the endonuclease and the potential effects of additional factors present in our partially purified fractions is limited. Thus, the basis for the effects of these mutations on the extent of the reaction may vary and might include (i) inappropriate reaction ordering or interaction of active sites; (ii) modification or sequestering of the substrates through competing specific or nonspecific interactions; and (iii) incomplete splicing complex assembly.

Mutations which affect the accuracy of IVS excision. Two of the mutations were found to affect the accuracy and, in one case, also the efficiency of endonuclease cleavage. These were Ai26, an insertion between the D and anticodon stems, and A37:13, a transition mutation within the IVS, three nucleotides from the 3′ splice site (Fig. 1). A comparison of the extent of conversion of the Ai26, A37:13, and *sup3-e* precursors to smaller products with increasing endonuclease concentration is shown in Fig. 4. Assays with both the Ai26 and A37:13 precursors yielded several species in addition to those observed for pre-*sup3-e*. All of these products were included in calculating the results shown in Fig. 4. The relative amounts and electrophoretic mobilities of these additional species are shown in Fig. 5. Individual reaction products were isolated from similar gels and their identities were determined by RNase T_1 digestion followed by two-dimensional oligonucleotide mapping (fingerprinting) and secondary digestions. Certain of the salient fingerprints are shown in Fig. 6. The results of each of these analyses are summarized below.

sup3-e. At the highest endonuclease concentration tested, more than 90% of the *sup3-e* precursor was converted to 3′

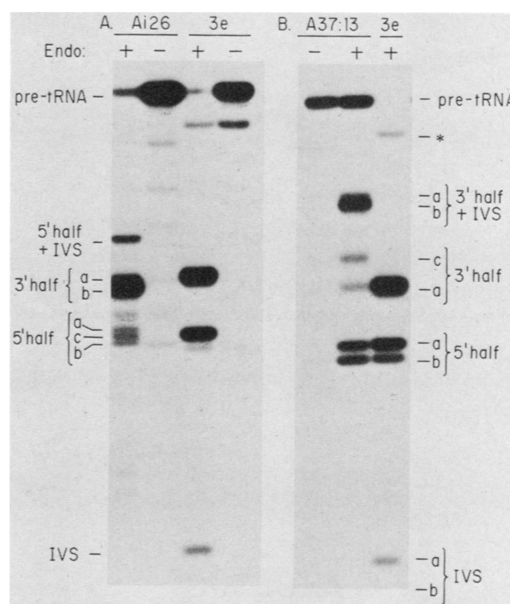

FIG. 5. Miscutting of the Ai26 and A37:13 precursors. Reactions (10 μl) contained 3.3×10^4 dpm of tRNA precursor with (lanes +) or without (lanes −) endonuclease (0.96 U/ml). Incubations were carried out as described in Materials and Methods, and products were analyzed by polyacrylamide gel electrophoresis followed by autoradiography. The identification of reaction products indicated by the notation at the outside edge of each panel is described in the text. The IVS b species, identified in other A37:13 incubations, migrated off the bottom of the gel shown here, and its approximate mobility is shown for reference. Note that the band 3′-half a in *sup3-e* and A37:13 incubations represents the authentic 3′ tRNA half and is not equivalent to 3′-half a in Ai26 incubations, which is missing the 5′-terminal A residue (see Fig. 1 and the text).

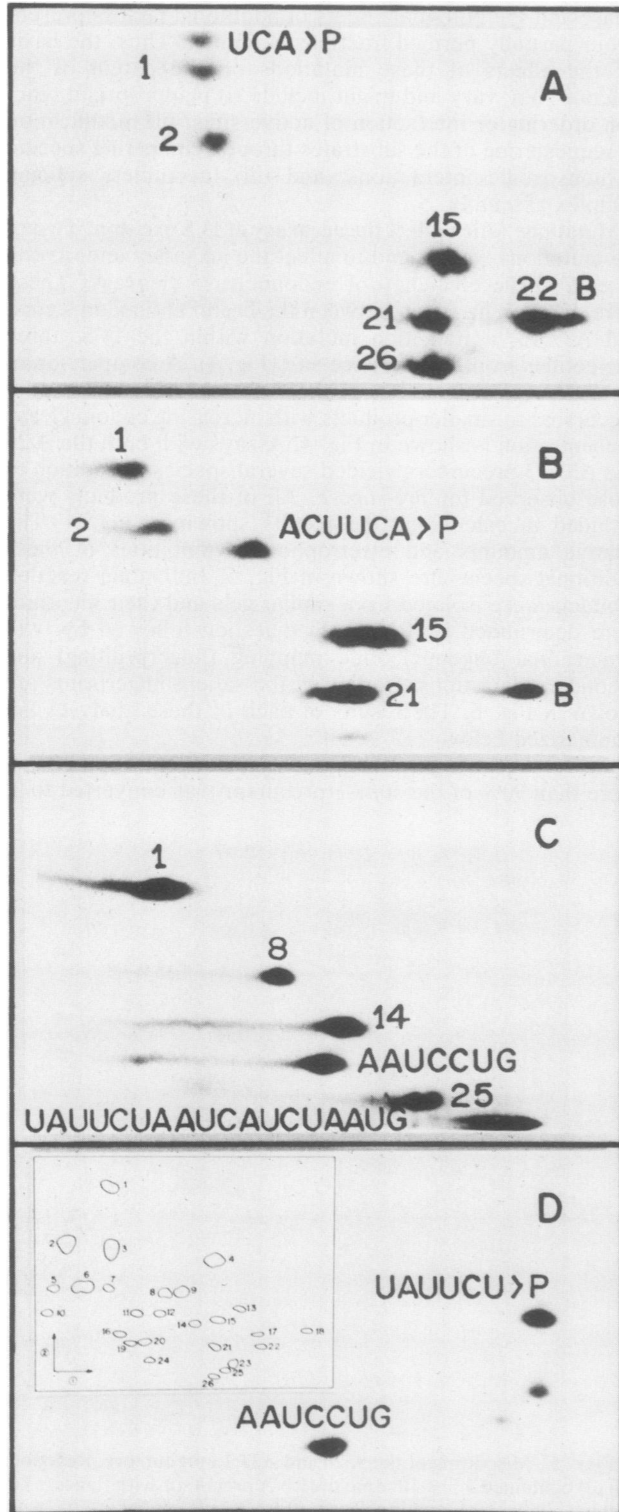

FIG. 6. Fingerprint analysis of splicing endonuclease cleavage products. Precursors labeled with $[\alpha$-^{32}P]UTP were incubated with splicing endonuclease, and the products were resolved and isolated from polyacrylamide gels prior to two-dimensional oligonucleotide mapping. The inset in panel D shows the direction of electrophoresis (direction 1) and homochromatography (direction 2) and, for comparison, illustrates schematically the pattern of $[\alpha$-^{32}P]GTP-labeled RNase T_1 oligonucleotides obtained for the *sup9-e* dimeric precursor (32). Numbered oligonucleotides and spot B (pGp) correspond to species identified previously (32). The sequences of new oligonucleotides are shown and were deduced from the following criteria: secondary analysis after digestion with RNase A and RNase T_2 or nuclease P1; expected mobility shifts; and size estimates from polyacrylamide gels such as the one shown in Fig. 5. The unidentified spots migrating below (in the second dimension) oligonucleotides containing 2',3'-cyclic phosphates are the corresponding 2'- and 3'-phosphate hydrolysis products (22). (A) Ai26 5'-half+IVS. Spot 15 is a mixture of UUAAG and AUUAG. The latter is a new oligonucleotide resulting from the Ai26 base insertion. (B) Ai26 5'-half band c. Spot 15 contains UUAAG and AUUAG as above. (C) A37:13 3'-half+IVS band a. (D) A37:13 IVS band b.

and 5' halves and the linear IVS (Fig. 4). In addition to the correct splicing intermediates, two minor species were seen (Fig. 5B). The amounts of these minor species varied (compare Fig. 5A and B and the inset in Fig. 2A). Both were found with and without added endonuclease, although the addition of endonuclease often increased the relative amounts. The identity of the largest of these is not known. The smaller, 5'-half b, was also found in incubations with the Ai26 and A37:13 precursors. RNase T_1 fingerprint analysis of the isolated 5'-half b fragment (not shown) revealed that it contained sequences from the 5' half minus two nucleotides at the 3' end. Thus, this fragment was generated by cleavage between positions 35 and 36 within the anticodon (site 1, Fig. 1). This minor species was not found in incubations with pre-*sup3*$^+$, suggesting that the anticodon base change in the suppressor alters the structure so as to create a site which is sensitive to hydrolysis. Notably, the cognate fragment expected for cleavage at this site (containing the 3'-half linked to the IVS) was not found. It is unlikely that this cognate fragment was simply degraded during the incubation since an equivalent fragment was observed among the products in A37:13 incubations (described below). Rather, it appears that the base change in the suppressor anticodon allows normal cutting at the 3' splice site, whereas cleavage at the 5' splice site is either occasionally inaccurate, is accurate but is followed by exonucleolytic removal of two 3' nucleotides, or competes with nonspecific cleavage at the anticodon site.

The efficiency of joining of *sup3-e* halves by ligase was examined as shown in the inset of Fig. 2B. The single band corresponding to spliced tRNA product was observed in incubations containing endonuclease and ligase. As the ligase concentration was increased, the amount of spliced product increased in direct proportion to a decrease in the amount of authentic 3' and 5' halves. No corresponding decrease in the amount of the 5'-half b fragment was observed. This result suggests that ligase can efficiently join only the correct splicing intermediates.

Mutation Ai26. The efficiency of conversion of the Ai26 precursor to smaller products in endonuclease incubations was comparable to that for the pre-*sup3-e* control (Fig. 4). However, only two of the resulting products were equivalent to those in the control (the authentic 5' half and 5'-half b generated by cleavage at site 1 within the anticodon). Three bands were observed which were not among the *sup3-e* products (Fig. 5A). These were a minor band, designated 5'-half c, a doublet (3'-half a and b), and a prominent band labeled 5'-half+IVS. Fingerprint analyses of the species corresponding to the 3'-half a and b and 5'-half+IVS (Fig. 6) revealed that these fragments were related. The 5'-half+IVS species contained oligonucleotides characteristic of the 5' half of the tRNA and the intervening sequence. Oligonucleotides corresponding to the 3' half were missing. In addition,

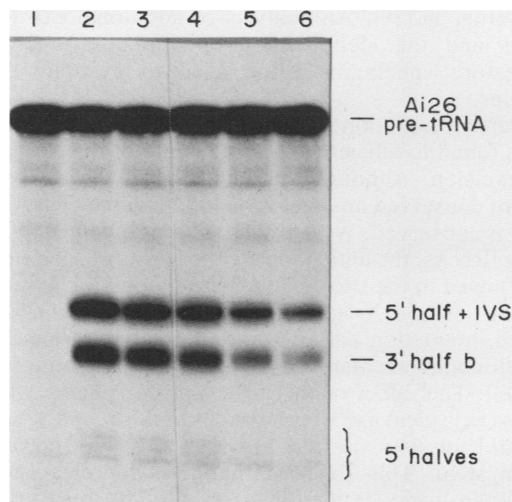

FIG. 7. Splicing endonuclease competition experiment. The ability of ^{32}P-labeled pre-Ai26 to compete for splicing endonuclease was examined in the presence of increasing amounts of tritiated pre-*sup3-e*. Lanes: 1, no enzyme; 2 to 6, 0.12 U of endonuclease per ml and no competitor (lane 2) or 0.25 (lane 3), 1.0 (lane 4), 4.0 (lane 5), or 8.0 (lane 6) times the amount of pre-Ai26 as tritiated pre-*sup3-e*.

a unique oligonucleotide (UCA>p) was found. These results indicate that this species was generated by a cut one nucleotide to the 3′ side of the normal 3′ splice site (site 4 in Fig. 1). Fingerprint analyses of 3′-half a and b indicated that both were cognate 3′ fragments generated by cleavage at this same site (the two closely spaced bands can be seen more clearly in Fig. 7). These two bands produced identical T_1 oligonucleotides (not shown) and probably differ as the result of 3′ CCA terminal heterogeneity. Thus, the 5′-half+IVS and the 3′-half a and b fragments are presumably generated by a single cut one base away from the 3′ splice site (site 4). The identity of the third novel species, 5′-half c, was also examined by fingerprint analysis (Fig. 6). All of the oligonucleotides expected for the 5′ tRNA half were present in good yield, although the 3′-terminal oligonucleotide (ACUUCA>p) was shorter by one nucleotide than expected for the correct splicing intermediate. Thus, this species was generated by a cut one nucleotide to the 5′ side of the normal 5′ splice site (site 3, Fig. 1). Note that cleavage at the normal splice sites and at sites 3 and 4 (Fig. 1) would generate IVS-containing fragments 15, 16, and 17 nucleotides in length, respectively. Fragments of this size were observed, although in amounts insufficient for further analysis.

Mutation A37:13. The efficiency of endonuclease cleavage of the A37:13 precursor was reduced relative to that of the *sup3-e* control (Fig. 4). The maximum extent of conversion of the A37:13 precursor to smaller products was less than 50%. As observed for the Ai26 precursor, only a portion of the A37:13 reaction products were equivalent to those in the *sup3-e* control (the authentic 3′ and 5′ halves and 5′-half b; Fig. 5B). The reduced ratio of 3′ to 5′ halves and the low yield of IVS suggest that this mutation has a greater effect on cleavage at the 3′ splice site than at the 5′ site. In addition to the bands corresponding to the products in the *sup3-e* control, three others were observed. Two of these (Fig. 5B, 3′-half+IVS bands a and b) were found to have similar structures. Fingerprint analysis of the larger of these, band a, is shown in Fig. 6. Oligonucleotides from the 3′ tRNA half and the IVS were present in good yield, whereas those from the 5′ half were absent. Note that the G to A change in A37:13 fused two RNase T_1 oligonucleotides, producing a characteristic 18-mer containing the 3′ splice site. The sequence of the 5′-terminal oligonucleotide (AAUCCUGp) indicates that this 3′-half+IVS species is the counterpart to the 5′-half b species; both may be generated by a single cut within the anticodon (site 1, Fig. 1). A similar analysis was carried out for 3′-half+IVS band b (not shown). The composition of this species differed from that of band a only in the oligonucleotide corresponding to the 5′ end. The sequence of the 5′-terminal oligonucleotide (UCCUGp) indicated that this 3′-half+IVS species is cognate to the authentic 5′ half and that both are generated presumably by a single cut at the 5′ splice site. The identity of the third aberrant product, labeled 3′-half band c in Fig. 5B, was also examined (not shown). This product contained oligonucleotides from both the 3′ half of the tRNA and the 3′ end of the IVS and was generated by cleavage within the IVS, four nucleotides from the 3′ splice site (site 2, Fig. 1). An IVS-containing fragment (IVS b) produced by cleavage at this same site was observed in other experiments and has been analyzed (Fig. 6). The 5′ end of this 13-nucleotide fragment was produced by cleavage within the anticodon (site 1, Fig. 1) and its 3′ end by cleavage with the IVS (site 2).

The effects of the anticodon base change and of mutations Ai26 and A37:13 can be summarized as follows. The anticodon base change in the suppressor resulted in inappropriate cutting within the anticodon, potentially as the result of a change in the structure of this region. However, this change alone had no significant effect on the efficiency of splicing. The Ai26 mutation had a similar effect in that it did not reduce the efficiency of conversion of pre-tRNA to smaller products. However, Ai26 did result in cutting at two inappropriate sites. The majority of the Ai26 reaction products were generated by a single cut within the 3′ tRNA half, one nucleotide from the 3′ splice site. A small amount of miscutting was also detected within the 5′ half, one nucleotide from the 5′ splice site. The A37:13 mutation affected both the efficiency and the accuracy of IVS excision. The majority of the reaction products were generated by a single cut at alternate sites; at the 5′ splice site or within the anticodon two nucleotides from the 5′ splice site. Miscutting within the IVS was also detected, one nucleotide from the position corresponding to the A37:13 mutation.

Incubation of the Ai26 and A37:13 precursors with both endonuclease and ligase (not shown) produced only small amounts of spliced tRNA product, as expected given the low level of accurate cutting of these precursors. The low yield of spliced product and the presence of potentially inhibitory cleavage products precluded analysis of the effects of the Ai26 mutation on the joining reaction.

Several lines of evidence suggest that the 5′- and 3′-half+IVS species in Ai26 and A37:13 incubations were produced through splicing endonuclease miscutting rather than by the action of a contaminating nuclease. First, the amounts of these products in the Ai26 and A37:13 incubations were directly correlated with endonuclease activity (Fig. 4). Second, the effect of ionic strength on the appearance of the half+IVS fragments was similar to that observed for the conversion of pre-*SUP6* to halves (not shown). The optimum ionic strength (approximately 25 mM NaCl) measured for the appearance of the half+IVS fragments and of *SUP6* halves was similar to that reported previously for another pre-tRNA substrate (22). Third, each of the half+IVS species and cognate halves had the appropriate combination of 5′-hydroxyl and 2′,3′-cyclic phosphate termini expected for splicing endonuclease cleavage products

(22). Finally, we have carried out a series of endonuclease competition reactions. In these experiments Ai26 pre-tRNA was incubated with a constant, limiting amount of endonuclease in the presence of various amounts of a tritiated pre-*sup3-e* competitor. The results demonstrate that Ai26 products, including the 5′-half+IVS species, decreased in direct proportion to the amount of competing pre-*sup3-e* (Fig. 7). At the highest concentration of pre-*sup3-e* competitor used (approximately an eightfold molar excess relative to Ai26), endonuclease cleavage of Ai26 was reduced by 75%. Thus, proper cutting of pre-*sup3-e* and miscutting of Ai26 (at sites 3 and 4, Fig. 1) requires a common component (splicing endonuclease) which is limiting in the reaction.

DISCUSSION

Our analysis of the effects of point mutations on splicing reveals that both the excision and joining steps involve interactions between the respective enzymes and specific elements of pre-tRNA sequence or structure. Among the mutations that were examined, some had a differential effect on the excision and joining steps and others affected these steps in parallel. These results suggest that endonuclease and ligase (or an endonuclease-ligase complex; see reference 7) recognize unique and overlapping sets of substrate features. These are described below.

The effects of two mutations within the extra arm were examined. The extra arm represents a hypervariable segment of tRNA structure and as such seems an unlikely candidate for a common recognition element. Among the IVS-containing precursors in *Saccharomyces cerevisiae*, the extra arm varies in length from 5 to 14 nucleotides, with a corresponding variation in the potential for forming base-paired secondary structures (20). In tRNAs with long extra arms, complementarity within this segment is thought to result in the formation of a stem-loop structure which precludes its participation in tertiary interactions which are charcteristic of shorter extra arm sequences (see reference 2 for a recent review of tRNA structure). A model for the structure of these tRNAs has been proposed in which the extra arm stem and loop point away from the acceptor stem, with the helical segment projecting slightly out of the plane formed by the arms of the L (1). The positions at the base of the stem are thought to be the only points for tertiary interactions, participating in the purine$_{15}$-pyrimidine$_{48}$ *trans* pair and the purine$_{26}$-pyrimidine$_{44}$ anticomplementary pair. With this structural model in mind, the effect of the A46 mutation on the excision and joining reactions is presumably due to disruption of the base-paired stem within the extra arm. The effect of the U47:7 mutation on the predicted structure of the extra arm is less dramatic. The C to U change created by this mutation places a G-U pair in a central position within the base-paired stem (Fig. 1). The resulting modified stem is marginally stable (calculated $\Delta G = +0.3$ kcal/mol [17]). This observation, together with the fact that the U47:7 mutation affects primarily endonuclease cleavage, suggests that the structural perturbation in this case may not involve disruption of the predicted secondary structure. Two other possibilities can be considered. First, the base change at this position might affect an unidentified tertiary interaction. Potential sites for such interactions based on the structural model are the D stem and loop and T loop. Second, this position may be part of a sequence-specific recognition site which is common to one or a few precursors. Note that the extra arms of *S. pombe sup3* tRNA and *Saccharomyces cerevisiae* $tRNA^{Ser}_{CGA}$ are essentially identical (Fig. 1) (20). An analysis of additional extra-arm mutations and the identification of third-site revertants which restore splicing is being used to examine these possibilities.

An insertion mutation (Ai26) at the top of the anticodon stem was found to affect the accuracy but not the efficiency of IVS excision. Although the sequence of the anticodon stem is not conserved among IVS-containing pre-tRNAs, its structure is conserved. With few exceptions among naturally occurring tRNAs, the anticodon stem consists of 5 base pairs and is followed in the tRNA by a 7-base loop (27). Insertion of an A at position 26 may disrupt the proposed G26-U44 conserved interaction and in addition creates the potential for a sixth complementary base pair in the anticodon stem (Ai26 U44). The effect of this insertion on splicing was to create two new cleavage sites adjacent to the 5′ and 3′ splice sites, with each new site one base closer to the top of the anticodon stem. This suggests that sites for cleavage by endonuclease might be identified, in part, by measuring a fixed distance from the top of the anticodon stem. Other mutations within or near the anticodon stem have been shown to affect splicing. These include mutations which disrupt pairing in the anticodon stem (18, 33) and those which disrupt the structure of the D stem nearby (6). Together, these results demonstrate that this region of the precursor represents an essential site for interaction with the splicing enzymes.

The A37:13 mutation within the intervening sequence was found to affect both the accuracy and efficiency of IVS excision. It is unlikely that this mutation alters a sequence-specific recognition site, since the IVS is not conserved and no counterpart to the sequence around this mutation is found among the *Saccharomyces cerevisiae* precursors (20). Potentially, this mutation either disrupts a structure which is essential for normal splicing or creates an alternate structure which is poorly spliced. With regard to the latter, a hypothesis has been suggested previously (33); however, the miscutting detected at the site adjacent to the IVS base change (site 2, Fig. 1) was neither predicted nor expected for the proposed alternate conformation. A candidate for the former possibility is the interaction of the IVS nucleotide with position 32, the first base in the anticodon loop. The potential for an equivalent interaction is found in eight of the nine *Saccharomyces cerevisiae* pre-tRNAs (20). In the exception, pre-$tRNA^{Pro}_{UGG}$, the putative position 32 complement would be four rather than three nucleotides from the 3′ splice site. Previously, Knapp and co-workers have proposed that cutting by endonuclease requires that the 3′ splice site be present in a single-stranded segment (M.-C. Lee, J. Milligan, and G. Knapp, manuscript in preparation). Potentially, the position 32-IVS interaction allows the formation of a compact structure within the active site, with the 3′ splice site present in a loop of defined size (three to four nucleotides). Notably, a splicing defect has been reported for a mutation at position 32 in pre-$tRNA^{Tyr}$ *SUP4* (18).

Excision of a linear IVS by endonuclease requires two events: cleavage at the 5′ and at the 3′ IVS boundaries (14). The Ai26 and A37:13 mutations had nearly inverse effects on these two events. Mutation Ai26 produced inefficient cutting at and around the 5′ splice site, whereas 3′ cutting proceeded efficiently (but at a site adjacent to the normal splice site), producing a 5′-half+IVS species. Mutation A37:13 reduced cutting at and around 3′ site to a much greater extent than near the 5′ site, resulting in the appearance of the 3′-half+IVS species. Thus, it seems that under these conditions the 5′ and 3′ cutting events can occur independently.

Position 33, adjacent to the anticodon, is highly conserved, with a U at this position in all noninitiator tRNAs. Interaction between the base at this position and the backbone phosphate at position 36 is thought to play an important role in the configuration of the anticodon bases in the mature tRNA (reviewed in reference 26). A mutation at this position (C33) was found to affect primarily joining by ligase. The effect of this mutation may be to alter the normal juxtaposition of the ends to be joined and thereby reduce the rate of ligation. Alternatively, the configuration of the anticodon loop may contribute to the affinity of ligase for its substrate. A method for measuring binding of ligase to pre-tRNA substrates has been developed (H.G. Belford and C.L. Greer, manuscript in preparation) and is being used to examine these possibilities.

The presence of a small (five- to seven-nucleotide) 5′-flanking segment prevented endonuclease cleavage of the end-extended *sup3-e* and *sup9-e* precursors. This is in contrast to the primary transcripts of the *Saccharomyces cerevisiae* $tRNA^{Leu}_{3}$ *SUP53* and $tRNA^{Tyr}$ *SUP6* genes which previously have been shown to be spliced in vitro, albeit with reduced efficiencies (M. Shapero and C. Greer, unpublished results). These *Saccharomyces cerevisiae* precursors differ from the 5′-end-extended *sup3-e* and *sup9-e* precursors in that both 5′ and 3′ extensions are present. Furthermore, the 5′ and 3′ extensions of the *SUP53* and *SUP6* transcripts are partially complementary and may form a compact, base-paired extension of the acceptor stem (5, 29). Thus, it may be the unpaired nature of the 5′ extension of pre-*sup3-e* which prevents endonuclease cleavage, perhaps through simple steric hindrance.

Mutations which alter conserved elements of sequence or structure without affecting splicing can provide a means for defining those features of the substrate which are not essential for splicing. Two of the mutations that were examined may fall into this category. The first is the anticodon base change in the nonsense suppressor. The presence of a segment within the IVS which is complementary to all or part of the anticodon is common to all of the IVS-containing precursors in *Saccharomyces cerevisiae* (20). Chemical and enzymatic structure probing have shown that these two segments are found within stable secondary structures in the wild-type pre-tRNA (16, 30). The base change in the nonsense suppressor anticodon is expected to destabilize this conserved structure. This expectation is confirmed by the presence of a unique hydrolysis-sensitive site in the *sup3-e* anticodon and by the reduced efficiency of processing of the *sup9-e* precursor (relative to $sup9^{+}$) by RNase P (32). The absence of an effect on splicing of the suppressor anticodon suggests that this conserved structure is not an essential recognition element. This result is consistent with previous observations on the splicing of two other suppressors, $tRNA^{Ser}$ *SUPRL1* (cited in reference 9) and $tRNA^{Tyr}$ *SUP6* (8).

The G14 mutation affects a position which is conserved in nearly all tRNAs (2). This conservation is thought to reflect an important tertiary interaction between A14 and U8 which contributes to the bend in the distinctive L shape of the molecule. The A to G change produced by this mutation had only a slight effect on splicing. The possibility that a G in this position allows an alternate tertiary interaction which mimics the normal structure has not been ruled out. Both solution structure probing and an analysis of additional mutations at this position will be required to demonstrate with certainty that this conserved structure is not required in splicing.

The results presented here together with prior work by others (3, 6, 9, 16, 18, 20, 21, 25, 30, 33) provide the outlines of a general model for the interaction of the tRNA-splicing enzymes with their substrates. Although this model is necessarily incomplete due to a lack of detailed structural information about the large extra arm in *sup3* tRNA and a requirement in this analysis for mutations which affect neither transcription nor prior end trimming, several important features of the interactions do emerge. Primary recognition sites for both endonuclease and ligase lie within the tRNA-like domain of the molecule and include the base-paired nature of the anticodon stem and elements of sequence or structure near the top of this stem, including the region occupied by the extra arm and the D stem nearby. The requirements for IVS sequence and structure in the excision reaction may be minimal. These may include the formation of a structure which is sufficiently compact to fit within the active site and which places the splice sites at central positions within defined single-stranded loops. The precise sites for cleavage may be determined in part by their distance from a conserved element within the tRNA-like portion of the molecule. Joining by ligase may include an additional requirement for tRNA-like configuration within the anticodon loop. This model is consistent with two general observations. The first is the ability of tRNA-splicing enzymes from organisms as diverse as yeast, amphibians, and humans to accurately process the same set of substrates (19, 29). All of these enzymes may bind to similar conserved regions. The second is the constant position of the intron relative to the coding sequences in nuclear intron-containing tRNA genes (see reference 28). Thus, splice sites may be determined to a large degree by their distance from a primary binding site.

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