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# Assembly of a tRNA Splicing Complex: Evidence for Concerted Excision and Joining Steps in Splicing In Vitro

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**Splicing of tRNA precursors in *Saccharomyces cerevisiae* extracts proceeds in two steps; excision of the intervening sequence and ligation of the tRNA halves. The ability to resolve these two steps and the distinct physical properties of the endonuclease and ligase suggested that the splicing steps may not be concerted and that these two enzymes may act independently in vivo. A ligase competition assay was developed to examine whether the excision and ligation steps in tRNA splicing in vitro are concerted or independent. The ability of either yeast ligase or T4 ligase plus kinase to join the tRNA halves produced by endonuclease and the distinct structures of the reaction products provided the basis for the competition assay. In control reactions, joining of isolated tRNA halves formed by preincubation with endonuclease was measured. The ratio of yeast to T4 reaction products in these control assays reflected the ratio of the enzyme activities, as would be expected if each has equal access to the substrate. In splicing competition assays, endonuclease and pre-tRNA were added to ligase mixtures, and joining of the halves that were formed was measured. In these assays the products were predominantly those of the yeast ligase even when the T4 enzymes were present in excess. These results demonstrate preferential access of yeast ligase to the endonuclease products and provide evidence for the assembly of a functional tRNA splicing complex in vitro. This observation has important implications for the organization of the splicing components and of the gene expression pathway in vivo.**

The synthesis of the mature, functional form of a tRNA requires a number of processing reactions which collectively form a discrete biosynthetic pathway. The mechanisms by which individual processing enzymes interact and are integrated into such a pathway are not well understood. One common mechanism for coordinating sequential enzymatic reactions is the formation of a multienzyme complex (34). In this work, coupling of the excision and joining steps in tRNA splicing through the assembly of a splicing complex was examined.

The mechanism of tRNA splicing in *Saccharomyces cerevisiae* extracts has been well defined. The reaction proceeds in two distinct steps (18, 25). First, the intervening sequence 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 form the mature-sequence tRNA. The endonuclease and ligase which carry out these two steps can be resolved in crude extracts (24) and have been partially purified (11, 24). These fractions contain four distinct enzymatic activities which are thought to be required for splicing in vitro (11, 24) (summarized in Fig. 1). Three of these activities, a cyclic phosphodiesterase, a polynucleotide kinase, and an RNA ligase, are required for joining of the halves produced by endonuclease. These three activities copurify and may be contained in a single polypeptide (11; E. Phizicky, R. Schwartz, and J. Abelson, manuscript in preparation). This putative multifunctional polypeptide could coordinate the three-step ligation event by restricting the process to the surface of a single enzyme molecule. Examples of this motif, the physical linkage of related enzymatic activities, have been found in other biosynthetic pathways in *S. cerevisiae* and include the ADE3 (30), HIS4 (16), and TRP5 (7) gene products.

A comparison of the physical properties of the ligase and endonuclease does not provide evidence for physical linkage of the intervening sequence excision reactions to the collective ligation reactions. The properties of the ligase are consistent with those of either soluble matrix or peripheral membrane proteins (11). The majority of the activity is found in the soluble fraction of a crude extract carried out in the presence of high salt concentration, and the ligase can be further purified from this fraction in the absence of detergent. Estimates of the native molecular weight by gel filtration and of the subunit molecular weight by sodium dodecyl sulfate-polyacrylamide gel electrophoresis both give a value of approximately 90 kilodaltons (11). In contrast, the endonuclease behaves as an integral membrane protein (24). The activity is associated with a particulate fraction in crude extracts and subsequently copurifies with a membrane fraction obtained by flotation on a sucrose density gradient. The endonuclease can be extracted from this fraction with nonionic detergents, and its further purification requires the continued presence of detergent.

The distinct physical properties of the splicing enzymes raised the possibility that the excision and joining steps are independent rather than concerted. For independent steps in splicing, the endonuclease and ligase need not be associated and could even be located in separate subcellular compartments. To distinguish between concerted and independent splicing mechanisms, a method was developed to determine whether the halves produced by endonuclease are released into solution prior to joining by ligase. The method is based on competition between yeast ligase and T4 RNA ligase. It had previously been shown that tRNA halves produced by yeast endonuclease could be joined either by yeast ligase or T4 RNA ligase in concert with T4 polynucleotide kinase (10). The proposed mechanisms for joining of halves by the yeast and T4 enzymes are similar (11, 31) and are shown in Fig. 1

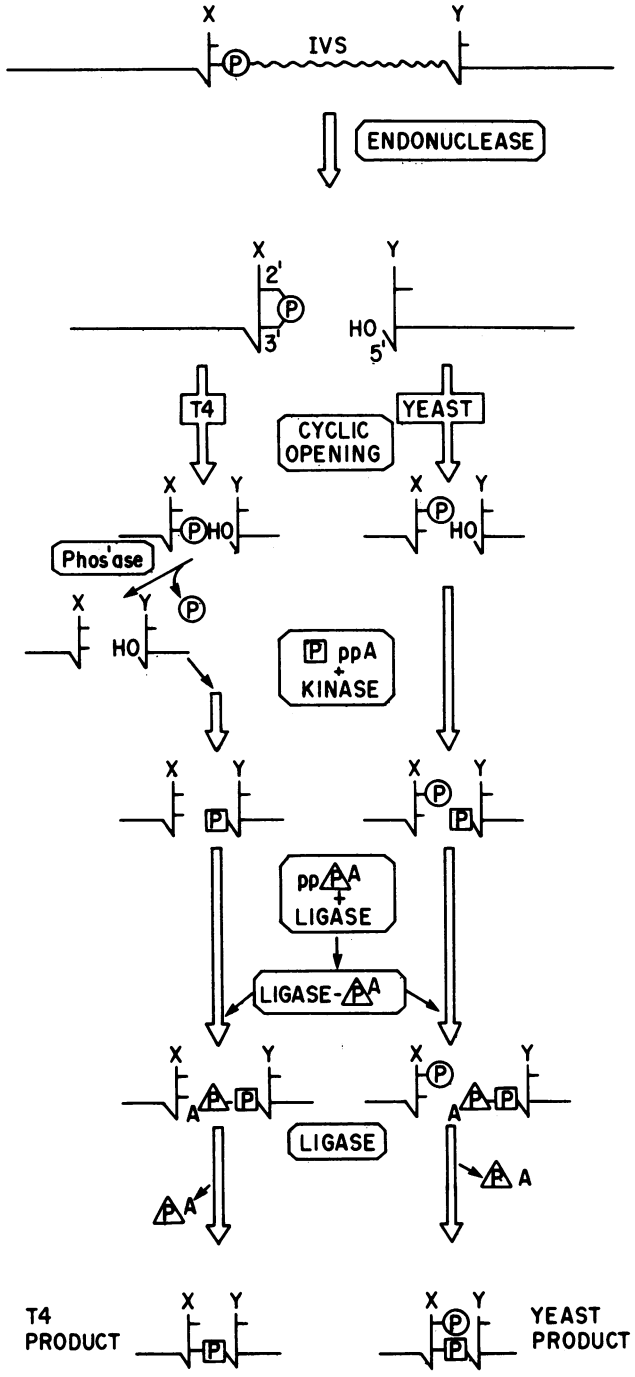


FIG. 1. Joining of tRNA halves. The pathways proposed for joining of tRNA halves by yeast ligase or T4 ligase plus kinase are summarized (cf. references 11, 31, 32). The first step shows the formation of halves from pre-tRNA by yeast endonuclease. The sequence of the subsequent reactions should be considered tentative since the precise order has not been determined for either the yeast or T4 enzymes. Symbols around the phosphates are to facilitate tracing each through the ligation pathways. Phos'ase, Phosphatase; IVS, intervening sequence; pA, AMP; pppA, ATP.

for comparison. A cyclic phosphodiesterase, a polynucleotide kinase, and an RNA ligase which proceeds through an adenylated enzyme intermediate are common to both. While in *S. cerevisiae* all of these activities may be associated with a single polypeptide, the T4 cyclic phosphodiesterase and polynucleotide kinase activities are associated with a multifunctional polypeptide (T. Weber, Ph.D. thesis, University of Illinois, Urbana, 1985) which is distinct from the RNA ligase. There is another important difference between these two pathways. In the T4 pathway, the phosphomonoester produced by the cyclic phosphodiesterase can be cleaved by the phosphatase activity of the T4 kinase phosphatase (4). No equivalent activity is found in the yeast ligase fraction. Therefore the yeast product has a 2′ phosphate which is absent in the T4 product. The nuclease resistance conferred by the 2′ phosphate provides a convenient means for distinguishing between the yeast and T4 ligase products.

The ability to distinguish between their respective products allowed measurement of competition between the yeast and T4 enzymes for halves being formed by endonuclease. The results of these experiments demonstrate preferential access of yeast ligase to the endonuclease products and provide evidence for the assembly of a functional tRNA splicing complex in vitro. This observation and the physical properties of the endonuclease suggest that a multienzyme complex, perhaps associated with the nuclear envelope, may be responsible for the process of tRNA splicing in vivo.

## MATERIALS AND METHODS

**Yeast strains.** Strain M304 (*rna1/rna1 ade1/+ tyr1/+*), obtained from J. Bossinger, was used for the preparation of pre-tRNA substrates. Strain EJ101 (α-*pep4-3 prb1 prc1 his*), provided by E. Jones, was used for the preparation of splicing enzyme fractions.

**Enzymes and chemicals.** Phage T4-encoded polynucleotide kinase was from New England BioLabs, Inc. Conventional units of activity and assay conditions similar to that used by the supplier have been described previously (29). Phage T4-encoded RNA ligase was from New England Nuclear Corp. Assay conditions and conventional activity units were as described previously (3). Nuclease P1 and RNase T2 were purchased from Calbiochem-Behring. Yeast endonuclease (24 U/ml) was equivalent to fraction VI of Peebles et al. (24). One unit of endonuclease produces $6 \times 10^{-6}$ g of tRNA halves in 10 min at 30°C. Yeast ligase (210 U/ml) was equivalent to fraction IV of Greer et al. (11). One unit of ligase joins 1 pmol of tRNA halves per min at 30°C. [γ-$^{32}$P]ATP (~9,000 Ci/mmol) was obtained from ICN Pharmaceuticals Inc. and [α-$^{32}$P]UTP (~3,000 Ci/mmol) was from Amersham Corp.

**Preparation of pre-tRNA substrates.** Unlabeled tRNA precursors were purified as described by Ogden et al. (23) except that precursors were visualized by staining with ethidium bromide after electrophoresis. Ethidium was removed from eluted precursors by isopropanol extraction.

Labeled pre-tRNA$^{Tyr}$SUP6 ($5 \times 10^6$ dpm/pmol) was prepared by in vitro transcription as described by Engelke et al. (8) in 100-μl reactions containing [α-$^{32}$P]UTP (10 μM; 100 Ci/mmol), plasmid pYSUP6 (33), and 40 μl of a yeast nuclear extract (prepared by the method of J. Topol and C. S. Parker as described previously [8]). Labeled pre-tRNA was purified by electrophoresis in gels containing 12% acrylamide, 0.4% bisacrylamide, and 8 M urea and eluted from gel slices by soaking in 20 mM Tris hydrochloride (pH 7.5)–0.3 M NaCl–1 mM EDTA–1% phenol for 8 to 12 h at 37°C.

**Preparation of tRNA halves.** Paired tRNA halves were prepared by preincubating endonuclease (1.3 U/ml) and

unlabeled pre-tRNA ($10^{-8}$ g) or unlabeled pre-tRNA ($10^{-8}$ g) plus $^{32}$P-labeled SUP6 transcript ($0.5 \times 10^{-9}$ g). Conditions for preincubations and the procedure for stopping reactions were as described for ligase assays below. Samples were then extracted three times with phenol-chloroform-isoamyl alcohol (25:24:1) and twice with ether and precipitated with the addition of 3 volumes of ethanol.

**Measurement of endonuclease and ligase activities.** Titrations of endonuclease and ligase activity were carried out in reactions (10 μl) containing 20 mM Tris hydrochloride (pH 8.0), 2.5 mM spermidine, 0.1 mM dithiothreitol, 40 mM NaCl, 10 mM $MgCl_2$, 0.1 mM ATP, 0.5% Triton X-100, and either $1 \times 10^{-8}$ g of unlabeled pre-tRNA$^{Tyr}$ plus $0.5 \times 10^{-9}$ g of $^{32}$P-labeled SUP6 transcript or equivalent amounts of the corresponding halves. Reactions were incubated for 10 min at 30°C, stopped with the addition of 0.2% sodium dodecyl sulfate–10 mM EDTA–0.2 mg of proteinase K per ml, and further incubated for 20 min at 50°C. Samples were then analyzed by electrophoresis in gels containing 20% acrylamide–0.6% bisacrylamide–4 M urea as described by Peebles et al. (24). Reaction products were quantitated by measuring Cerenkov radiation in gel slices.

**Competition assays.** Ligase activities in mixtures of yeast ligase and T4 kinase plus ligase were measured by incorporation of label from [γ-$^{32}$P]ATP into tRNA products. Reaction conditions were as described for ligase assays with the addition of [γ-$^{32}$P]ATP (50 μM; 500 Ci/mmol). Coupled competition assays contained constant amounts of endonuclease (1.3 U/ml) and unlabeled pre-tRNA ($10^{-8}$ g). Uncoupled assays contained an equivalent amount of tRNA halves ($10^{-8}$ g) and no endonuclease. Reactions were stopped with sodium dodecyl sulfate-EDTA-proteinase K as above and brought to 100 μl with the addition of 0.4 M ammonium acetate (pH 7.0) and 20 μg of glycogen carrier (Type II; Sigma Chemical Co.). Samples were extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) and once with ether and precipitated with the addition of 3 volumes of ethanol. The products of single-substrate reactions were purified by gel electrophoresis (20% acrylamide–0.6% bisacrylamide–4 M urea) and eluted from gel slices as described above. The products of two-substrate reactions were purified by two-dimensional gel electrophoresis. The first dimension gel contained 10% acrylamide–0.3% bisacrylamide–4 M urea. The second dimension gel contained 20% acrylamide, 0.6% bisacrylamide, and 4 M urea.

The concentration of pre-tRNA or halves in these assays was approximately 40 nM (estimated from the $A_{260}$ of the substrate preparation). The enzyme concentrations were estimated from the molecular weights and specific activities reported for the purified proteins. For T4 ligase, the reported values for molecular weight (43,000 [20]) and specific activity (2,000 U/mg in an assay similar to that used by the supplier [3]) translate into concentrations of 1.15 and 2.3 μM ligase at 100 and 200 U/ml, respectively. For T4 kinase, the reported values of 68,000 daltons and 140,000 U/mg (29) (the specific activity was adjusted for the ATP concentration used in assays by the supplier [29]) give estimated concentrations of 5 and 10 nM kinase at 100 and 200 U/ml, respectively. For yeast ligase, a molecular weight of 90,000 (11) and a specific activity for the purified protein of 22,000 U/mg (Phizicky et al., manuscript in preparation) have been reported. These values give estimated concentrations of 2 to 10 nM ligase at 4 to 21 U/ml. The concentration of endonuclease cannot be estimated from the available data.

**Yeast-to-T4 product ratios.** The ratio of yeast ligase to T4 kinase plus ligase products was measured as follows. Carrier RNA (10 μg) was added to the eluted tRNA products from competition and control incubations. Eluted samples were digested with RNase T2 followed by nuclease P1 as described previously (10). Digestion products were then resolved by chromatography on polyethyleneimine-cellulose thin-layer plates (Macherey-Nagel) developed with 1 M LiCl. After autoradiography, individual spots were cut out of the plate, and radioactivity was measured in scintillation fluid (Liquifluor; New England Nuclear).

## RESULTS

**Measurement of yeast-to-T4 ligase product ratios.** A method for measuring the ratio of yeast to T4 ligase products was required prior to examining competition between these two enzymes in in vitro splicing reactions. Previously it had been shown that the tRNA produced by joining of halves by T4 ligase plus kinase was identical to that produced by yeast ligase except for a 2′ phosphate unique to the yeast product (10). The method developed here to differentiate between the reaction products involves selective labeling of the ligated junction in both the yeast and T4 products and nuclease digestion to probe the structure of the two labeled junctions.

The phosphate in the newly formed phosphodiester bond in both the yeast and T4 ligase products is derived from the γ position of ATP (11, 31). In the competition assays described below, ligase activity was measured by incorporation of label from [γ-$^{32}$P]ATP into unlabeled pre-tRNA substrates yielding tRNA products in which the only labeled internal position is the ligated junction (11). A potential source of background labeling in this method is phosphorylation of preexisting 5′-hydroxyl termini by the kinase activities in the yeast and T4 fractions. To examine whether this source of background labeling was significant, labeled tRNA products from reactions containing pre-tRNA$^{Tyr}$, endonuclease, [γ-$^{32}$P]ATP, and yeast ligase or T4 ligase plus kinase were isolated by electrophoresis and treated with phosphatase. Under the conditions described by Schwartz et al. (27) for complete dephosphorylation by calf intestinal alkaline phosphatase, less than 1% of the label was converted to an acid-soluble form (data not shown). The results of the experiments shown in Fig. 2 and 4 and described in the text below provide additional evidence that the label in tRNA-sized products is internal rather than at phosphorylated 5′ termini. In these experiments, the products of nuclease digestion of molecules containing labeled 5′ phosphates would be labeled 5′ nucleoside monophosphates. No significant amounts of labeled nucleoside monophosphates were found among the nuclease digestion products.

Differentiation of the yeast and T4 ligase products on the basis of nuclease resistance was examined by sequential digestion of the purified reaction products with RNase T2 followed by nuclease P1. RNase T2 is a nonspecific ribonuclease whose cleavage products have 3′ phosphates (26). Nuclease P1 is both a nonspecific nuclease which produces 5′ phosphates and a 3′ phosphatase (9). The halves joined by T4 ligase in the presence of [γ-$^{32}$P]ATP are expected to yield labeled 3′ AMP after T2 digestion and labeled $P_i$ after subsequent P1 treatment (the bases adjacent to the spliced junction in tRNA$^{Tyr}$ are adenine residues). The spliced junction in the yeast ligase product is resistant to both T2 and P1 and is expected to yield labeled junction dinucleotides with a 2′-phosphomonester, 3′,5′-phosphodiester linkage $A^Pp^*Ap$ after T2 and $A^Pp^*A$ after

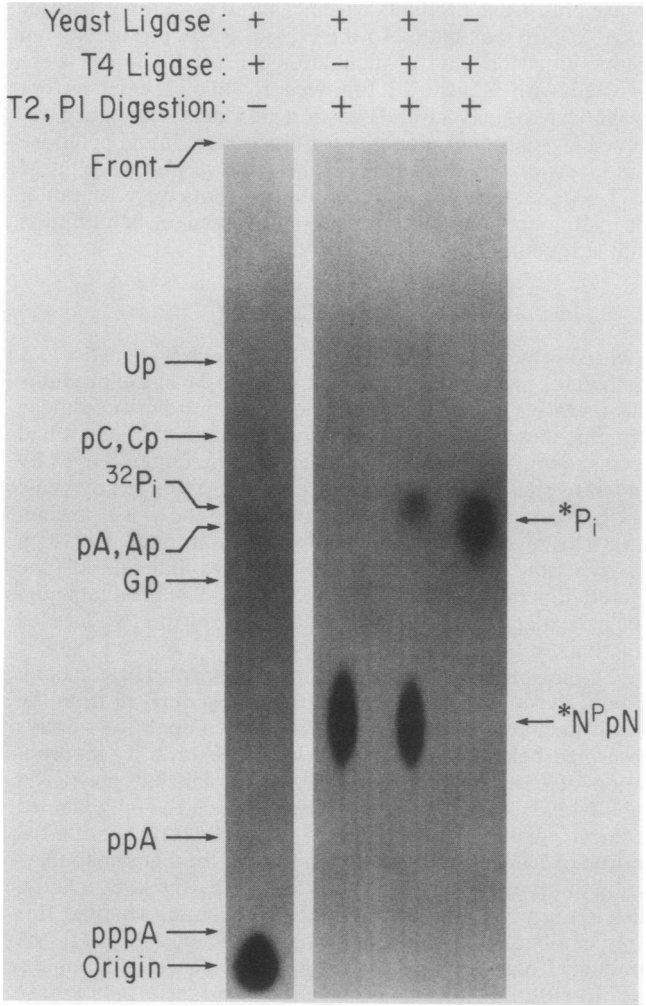


FIG. 2. Measurement of yeast and T4 ligase products. Splicing reactions were carried out with endonuclease, pre-tRNA$^{Tyr}$, and [$\gamma$-$^{32}$P]ATP as described in Materials and Methods. Yeast ligase (6 U/ml), T4 ligase plus kinase (75 U/ml each), or both the yeast and T4 enzymes were added as indicated at the top of the figure. The results shown are an analysis by thin-layer chromatography of the purified reaction products either run directly (left lane only) or after sequential digestion with RNase T2 and nuclease P1. The mobilities of authentic markers are indicated at the left edge. The identification of labeled species, indicated at the right edge, is described in the text. *, Labeled phosphate; N$^{p}$pN, nuclease-resistant junction dinucleotide; Up, Cp, Ap, and Gp, nucleoside 3′-monophosphates; other abbreviations, as for Fig. 1.

P1 digestion (labeled phosphates are marked with an asterisk).

Splicing reactions were carried out with endonuclease (1.3 U/ml), unlabeled pre-tRNA$^{Tyr}$ ($10^{-8}$ g), [$\gamma$-$^{32}$P]ATP (500 Ci/mmol), and yeast ligase, T4 ligase and T4 kinase, or a mixture of both the yeast and T4 enzymes. Incubation conditions were as described in Materials and Methods, and reaction products were purified by polyacrylamide gel electrophoresis after phenol extraction and ethanol precipitation.

The products of sequential nuclease digestions, resolved by thin-layer chromatography and visualized by autoradiography, are shown in Fig. 2. Chromatography of purified reaction products without prior nuclease digestion produced label only at the origin. Sequential T2 and P1 digestion of the yeast tRNA product yielded a single, slow-migrating species. Identification of this species as the resistant junction dinucleotide is based on a comparison with previous analyses (10, 11, 27) and the ability of phosphatase treatment of T2-resistant material to render the label sensitive to nuclease P1 digestion (data not shown). Digestion of the T4 product yielded a single species which comigrates with authentic $P_i$ marker. Digestion of the tRNA products from a mixed yeast and T4 ligase incubation yielded both junction dinucleotide and $P_i$.

Together, these results demonstrate that the ratio of yeast to T4 ligase products in mixtures can be measured as the ratio of label in junction dinucleotide to that in $P_i$ after sequential digestion to completion with RNase T2 followed by nuclease P1.

**Conditions for competition assays.** The following rationale was used in setting the conditions for competition assays. If the endonuclease and ligase reactions could be coupled through the assembly of a functional splicing complex, yeast ligase should have preferential access to halves being formed by endonuclease even in the presence of excess T4 ligase. If, on the other hand, the halves are released into solution prior to joining, the yeast and T4 enzymes should have equal access to halves, and the resulting product ratio should be a function of the ratio of the two enzyme concentrations. The conditions appropriate for the competition assay are as follows. First, the T4 enzymes should be present in excess, whereas the yeast ligase should be in a limiting amount. This is to enhance the probability that any halves released by endonuclease will be joined by the T4 enzymes. Additionally, the endonuclease should be present in a limiting amount. This is to avoid the possibility of tying up limiting amounts of yeast ligase in nonproductive complexes with excess endonuclease.

An appropriate limiting amount of endonuclease was determined by titrating enzyme levels under standard reaction conditions. Titration reactions contained a constant amount of unlabeled pre-tRNA$^{Tyr}$ ($1 \times 10^{-8}$ g) plus a small amount ($0.5 \times 10^{-9}$ g) of pre-tRNA$^{Tyr}$ SUP6 labeled by in vitro transcription as tracer. The reaction products were analyzed by gel electrophoresis and quantitated by measuring the radioactivity in gel slices. The results of these assays (data not shown) were used to select a concentration of endonuclease (1.3 U/ml) for all subsequent reactions. This level of endonuclease gives substantial (~70%) conversion of pre-tRNA to halves but remains within a range in which the measured activity is proportional to the amount of enzyme added.

Appropriate levels of yeast ligase and T4 ligase plus kinase were determined in a coupled splicing assay under the same conditions as were used for endonuclease assays. Reactions contained constant amounts of endonuclease and labeled plus unlabeled pre-tRNA$^{Tyr}$ as described above with the addition of increasing amounts of yeast ligase or T4 ligase plus kinase. The results are shown in Fig. 3. The yeast ligase concentrations chosen for subsequent competition assays (4 to 21 U/ml) were within a range in which the extent of joining (15 to 50%) was proportional to the amount of enzyme added. The T4 enzyme concentrations chosen for competition assays (100 and 200 U/ml each) were within a range in which no significant increase in joining was obtained with increased enzyme concentrations.

Note that in all incubations with T4 enzymes, equal units of T4 ligase and T4 kinase were added. This choice is intended to provide equal activities of these two enzymes

and not necessarily equimolar amounts. The choice of equal units of activity was based on the similarity of the methods used to measure the kinase and ligase activities (incorporation of label into acid-insoluble form with bulk nucleic acid as acceptor in a 30-min incubation at 37°C) and the equivalent form used in expressing units of activity (nanomoles of acid-insoluble $^{32}P$ per 30 min). The identity of the limiting T4 component was not determined in this titration series.

The activities of both the yeast and T4 enzymes were also measured with an uncoupled splicing reaction. In the uncoupling splicing assay tRNA halves, formed by preincubation with endonuclease and isolated together with residual pre-tRNA by phenol extraction and ethanol precipitation, are used as substrate in ligase reactions. Since endonuclease is not present, the yeast and T4 enzymes are expected to have equal access to the halves in solution, and the uncoupled assay could provide a control for the competition assays. To determine whether the coupled and uncoupled assays provided equivalent measures of joining activity, the results of coupled and uncoupled assays of yeast ligase (Fig. 3A) and T4 ligase plus kinase (Fig. 3B) were compared. Of concern was whether the effective substrate concentrations in identical coupled and uncoupled assays might be expected to differ. In the uncoupled assay, all of the substrate is available at the start of the reaction. In the coupled assay, halves are available only as they are formed by endonuclease during the incubation. If the rate of endonuclease cleavage is limiting, the effective concentration of halves might be lower in the coupled assay, particularly at early time points. Potentially, this could be reflected in a reduced rate of joining. The result would be a reduced amount of tRNA product in the coupled assay at limiting yeast ligase or T4 enzyme concentrations. The coupled and uncoupled assays give essentially identical measures of ligase activity throughout the concentrations of yeast and T4 enzymes tested (Fig. 3). Thus the endonuclease reaction is not limiting in the coupled assay. Apparently, any relative reduction in the effective concentration of halves is sufficiently small or transient so as to preclude any reduction in the extent of joining even at limiting enzyme concentrations. These results demonstrate that the two methods provide an equivalent measure of ligase activity and that the uncoupled assay can provide a control for competition experiments.

**Single-substrate competition assays.** Competition between yeast ligase and T4 ligase plus kinase for joining of tRNA halves was measured with the reaction conditions and the sequential nuclease digestion method described above. The competition assays were carried out with both the uncoupled splicing reaction (in which halves are formed by preincubation with endonuclease) and the coupled reaction (in which halves are formed by endonuclease during the ligase incubation). Assays contained constant amounts of unlabeled pre-tRNA$^{Tyr}$ ($10^{-8}$g), endonuclease (1.3 U/ml), T4 ligase plus kinase (100 or 200 U/ml each), and [$\gamma$-$^{32}P$]ATP and increasing amounts of yeast ligase (0 to 21 U/ml). The reaction products were purified by electrophoresis and subjected to sequential digestion with RNase T2 followed by nuclease P1.

The nuclease digestion products from coupled and uncoupled assays containing 200 U/ml of each of the T4 enzymes are shown in Fig. 4. With no added yeast ligase, the nuclease digestion products contained only labeled $P_i$ as expected for the T4 ligase product alone. With increasing yeast ligase, both labeled junction dinucleotide and $P_i$ are found in the digestion products, indicating that both the yeast and T4 enzymes are contributing to labeled tRNA products. However, the ratio of junction dinucleotide to $P_i$ appeared to be different for the uncoupled and coupled assay methods (compare lanes 4 and 5 in Fig. 4A and B).

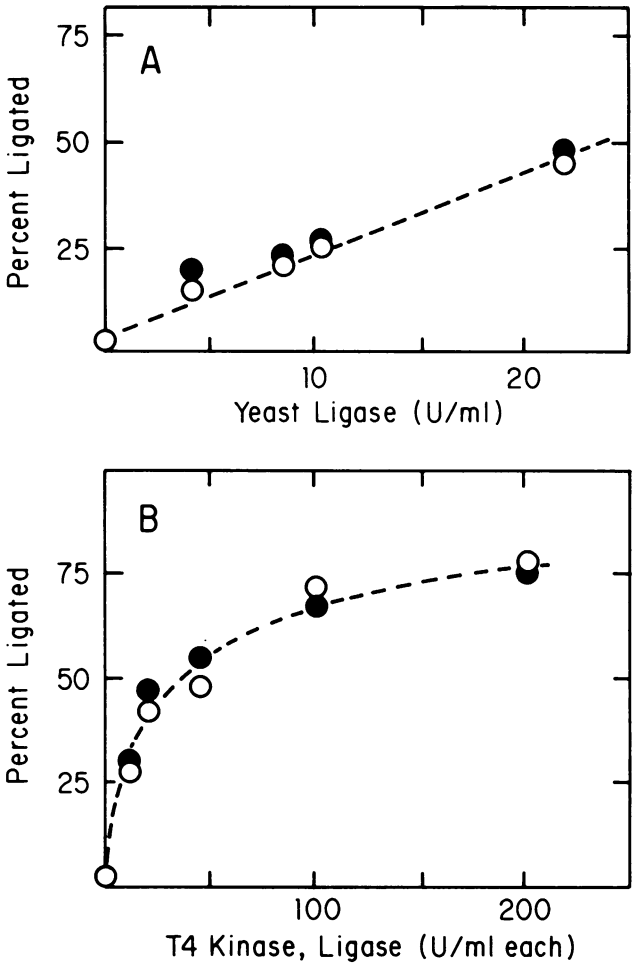


FIG. 3. Ligase titrations. The yield of tRNA products in reactions containing increasing concentrations of yeast ligase (A) or T4 ligase plus kinase (B) are shown. Coupled splicing reactions (○) contained constant amounts of endonuclease (1.3 U/ml) and unlabeled pre-tRNA$^{Tyr}$ ($1 \times 10^{-8}$ g) plus labeled pre-tRNA$^{Tyr}$ SUP6 ($0.5 \times 10^{-9}$ g). Uncoupled reactions (●) contained equivalent amounts of labeled and unlabeled tRNA halves produced by endonuclease preincubation as described in Materials and Methods. After incubation at 30°C for 10 min, halves and tRNA products were isolated by electrophoresis and quantitated by measuring radioactivity in gel slices. The percentage of ligated products was defined as: (counts per minute of tRNA)/(counts per minute of tRNA + halves) × 100.

The ratio of yeast to T4 ligase products was measured as the ratio of radioactivity in junction dinucleotide to that in $P_i$ after excising individual spots from thin-layer plates. The results obtained with both the coupled and uncoupled assays at two different T4 enzyme concentrations are shown in Fig. 5. The ratio of yeast ligase to T4 ligase plus kinase activities is plotted in these graphs for reference. The yeast-to-T4 activity ratio was calculated by converting the levels of each into equivalent arbitrary units defined as the amount required to join 50% of the halves in a standard reaction as described above. With the data shown in Fig. 3, one arbitrary unit corresponds to 0.35 conventional units each of T4

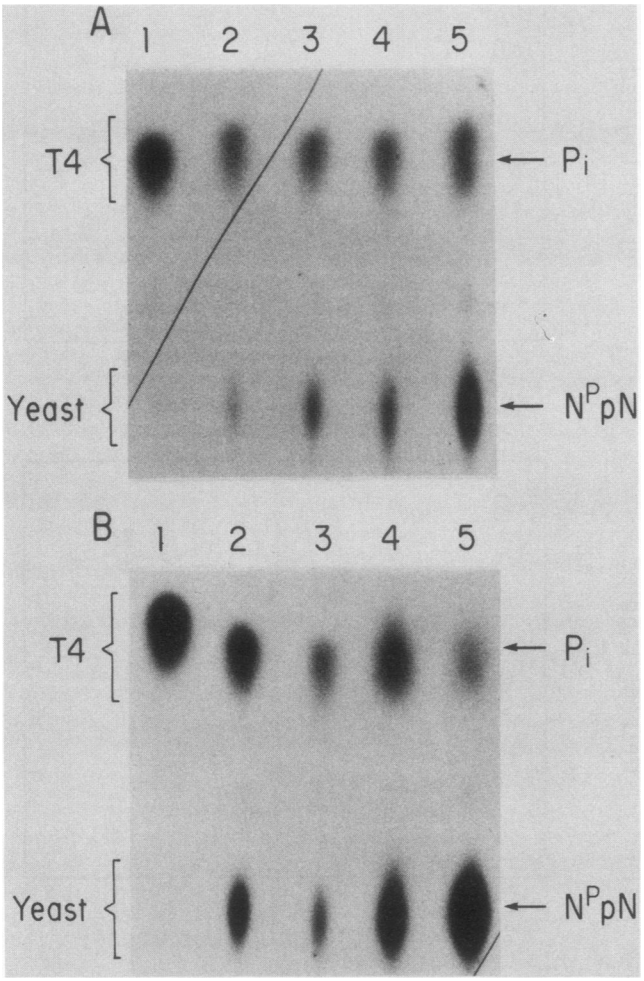


FIG. 4. Nuclease digestion products. (A) Competition between yeast ligase and T4 ligase plus kinase was measured in uncoupled assays containing isolated tRNA halves and [$\gamma$-$^{32}$P]ATP as described in Materials and Methods and 200 U each of T4 ligase and T4 kinase per ml. Coupled assays (B) were carried out under identical conditions except that unlabeled pre-tRNA and endonuclease were added in place of isolated halves. The concentration of yeast ligase (in U/ml) in each sample was: 1, 0; 2, 4.2; 3, 8.3; 4, 10.4; and 5, 20.8. After incubation for 10 min at 30°C, tRNA products were purified by electrophoresis and digested with RNase T2, followed by nuclease P1. The results of thin-layer chromatography of nuclease digestion products are shown. The positions of $P_i$ and nuclease-resistant junction dinucleotide ($N^pN$) corresponding to the T4 and yeast ligase products, respectively, are indicated at the edges.

ligase and T4 kinase and to 0.23 conventional units of yeast ligase (conventional units are defined in Materials and Methods).

In the uncoupled assay (joining of preformed halves), the yeast and T4 enzymes are expected to have equal access to substrate, and the product ratio should be a function of the yeast to T4 activity ratio (detailed in the Discussion below). The effect of increasing yeast ligase on the observed product ratio in uncoupled assays at two different T4 enzyme concentrations is shown by the solid circles in Fig. 5A and B. In each case, the observed increase in the product ratio corresponded closely to the calculated increase in activity ratios. The uncoupled assays in the two panels differ only in the concentration of T4 enzymes, 100 U/ml in Fig. 5A and 200 U/ml in Fig. 5B. The slopes of the two product ratio curves differ approximately in proportion to the difference in T4 enzyme activities. These results are consistent with simple competition between the yeast and T4 enzymes for joining of a common substrate. Thus the assay method in combination with the conditions of enzyme and substrate concentrations selected can provide an accurate measure of competition between the yeast and T4 enzymes.

Two distinct types of results would be predicted in the coupled splicing assay for independent versus concerted steps in splicing. First, if yeast endonuclease and ligase act independently, the yeast ligase and the T4 enzymes should

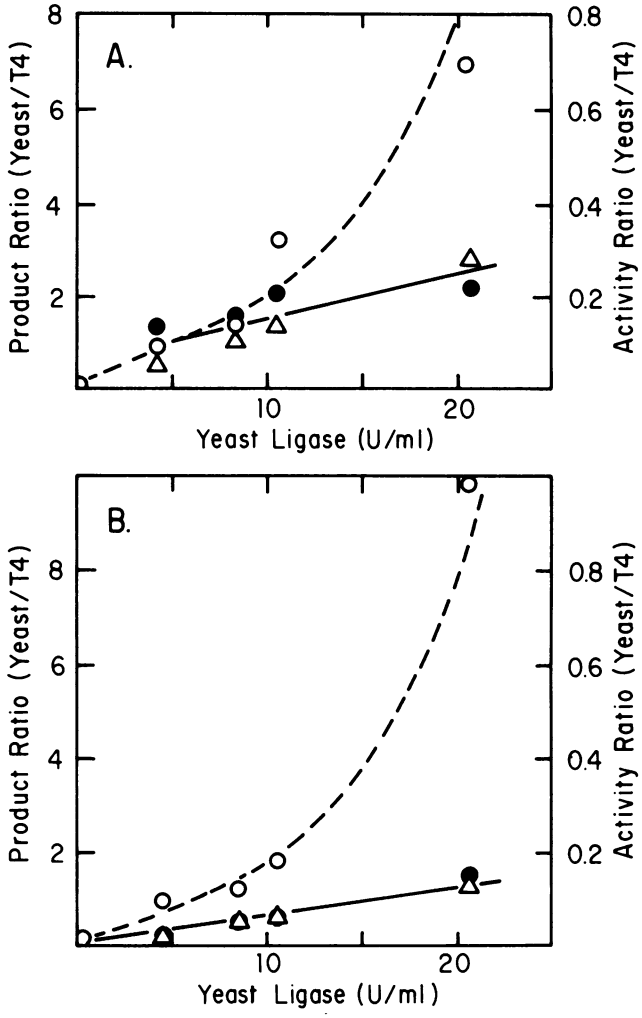


FIG. 5. Single-substrate competition assays. The product ratios (yeast ligase to T4 ligase plus kinase) were measured in competition assays containing [$\gamma$-$^{32}$P]ATP and isolated tRNA halves (○) or pre-tRNA$^{Tyr}$ plus endonuclease (●) as described in Materials and Methods. Increasing concentrations of yeast ligase (0 to 21 U/ml) were added to reactions containing constant amounts of T4 ligase plus kinase (A, 100 U/ml; B, 200 U/ml. The ratio of yeast to T4 enzyme activities (△), calculated by converting each to equivalent tRNA joining units as described in the text, is shown in each panel for reference. After incubation for 10 min at 30°C, tRNA products were isolated by electrophoresis and digested with RNase T2 followed by nuclease P1, and the digestion products were resolved by thin-layer chromatography. The yeast-to-T4 product ratio was then measured as the radioactivity in junction dinucleotide to that in $P_i$ after excising individual spots from thin-layer plates.

compete for joining of halves. In this instance the product ratios should be similar in the coupled and uncoupled assays, and both should reflect the yeast-to-T4 activity ratios. Second, if the endonuclease and ligase steps are concerted through the formation of a splicing complex, yeast ligase should have preferential access to halves being formed by endonuclease. In this case, the yeast-to-T4 product ratios in coupled assays should exceed those in equivalent uncoupled assays. Additionally, the product ratio should increase as a function of the ratio of yeast ligase to yeast endonuclease and should be independent of T4 enzyme concentrations (so long as the T4 enzymes are present in excess). In other words, the T4 enzymes will have access only to those halves produced in excess of the amount of yeast ligase available.

The results of the coupled competition assays at two different T4 enzyme concentrations are also shown in Fig. 5. The observed yeast-to-T4 product ratios with increasing yeast ligase exceed the ratios measured with the uncoupled assay. The ratios obtained with the two assays differ by a maximum of sixfold at a yeast-to-T4-ligase activity ratio of 0.16. Additionally, comparison of the product ratio curves at two different T4 enzyme concentrations (compare Fig. 5A and B) reveals that the measured product ratios do not vary in proportion to the T4 enzyme concentrations. These results are consistent with the second possibility, that the steps in the splicing reaction are concerted and not independent.

**Two-substrate competition assays.** Competition assays with two substrates were carried out as an additional means for demonstrating preferential access of yeast ligase to endonuclease products and to examine whether this is a general property of yeast ligase or unique to a particular pre-tRNA. Additionally, assays in which one substrate is added as pre-tRNA and the other as preformed halves can be used to eliminate the possibility that any difference in the observed product ratios is due to a slight difference in reaction conditions.

The substrates in these competition assays were unlabeled pre-tRNA$^{Trp}$ and pre-tRNA$^{Ile}$ ($0.5 \times 10^{-8}$ g each). Reactions contained constant amounts of endonuclease and [$\gamma$-$^{32}$P]ATP as above with 100 U of T4 ligase and kinase each per ml and 21 U of yeast ligase per ml. The reaction products were purified by two-dimensional gel electrophoresis as described in Materials and Methods. Separation of the tRNA products in this gel system may be due primarily to differences in conformation. The tRNA$^{Trp}$ and tRNA$^{Ile}$ products (which differ in length by one nucleotide) ran closely together but could be resolved. However, the yeast ligase and T4 enzyme products corresponding to a single tRNA species (which differ only by the presence of an additional phosphate in the yeast product) were not resolved (data not shown).

TABLE 1. Product ratios in two-substrate competition assays

| Product | Ratio[a] when substrates added as follows[b]: | | | |
|---|---|---|---|---|
| | Ile as pre-tRNA plus: | | Ile as halves plus: | |
| | Trp as pre-tRNA | Trp as halves | Trp as pre-tRNA | Trp as halves |
| tRNA$^{Ile}$ | 6.5 | 4.8 | 2.2 | 2.0 |
| tRNA$^{Trp}$ | 8.7 | 2.5 | 5.9 | 2.4 |

[a] Product ratios are expressed as the ratio of yeast ligase to T4 ligase plus kinase products.

[b] Substrates were added as pre-tRNA or as halves formed by preincubation with endonuclease and isolated together with residual pre-tRNA by phenol extraction.

The ratio of yeast ligase to T4 ligase products in each of the gel-purified tRNAs was measured by sequential nuclease digestion as before. The results of the two-substrate assays are summarized in Table 1. The measured yeast-to-T4 product ratio was approximately 3.5-fold higher when both were added as pre-tRNA rather than as preformed halves. In assays in which one substrate was added as pre-tRNA and the other as preformed halves, the yeast-to-T4 product ratio for the tRNA derived from pre-tRNA substrate was 2- to 2.5-fold higher than that for the tRNA derived from preformed halves.

These results demonstrate that preferential formation of the yeast ligase product in the coupled splicing reaction cannot be attributed to some aspect of the reaction conditions and is not unique to a particular pre-tRNA substrate. The results of both the single and two-substrate competition assays provide evidence for concerted steps in tRNA splicing through the assembly of a tRNA splicing complex.

## DISCUSSION

The yeast endonuclease and ligase involved in tRNA splicing in vitro had been shown previously to have distinct physical properties (11, 24). This observation suggested that the two steps in splicing, excision of the intervening sequence and joining of the tRNA halves, might normally occur independently. Competition between yeast ligase and T4 ligase plus kinase was used to differentiate between concerted and independent splicing mechanisms.

The results of the competition assays can be compared with the kinetic behavior expected for concerted versus independent splicing mechanisms. The following paragraphs describe models for equal versus preferential access to halves and compare the expected and observed results.

For a mechanism in which the splicing steps are independent, yeast ligase and T4 ligase plus kinase are expected to have equal access to halves being formed by endonuclease. In this instance, the kinetic behavior can be predicted as the ratio of the yeast and T4 reaction velocities. The Michaelis-Menten equation, commonly used to estimate reaction velocities, is subject to considerable error under conditions of high enzyme concentration ($E_t$) and low substrate concentration ($S$) relative to the Michaelis constant ($K_m$) (5). The conditions for competition assays (described in Materials and Methods) include yeast ligase and T4 kinase concentrations which are close to that of the substrate. The concentration of T4 ligase is considerably greater than that of the substrate. Thus an approximation of the complete rate equation derived by Cha and Cha (6) which is less prone to error in this range of enzyme and substrate concentrations should be considered. Using the Cha equation, the initial ratio of yeast ligase-substrate complex ($ES^Y$) to T4 enzyme-substrate complex ($ES^{T4}$) for a simple competition reaction is given by the following relation:

$$\frac{ES^Y}{ES^{T4}} = \left(\frac{E_t^Y}{E_t^{T4}}\right)\left(\frac{K_m^{T4} + S + E_t^{T4}}{K_m^Y + S + E_t^Y}\right)$$

The use of this equation in predicting the behavior in the competition assay is based on the following. The enzyme-substrate complex described by this equation would correspond to those in the first reactions in the yeast and T4 ligation pathways (Fig. 1) in which the products are unique or are committed to that pathway. In both pathways, this is probably equivalent to the first step. For the yeast ligation

pathway, the reactions may be concerted through the association of all of the corresponding activities with a single polypeptide chain (11; Phizicky et al., manuscript in preparation). In the T4 pathway, phosphorylation of the 5′ end and removal of the phosphate from the 3′ end may be effectively concerted since the ends are in close proximity in the tRNA halves and the corresponding activities are associated with a single polypeptide chain (29; Weber, Ph.D. thesis). The products of the T4 kinase phosphatase are not expected to serve as substrates for the yeast ligase (by analogy to wheat germ ligase [28]). Thus, initial binding to either yeast ligase or T4 kinase phosphatase may commit a substrate molecule to the corresponding ligation pathway.

With the assumptions described in the preceding paragraph, the above equation can be used to predict the product ratios expected for simple competition in which the yeast and T4 enzymes have equal access to halves. Under the conditions used in the competition assays, a simplified form of this equation can be derived as follows. At equal estimated concentrations of yeast ligase and T4 kinase (21 U of ligase and 100 U of kinase per ml based on the values above) the observed yeast-to-T4 product ratio was 1.5 in an uncoupled assay with preformed halves as substrate (data from Fig. 5). This result suggests that the values of $K_m$ for the yeast and T4 enzymes are similar (from primary equation above, $K_m{}^Y = 0.7\ K_m{}^{T4}$). Values for the $K_m$ of the kinase, phosphatase, and phosphodiesterase activities of T4 kinase have been estimated for a number of substrates (29; Weber, Ph.D. thesis). The lowest of the reported values was 3 μM for phosphorylation of the 5′ ends of short DNA chains (29). Assuming the $K_m$ for tRNA halves as substrate is not substantially less than these reported values, the concentrations of T4 kinase (and therefore of yeast ligase) and of the substrate in competition assays were much less than the $K_m$ ($E + S << K_m$). Under these conditions, the product ratio curve can be approximated by a straight line corresponding to the simplified form of the primary equation shown in Fig. 6. Thus for equal access of the yeast and T4 enzymes to halves in the competition assays, the product ratio is expected to be proportional to the yeast-to-T4 enzyme ratio times a constant, $C$, where $C = K_m{}^{T4}/K_m{}^Y$.

The experimental results obtained with coupled and uncoupled assays (Fig. 5) can be compared with the curve predicted by the simple competition model (Fig. 6). The results of the uncoupled assays are consistent with the behavior predicted for simple competition for a common substrate. The measured product ratios in coupled assays are not consistent with this simple competition model.

For concerted steps in splicing, yeast ligase would have preferential (or exclusive) access to halves being formed by endonuclease. The T4 enzymes, though present in amounts sufficient to join all of the substrate, would have access only to those halves formed in excess of the available yeast ligase ($P^{T4} = S_t - P^Y$). In this instance, the yeast-to-T4 product ratio ($R$) would be a function of the total amount of substrate ($S_t$) and the amount of yeast product formed [$R = P^Y/(S_t - P^Y)$]. The predicted product ratio curve for competition assays is shown in Fig. 6. This model predicts a product ratio curve in which the ratio goes to infinity as sufficient yeast ligase is added to join all of the halves produced in the incubation ($P^Y = S_t$). Under the conditions used in the competition assay, the product ratio would be independent of T4 enzyme concentrations. The product ratios measured in the coupled competition assays shown in Fig. 5 are consistent with the behavior predicted by the preferential access model. These results demonstrate that the excision and joining steps in tRNA splicing in vitro are concerted and provide evidence for the assembly of a functional tRNA splicing complex.

Two distinct models can be proposed for the mechanism of complex assembly. These are (i) the association of pre-tRNA with a preformed complex and (ii) the active participation of the substrate in complex assembly. In the first model, a direct interaction between endonuclease and ligase might be sufficient for complex formation. Alternatively, additional proteins present in the partially purified yeast fractions might mediate complex assembly or serve as necessary components of some larger complex. In the second model, the pre-tRNA itself would be required to promote the assembly of a protein-nucleic acid complex which might include endonuclease or ligase. The complex that is formed need not be stable and might involve the transient association of the splicing enzymes with some core particle. It should be possible to differentiate between each of these possibilities by carrying out competition assays with sequential addition of two unique substrates, by using more highly purified splicing fractions, and by probing the interaction of particular protein and nucleic acid species with appropriate cross-linking reagents.

The formation of a splicing complex is not unique to tRNA splicing. The splicing of mRNA precursors has been shown to proceed with the assembly of a 40S splicing complex in soluble whole cell extracts of yeast (2). One of the functions attributed to the mRNA splicing complex, holding cognate exons together to ensure proper joining (2), is obviated for tRNA splicing by the base-paired structure of the tRNA halves. Potentially, both the mRNA and tRNA splicing

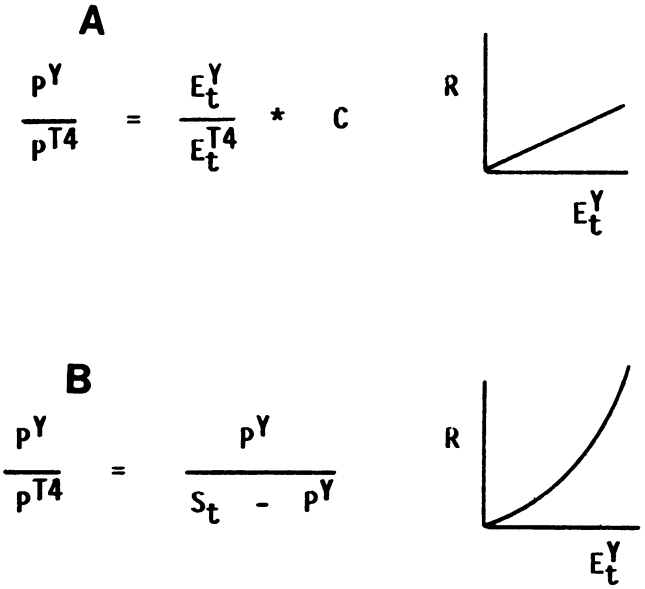


FIG. 6. Predicted kinetic behavior. A comparison of the relationship between the product ratio and the concentration of yeast ligase expected for alternate competition reaction models is shown. These models show equal access of the yeast and T4 enzymes to substrate (A) and preferential access of yeast ligase to tRNA halves (B). The equations for calculating the ratio (R) of yeast ($P^Y$) to T4 ($P^{T4}$) products and the nature of the constant C are described in the text. The product ratio curves shown are for competition assays in which the amount of substrate ($S_t$) and the T4 enzyme concentrations ($E_t{}^{T4}$) are held constant while the concentration of yeast ligase ($E_t{}^Y$) is varied.

complexes serve similar functions in ensuring that the complete splicing process proceeds efficiently.

The formation of a tRNA splicing complex in vitro has important implications for the organization of the splicing components and of the gene expression pathway in vivo. A relationship between tRNA splicing and nuclear transport has been suggested previously on the basis of two types of observations. The first is the processing of transcripts of tRNA genes microinjected into *Xenopus* oocytes. Analysis of the structure of processing intermediates revealed that splicing follows 5′ and 3′ end trimming (19) and the addition of most of the base modifications found in the mature tRNA (21). A nuclear location for splicing was suggested by the finding that reinjection of end-mature, intervening sequence-containing pre-tRNA into the nucleus resulted in splicing, whereas cytoplasmic injection did not (19). Together these results suggest that tRNA splicing in *Xenopus* oocytes occurs late in the processing pathway, at or about the same time as nuclear transport. The second set of observations concerns the phenotype of the *rna1-1* mutation in *S. cerevisiae*. Among the pleiotropic effects of this mutation (13, 15) is the accumulation of intervening sequence-containing pre-tRNAs (14, 17, 18, 22, 25). It has been proposed that the *rna1-1* mutation results in a block in RNA transport (13, 15, 28) perhaps due to a general defect in nuclear structure (1). The apparent correlation between tRNA splicing and transport defects in this mutant suggests that the two processes may be related. The copurification of yeast endonuclease with a membrane fraction (24) and the results presented here raise the possibility that, in vivo, the splicing enzymes form a complex associated with the nuclear envelope. An arrangement of this sort would include the potential for direct coupling of the splicing and transport processes. Further support for this possibility will require determining the intracellular localization of the yeast splicing enzymes and defining the structure of the complex assembled in vitro. Potentially, exploring the use of artificial phospholipid bilayers in place of the detergent present in the standard splicing assay could be used to pursue this possibility.

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