Binding Interactions between Yeast tRNA Ligase and a Precursor Transfer Ribonucleic Acid Containing Two Photoreactive Uridine Analogues†

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ABSTRACT: Yeast tRNA ligase, from *Saccharomyces cerevisiae*, is one of the protein components that is involved in the splicing reaction of intron-containing yeast precursor tRNAs. It is an unusual protein because it has three distinct catalytic activities. It functions as a polynucleotide kinase, as a cyclic phosphodiesterase, and as an RNA ligase. We have studied the binding interactions between ligase and precursor tRNAs containing two photoreactive uridine analogues, 4-thiouridine and 5-bromouridine. When irradiated with long ultraviolet light, RNA containing these analogues can form specific covalent bonds with associated proteins. In this paper, we show that 4-thiouridine triphosphate and 5-bromouridine triphosphate were readily incorporated into a precursor $tRNA^{Phe}$ that was synthesized, in vitro, with bacteriophage T7 RNA polymerase. The analogue-containing precursor tRNAs were authentic substrates for the two splicing enzymes that were tested (endonuclease and ligase), and they formed specific covalent bonds with ligase when they were irradiated with long-wavelength ultraviolet light. We have determined the position of three major cross-links and one minor cross-link on precursor $tRNA^{Phe}$ that were located within the intron and near the 3′ splice site. On the basis of these data, we present a model for the in vivo splicing reaction of yeast precursor tRNAs.

The yeast *Saccharomyces cerevisiae* contains approximately 350 genes coding for tRNAs. About 10% of these contain an intervening sequence (intron) that interrupts the coding region of the gene (Ogden et al., 1984). These introns are removed from the transcribed precursor ribonucleic acid (RNA)[1] by a reaction known as splicing. Splicing occurs through a series of protein-mediated reactions that involve an endonuclease, a tRNA ligase, and an unidentified phosphatase (Figure 1; Greer et al., 1983). Ligase is a particularly interesting protein because it has three distinct enzymatic activities. It functions as a polynucleotide kinase, a cyclic phosphodiesterase, and an RNA ligase (Phizicky et al., 1986). Yeast tRNA ligase has been purified, and the gene has been cloned, sequenced, and overexpressed in *Escherichia coli* (Phizicky et al., 1986; Westaway et al., 1988). It is a 95-kDa protein consisting of a single polypeptide chain.

We are interested in knowing which features of the pre-tRNA are recognized by ligase. Previous studies have concentrated on the effects of nucleotide changes within the pre-tRNA on the splicing reactions in vivo and in vitro [see Szekely et al. (1988)]. This strategy has been effective, but it is limited in that it does not distinguish between changes that have specifically affected contact points with the protein(s) and those that have somehow altered the overall structure of the pre-tRNA. Furthermore, with only one exception, the variant pre-tRNAs are deficient in the first step in splicing—excision of the intron by endonuclease—more than in subsequent steps (Greer et al., 1987). Thus, these experiments have done little to elucidate the ligase binding site requirements.

We have used two photoreactive uridine analogues, 5-bromouridine and 4-thiouridine, to directly study the contact sites between ligase and the pre-tRNA. Nucleic acid polymers containing these analogues are known to make specific covalent bonds with associated proteins when irradiated with long-wavelength UV light [reviewed by Shetlar (1980)]. The binding interactions between ligase and the pre-tRNA can thereby be frozen and the products subsequently analyzed to determine the location of the interactions. We used pre-$tRNA^{Phe}$ for this study because it can be readily transcribed, in vitro, with a synthetic pre-$tRNA^{Phe}$ gene (Reyes & Abelson, 1987). Ligase is known to bind pre-$tRNA^{Phe}$ and mature $tRNA^{Phe}$ (B. Apostol, H. Belford, and C. Greer, personal communication). Also, except for elements of secondary or tertiary structure near the 5′ and 3′ splice sites, no particular feature of the intron sequence and structure is important in the splicing reaction (Johnson et al., 1981; Raymond & Johnson, 1983; Strobel & Abelson, 1986a,b; Greer et al., 1987; Szekely et al., 1988; Reyes & Abelson, 1988).

In this paper, we show that BrUTP and thioUTP were readily incorporated into pre-$tRNA^{Phe}$, in vitro, by T7 RNA polymerase. We show that the analogue-incorporated pre-tRNAs were accurately processed, in vitro, by partially purified endonuclease and purified ligase. The modified pre-tRNAs formed specific covalent cross-links with ligase when the samples were irradiated with long-wavelength UV light. We

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[1] Abbreviations: BSA, bovine serum albumin; BrUTP, 5-bromouridine 5′-triphosphate; BrU-pre-tRNA, 5-bromouridine-incorporated pre-$tRNA^{Phe}$; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; kDa, kilodalton(s); NTP, nucleoside triphosphate; pre-tRNA, precursor transfer RNA; RNA, ribonucleic acid; rRNA, ribosomal RNA; TEAB, triethylammonium bicarbonate; thioUTP, 4-thiouridine 5′-triphosphate; thioU-pre-tRNA, 4-thiouridine-incorporated pre-$tRNA^{Phe}$; TLC, thin-layer chromatography; NAD, β-nicotinamide adenine dinucleotide; Tris, tris(hydroxymethyl)-aminomethane; SDS, sodium dodecyl sulfate; U-pre-tRNA, uridine-incorporated pre-$tRNA^{Phe}$; UV, ultraviolet.

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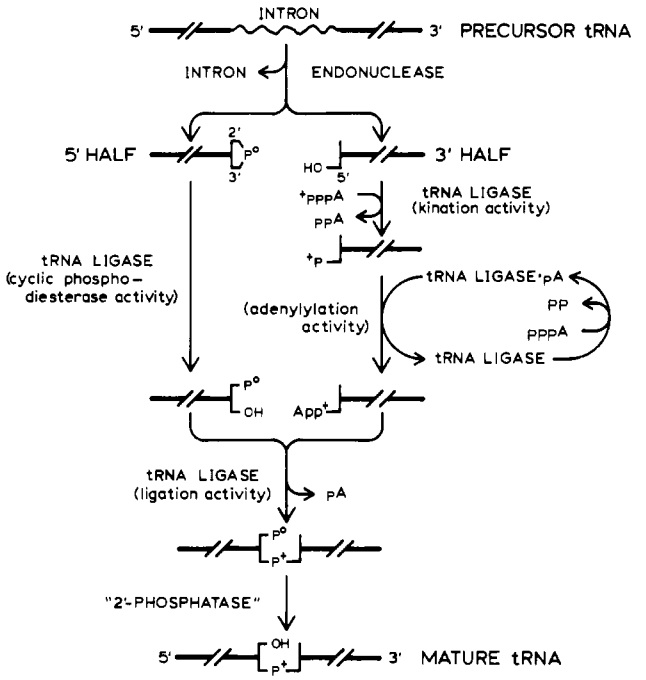

FIGURE 1: Splicing pathway for pre-tRNA maturation. The bold lines represent the mature domains of the tRNA, and the squiggly line signifies the intron. Endonuclease cleaves the phosphodiester bonds at the 5′ and 3′ splice sites and forms a 5′ half-molecule with a 2′,3′-cyclic phosphate and a 3′ half-molecule with a 5′-hydroxyl. tRNA ligase cleaves the 2′,3′-cyclic phosphodiester bond, leaving a 2′-phosphate, and adds a γ-phosphate from an ATP molecule to the 5′-hydroxyl. tRNA ligase then adenylylates the 5′-phosphate with another ATP molecule and ligates the two half-molecules together. (We consider the adenylylation activity of ligase to be part of its ligation activity.) Finally, the 2′-phosphate is removed by an uncharacterized 2′-phosphatase (Greer et al., 1983; Phizicky et al., 1986).

have determined the major sites of cross-linking with ligase on the pre-tRNA by using RNA sequencing methodology. On the basis of these results, we present a model for pre-tRNA splicing in vivo.

Experimental Procedures

Synthesis of 4-Thiouridine Triphosphate. 4-ThioUTP was synthesized by enzymatic addition of a phosphate from 3-phosphoglycerol phosphate to 4-thioUDP (Sigma) in a manner similar to the glycolytic synthesis of ATP from ADP (Imazawa & Eckstein, 1979). This method has the advantage of allowing the isolation of 4-thioUTP which is completely free of ATP. We will describe the preparation of 0.5 μmol of 4-thioUTP; however, this synthesis has been successfully scaled up 10-fold with corresponding yields. All steps were carried out on ice or at 4 °C unless otherwise specified. Sodium pyruvate and cysteine solutions must be prepared immediately before use. Aqueous solutions of L-glycerol 3-phosphate and NAD may be stored at −20 °C for up to 6 months.

An ammonium sulfate suspension of enzymes was prepared that contained 0.1 mg of glycerol-3-phosphate dehydrogenase, 0.001 mg of triosephosphate isomerase, 0.1 mg of glyceraldehyde-3-phosphate dehydrogenase, 0.01 mg of 3-phosphoglycerate kinase, and 0.05 mg of lactate dehydrogenase. Enzymes were purchased from Boehringer Mannheim Biochemicals. Just prior to use, the enzyme solution was spun for 5 min in a microcentrifuge, and the supernatant solution was removed by aspiration. A 60 mM cysteine stock solution (pH 8–9) was prepared by dissolving 10 mg of cysteine hydrochloride and 12.5 mg of Tris-HCl in 1.0 mL of water. An enzyme–cysteine mixture (solution A) was prepared by resuspending the enzyme pellet in 0.333 mL of 0.15 M Tris-HCl, pH 9.0, and then adding 0.667 mL of the 60 mM cysteine stock solution.

A 40 mM sodium pyruvate solution was prepared by dissolving sodium pyruvate in water. All remaining steps were carried out in dim light. Solution B contained the following in a 3.5-mL volume: 0.15 M Tris-HCl, pH 9.0, 35 mM $MgCl_2$, 15 mM DTT, 1.2 μmol of L-glycerol 3-phosphate, 5 μmol of NAD, 0.5 μmol of 4-thioUDP, and 0.01 mmol of sodium pyruvate. The final reaction solution was prepared by adding 0.875 mL of solution A and 4.4 mL of 0.6 mM phosphoric acid (HPLC grade) to solution B. The mixture was incubated for 1 h at 25 °C, and it was then extracted twice with phenol to remove the enzymes.

Purification of 4-ThioUTP. The reaction mix was diluted to 100 mL with water and loaded onto a 1.75 × 25 cm DE-52 column which was equilibrated in 0.1 M TEAB, pH 8.0. The column was washed with 2 column volumes of 0.1 M TEAB, and the product was then eluted with a linear gradient from 0.1 to 0.5 M TEAB (500 mL of each). 4-ThioUTP eluted at approximately 0.4 M TEAB. The product fractions were located (by measuring the absorbance at 330 nm), pooled, and lyophilized to remove solvent. The product was dissolved in water and lyophilized 2 or 3 more times until all the salt was removed. The product was then dissolved in water. The concentration was determined by diluting an aliquot into 0.01 M phosphate buffer, pH 7, and measuring the absorbance at 330 nm using an extinction coefficient of 21.2×10^3 M^{-1} cm^{-1} (Pleiss et al., 1969). The yields were typically greater than 95%.

Preparation of Precursor tRNA. RNA was transcribed from pUC13Phe plasmid DNA, which contains a bacteriophage T7 promoter and the complete precursor $tRNA^{Phe}$ sequence (Reyes & Abelson, 1987). Transcription reactions (10–50-μL volumes) contained 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 10 mM DTT, 2–4 mM spermidine, 1 mM NTPs, 1–5 μg of pUC13Phe DNA (linearized with *Bst*NI), 50 units of T7 polymerase (U.S. Biochemicals or NE Biolabs) per microgram of DNA, and 1 unit/μL RNasin (Promega). Reaction mixtures were incubated for 2 h at 37 °C and then quenched on ice. The major product of the transcription reaction was a 95-nucleotide-long, unmodified, pre-tRNA containing the sequence expected for pre-$tRNA^{Phe}$. BrUMP- and thioUMP-incorporated pre-tRNAs were made by replacing the UTP in the transcription mix with 1 mM BrUTP (Sigma) or thioUTP. Samples containing 5-bromouridine and 4-thiouridine nucleotides were exposed only to dim room light and were stored in foil-wrapped brown Eppendorf tubes (West Coast Scientific).

Pre-tRNA was labeled and its 5′ end by replacing the GTP in the transcription mix with 0.50–0.75 mM [γ-^{32}P]GTP (15–30 Ci/mmol; Amersham or ICN). This labeling technique was found to be very inefficient. Pre-tRNA was internally labeled with ^{32}P by transcribing the RNA in the presence of [α-^{32}P]ATP (2.0–3.0 Ci/mmol). Pre-tRNA was labeled at its 3′ end by first transcribing the RNA in the presence of [^{3}H]CTP (3.5–4.0 Ci/mmol). The transcribed, ^{3}H-labeled, RNA was separated from the unincorporated NTPs by chromatography on a 8-cm-long Sephadex G50-150 column (in 10 mM Tris-HCl, pH 7.5, 250 mM sodium acetate, and 1 mM EDTA). The RNA was then ethanol precipitated and resuspended in 10 mM Tris-HCl, pH 7.5, and 0.1 mM EDTA. Finally, 10–15 pmol of this material was incubated overnight at 4 °C in the presence of 50 mM HEPES, pH 8.3, ~150 μM ATP, 3 mM DTT, 10 μg/mL BSA, 20 mM $MgCl_2$, 10% DMSO, ~30 pmol of [^{32}P]pCp (2000–3000 Ci/mmol),

and 400 units/mL T4 RNA ligase (NE Biolabs). This protocol is similar to that described by England et al. (1980).

The pre-tRNA was purified by first adding an equal volume of urea loading buffer containing 10 M urea, 20% sucrose, 0.02% xylene cyanol, 0.02% bromophenol blue, 0.5% SDS, 10 mM Tris base, 8.3 mM boric acid, and 0.1 mM EDTA and then loading the material on an 8% polyacrylamide gel containing 8 M urea. The RNA was separated by electrophoresis with a buffer containing 100 mM Tris base, 83 mM boric acid, and 1 mM EDTA. The position of the pre-tRNA band in the gel was determined by subjecting the gel to autoradiography. The portion of the gel containing the pre-tRNA was isolated and then thoroughly crushed with a glass rod in a buffer containing 500 mM ammonium acetate, 0.1% SDS, and 0.1 mM EDTA. This material was allowed to soak overnight at 4 °C. The supernatant was collected and the RNA precipitated with ethanol overnight at −20 °C. The RNA was resuspended in 10 mM Tris-HCl, pH 7.5, 250 mM sodium acetate, and 1 mM EDTA and further purified either by chromatography on a Sephadex G50-150 column (as described above) or by a second ethanol precipitation. Pre-tRNA was stored in 10 mM Tris-HCl, pH 7.5, and 0.1 mM EDTA at −20 °C.

Splicing Reaction Conditions. Pre-tRNA (0.20–1.5 pmol) that was internally labeled with ^{32}P was incubated in 10-μL volumes in a buffer containing 20 mM Tris-HCl, pH 8.0, 0.1 mM DTT, 25 mM NaCl, 5 mM $MgCl_2$, 2.5 mM spermidine, 0.4% Triton X-100, 2 mM ATP, and 10% glycerol. Partially purified yeast endonuclease [$(0.4–4) \times 10^{-3}$ units; Green and Abelson, unpublished data] and purified yeast ligase (0.1–1.0 pmol; Phizicky et al., 1986) were added to the reactions. The reaction mixtures were incubated for up to 60 min at 30 °C and then quenched on ice. An equal volume of urea loading buffer was added, the material was separated by electrophoresis on a 0.5-mm-thick, 12% polyacrylamide gel containing 7 M urea, and the resulting gel was subjected to autoradiography with Kodak X-ray film (X-Omat AR) at −70 °C. High protein concentrations caused the RNA to smear on the gel somewhat; this was alleviated by adding 1 μL of a solution containing 0.2% SDS, 10 mM EDTA, and 0.2 mg/mL proteins K to the reaction mixture and then incubating for 20 min at 50 °C (Greer et al., 1983). Results were quantified by cutting out gel slices corresponding to the pre-tRNA, tRNA, half-molecules, and intron (using the autoradiogram as a guide), adding 4 mL of Safety-Solve cocktail (Research Products) to the slices, and determining the radioactivity by counting in a Beckman LS 7800 scintillation counter.

Two-Dimensional Chromatography of Nucleotides. The nucleotide composition of the pre-tRNA and analogue-incorporated pre-tRNA, which were transcribed in the presence of [α-^{32}P]ATP, was determined as described by Nishimura (1972) except that the RNA was digested with RNase T2 and the elution buffers contained 1 mM EDTA.

Cross-Linking Reaction Conditions. ^{32}P-Labeled pre-tRNA (0.1–1.0 pmol) was irradiated by long-wavelength UV light in the presence of ligase (1–3 pmol) in a 10-μL volume of a solution containing 20 mM Tris-HCl, pH 8.0, 75 mM NaCl, 0.4% Triton X-100, 0.1 mM EDTA, 1.5 mM 2-mercaptoethanol, 0.1 mM DTT, 2.5 mM spermidine, and 12% glycerol. Ligase, which was purified from *E. coli* cells containing the ligase plasmid pK223-3-RLGX (Phizicky et al., 1986), was a kind gift of M. Clark. Samples were contained in 1.5-mL brown Eppendorf tubes and kept cold in an ice bath. Samples were irradiated, from above through the open Eppendorf tubes, at a distance of 4.5 cm from the light source. A polystyrene petri dish over the tubes was used to filter out most of the emission below 290 nm (>95% between 200 and 290 nm) and to prevent excess condensation from forming on the walls of the tubes. Ligase was noticeably degraded in samples that did not have the filter. After 3–4 h of irradiation, the ice bath and petri dish filter were renewed. The light source was a UV Products, 15-W, midrange, UV fluorescent, light bulb with a broad UV light emission centered around 302 nm. The intensity at the surface of the bulb was $\sim 10000\ \mu W/cm^2$. The cross-linking reaction is probably promoted by an $n-\pi^*$ transition in the region of 308 nm (Dietz et al., 1987).

After irradiation, an equal volume of SDS loading buffer containing 0.01% bromophenol blue, 5% 2-mercaptoethanol, 3% SDS, and 63 mM Tris-HCl, pH 6.8, was added to the samples. The material was heated at 65 °C for 2 min and then electrophoresed on a 1.5-mm-thick, 11-cm-long, SDS Laemmli gel (Laemmli, 1970) consisting either of a 4% stacking and 9% separating gel or of a 4% stacking and 5–15% gradient separating gel. The un-cross-linked RNA migrated as a more discrete band on the latter SDS gel. Protein bands were visualized by silver staining (Wray et al., 1981). The gel was dried and subjected to autoradiography at −70 °C. Results were quantified by cutting out gel slices corresponding to bands on the autoradiogram, adding 4 mL of Safety-Solve cocktail to the slices, and determining the radioactivity by counting in a scintillation counter.

Localization of Cross-Links. Analogue-incorporated pre-tRNA, which was labeled at its 5′ or 3′ end with ^{32}P, was irradiated in the presence of ligase and purified on a 4–15% gradient, SDS Laemmli gel. The gel slice containing the cross-linked RNA was isolated and the RNA electroeluted using SDS electrophoresis buffer. The cross-linked RNA did not readily elute from the gel even after multiple extractions. Best results were obtained if the gel slice was first soaked in 125 mM Tris-HCl, pH 6.8, and 0.1% SDS. Carrier RNA was added (2.5–5.0 μg) to the eluted RNA, and it was ethanol precipitated at −20 °C. The material was resuspended in 10 mM Tris-HCl, pH 7.5, and 0.1 mM EDTA. Generally, the cross-linked RNA was then digested twice with 100 μg/mL proteinase K for 30 min at 50 °C. The digested RNA was extracted with phenol, extracted with chloroform/isoamyl alcohol (24:1), ethanol precipitated, and stored in 10 mM Tris-HCl, pH 7.5, and 0.1 mM EDTA. The sites of cross-linking were located by subjecting the RNA to limited (<1 cut per pre-tRNA) alkali hydrolysis and separating the products on a 12% polyacrylamide sequencing gel containing 7 M urea.

RESULTS

Uridine Analogues Are Incorporated into Pre-tRNA. BrUTP and thioUTP were readily incorporated into the pre-$tRNA^{Phe}$ by bacteriophage T7 RNA polymerase in the absence of UTP (data not shown). The product of the transcription reactions, for both analogues, was a single, intense ^{32}P band on a urea–polyacrylamide gel which comigrated with the UTP-incorporated pre-tRNA. Generally, 70–80% as much product was obtained in transcription reactions containing BrUTP as was obtained with reactions containing UTP. Up to 50% as much product was obtained in transcription reactions containing thioUTP. No premature termination or increased smearing on the gel was found for either analogue.

The nucleotide composition of the purified pre-tRNA was determined by two-dimensional chromatography on poly(ethylenimine)-impregnated cellulose plates (Nishimura, 1972). The results indicated that all of the uridine nucleotides were replaced by BrU (>95%). The thiouridine content was

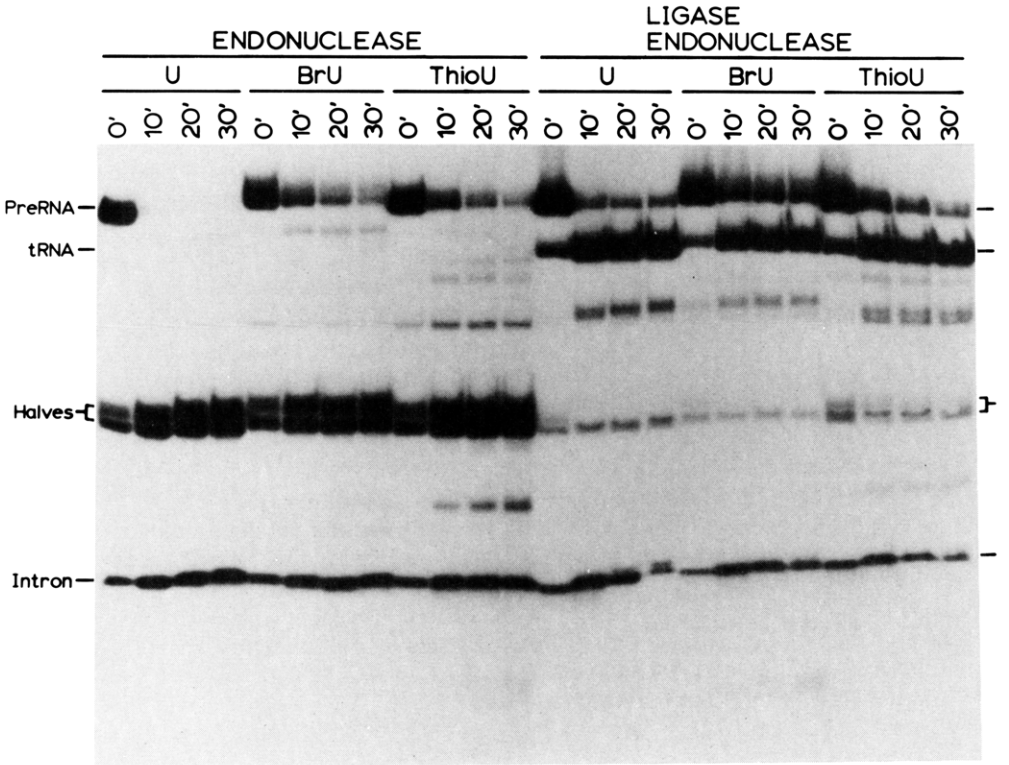

FIGURE 2: Processing of the analogue-incorporated pre-tRNA. Uridine-incorporated (U), bromouridine-incorporated (BrU), and thiouridine-incorporated (ThioU) pre-tRNAs, which were labeled with ^{32}P, were incubated under splicing conditions for the indicated times (minutes) with partially purified endonuclease and with purified ligase. The products were separated on a 12% polyacrylamide gel containing 7 M urea, which was then subjected to autoradiography. A small amount of processing occurred while the samples were being prepared and stored on ice. The tRNA half-molecules were not well resolved on this gel. The band that migrated between the half-molecules and the tRNA consisted of a half-molecule with the intron still attached. It resulted from partial processing by endonuclease.

variable, but at least 60% of the uridines were replaced by thioU. Some uridine was probably formed from thiouridine during the analysis.

The pre-RNA^{Phe}, which was labeled at the 3′ end with ^{32}P, was also analyzed by enzymatic RNA sequencing; that is, the RNA was partially digested under denaturing conditions with G-specific RNase T1, A-specific RNase U2, or U- and A-specific RNase PhyM or by nonspecific alkaline hydrolysis (Donis-Keller et al., 1977; Donis-Keller, 1980). The cleavage products were separated by gel electrophoresis and located by subjecting the gel to autoradiography (see Figure 6, data not shown). The results indicate that the pre-tRNA^{Phe} gene was accurately transcribed in the presence of BrUTP. We were less confident of the sequence of the thiouridine-incorporated pre-tRNA because it tended to smear somewhat on the sequencing gel. However, the sequence obtained was consistent with that expected. Smearing was minimized by first heating the samples with 1 mM DTT before loading on the gel.

Analogue-Incorporated Pre-tRNAs Are Spliced. Bromouridine and thiouridine are known to have different hydrogen-bonding properties from uridine (Katritzky & Waring, 1962; Kyogoku et al., 1967; Iwahashi & Kyogoku, 1977). Therefore, it was possible that the analogue-incorporated pre-tRNAs would not be substrates for the splicing enzymes. We tested this possibility by incubating the pre-tRNAs under splicing conditions with endonuclease and with ligase (Figure 2). Both BrU–pre-tRNA and thioU–pre-tRNA were cleaved and ligated by the splicing enzymes. The products all comigrated with those produced from processing of U–pre-tRNA; this indicated that the analogue-incorporated pre-tRNAs were accurately processed by endonuclease and ligase.

While most of the analogue-incorporated pre-tRNA was cleaved within the first 10 min of incubation (>80%), there was some material that was cleaved less rapidly (Figure 2). These pre-tRNAs may not have initially folded into a conformation that could be spliced. All of the pre-tRNA was processed after 1 h of incubation (data not shown). The pre-tRNAs were also not cleaved by endonuclease as readily in the presence of ligase (Figure 2). This was in contrast with other results which indicate that ligase actually enhances cleavage of pre-tRNA by endonuclease (C. Greer, personal communication). However, our experiments were done with much higher concentrations of ligase than endonuclease. The excess ligase may have made the pre-tRNA less accessible to the endonuclease.

Pre-tRNA Stability under Irradiation Conditions. In order to ensure that cross-links were only the result of intact pre-tRNAs interacting with ligase, we tested the stability of the analogue-incorporated pre-tRNAs to the cross-linking conditions. We did this by irradiating the pre-tRNAs in the splicing buffer in the absence of ligase and then separating the products on a urea–polyacrylamide gel (Figure 3). The BrU–pre-tRNA was mostly unaffected by the irradiation conditions, but there was a faint, irradiation time dependent, band that migrated slower than the pre-tRNA. This material may have had an intrastrand cross-link. There was another faint band that migrated slightly faster than the pre-tRNA.

The thioU–pre-tRNA formed an intense band, which comigrated with that produced with the BrU–pre-tRNA. There were also other bands that may have resulted from other intrastrand cross-link(s) or even from an interstrand cross-link. These reaction products formed at about the same rate as the products from ligase cross-linking (see below); however, this was not a problem because other experiments indicated that only a negligible amount of these reaction products formed when the thioU–pre-tRNA was irradiated in the presence of ligase (see Figure 5B). Very little RNA degradation occurred during the irradiation period. The unmodified pre-tRNA was

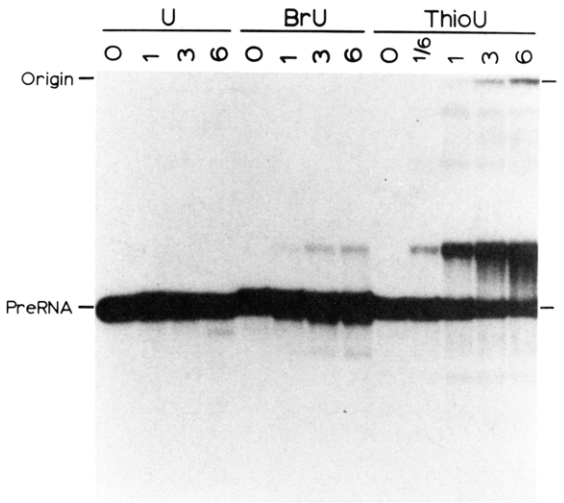

FIGURE 3: Stability of the modified pre-tRNAs to long-wavelength UV irradiation. Modified pre-tRNA and unmodified pre-tRNA, which were labeled with ^{32}P, were irradiated for the indicated times (hours) in a reaction mixture containing splicing buffer and ligase storage buffer. The RNA was separated on a 12% polyacrylamide gel containing 7 M urea. The gel was then subjected to autoradiography. There was little RNA degradation, although it was possible that strand scission occurred within intrastrand-cross-linked thioU–pre-tRNA.

unaffected by the irradiation conditions.

Cross-Linking Pre-tRNA to Ligase. Cross-links were formed by incubating ^{32}P-labeled BrU–pre-tRNA or thioU–pre-tRNA with ligase in splicing buffer and irradiating them with long-wavelength UV light. Samples were analyzed on an SDS–polyacrylamide gel (Figure 4A). The results showed that there was an irradiation time dependent formation of ligase-cross-linked RNA. U–pre-tRNA did not form these products. The cross-linked BrU–pre-tRNA migrated as a major band at a position equivalent to ~120 kDa. This was consistent with the combined molecular weight of ligase and the pre-tRNA. Another faint, but discrete, band migrated at a position equivalent to ~200 kDa. This band may have been an aggregate consisting of two ligase molecules cross-linked to the same pre-tRNA (predicted molecular weight of 210K). This was possible because, in order to obtain efficient cross-linking, a 5–10-fold molar excess of ligase to pre-tRNA was used in the reactions. This also minimized the amount of free pre-tRNA present, which otherwise might have made nonspecific associations with ligase.

The cross-linked thioU–pre-tRNA migrated as a broad band equivalent to 100–180 kDa. The upper band migrated at 200–300 kDa. The broader bands may have resulted because thioU is more reactive than BrU; it may have formed cross-links with ligase at more positions. These products may then migrate slightly differently on the gel. Intrastrand, RNA–RNA, cross-links may also have been present, which might have affected the mobility. These cross-linked products did not appear when BrU–pre-tRNA and thioU–pre-tRNA were irradiated in the absence of ligase (data not shown).

Faint bands were present on the silver-stained gel that corresponded to the ^{32}P-labeled products on the autoradiogram (Figure 4B). These bands were not present in the lanes with U–pre-tRNA or in the lanes where the material was not irradiated (zero time). Thus, they represented ligase molecules cross-linked to pre-tRNA. There was no degradation of the ligase.

The major cross-linked products comigrated with ligase (at ~90 kDa) when most of the RNA was removed with RNase T1 (Figure 4C). There were some faint higher molecular weight bands; this RNA may have been resistant to RNase T1 cleavage. The largest RNA fragment that would be generated from RNase T1 digestion of pre-tRNA^{Phe} is ~3.5 kDa (11 nucleotides long). Thus, any RNA still attached to the ligase should have had little effect on the mobility of ligase on the gel. In addition, the slowly migrating bands on the silver-stranded gel also disappeared after RNase T1 digestion (data not shown). These data indicated that the altered mobility of ligase on the gel was due to the covalent addition of the pre-tRNA molecule.

The ^{32}P bands shown in Figure 4A were isolated and quantified (Figure 4D). More than 50% of the BrU–pre-tRNA and ~90% of the thioU–pre-tRNA were cross-linked to ligase after 6 h of irradiation. The distribution of the cross-linked products did not change appreciably with time; that is, there was not a tendency for larger cross-linked aggregates to form. There was no reaction of the U–pre-tRNA.

Competition for Ligase Binding. It was possible that the cross-linked products were a result of random collisions between the analogue-incorporation pre-tRNAs and ligase rather than due to specific binding interactions. This possibility was tested by irradiating the analogue-incorporated pre-tRNA–ligase mixture with different concentrations of unlabeled tRNA^{Phe}. Since ligase can bind both pre-tRNA^{Phe} and mature tRNA^{Phe} (B. Apostol, H. Belford, and C. Greer, personal communication), we expected that fewer ligase molecules would be available to bind the modified pre-tRNA and that this would result in less cross-linking. Figure 5 shows that for both BrU–pre-tRNA and thioU–pre-tRNA, the amount of cross-linked products formed decreased with increasing amounts of tRNA^{Phe}. An equivalent amount of rRNA (based on the adsorption at 260 nm) had no affect on the amount of ligase cross-linked to BrU–pre-tRNA up to a 10-fold excess. At a 100-fold excess, the cross-linked products were smeared down the length of the gel (data not shown). Unlabeled U–pre-tRNA also reduced ligase cross-linking to BrU–pre-tRNA (data not shown). Thus, by these criteria, we are confident that the cross-links were a direct result of specific binding interactions between ligase and pre-tRNA.

Location of Cross-Links on Pre-tRNA. To determine the position of the cross-links between ligase and the BrU–pre-tRNA, the 5′ end-labeled pre-tRNA was cross-linked to ligase and then gel purified. The cross-linked RNA was partially base hydrolyzed, and the products were separated by sequencing gel electrophoresis (Figure 6, X-linked lane). RNA fragments that were 5′ to the position of the ligase cross-link on the pre-tRNA comigrated with the corresponding fragments generated from alkali hydrolysis of un-cross-linked RNA: radioactively labeled RNA fragments that contained the protein cross-link migrated as bands reflecting the combined molecular weight of the RNA and protein fragment. Thus, a gap appeared in the alkali hydrolysis ladder at the position corresponding to the cross-link. The major cross-link occurred at the ninth residue of the intron (U37:9).

Because most of the protein was removed from the RNA by protease K, the fragments containing the ligase cross-link shown in Figure 6 were displaced by only seven nucleotides. However, these bands were not as distinct as the bands corresponding to the un-cross-linked RNA fragments. In addition, the intact (unhydrolyzed), cross-linked, pre-tRNA was smeared on the gel. Evidently, a distinct proteolytic fragment was not present on the RNA. More extensive digestions with proteinase K or with a mixture of bromelain and proteinase K did not substantially improve the resolution (data not shown). This made it difficult to detect any cross-links on the

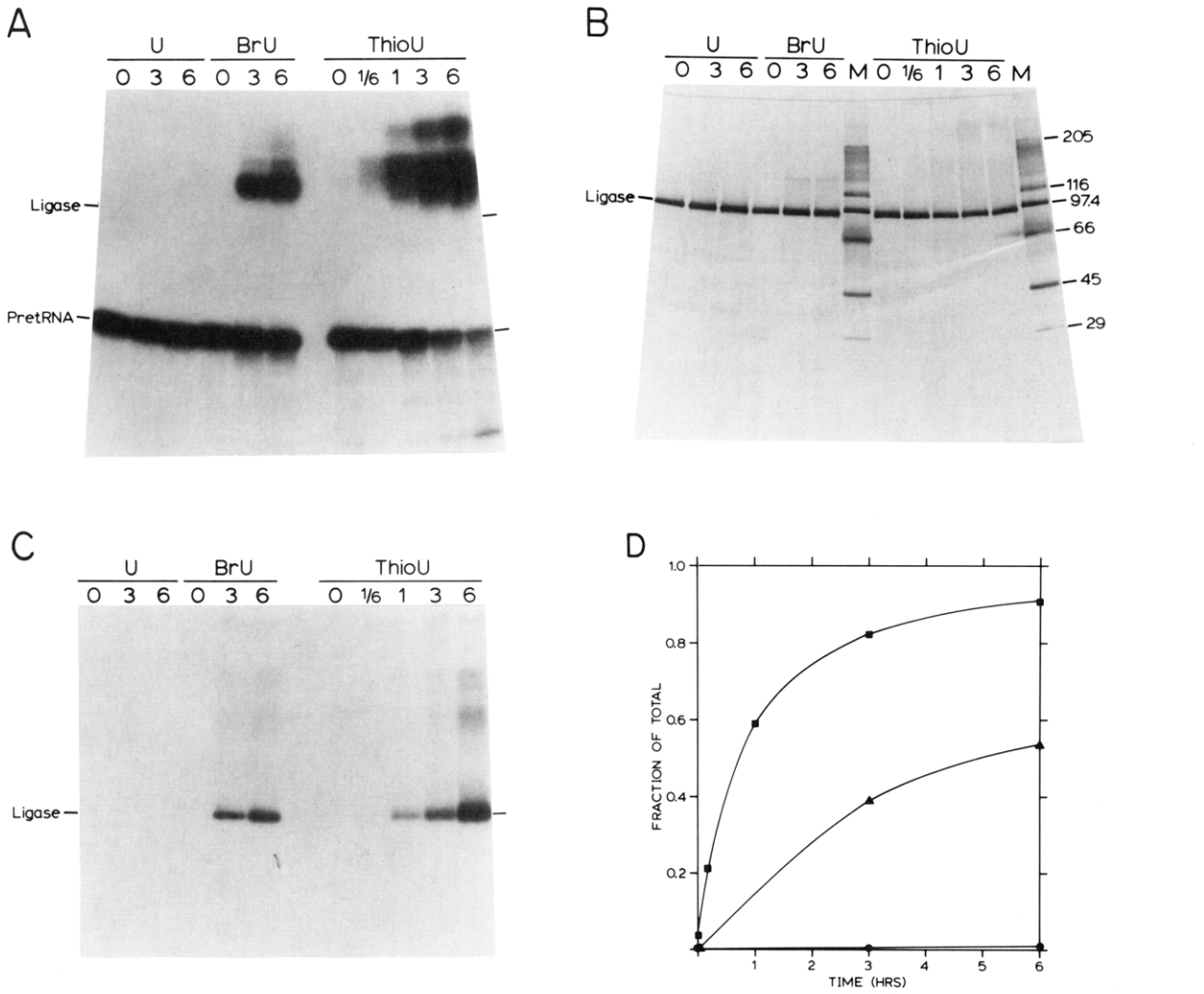

FIGURE 4: Cross-linking ^{32}P-labeled pre-tRNA to ligase. (A) Autoradiogram of unmodified (U), 5-bromouridine-incorporated (BrU), and 4-thiouridine-incorporated (ThioU) pre-tRNAs that were irradiated for the indicated times (hours) with long-wavelength UV light in the presence of ligase. The products were separated on a 5–15% gradient, SDS gel. The un-cross-linked pre-tRNA migrated at a position equivalent to ~30 kDa. (B) Photograph of the same gel shown in (A) that was stained with silver. Faint bands were apparent on the gel just above ligase that comigrated with the ^{32}P label. A large excess of ligase to pre-tRNA was used in this experiment, so the intensity of the ligase band was not substantially decreased by the cross-linking reaction. (C) Autoradiogram of aliquots of the same samples shown in (A) that were digested with RNase T1 and separated on a 5–15% gradient, SDS gel. Most of the radioactivity comigrated with ligase (the silver-strained gel is not shown), but some slower migrating material was still apparent. The un-cross-linked pre-tRNA was reduced to small fragments that were run off the gel. (D) Pre-tRNA and ligase-cross-linked pre-tRNA shown in (A) were isolated, quantified, and plotted as the fraction of the total counts that were cross-linked to ligase against time: (■) thioU–pre-tRNA; (▲) BrU–pre-tRNA; (●) pre-tRNA.

pre-tRNA that were 3′ to U37:9.

Faint bands were present in the gapped region of the original autoradiogram of Figure 6. Apparently, a small number of pre-tRNA molecules were cross-linked to ligase only at residues 3′ to U37:9. Alternatively, these bands may have resulted if the cross-link somehow was cleaved during purification or during the subsequent analysis of the RNA.

Ligase-cross-linked BrU–pre-tRNA that was 3′ end labeled was also subjected to limited alkali hydrolysis (Figure 7, WT lane). Two major gaps were obvious on this hydrolysis ladder. The uppermost gap resulted from a cross-link to the intron at U37:9, as was found with the 5′ end-labeled pre-tRNA. The lower gap resulted from a cross-link near the 3′ splice site at U39. There was a third, less obvious, cross-link within the intron at U37:13. We are less confident of this cross-link because it corresponded to a position within the alkali ladders of the un-cross-linked pre-tRNA (Alk) where there was less hydrolysis of the phosphodiester backbone (less intense bands within the hydrolysis ladder) of the pre-tRNA. The other two cross-links correspond to positions within the alkali ladders (Alk) where the bands were actually more intense. We have no explanation for why the phosphodiester bonds of the pre-tRNA were hydrolyzed at different rates. A minor cross-link was located at either U37:18 or U37:19.

The position of the uppermost cross-link shown in Figure 7 (U37:9) was actually displaced approximately two nucleotides relative to the sequencing ladder. This would place the cross-link at C37:7 (a nonreactive residue). However, the gap within the alkali ladder for the cross-link at U39 was two to three nucleotides wide; the peptide fragment(s) on the pre-tRNA has(have) apparently shifted the mobility of the RNA by at least this amount. The alkali hydrolysis ladder was less distinct after U39, which again indicated that a discrete proteolytic fragment was not left on the RNA. Further, the sample may have consisted of a combination of species with cross-links at one or both positions within the pre-tRNA. Cross-linking may have preferentially occurred at position U37:9. This would explain why the cross-link at position U39 was not apparent on the autoradiograms of the pre-tRNA that was labeled on its 5′ end. The minor cross-link at U37:18/19 was also displaced one to two nucleotides (toward G37:17) relative to the sequencing ladder.

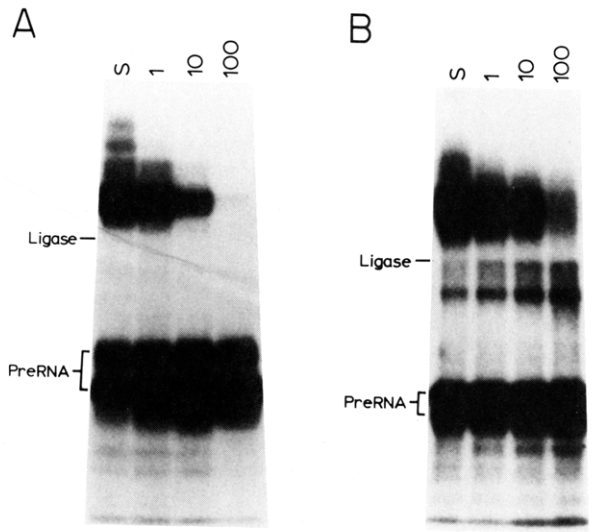

FIGURE 5: Competition between pre-tRNA and $tRNA^{Phe}$ for ligase binding. (A) Autoradiogram of ^{32}P-labeled, BrU–pre-tRNA that was irradiated for 6 h with ligase in the presence of various concentrations of unlabeled $tRNA^{Phe}$ (as an approximate molar ratio of $tRNA^{Phe}$ to ligase; there was approximately 5-fold more ligase than pre-tRNA). Lane S contained only pre-tRNA. The samples were separated on a 5–15% gradient, SDS gel. (B) Autoradiogram of ^{32}P-labeled thioU–pre-tRNA that was irradiated for 1 h with ligase and $tRNA^{Phe}$, as in (A). Notice that there is a band, which migrated just below the ligase, that increases in intensity with increasing amounts of carrier tRNA. This was probably an intrastrand or interstrand cross-link of the thioU–pre-tRNA. Probably only unbound pre-tRNAs were capable of forming this product, although ligase-cross-linked pre-tRNA may also have had an intrastrand cross-link. The pre-tRNA migrated as two bands on this gel; the RNA may not have been completely denatured.

There was an anomaly in the alkali ladder of the cross-linked pre-tRNA at a position corresponding to C48 in the sequencing ladders. The bands equivalent to C48 and C49 were missing, and another band was present which migrated between these two nucleotides. Bands corresponding to positions U50 and U47 were not affected. The bands within the alkali ladders for both the cross-linked and un-cross-linked RNA were less intense between positions C48 and G45, but they remain distinct. Therefore, this anomaly was probably not due to a ligase–pre-tRNA cross-link. It is possible that it was caused by an RNA–RNA cross-link or some other photochemical reaction.

Ligase-cross-linked thioU–pre-tRNA that was 3′ end-labeled was also analyzed. Hydrolysis ladders had a very similar pattern to those obtained with the ligase-cross-linked BrU–pre-tRNA. However, because the thioU–pre-tRNA tended to smear on the sequencing gel, we were not able to accurately map the positions of the ligase cross-links.

Cross-Links on Mutant Pre-tRNAs. The pre-tRNA introns vary in length and in sequence (Odgen et al., 1984). The only common feature is that they frequently contain sequences that are complementary to the anticodon, although this latter feature is not required for splicing (Strobel & Abelson, 1986a,b). Therefore, it was unlikely that ligase could recognize specific residues within the intron. We tested the specificity of the cross-links on the pre-tRNA by mapping the positions of ligase cross-links with two mutant pre-tRNAs, GC▽ and poly[U(G)] (Reyes & Abelson, 1988). The GC▽ variant has a GC base pair inserted between the second and third base pairs of the anticodon stem (G28:1, C41:1). This lengthens the anticodon stem by one base pair and rotates the helix by 33°. Thus, residues U37:9, U37:13, and U39 should be shifted by this amount relative to the wild-type pre-tRNA. The unmodified GC▽ pre-tRNA is processed by both endonuclease and ligase, although the endonuclease cleavage occurs one nucleotide upstream and one nucleotide downstream from the normal 5′ and 3′ splice sites, respectively (Reyes & Abelson, 1988). We found that the BrU–pre-tRNA of this mutant was also processed. In the cross-linking reaction, slightly less product was formed with ligase in comparison with wild-type BrU–pre-tRNA (data not shown).

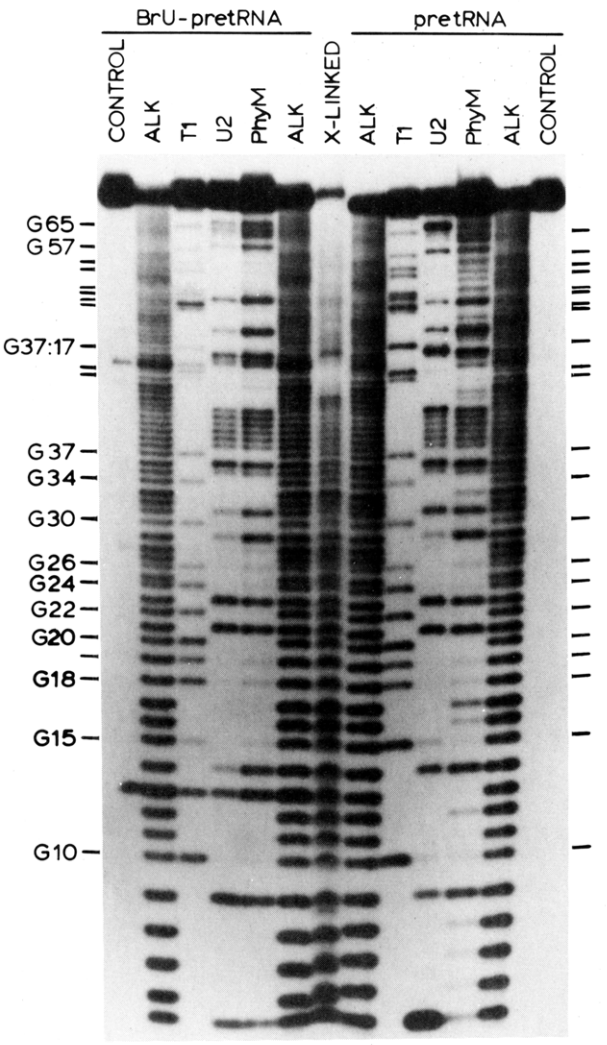

FIGURE 6: Locating the position of the ligase cross-link on 5′ end-labeled BrU–pre-tRNA. The purified, cross-linked pre-tRNA was extensively digested with proteinase K, extracted with buffer-saturated phenol, and then lightly cleaved by alkali hydrolysis. The material was separated by a 12% polyacrylamide gel containing 7 M urea, and the gel was subjected to autoradiography (X-Linked). The RNA oligomers that contained the proteolytic fragment(s) of ligase migrated slower on the gel than the corresponding oligomers generated from the alkali hydrolysis of un-cross-linked RNA (Alk). This created a gap in the alkali ladder at the position of the cross-link. Sequencing ladders were made by digesting the un-cross-linked pre-tRNA with G-specific RNase T1 (T1), with A-specific RNase U2 (U2), and with A- and U-specific RNase PhyM (PhyM). The sequencing ladders for the BrU-incorporated and U–pre-tRNAs were identical except that RNase PhyM would sometimes fail to cleave the phosphodiester bond following a bromouridine.

The poly(U) variant has the anticodon loop and most of the intron replaced with an oligonucleotide containing 14 uridines and 1 guanosine (U_6GU_8 replaced nucleotides G34 through A37:16). The position of the cross-links between the intron of the pre-tRNA and ligase should not have been limited by the availability of reactive functionalities. Poly(U) pre-tRNA is cleaved only at the 3′ splice site by endonuclease (Reyes & Abelson, 1988). We found that the BrU–pre-tRNA of this mutant was also only cleaved at the 3′ splice site. In the

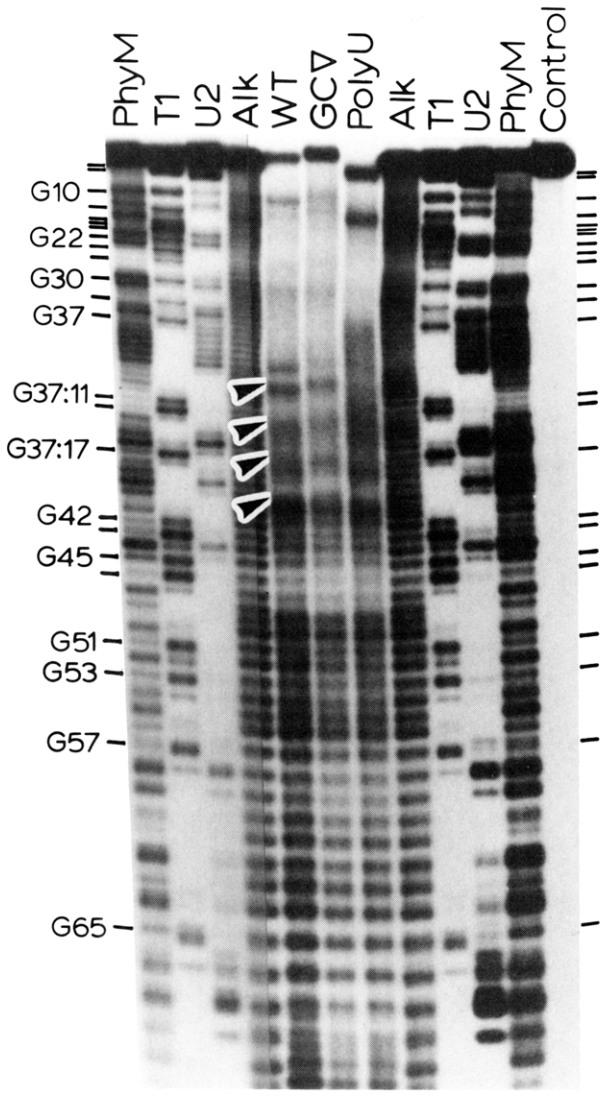

FIGURE 7: Locating the positions of the ligase cross-links on 3′-[^{32}P]pCp end-labeled BrU–pre-tRNA. Material was analyzed as described in Figure 6. The arrowheads indicate the positions of the major and minor sites of cross-linking. WT, wild-type pre-tRNA. GC∇, a variant pre-tRNA with a GC base pair inserted into the anticodon stem. This RNA was 97 nucleotides long. PolyU, a variant pre-tRNA where the anticodon and most of the intron were replaced with the sequence U_6GU_8. This RNA was 90 nucleotides long. Faint secondary bands were present in the sequencing ladders. This was due to 3′ end heterogeneity as was described previously (Reyes & Abelson, 1987).

cross-linking reaction, slightly more cross-linked product was formed in comparison with wild-type pre-tRNA (data not shown). The cross-linked products of both variants migrated the same as the cross-linked products for wild-type BrU–pre-tRNA on an SDS Laemmli gel. We have not attempted to incorporate thioUTP into these variants.

The purified, ligase-cross-linked, variant BrU–pre-tRNAs, which were labeled on their 3′ ends with ^{32}P, were randomly cleaved by the alkali hydrolysis and the products separated on a sequencing gel (Figure 7, lanes GC∇ and PolyU). The ligase-cross-linked, poly(U) BrU–pre-tRNA had a gap in the ladder corresponding to a cross-link to U39. The poly(U) variant did not show any discrete gaps in the alkali ladder beyond this point. This indicated that cross-links were forming to many different positions within the intron. The ligase-cross-linked, GC∇ BrU–pre-tRNA had a gap in the ladder at a position one nucleotide higher than the corresponding cross-link to U39 for the wild-type pre-tRNA. Cross-links at

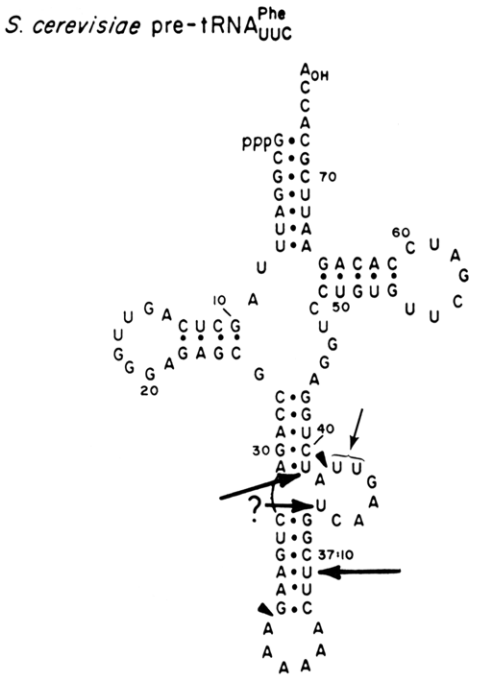

FIGURE 8: Location of the ligase cross-links on the pre-tRNA. The major cross-links mapped to U37:9, U39, and U37:13 (large arrows), and a minor cross-link mapped to U37:18/19 (small arrow). We are not completely confident of the cross-link to U37:13. Arrowhead, the 5′ and 3′ splice sites.

positions U37:9, U37:13, and U37:18/19 were also one nucleotide higher. This was consistent with the pre-tRNA sequence containing one additional nucleotide 3′ to the cross-links. Thus, the insertion of a GC base pair did not change the cross-links at positions U37:9, U37:13, U37:18/19, and U39.

Discussion

We have shown that the photoreactive uridine analogues BrUMP and thioUMP can be used to study the interactions between yeast tRNA ligase and pre-tRNA^{Phe}. These analogues were readily and accurately incorporated into the RNA by bacteriophage T7 RNA polymerase. It has been shown that the sequence and structural integrity of the mature domain of the pre-tRNA are important for accurate splicing (Nishikura et al., 1982; Gandini-Attardi et al., 1985; Strobel & Abelson, 1986a,b; Greer et al., 1987; Reyes & Abelson, 1988). The fact that the analogue-incorporated pre-tRNAs were accurately spliced strongly argues that these analogues were not causing major perturbations to the pre-tRNA structure and that these pre-tRNAs were good substrates for studying the binding interactions with ligase. Partially thiolated tRNA^{Phe} (five to six modified uridine residues per tRNA molecule) will also bind phenylalanyl-tRNA synthetase about as well as unmodified tRNA^{Phe} (Baltzinger et al., 1979).

The major cross-links on the BrU-incorporated pre-tRNA mapped to positions U37:9, U37:13, and U39 (Figure 8). A minor site was located at either U37:18 or U37:19. There may be other minor sites of cross-linking as well. However, our assay was probably not sensitive enough to detect them because the small number of molecules containing these cross-links would cause only a slight decrease in the intensity of the bands in the hydrolysis ladder at the position(s) corresponding to the cross-link(s). We were not able to accurately map the ligase cross-links on the thioU-incorporated pre-tRNA. However, the hydrolysis ladders of ligase-cross-linked thioU–pre-tRNA were similar to those obtained with the ligase-cross-linked BrU–pre-tRNA.

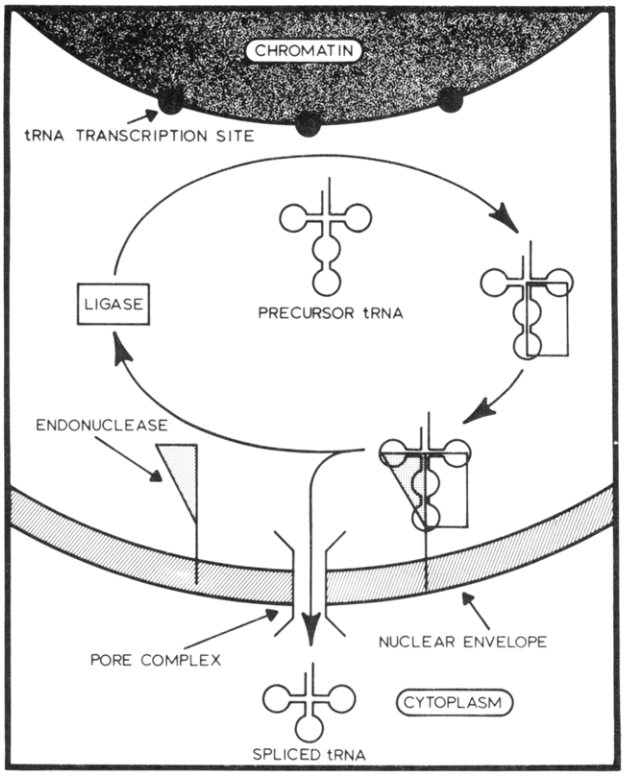

FIGURE 9: Model for pre-tRNA splicing within yeast. The details of the model are described in the text. A similar model has been independently proposed by C. Greer (personal communication).

The fact that the pre-tRNA was processed and that cross-linking was inhibited by the addition of $tRNA^{Phe}$ or U–pre-tRNA, and not by rRNA, indicated that these cross-links were the result of specific binding interactions between ligase and pre-tRNA and were not due to random collisions. While the experimental evidence was consistent with these nucleotides being in close proximity to regions of the ligase, we do not know whether or not these particular bases were actually recognized by ligase as binding elements (see below).

Cross-links were conspicuously absent from most of the mature domain of the pre-tRNA. This was a surprising result because it is generally thought that ligase mostly recognizes the mature domain of the pre-tRNA instead of the variable sequence and structure of the intron. Also, most of the splicing-defective tRNA mutants that have been analyzed have changes within the mature domain of the tRNA [see Szekely et al. (1988)]. However, the contacts within the central domain may not have involved, or have been close to, uridine analogue residues that were capable of forming cross-links with ligase.

The GC∇ variant pre-tRNA, where the anticodon stem and intron were rotated 33° and elongated by one base pair relative to the wild-type pre-tRNA, cross-linked to ligase only slightly less than wild-type pre-tRNA, and the cross-links were all to the same positions within the pre-tRNA. The poly(U) variant, where the intron was replaced with mostly uridines, cross-linked to ligase slightly better. In this case, the cross-links seemed to be located at various positions within the intron. These results indicated that there was a fair amount of flexibility in the interactions between ligase and the precursor but that the interactions were mostly with the anticodon stem and intron. Most of the uridine residues within the anticodon arm and intron were located within the intron. This dictated in part where cross-links could form. However, it is important to note that the major cross-links were all located near the splice sites. This would be expected because ligase must bind portions of the pre-tRNA in these regions in order to align the ends for the subsequent ligation reactions. Since ligase has three distinct enzymatic activities, it will be interesting to determine whether the cross-links on ligase correspond to a single binding site or to three separate binding sites. Recent data indicate that each catalytic site is located on a different domain of ligase (Qi and Abelson, unpublished results). It remains to be seen whether these catalytic sites are brought together by the tertiary structure of ligase to form a single binding site.

Cross-links within the intron of the pre-tRNA might not be expected because the substrate for ligase has first had the intron removed by endonuclease. However, this result was consistent with a model we have for pre-tRNA splicing, in vivo. The bases for this model are as follows: First, ligase has the properties of both a peripheral membrane protein and a soluble protein (Greer et al., 1983; Phizicky et al., 1986). Endonuclease has the characteristics of an integral membrane protein (Peebles et al., 1983; Green and Abelson, unpublished results). Second, Greer (1986), using competition assays, found a functional association between ligase and endonuclease and that the endonuclease activity was more efficient in the presence of ligase (C. Greer, personal communication). Finally, a cytological study, using antibodies to yeast ligase in an immune electron microscopic analysis, has determined that the primary location of ligase within yeast is at the nuclear membrane, near the nuclear pores. Cells which overexpress ligase do not show an increase in staining of the nuclear envelope, which indicates that there is a limited number of binding sites available for ligase (Clark & Abelson, 1987). From these data, it appears that splicing occurs on the nuclear envelope (Clark & Abelson, 1987). Moreover, there is also a secondary location of ligase within the cell that is 100–200 nm within the nucleoplasm in an annular region that is parallel to the nuclear membrane (Clark & Abelson, 1987).

On the basis of these observations, we propose a model for the in vivo splicing reaction of yeast pre-tRNAs (Figure 9). In this model, the newly synthesized and partially modified pre-tRNA is bound to a ligase molecule within a space that is approximately 200 nm from the nuclear membrane. Ligase escorts the precursor to the nuclear membrane, during which time modifications of the tRNA residues may occur, where a ternary complex is formed with endonuclease. Splicing then takes place within this complex, and the spliced tRNA is transported through the nuclear pore into the cytoplasm. Our observations that the major cross-links with ligase are within or near the intron of the pre-tRNA support this model of the pre-tRNA transport function of ligase.

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Registry No. BrUTP, 3398-50-3; thioUTP, 31556-28-2; endonuclease, 9055-11-2; tRNA ligase, 106640-78-2.

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Altered Catalytic Activity of and DNA Cleavage by DNA Topoisomerase II from Human Leukemic Cells Selected for Resistance to VM-26†

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ABSTRACT: The simultaneous development of resistance to the cytotoxic effects of several classes of natural product anticancer drugs, after exposure to only one of these agents, is referred to as multiple drug resistance (MDR). At least two distinct mechanisms for MDR have been postulated: that associated with P-glycoprotein and that thought to be due to an alteration in DNA topoisomerase II activity (at-MDR). We describe studies with two sublines of human leukemic CCRF-CEM cells ≈50-fold resistant (CEM/VM-1) and ≈140-fold resistant (CEM/VM-1-5) to VM-26, a drug known to interfere with DNA topoisomerase II activity. Each of these lines is cross-resistant to other drugs known to affect topoisomerase II but not cross-resistant to vinblastine, an inhibitor of mitotic spindle formation. We found little difference in the amount of immunoreactive DNA topoisomerase II in 1.0 M NaCl nuclear extracts of the two resistant and parental cell lines. However, topoisomerase II in nuclear extracts of the resistant sublines is altered in both catalytic activity (unknotting) of and DNA cleavage by this enzyme. Also, the rate at which catenation occurs is 20–30-fold slower with the CEM/VM-1-5 preparatons. The effect of VM-26 on both strand passing and DNA cleavage is inversely related to the degree of primary resistance of each cell line. Our data support the hypothesis that at-MDR is due to an alteration in topoisomerase II or in a factor modulating its activity.

We have described a human leukemic cell line selected for resistance to VM-26 (CEM/VM-1)[1] that has an "atypical" multiple drug resistance (at-MDR) phenotype (Danks et al., 1987; Beck et al., 1987). Cells with this phenotype are cross-resistant to many of the same natural product drugs used to select P-glycoprotein-overexpressing MDR cells, but other characteristics of these cells differ markedly from those of the

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[1] Abbreviations: MDR, multiple drug resistance, multiple drug resistant; at-MDR, MDR associated with altered topoisomerase II activity; VM-26, teniposide [9-[4,6-*O*-(2-thenylidene)-β-D-glucopyranosyl]-4′-demethylepipodophyllotoxin]; VP-16, VP-16-213, etoposide [9-(4,6-*O*-ethylidene-β-D-glucopyranosyl)-4′-demethylepipodophyllotoxin]; DMSO, dimethyl sulfoxide; mAMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; CEM/VM-1, cloned CEM subline, ≈50-fold resistant to VM-26; CEM/VM-1-5, CEM/VM-1 subline, ≈140-fold resistant to VM-26; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BCIP, 5-bromo-4-chloro-3-indolyl phosphate, *p*-toluidine salt; BSA, bovine serum albumin; Pgp, P-glycoprotein; VCR, vincristine.

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