Preferential binding of yeast tRNA ligase to pre-tRNA substrates

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Received December 3, 1990; Revised and Accepted February 8, 1991

ABSTRACT

Joining of tRNA halves during splicing in extracts of *Saccharomyces cerevisiae* requires each of the three enzymatic activities associated with the tRNA ligase polypeptide. Joining is most efficient for tRNA as opposed to oligonucleotide substrates and is sensitive to single base changes at a distance from splice sites suggesting considerable specificity. To examine the basis for this specificity, binding of ligase to labeled RNA substrates was measured by native gel electrophoresis. Ligase bound tRNA halves with an association constant 1600-fold greater than that for a nonspecific RNA. Comparison of binding of a series of tRNA processing intermediates revealed that tRNA-structure, particularly in the region around the splice sites, contributes to specific binding. Finally, the ligase was shown to form multiple, discrete complexes with tRNA substrates. The basis for recognition by ligase and its role in a tRNA processing pathway are discussed.

INTRODUCTION

The mechanism of tRNA splicing in extracts of *Saccharomyces cerevisiae* has been extensively characterized (see ref. 1 for a review). The reaction proceeeds in two steps (2). The first is cleavage at the 5′ and 3′ splice junctions by a site-specific endonuclease to produce the linear intervening sequence (IVS) and paired tRNA half molecules (3). The second is joining of the halves by an ATP-dependent tRNA ligase. The properties of this latter enzyme, RNA ligase, and its interaction with RNA substrates are the subjects of this research.

In yeast, only a subset of the tRNA genes contain intervening sequences (reviewed in ref. 4). The pre-tRNA transcripts of these genes share several features in common. All contain small IVSs, ranging from 14 to 60 nucleotides. These IVSs interrupt the coding sequences in the same relative position, one nucleotide 3′ to the anticodon. Both lowest free energy calculations (5) and solution structure probing (6, 7) suggest a common general structure among pre-tRNAs organized into two domains. These are an exon domain with many of the features found among mature tRNAs and an IVS-containing domain forming an extension of the anticodon stem. Accurate cleavage at splice sites by yeast endonuclease requires a high degree of specificity. This specificity is achieved, in part, by the recognition of elements of tRNA-like structure conserved within exon domains of substrates (8, 9, 10, 11, 12). In contrast to the cleavage reaction, accuracy in the joining step is ensured by the structure of substrates—the product of cleavage by endonuclease is equivalent to a tRNA with a nick in the anticodon loop. Thus phage T4 and wheat germ RNA ligases, both non-specific enzymes capable of joining a wide variety of oligonucleotide substrates, complement yeast endonuclease *in vitro* to produce the correct spliced product (13, 14). This result demonstrates that accurate splicing includes no intrinsic requirement for specificity on the part of an RNA ligase. Despite this, yeast ligase shows a high degree of specificity. Joining of tRNA halves by the yeast enzyme is 10^4-fold more efficient than joining of oligonucleotide substrates (15). Additionally, single base changes at sites distant from splice junctions have been shown to affect joining by ligase (10). These results suggest yeast ligase recognizes specific structural features which are conserved among its natural substrates.

Specific recognition of pre-tRNA substrates by yeast ligase, while not required for accurate splicing, might have other important functions. Potentially, ligase might perform an editing function by preventing the joining of inaccurate or partially-degraded cleavage products as well as to preclude joining of unrelated RNA species. Additionally, specific binding of substrates by ligase might function to ensure efficient splicing. This could be accomplished through the stepwise assembly of a splicing complex in which binding of ligase to pre-tRNA substrates precedes association with endonuclease. This latter possibility is suggested by two types of observations. First, addition of ligase to endonuclease incubations stimulates cleavage in an ATP-independent manner (data not shown). The magnitude of this effect shows a strong dependence on the ratio of ligase to both endonuclease and pre-tRNA substrate suggesting the formation of a ternary or higher order complex may be involved. Second, the cleavage and joining steps in reconstituted splicing reactions are effectively concerted (10). This is consistent with the formation of a functional complex of ligase and endonuclease in which the substrate is available to both enzymes.

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To examine each of these possibilities further, it was first necessary to determine whether ligase was capable of specific binding to pre-tRNA substrates. Native gel electrophoresis was used to demonstrate that ligase binds to tRNA halves with an association constant 1600-fold greater than that for a non-specific polynucleotide ligand. Analysis of a series of tRNA processing intermediates suggested that binding by ligase involved the recognition of specific elements of tRNA-like structure.

EXPERIMENTAL PROCEDURES

Materials

Phage T4 polynucleotide kinase was obtained from USB and calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim. Poly(rA) RNA was from Pharmacia. *Saccharomyces cerevisae* endonuclease (23 U/ml) was equivalent to fraction IV of Peebles *et al.* (16). Both [α-^{32}P] UTP (3000 Ci/mmole) and [γ-^{32}P] ATP (3000 Ci/mmole) were obtained from Amersham.

Yeast tRNA Ligase

Saccharomyces cerevisiae ligase used in the binding assays was equivalent to fraction IV of Greer *et al.* (17) except that it was purified from yeast cells containing the ligase gene on a high copy number plasmid, called pBM150-RLGX (15) and was kindly provided by M. Clark. The total protein concentration as determined by the method of Bradford (18) was 0.044 mg/ml with ligase constituting 26% of the total protein based on densitometric scanning of silver stained SDS-PAGE gels (19, 20). From these estimates the total concentration of ligase in this fraction was 1.22×10^{-7} M. The tRNA joining activity as defined by Greer *et al.* (17) and utilizing pre-tRNA^{SUP6} (21) as a substrate was 434 U/mg. Using the value of 6,000 U/mg for ligase purified to apparent homogeneity from an *E. coli* expression system (manuscript in preparation) yields a concentration of enzymatically active ligase of 1.9×10^{-8} M. Based on pre-tRNA binding activity the concentration of ligase was determined to be 1.8×10^{-8} M (see results).

Plasmids

pUC8-SUP53 contains the SUP53 gene (22) inserted into the EcoRI/HindIII site of pUC8. The SUP53 gene encodes an amber suppressor derivative of tRNA$_3^{Leu}$ (22, 23). Plasmid pUC8-SUP53ΔIVS contains a derivative of the SUP53 gene from which the intron has been precisely deleted (24).

Preparation of RNA Substrates

Labeled tRNA substrates were prepared by *in vitro* transcription using plasmid pUC8-SUP53 or plasmid pUC8-SUP53ΔIVS as template. Transcription reactions were carried out essentially as described by Engelke *et al.* (25) and contained [α-^{32}P]UTP and a yeast nuclear extract fraction. This extract contained RNA polymerase III and the tRNA processing activities required for removal of 5′ leader and 3′ trailer segments, introduction of certain base modifications and addition of the 3′ CCA sequence (24, 25). Incubations carried out at 30°C resulted in the production of IVS-containing, end-mature pre-tRNA (designated *pre-tRNA*) as the predominant product. Incubations at 12°C resulted in pre-tRNA retaining 5′ leader and 3′ trailer segments (designated *primary transcript*). Mature sequence tRNA [designated *tRNA*(ΔIVS)] was produced by transcriptions using plasmid pUC8-SUP53ΔIVS. Following transcription incubations, reaction products were purified by electrophoresis into 12% polyacrylamide, 8 M urea gels and recovered as previously described (17).

Paired tRNA halves were prepared by excision of the IVS from labeled pre-tRNA substrates with yeast endonuclease. Preparative reactions (100 μl) containing yeast endonuclease (0.7 U/ml) and labeled pre-tRNA (44 fmole) were incubated for 20 minutes at 30°C using the buffer conditions described previously (14). Samples were then extracted twice with phenol:chloroform (1:1, v/v), once with chloroform:isoamyl alcohol (24:1, v/v), and twice with ether. The products were recovered by ethanol precipitation and resuspended in H_2O. An aliquot of each preparation of tRNA halves was removed and assayed for joining by yeast ligase as described previously (17). Using this procedure we found that 75−80% of the input pre-tRNA was cleaved by endonuclease to produce tRNA half molecules and 75−90% of these halves could be joined by yeast ligase. Spliced tRNA (designated *spl-tRNA*) was generated by preparative splicing reactions with labeled pre-tRNA as described above for preparation of tRNA halves except that 1 mM ATP and yeast RNA ligase (0.02 U/ml) were included in the reaction (17). RNA was then recovered by precipitation with ethanol and spliced products were purified by gel electrophoresis and recovered as described previously (17).

The specific activities of the tRNA substrates were as follows: primary transcript, 8.8×10^3 dpm/fmole; pre-tRNA, 6.8×10^3 dpm/fmole; and tRNA(ΔIVS), spl-tRNA, and the tRNA half molecules, 5.7×10^3 dpm/fmole. Where indicated, low specific activity tRNA substrates were produced by adjusting UTP concentrations in transcription reactions so as to yield products with specific activities 1000-fold lower than those listed above.

Labeled poly(rA) RNA was prepared as follows. Samples were treated with calf intestinal alkaline phosphatase prior to labeling with phage T4 polynucleotide kinase. Labelling reactions containing [γ-^{32}P] ATP and polynucleotide kinase were carried out as specified by the manufacturer. The radiolabeled poly(rA) RNA was resolved on polyacrylamide gels and RNA approximately equivalent to pre-tRNA in size (i.e. 110−120 nucleotides) was excised and recovered as described previously (17). The specific activity of labeled poly(rA) fractions ranged from $0.1-3.0\times10^3$ dpm/fmole.

Gel Shift Assay for Binding

Typical binding reactions (10 μl) contained 6.8 fmoles of labeled tRNA substrate or labeled, size fractionated, poly(rA) RNA and buffer containing 28 mM Hepes (pH 7.5), 2.5 mM spermidine, 0.5 mM dithiothreitol, 5 mM $MgCl_2$, 0.2 mM EDTA, 10% (v/v) glycerol and 40 mM NaCl unless otherwise indicated. In addition, all reactions with tRNA substrates contained an excess (1 mg/ml) of unfractionated, unlabeled poly(rA) RNA. Binding reactions were initiated by addition of ligase to the concentrations indicated followed by gentle mixing. After incubation for 5−10 minutes on ice the reactions were loaded directly on vertical 4% polyacrylamide [acrylamide:bisacrylamide, 39:1 (wt/wt)] gels (20×20×0.08 cm) containing 50 mM Tris-borate (pH 8.3), 1 mM EDTA and 5% (v/v) glycerol (26). Electrophoresis was carried out at 4°C, (250 volts, 10 milliamps) for 4 hours. Gels were dried onto 3MM Whatmann paper and subjected to autoradiography. Individual bands were excised from gels and radioactivity was measured by Cerenkov counting.

Ligase Immunoblots

Preparative binding reactions (50 μl) contained varying amounts of tRNA substrates labeled to a low specific activity (13.6

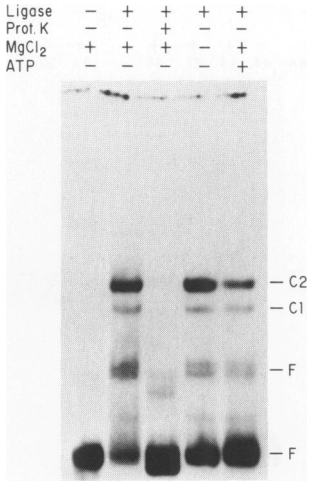

Figure 1: tRNA ligase binding to pre-tRNA. Pre-$tRNA_3^{Leu}$ (3.3×10^4 dpm) was incubated for 10 min at 0–4°C in the absence or presence of tRNA ligase (2.3×10^{-9} M), proteinase k (0.2 mg/ml), ATP (2 mM), or $MgCl_2$ (5 mM) as indicated. Samples were resolved by native gel electrophoresis and visualized by autoradiography as described in Experimental Procedures. The identification of individual species is indicated at the right edge and is described in the text. C1 and C2 refer to bound pre-tRNA complexes 1 and 2; F to free pre-tRNA.

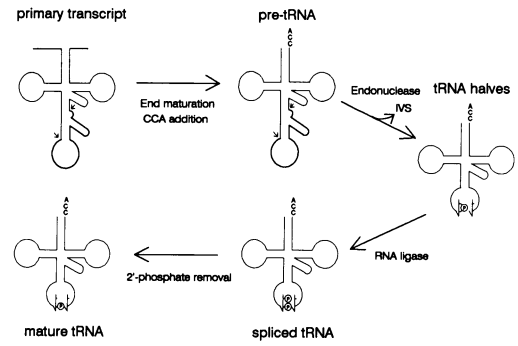

Figure 2: Pre-tRNA processing intermediates. A schematic representation is shown of potential tRNA processing intermediates based on the maturation pathway proposed by Melton *et al.* (32). First, transcription in the nucleus yields the primary transcript. The 5′ leader and 3′ trailer segments are removed and the CCA sequence is added to the 3′ end to give the intermediate designated pre-tRNA. The intervening sequence (IVS) is excised by an endonuclease to produce tRNA halves which are subsequently ligated by tRNA ligase yielding the spliced tRNA. In yeast, generation of the mature tRNA requires the removal of the 2′-phosphate at the site of joining by a dephosphorylating activity (33). Thin lines denote regions of the mature domain of the pre-tRNA and thick lines represent regions removed by RNA processing. Arrows indicate 5′ and 3′ splice sites. The structure of the splice site is enlarged in the figure with vertical lines representing ribose and the phosphates are circled.

dpm/fmol) and 1.7×10^5 dpm of high specific activity (6.8×10^3 dpm/fmol) tRNA as indicated. Bound complexes and free RNA were separated by native gel electrophoresis as described above and then electrotransfered (30 volts, 25 milliamps) in 50 mM Tris-borate (pH 8.3), 1 mM EDTA and 5% (v/v) glycerol at 4°C onto nitrocellulose membranes backed with nylon membrane. Under these conditions both the tRNA and the ligase polypeptide were transfered with high efficiency to nitrocellulose placed on the cathode side of the gel. Regions of the nitrocellulose which contained the lanes with the high specific activity tRNA were excised and complexes were visualized by autoradiography. The remaining portion of the nitrocellulose was probed with an anti-ligase antibody fraction designated anti-tLE [(27) kindly provided by M. Clark] and immune complexes were visualized by conjugation with ^{125}I protein A (28) followed by autoradiography.

RESULTS

tRNA Ligase Binding to tRNA Substrates

A gel shift assay was used to examine the ability of yeast tRNA ligase to bind to pre-$tRNA_3^{Leu}$ substrates. In this assay specific protein-nucleic acid interactions can be detected by a reduction in electrophoretic mobility in native polyacrylamide gels (29, 30). Radiolabeled pre-tRNA was incubated with a partially purified tRNA ligase fraction (~25% pure). In the presence of tRNA ligase two major complexes (designated C1 and C2, Fig. 1) and several minor complexes (unlabeled, Fig. 1) with reduced electrophoretic mobilities were resolved. Complex formation was not observed when proteinase k was added to the preincubation and was not dependent on Mg^{2+} or ATP, though both are essential for tRNA ligase joining activity (17). A 3000- and 8000-fold excess of poly(rA) RNA as a nonspecific competitor was required to inhibit 50% of C1 and C2 formation respectively. These results are consistent with the formation of specific pre-tRNA/protein complexes.

Similar assays were performed using a partially purified endonuclease fraction. However, in the presence of the endonuclease fraction the majority of the pre-tRNA did not enter the gel. Addition of both endonuclease and ligase also resulted in most of the pre-tRNA failing to enter the gel. The small amount of pre-tRNA which did enter the gel comigrated with complexes C1 and C2 which are observed in the absence of the endonuclease (data not shown). These technical difficulties in measuring binding by endonuclease using native gel electrophoresis may reflect the physical properties of the enzyme. The endonuclease has the properties of an integral membrane protein and is insoluble in the absence of nonionic detergents (16).

It has previously been proposed that the specificity of tRNA ligase for pre-tRNA substrates might reflect a transport function of the protein in which pre-tRNAs are delivered to the endonuclease at the nuclear envelope (cf. 31). In this model the tRNA ligase would be required to specifically recognize unspliced precursors. One prediction of this model is that ligase might exhibit different affinities for distinct pre-tRNA processing intermediates in the expression pathway. In order to address this possibility the ability of tRNA ligase to bind to a series of pre-tRNA processing intermediates was examined. Potential intermediates shown schematically in Fig. 2 are based on the proposed order of processing events in a pre-tRNA expression pathway (32). Intermediates found prior to the removal of the intervening sequence include the intron-containing pre-tRNA with 5′-leader and 3′-trailer segments (referred to as *primary transcript*) and the end-trimmed intron-containing pre-tRNA (referred to as *pre-tRNA*). The intermediates of the splicing reaction were also examined; including the paired tRNA half molecules generated by yeast endonuclease (referred to as *halves*) and the spliced tRNA (referred to as *spl-tRNA*). The product of the splicing reaction contains a 2′-phosphate at the site where ligase has acted (17). This phosphate is absent from mature tRNAs *in vivo* suggesting that it is removed by a dephosphorylating activity (33). To mimick the mature tRNA,

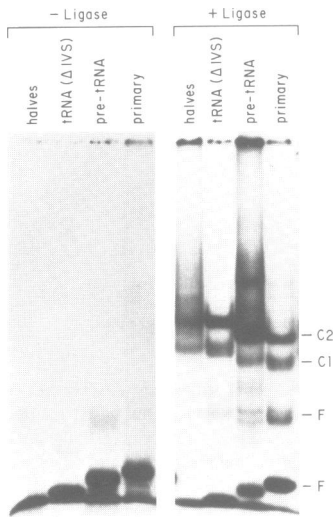

Figure 3: tRNA ligase binding to tRNA processing intermediates. Binding assays contained the indicated $tRNA_3^{Leu}$ substrates (3.3×10^4 dpm) and the tRNA ligase at the following concentrations: halves, 7.7×10^{-10} M; tRNA(ΔIVS), 5.7×10^{-9} M; pre-tRNA, 2.3×10^{-9} M; and primary, 3.4×10^{-9} M. Complexes were resolved by native gel electrophoresis and visualized by autoradiography. For a description of the substrates see the Results section and Fig. 2. Complexes C1, C2 and free pre-tRNA, F, are indicated at the right edge and are described in the text.

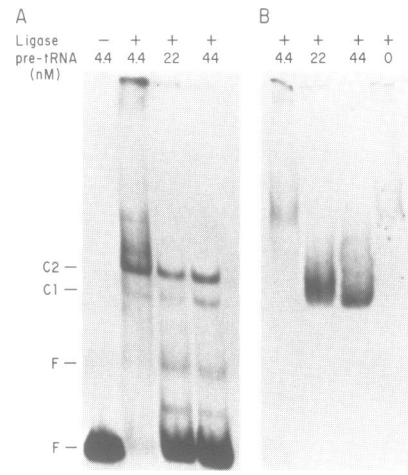

Figure 4: Immunoblot of pre-tRNA binding. Preparative binding assays with ligase and pre-$tRNA_3^{Leu}$ substrate were carried out as described in the Experimental Procedures and bound and free forms were resolved by native gel electrophoresis. A) High specific activity pre-$tRNA_3^{Leu}$ (1.75×10^5 dpm) and tRNA ligase (2.3×10^{-9} M) were incubated with increasing amounts of low specific activity pre-$tRNA_3^{Leu}$ (6.8 dpm/fmole). The concentrations of the low specific activity pre-tRNA are indicated at the top. Following electrophoresis samples were electrotransferred to nitrocellulose membranes and visualized by autoradiography. B) Reaction conditions were the same as in panel A except that only low specific activity pre-tRNA was present and nitrocellulose membranes were probed with an anti-ligase antibody and visualized with ^{125}I protein A conjugate followed by autoradiography. C1, C2, and F refer to bound and free forms as described in the legend to Fig. 1.

ligase binding to a tRNA transcribed from a gene in which the intron was deleted [referred to as *tRNA*(ΔIVS)] was examined.

The various tRNA substrates were synthesized using a yeast nuclear extract (25) as described in the Experimental Procedures and binding assays similar to the above were performed. In all cases two major complexes with reduced electrophoretic mobilities predominated (referred to as C1 and C2, Fig. 3). Note that both size and net charge contribute to mobility in this native gel system. Thus the differences in mobilities observed among the various complexes and free RNA substrates may reflect proportionately different contributions of size and net charge to relative mobility. Potentially, then, size differences may be the predominant factor for small RNAs while net charge may predominate for the larger complexes. The amount of the partially purified tRNA ligase fraction necessary to detect binding varied significantly suggesting differences in affinity for the various substrates (see legend to Fig. 3). Binding assays with spl-tRNA had a pattern of bands identical to tRNA(ΔIVS) and the tRNA half molecules (data not shown).

Since the tRNA ligase fraction used in these experiments was only partially pure it was necessary to determine whether tRNA ligase was a component of the C1 and C2 complexes. To this end preparative binding assays were resolved on native gels and immunoblotted utilizing an anti-ligase antibody. Fig. 4A and B show an example of such an experiment with pre-tRNA substrate. Although some of the resolution is lost during electrotransfer of native gels, it can be seen that as the concentration of pre-tRNA is increased, anti-ligase immunoreactive material is shifted to a position similar to that of the C1 and C2 complexes. Immunoblots of gels containing binding assays with primary transcript and tRNA(ΔIVS) resulted in a shift of anti-ligase immunoreactive material to new regions of the gel corresponding to the unique mobilities of the C1 and C2 complexes characteristic for these substrates (data not shown). Together these results suggest that ligase was present in the C1 and C2 complexes for all these substrates. The tRNA halves and spl-tRNA substrates exhibit a pattern of complexes identical to tRNA(ΔIVS) suggesting that tRNA ligase is also present in the C1 and C2 complexes for these substrates.

Certain additional minor complexes are also observed with the various tRNA substrates. The possibility that these complexes are due to the presence of additional tRNA binding proteins in the partially-purified ligase fraction is supported by the following. First, the presence of additional tRNA binding proteins in an equivalent fraction has been demonstrated by fractionation and partial heat inactivation (H. Belford and C. Greer, unpublished observation) Second, these minor complexes are not observed for binding of a more highly-purified ligase fraction. Finally, these minor complexes are not detected by immunoblotting with anti-ligase antibody.

Effect of Ionic Strength on Binding by tRNA Ligase

The effect of the type and concentration of electrolyte in solution on binding of pre-tRNA and size-fractionated (i.e. ~100 bases) poly(rA) RNA was examined. Assays were performed with sodium chloride, potassium chloride or potassium glutamate at concentrations up to 500 mM and the products were resolved on native gels (data not shown). Formation of the C1 complex with pre-tRNA substrates was unaffected by high salt while C2 formation was only slightly inhibited. Similar results were obtained with other tRNA substrates except that C1 formation with tRNA (ΔIVS) and spl-tRNA substrates was slightly inhibited at high salt. The formation of a single complex observed with poly(rA) RNA was strongly inhibited by high salt suggesting that a distinct type of interaction was being measured for this non-

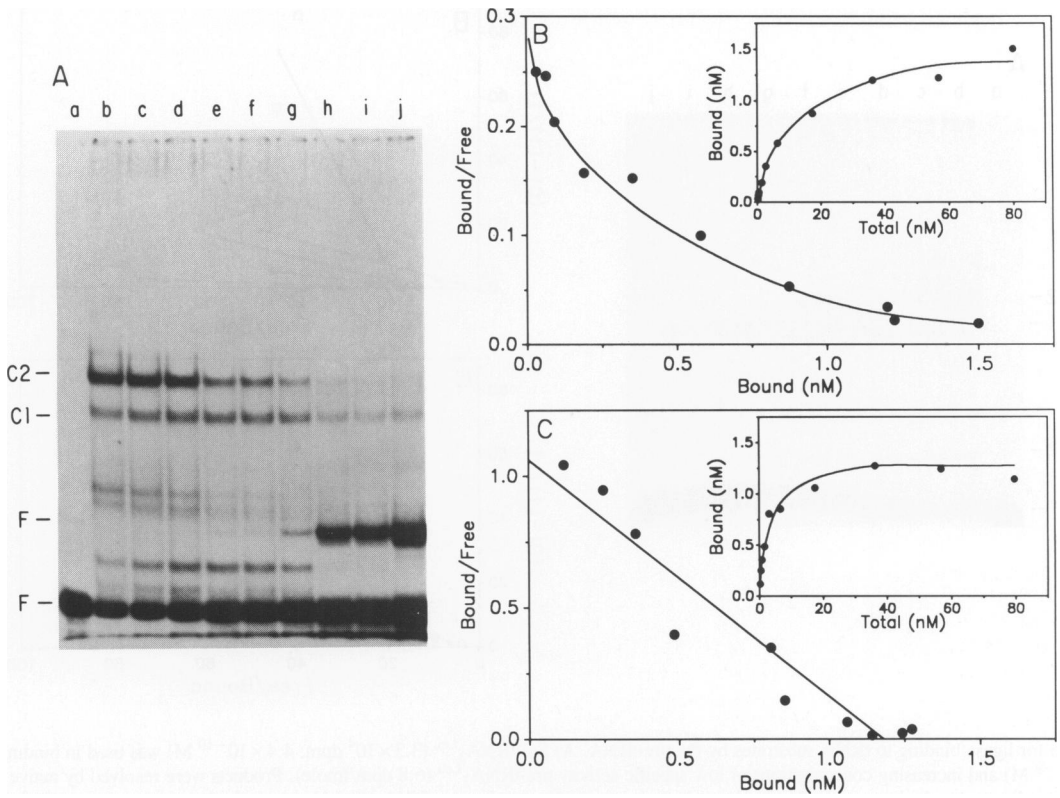

Figure 5: Effect of pre-tRNA substrate concentration on binding by ligase. A) High specific activity pre-$tRNA_3^{Leu}$ (3.3×10^4 dpm) was mixed with increasing amounts of low specific activity pre-$tRNA_3^{Leu}$ (6.8 dpm/fmole) and tRNA ligase (2.3×10^{-9} M). Products were resolved by native gel electrophoresis and visualized by autoradiography. In lane a), no ligase; lanes b–j), low specific activity pre-$tRNA_3^{Leu}$ added to a final concentration of 0, 0.44, 0.88, 2.2, 4.4, 8.8, 22, 44, and 66 nM respectively. C1, C2 and F refer to bound and free forms as described in the legend to Fig. 1. B) and C) Bands from assays as in panel A for C1 and C2, respectively, were excised and quantitated as described in the legend to Fig. 5B. Results were averaged from 5 experiments and the saturation curves (insets) were plotted as the concentration of bound pre-tRNA as a function of increasing total precursor concentration. The same data was used to produce Scatchard plots using the Munson and Rodbard Ligand Program (48, 49).

specific substrate. In subsequent binding assays 300 mM potassium glutamate was included in the reactions.

Effect of Substrate Concentration on Pre-tRNA Binding

The binding assays above were done with a large excess of protein over RNA. To determine the affinity of tRNA ligase for pre-tRNA substrates, titrations were performed in which increasing amounts of pre-tRNA radiolabeled to a low specific activity were added to a constant amount of pre-tRNA radiolabeled to a high specific activity. An example of such an assay is show in Fig. 5A. Formation of both the C1 and C2 complexes approached saturation at a pre-tRNA concentration of 1.0–1.5 nM (see inset Fig. 5B and C). To estimate binding constants from this data, Scatchard plot analysis was performed using the Munson and Rodbard Ligand program (48, 49). From this analysis, the simplest model which provided a good fit to the data was chosen and is represented by the curves in Figures 5B and 5C. For C1 the data was best fit by a two site as opposed to a one site model with association constants (Ka's) of 5.1×10^8 M^{-1} and 4.2×10^7 M^{-1}. Values for the maximum amount of pre-tRNA bound (Bmax) were 3.9×10^{-10} M and 1.4×10^{-9} M for high and low affinity binding sites respectively. The data for C2 can be fit to a single site model with a Ka of 9.3×10^8 M^{-1} and a Bmax of 1.1×10^{-9} M.

At high RNA-protein ratios the formation of new complexes with altered electrophoretic mobilities was not observed suggesting that higher order complexes are not being formed. The concentration of binding sites can then be obtained simply by summing the binding curves for C1 and C2 to obtain a value of 1.8×10^{-8} M. This is in good agreement with the concentration of tRNA ligase based on pre-tRNA joining activity (i.e. 1.9×10^{-8} M, see Experimental Procedures).

Relative Affinity Constants of RNA Substrates

The concentration of tRNA ligase required to obtain complex formation with the different tRNA substrates varied significantly. This suggests that the tRNA ligase has different affinities for these substrates (see legend to Fig. 3). As a means of examining these differences, competition assays were performed using increasing amounts of pre-tRNA radiolabeled to a low specific activity as the competitor. An example of such an experiment is shown in Fig. 6A for the spl-tRNA substrate.

The following simple exchange reaction can be used to model competition for binding to a common site in the formation of a discrete complex (41):

$$C + S\text{-}L \rightleftharpoons C\text{-}L + S$$

where S and C are the free substrate and competitor and S-L and C-L are the complexed substrate and competitor respectively. The equilibrium constant for this reaction is the exchange constant, (Kex), which is the ratio of the association constants for the substrate (Ka^s) and competitor (Ka^c).

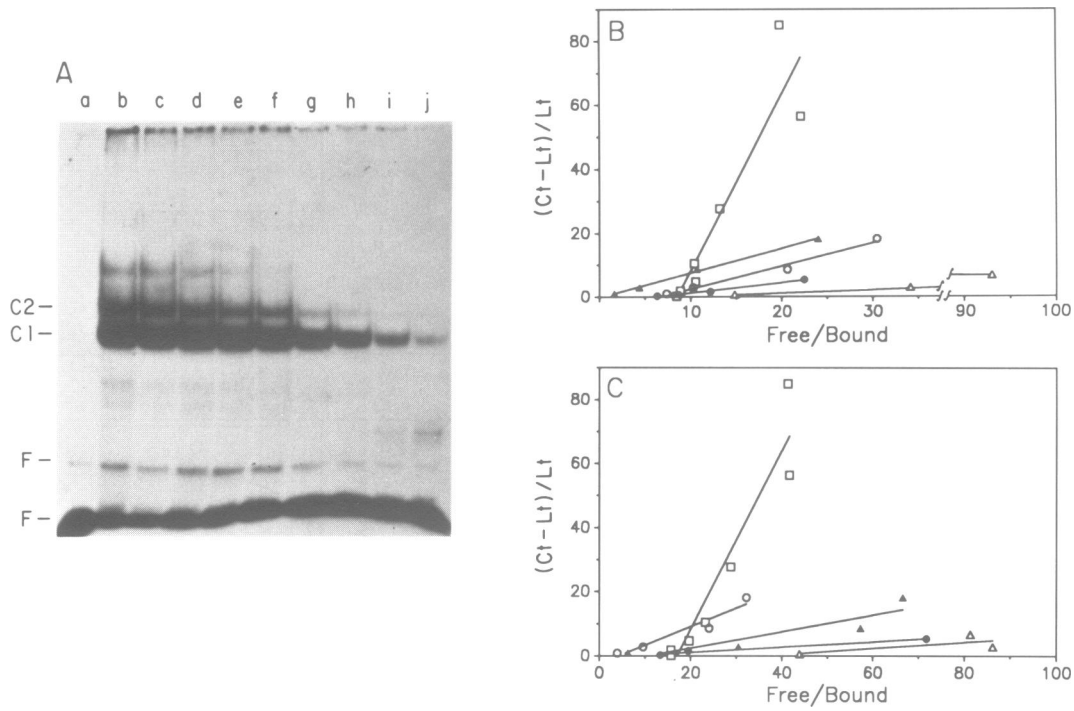

Figure 6: Competition for ligase binding to tRNA substrates by the pre-tRNA. A) Spl-$tRNA_3^{Leu}$ (3.3×10^4 dpm; 4.4×10^{-10} M) was used in binding assays containing tRNA ligase (2.3×10^{-9} M) and increasing concentrations of low specific activity pre-$tRNA_3^{Leu}$ (6.8 dpm/fmole). Products were resolved by native gel electrophoresis and visualized by autoradiography. In lane a), no ligase; lanes b-j), low specific activity pre-$tRNA_3^{Leu}$ added to a final concentration of 0, 0.44, 0.88, 2.2, 4.4, 8.8, 22, 44 and 66 nM respectively. C1, C2 and F refer to the bound and free spl-tRNA forms as described in the legend to Fig. 1. B) and C) Bands from competition assays as in panel A for C1 and C2, respectively, were excised and quantitated as described in the legend to Fig. 5B. Results were averaged from at least 3 experiments for each of the tRNA substrates. Ct, total concentration of competitor (i.e. pre-tRNA) Lt, total concentration of tRNA ligase in the reaction as follows: pre-tRNA (○), 2.3×10^{-9} M; primary (●), 3.4×10^{-9} M; tRNA(ΔIVS) (△), 5.5×10^{-9} M; spl-tRNA (▲), 2.3×10^{-9} M and halves (□) 7.7×10^{-10} M. The affinity constants (Ka^s) were derived from the slopes of the curves and are given in Table 1 and described in the Results section.

$$(1)\quad Kex = \frac{Ka^c}{Ka^s} = \frac{[S][C\text{-}L]}{[S\text{-}L][C]}$$

The values for [S] and [S-L] are obtained directly from the data. This equation can be simplified by considering only those reactions where the total competitor concentration, [Ct], is much greater than the total tRNA ligase concentration, <. Under these conditions most of the tRNA ligase is bound to the competitor and [C-L] and [C] can be approximated by < and [Ct−Lt] respectively. Substituting these values into equation (1) above gives the following equation:

$$(2)\quad Kex = \frac{[S][Lt]}{[S\text{-}L][Ct-Lt]}$$

Further substituting Ka^c/Ka^s for Kex and rearranging to give a linear form gives equation (3) below:

$$(3)\quad \frac{[Ct-LtI]}{[Lt]} = \frac{Ka^s[S]}{Ka^c[S\text{-}L]}$$

A plot of this equation will have a slope equal to the ratio of the association constants (Ka^s/Ka^c) in which Ka^c is obtained by summing the association constants for the pre-tRNA from the previous section. The competition data presented in this form are shown in Fig. 6B and 6C for C1 and C2 and Ka^s values are given in Table 1. Competitor concentrations were restricted to a range in which [S]/[S-L] < 100 due to the inability to accurately quantitate the value of S-L at higher ratios.

When the data for the pre-tRNA is analyzed as a competition assay Ka^s values for C1 and C2 are 1.0×10^9 M^{-1} and 7.8×10^8 M^{-1} respectively (see Table 1). The discrepancy in Ka values obtained in the competition versus the Scatchard plot analysis is due in part to a higher inherent error in the method used to analyze data from competition experiments. Also, the competition approach is best suited to studying a single high affinity interaction since the ability to detect additional low affinity interactions is affected by the choice of substrate and competitor concentrations. Despite the uncertainty in the absolute values of association constants from these competition experiments, the relative affinity for each of these ligands can be reliably deduced from this data.

From this analysis the order of decreasing affinity (i.e. decreasing Ka^s) for C1 is: halves > spl-tRNA > pre-tRNA > primary > tRNA(ΔIVS) and for C2: halves > pre-tRNA > spl-tRNA > tRNA(ΔIVS) > primary. Notably, the tRNA ligase has at least a 5-fold higher affinity for the tRNA halves than for any of the other tRNA substrates. This is particularly significant considering that the tRNA halves differ from the spl-tRNA and the mature tRNA substrate only at the site of joining. This result suggests that specific elements, particularly in the region of the splice sites, contribute to the tRNA ligase's substrate recognition properties.

As a means of examining how well a nonspecific RNA competes for tRNA ligase, pre-tRNA binding was titrated with increasing amounts of unfractionated poly(rA) RNA (see Table 1). The relative affinity constant for ligase binding poly(rA) RNA competitor was at least 20-fold lower than for ligase binding any of the tRNA substrates. Furthermore, the tRNA ligase bound the tRNA halves in C1 with a 1600-fold higher affinity than the poly(rA) RNA. These results demonstrate that the tRNA ligase

recognizes general elements which are common among all of the tRNA substrates. Therefore ligase recognition seems to be due to a combination of general 'tRNA'-like features conserved in all the processing intermediates examined and specific features present around the splice sites.

DISCUSSION

The results of these experiments demonstrate tRNA ligase binds a variety of tRNA substrates with an affinity significantly greater than that for a nonspecific polynucleotide. This suggests that the specificity of the tRNA ligase for tRNA halves in the joining reaction might be due in part to the enzyme's specific binding properties. Specific binding seems to be largely the result of features conserved in all the tRNA processing intermediates and not unique in the tRNA halves. The mature domain in both intron-containing pre-tRNAs and primary transcripts has previously been shown to be largely conserved (42, 43). Therefore general features conserved in a 'tRNA like' conformation are likely to be the primary feature important for ligase recognition.

A number of observations suggest that the different tRNA substrates bind at similar sites on the ligase polypeptide. First, the electrophoretic mobilities of complexes C1 and C2 for the different tRNA substrates are similar and do not suggest a significant difference in composition. Second, in the competition assays, the formation of new complexes with altered electrophoretic mobilities was not observed. Third, values of association constants determined by substrate titrations or competition assays were similar. Collectively, these observations suggest the two substrates are competing for the same type of interaction.

A particularly intriguing observation is that ligase binds the tRNA halves, spl-tRNA and tRNA(ΔIVS) substrates with significantly different affinities. Notably the substrate for ligase enzymatic activity, paired tRNA half molecules, was the best ligand. The differences in binding affinities among the substrates could be due to a number of factors including: (1) differences in base composition, (2) differences in conformation, and (3) differences in structure at the splice site junction.

First, these substrates are likely to differ in their composition of base modifications. Although the base modifications of these substrates were not specifically examined here, Strobel and Abelson (24) found that SUP53ΔIVS transcribed in a yeast nuclear extract contained additional modified bases compared to the pre-tRNA. Furthermore although the spl-tRNA and tRNA halves substrates are derived from the pre-tRNA and should contain similar base modifications the paritially purified endonuclease and ligase fractions may contain base modifying activites as well. For example, the partially purified ligase fraction used in these experiments contains pseudouridylate synthase activity (M. Shapero and C. Greer, personal communication).

Second, substrates lacking an intervening sequence may have different conformations unrelated to the presence of base modifications. The nick at the splice site in the tRNA halves is likely to result in a more flexible conformation of the anticodon stem and loop and might also affect distant structures in the molecule. Based on chemical structure probing of the end-extended forms of pre-tRNA$_3^{Leu}$ containing and lacking an intron, Leontis *et al.* (43) concluded that the absence of an intron resulted in a more nuclease-resistant structure relative to the molecule containing an intron. Furthermore, this molecule was an inefficient substrate for RNase P cleavage. The insertion of an intron of only two bases converted the molecule to a 'loose' conformation and an efficient substrate for RNase P. In these experiments ligase may have a reduced affinity for the spl-tRNA and tRNA(ΔIVS) substrates as the result of a more rigid conformation of these molecules. Therefore while the mature domain seems to be important for ligase binding, conformation within this domain, such as a less 'compact' structure, may modulate binding efficiency.

Third, differences in ligase affinity for these substrates may be due to the specific structures at splice sites not directly related to overall conformation. The ligase must be able to correctly align the free ends of the tRNA halves generated by endonuclease in order for efficient joining to occur. Additionally, the cyclic phosphodiesterase function of the polypeptide might include a binding pocket for the 2′,3′-cyclic phosphate termini of 5′ tRNA half molecules. Similarily, the region of the protein responsible for polynucleotide kinase activity might contain a binding pocket for the 5′-hydroxyl group of the 3′ tRNA half. Using photoreactive RNA substrates, Tanner *et al.* (31) found that ligase was specifically crosslinked to pre-tRNA^{Phe} in the region of the 3′ splice site suggesting a close physical proximity of ligase to this region.

In these experiments complex 1 formation was not dependent on ionic strength while complex C2 formation was only slightly reduced at higher salt concentrations. These results suggest that electrostatic interactions are not the primary mode by which ligase binding specificity is achieved *in vitro*. Other examples exist in which protein/RNA interactions are not dependent on the ionic environment. For example binding of the *E. coli* small ribosomal protein S4 to α operon mRNA exhibits only a low to moderate salt dependence which is related to the particular anion present (44). Furthermore the *E. coli* S1 protein shows a positive salt dependence in RNA binding (45). If hydrophobic interactions play a role in the formation of ligase/tRNA complexes, increasing salt concentrations could enhance protein affinity (46). This would result in a reduction in the apparent salt dependence of binding.

The existence of two complexes with different electrophoretic mobilities could be due to a number of factors. First, the presence of multiple complexes could be due to differences in the ratio of protein to tRNA. The fact that a variation in the pattern of bands at unbalanced protein:tRNA ratios was not observed suggests that this is not the case. Second, complexes with different

Table 1. Relative association constants of RNA substrates

Substrate[a]	Competitor[a]	Ka^s (1×10^9 M^{-1})[b] Complex 1[c]	Complex 2[c]
pre-tRNA	pre-tRNA	1.10	0.78
primary	pre-tRNA	0.47	0.12
tRNA(ΔIVS)	pre-tRNA	0.11	0.14
spl-tRNA	pre-tRNA	1.14	0.38
halves	pre-tRNA	8.16	4.11
		Ka^c (1×10^9 M^{-1})[d] Complex 1	Complex 2
pre-tRNA	poly(rA) RNA	0.005	0.001

[a] Substrate and competitor designations are described in the Results.
[b] Relative association constants for the substrates (i.e. Ka^s) were derived from the slopes of the curves plotted in Fig. 7B and C and described in the Results section.
[c] Complex 1 and Complex 2 refer to tRNA complexes with reduced electrophoretic mobilities observed when binding assays containing tRNA ligase were resolved by native gel electrophoresis.
[d] The relative association constants for poly(rA) competitor (i.e. Ka^c) were derived from graphs as shown in Fig. 7A and B and described in the Results.

electrophoretic mobilities might be due to differences in conformation of the ligase protein. The ligase may contain multiple binding sites for tRNAs which, when occupied, produce complexes with different conformations and correspondingly different mobilities. These distinct binding sites might be correlated with the different enzymatic activities of the ligase polypeptide (47). These catalytic sites could contain their own binding sites or may utilize a common binding site formed by tertiary folding of the ligase polypeptide.

A final possibility is that complexes C1 and C2 differ in their protein compositions. In the binding assays described here a partially purified ligase fraction was used. In binding assays containing a highly purified ligase fraction a single complex which comigrated with complex C1 was formed. Therefore while complex C2 seems to contain the tRNA ligase protein it is likely to contain other factors as well. It should be noted that the partially purified ligase fraction was obtained from yeast cells overexpressing the protein on a high copy number plasmid. If an additional pre-tRNA/ligase binding factor(s) copurified with the tRNA ligase it might be expected to be present in reduced or limiting amounts relative to the overproduced ligase. If present in limiting amounts two complexes might be expected: one complex containing ligase alone and another containing ligase and an additional factor(s). Binding assays were previously performed using a partially purified ligase fraction analogous to that used here except that it was obtained from yeast cells expressing only the genomic copy of the ligase gene. In these assays a single complex was resolved on native gels which did not comigrate with any of the complexes observed with the highly purified protein fraction (H. Belford and C. Greer, unpublished observation). These observations suggest that a protein(s) in the extract binds to either ligase or pre-tRNA substrates and raises the possibility that such a factor might contribute to processing *in vivo*.

As previously discussed, a proposed additional function of tRNA ligase *in vivo* is to transport pre-tRNAs to the endonuclease at the nuclear envelope. The specific binding properties of ligase might reflect this property of the enzyme. Furthermore, *in vivo* an additional factor which recognizes pre-tRNA/ligase complexes might be required specifically for shuttling pre-tRNAs or possibly for other processing steps. However, the observation that ligase has the highest affinity for the tRNA halves in both complexes C1 and C2 suggests that a transport function is not the sole purpose for the specific binding properties of the enzyme.

ACKNOWLEDGEMENTS

We thank Alexandra Charlesworth for expert technical assistance, Ian Willis for helpful advice and suggestions, and Michael Clark, Eric Phizicky, Reinhard Rauhut and Shawn Westaway for providing reagents and communicating their results prior to publication. This work was supported by NSF grant DMB-8917393 to CLG and by NIH predoctoral fellowship (GM07311-16) to BLA. This work was done during the tenure of an Established Investigatorship from the American Heart Association to CLG.

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