In Vitro Biosynthesis of Functional *Escherichia coli* su_3^+ Tyrosine Transfer RNA†

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ABSTRACT: Biologically active *Escherichia coli* su_3^+ tyrosine tRNA can be synthesized in vitro by transcription of phage ϕ80psu_3^+ DNA with *E. coli* RNA polymerase and treatment of the resulting precursor tRNA with a partially enriched preparation of processing nucleases. Results from competition hybridization analyses indicate that there is preferential expression of the single tRNA gene which occurs in this DNA. From 2 to 15% of the RNA formed was tRNA-like RNA, although the DNA corresponding to mature $tRNA^{Tyr}$ constitutes only 0.2% of the phage chromosome. Analysis of the transcription products by acrylamide gel electrophoresis revealed the occurrence of several species of tRNA-containing RNA with molecular weights which ranged from 55 000 to 200 000. However, when the transcripts were analyzed under denaturing conditions, only one tRNA species of 55 000 daltons could be detected. This pre-tRNA is about 50 nucleotides larger than the largest su_3^+ $tRNA^{Tyr}$ precursor known to occur in vivo and is markedly smaller than the pre-tRNA species produced in other in vitro systems. The occurrence of a single precursor tRNA and the finding that the termination factor, ρ, influences the yield of tRNA, but, not the size of the monomeric gene product, suggests that transcription of the su_3^+ gene may be accurate. This view is supported by results which show that the pre-tRNA is a suitable substrate for at least two of the base modifying enzymes and for posttranscriptional processing RNases. These conclusions come from the findings that: (1) the pre-tRNA can be converted to 4S tRNA by treatment with a partially enriched preparation of soluble enzymes and, (2) ribothymidine and 2′-*O*-methylguanosine can be formed when the pre-tRNA is incubated with tRNA methylase and *S*-adenosylmethionine. tRNA synthesized and processed in this manner is biologically active as determined by its ability to accept tyrosine in an in vitro aminoacylation assay.

It has become clear in recent years that the biosynthesis of transfer RNA occurs by a complex, multistep process. In the first step, tRNA genes are transcribed by DNA-dependent RNA polymerase to yield precursor molecules which are, in every case studied thus far, larger than the final product and devoid of the modified nucleosides characteristic of the mature tRNA. Once transcription has been completed—and possibly starting sooner—a variety of site-specific processing RNases and base modifying enzymes act to convert the precursors to mature 4S tRNA (see reviews by: Altman and Robertson, 1973; Schäfer and Söll, 1974; Smith, 1976).

Nucleolytic processing of the precursor species occurs extremely rapidly in vivo, however, and it has only been possible to detect and characterize such intermediates in bacteria by using very rapid pulse-labeling techniques (Altman, 1971; Altman and Smith, 1971) or mutants which are conditionally defective for a specific processing activity (Schedl and Primakoff, 1973; Sakano et al., 1974b). While precursors obtained in this way have proven to be very valuable in analyzing tRNA gene structure and the maturation process (Smith, 1976), it has not yet been established that any of the precursors described thus far correspond to a primary gene product. Clearly, it will be necessary to obtain uncleaved, unmodified precursor tRNAs before our understanding of the process and products of tRNA biosynthesis is complete. To this end and with a long range view toward establishing a completely defined in vitro system capable of producing biologically active tRNA, a number of laboratories have been studying the transcription of tRNA genes in vitro.

At this writing there have been a number of reports on the successful in vitro transcription of the *E. coli* su_3^+ tyrosine tRNA gene contained in the DNA of the transducing phage, ϕ80su_3^+, using preparations of *E. coli* RNA polymerase. However, it is not clear if the tRNA-like species formed were products of accurate transcription. Where dispersity and sizing experiments have been done, the tRNA-containing species have been found to be heterogeneous in some cases and, in every case, considerably larger than the largest su_3^+ $tRNA^{Tyr}$ precursor known to occur in vivo (Altman and Smith, 1971). The in vivo precursor isolated by Altman and Smith is 41 nucleotides longer at the 5′ terminus than mature $tRNA^{Tyr}$ and has three extra nucleotides at the 3′ end. The precursor starts with pppG at the 5′ end indicating that that portion of the molecule corresponds to the original 5′ terminus of the primary transcript. Overall, this pre-tRNA contains about 120 nucleotides and has a molecular weight of about 43 000.

Daniel et al. (1970) reported that the tRNA-like RNA produced in their in vitro system was polydisperse when analyzed by sucrose gradient centrifugation and had a sedimentation value of 7-8 S. Using gel electrophoresis to analyze the in vitro derived su_3^+ transcripts, Ikeda (1971) observed that three species of tRNA-like RNA had been formed with molecular weights of 2.8×10^5, 1.7×10^5, and 8×10^4. The smallest of these species was about twice the size of the in vivo Altman–Smith precursor. In that report it was suggested that the occurrence of multiple tRNA-containing transcripts might reflect the presence of multiple initiation or termination sites. Bikoff and her colleagues (Bikoff and Gefter, 1975; Bikoff et al., 1975) have recently reported that the largest pre-tRNA formed in their reconstituted transcription system is at least 100 nucleotides longer at the 3′ end than the in vivo precursor. Finally, Küpper et al. (1975) reported that transcription of a fragment of su_3^+ tDNA prepared from intact phage DNA with restriction nucleases resulted in the formation of a pre-tRNA species containing 175 nucleotides or about 50 nucleotides

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more than the in vivo precursor. In this case, the size of the product corresponds precisely to the distance between the promoter and the end of the DNA fragment.

Thus, while some of the in vitro su_3^+ precursors and multimeric precursors to other tRNAs can be converted to 4S RNA by treatment with crude preparations of processing enzymes (Zubay et al., 1971; Nierlich et al., 1973; Daniel et al., 1975; Bikoff and Gefter, 1975) and certain of the species matured in this way have been shown to be biologically active (Küpper et al., 1975; Zeevi and Daniel, 1976), the number and size(s) of the primary products produced from the monomeric su_3^+ tRNA transcription unit are still unclear. For this reason, many questions also remain about the early events in the maturation process.

In this report, we present results from studies of an in vitro transcription system which produces only one su_3^+ tRNA precursor of about 175 nucleotides. This precursor can be modified and processed in vitro to yield functional tyrosine tRNA.

Experimental Section

Materials

Yeast extract and bactotryptone were obtained from Difco; dextran sulfate was from Pharmacia; poly(ethylene glycol) 6000 was from Baker Chemical Co.; cesium chloride (biological grade) was from Schwarz/Mann; ribonucleoside triphosphates (disodium salts) were from P-L Biochemicals; and acrylamide and bisacrylamide were from Eastman Kodak. DEAE-cellulose (Whatman-52) was obtained from Reeve-Angel and prewashed with 0.5 N HCl and 0.5 N NaOH. Calf thymus DNA, electrophoretically purified DNase I (RNase-free), and pancreatic RNase A (five times crystallized) were obtained from Worthington Biochemical Corp. The RNase was incubated for 10 min at 90–95 °C at a concentration of 10 mg/mL in 0.15 M NaCl–0.015 M sodium citrate, pH 7.0, to inactivate any possible DNase activity. RNase CB (a mixture of T_1 and T_2 RNases) was obtained from Calbiochem. Nitrocellulose filters for nucleic acid hybridization experiments were purchased from Schleicher and Schuell (Selectron filters, 0.45 μm). [^{32}P]Orthophosphate (carrier-free) was obtained from ICN. ^{125}I as sodium iodide (carrier-free, low pH), [5,6-^{3}H]UTP (30–35 Ci/mmol), [*methyl*-^{3}H]-*S*-adenosylmethionine (3–12.6 Ci/mmol), [3,5-^{3}H]tyrosine (38.3 Ci/mmol), and [^{14}C]ATP (53.4 mCi/mmol) were purchased from New England Nuclear Corp. The [^{3}H]UTP and [^{14}C]ATP were adjusted to specific activities of 50 mCi/mmol and 10 mCi/mmol, respectively, before use.

Methods

Bacteria, Phage, and Culturing Conditions. Phages ϕ80psu$_3^+$ (MRC-Cambridge) and ϕ80 (provided by M. Gefter and M. Gottesman, respectively) were grown on *E. coli* CA 274 (Hfr C $lac^-_{125\,amber}$ $tryp^-_{amber}$ su^-) supplied by M. Gefter. *E. coli* PR-7 (thr^-, leu^-, RNase I^-, Pnp^{def}, gal^-, mal^-, λ^R, xyl^-, mtl^-) obtained from A. Reiner, was used as the source of RNA polymerase and the termination factor ρ. *E. coli* B was used for the preparation of tRNA and *E. coli* MRE-600 (RNase I^-) was the source of tRNA methylase and crude tRNA processing enzymes. *E. coli* CA 274 was grown in medium containing per liter: 20 g of bactotryptone, 15 g of yeast extract, and 5 g of NaCl. Culturing of *E. coli* PR-7 was performed in medium that contained per liter: 10 g of bactotryptone, 5 g of yeast extract, and 10 g of NaCl. *E. coli* MRE 600 was cultured in medium that contained per liter: 5.6 g of KH_2PO_4, 28.9 g of K_2HPO_4, 10 g of yeast extract, 5 g of NaCl, 1% glucose. All culturing was done at 37 °C in a rotary-action shaking incubator or in a 14-L New Brunswick Microferm fermentor. When the fermentor was used, the stirrer was rotated at 200 rpm and the medium aerated at a rate of 8 L per min; antifoam was added as needed.

Preparation of Bacteriophage. Cultures of *E. coli* CA 274 were infected with phage at a multiplicity of 0.1 when the turbidity was between 0.3 and 0.5 A_{650} (3–5 × 10^8 cells/mL; full growth corresponded to A_{650} = 0.9). After lysis, 0.05 volume of chloroform was added to ensure that lysis was complete and the lysate was clarified either by centrifugation at 10 000*g* for 15 min in a fixed angle rotor or continuous flow centrifugation at 10 000*g* at a flow rate of 200 mL/min (Sorvall SZ-14). Phage were isolated from the supernatant by the dextran sulfate–poly(ethylene glycol) extraction procedure of Albertsson (1967). The resulting phage precipitate was solubilized in buffer that was 0.01 M Tris[2]-HCl, pH 7.5, 5 mM $MgCl_2$, 0.1 M NaCl and incubated with DNase at a final concentration of 10 μg/mL for 1 h at room temperature. Solid CsCl (0.776 g per mL of suspension) was added to the phage suspension to adjust the density to 1.5 g/mL and the suspension centrifuged in a Beckman SW 27 rotor at 60 000*g* for 20 h at 15 °C. After tapping the tube from the bottom, the phage-containing fractions (identified visually or spectrophotometrically) were pooled and dialyzed against 0.01 M Tris-HCl, pH 7.5, 0.05 M NaCl, mM EDTA.

Preparation of Phage DNA. Sodium dodecyl sulfate was added to the phage preparation to a final concentration of 0.5% and the phage incubated at 65 °C for 10–15 min. KCl was added to a final concentration of 0.5 M, the solution chilled on ice, and the resulting potassium–sodium dodecyl sulfate precipitate removed by centrifugation at 4 °C. The supernatant was dialyzed against 0.01 M Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 M NaCl, extracted three times with equal volumes of 90% phenol and one time with an equal volume of chloroform–isoamyl alcohol (24:1). The DNA was dialyzed against 0.1 × SSC (SSC = 0.15 M NaCl–0.015 M sodium citrate, pH 7.0) and stored at 4 °C over chloroform.

Preparation of ^{32}P- and ^{125}I-Labeled E. coli tRNA. The procedure used for the preparation of [^{32}P]tRNA will be described elsewhere (Kitchingman and Fournier, in preparation). The specific activity was adjusted to 0.6–1.0 × 10^6 cpm per μg. [^{125}I]tRNA was prepared from acrylamide gel purified tRNA by the method of Prensky (1976) except that the labeled tRNA was repurified by chromatography on DEAE-cellulose. The specific activity was adjusted to 4–6 × 10^5 cpm per μg.

Preparation of RNA Polymerase. DNA-dependent RNA polymerase was prepared from *E. coli* PR-7 by the method of Berg et al. (1971) through the DEAE-cellulose chromatography step. In different preparations, the enzyme had a specific activity of 100–140 units per mg with ϕ80su$_3^+$ DNA where 1 unit is equal to the incorporation of 1 nmol of UTP per 10 min at 37 °C under the assay conditions described below. The ratio of activities on poly[d(AT)] and ϕ80su$_3^+$ templates ranged from 5:1 to 3:1 for different preparations. No RNase activity was detected when the enzyme was incubated with [^{32}P]tRNA under the conditions used for transcription and the release of acid-soluble radioactivity determined by precipitation with trichloroacetic acid.

Preparation of ρ. The termination factor ρ was prepared from *E. coli* PR-7 by a modification (B. de Crombrugghe,

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl. SSC, 0.015 M sodium citrate, 0.15 M sodium chloride, pH 7.0.

personal communication) of the method of Roberts (1969). Following ammonium sulfate precipitation of the pooled phosphocellulose fractions, the protein was solubilized and dialyzed in buffer that was 0.05 M potassium phosphate, pH 7.5, 0.1 mM dithiothreitol, 0.1 mM EDTA, and 5% glycerol. One-half-milliliter portions of the sample (~5 mg of protein) were layered on a 10–30% glycerol gradient in the same buffer and centrifuged in a Beckman SW 41 rotor for 23 h at 41 000 rpm and 4 °C in a Beckman L-2 65-B centrifuge. After bottom-tapping the gradient, the fractions which contained ρ activity were located using the standard transcription assay with $\phi 80su_3^+$ DNA. ρ activity was detected in fractions 6–12 of the 30 fractions collected and was coincident with an almost completely resolved A_{280} peak. Analysis of the protein by sodium dodecyl sulfate–acrylamide gel electrophoresis (0.1% sodium dodecyl sulfate–7.5% gel) revealed the preparation to be better than 90% pure with only one band of contaminating protein. The molecular weight of ρ was estimated to be 48 000 using H1 and H4 bovine histones (obtained from S. Maxwell and M. Fischer) as markers. In a saturation assay, 1 μg of ρ was required to obtain maximal depression (65%) of transcription with 1.2 units of RNA polymerase under the assay conditions described below. About 400 μg of ρ was obtained per gradient tube.

Preparation of tRNA Methylase, Pre-tRNA Processing Enzymes, and Aminoacyl-tRNA Synthetase. Crude tRNA methylase was prepared from *E. coli* MRE-600 essentially by the method described earlier for bulk aminoacyl-tRNA synthetase (Fournier and Peterkofsky, 1975). Fresh cells were disrupted by grinding with alumina and extracted with 3 volumes of buffer that was 0.05 M Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM KCl, and 5 mM β-mercaptoethanol. The extract was centrifuged twice at 12 000g for 30 min in a Sorvall SS-34 rotor and the resulting supernatant centrifuged at 100 000g for 90 min in a Beckman No. 65 rotor. The high-speed supernatant from 12 g of cells was applied to a column of DEAE-cellulose (1 × 26 cm) preequilibrated in buffer that was 0.02 M potassium phosphate, pH 7.5, 1 mM $MgCl_2$, 5 mM β-mercaptoethanol, and 10% glycerol. After washing with 2 column volumes of the same buffer, the methylase was eluted with buffer that was: 0.25 M potassium phosphate, pH 6.5, 1 mM $MgCl_2$, 5 mM β-mercaptoethanol, and 10% glycerol. The fractions which contained the bulk of the protein were combined and the protein was precipitated by the slow addition of solid ammonium sulfate to 75% saturation (48 g/100 mL, 4 °C). Ammonium carbonate (1 g/100 mL) was added with the ammonium sulfate to stabilize the pH at about neutrality. After stirring for 30 min, the protein precipitate was recovered by centrifugation at 15 000g for 30 min (Sorvall SS-34 rotor), solubilized in 2 mL of buffer that was 0.05 M Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, 5 mM KCl, 5 mM β-mercaptoethanol and 10% glycerol, and dialyzed against the same buffer. After adjusting the glycerol concentration to 50%, the preparation was stored at −20 °C. The final concentration of protein was 22 mg per mL.

Freshly prepared high-speed supernatant which had been passed through a DEAE column equilibrated in the 0.25 M phosphate buffer (see above) was used as the source of crude tRNA processing nuclease.

Unfractionated aminoacyl-tRNA synthetase was prepared by the DEAE-cellulose chromatography procedure described above for methylase except that the material from the DEAE-cellulose column was used without further purification. Glycerol was added to the pooled fractions to a final concentration of 50% and the enzyme stored at −20 °C.

Transcription of Phage DNA. Phage DNA was transcribed in a reaction mixture that contained per mL: 0.04 M Tris-HCl, pH 8, 150 μg of DNA, 16 units of RNA polymerase, 0.4 mM each CTP, ATP, GTP, and [^{3}H]UTP (50 mCi/mmol) or unlabeled UTP as indicated, 0.01 M $MgCl_2$, 0.01 M β-mercaptoethanol and, except where indicated, 0.06 M KCl. RNA synthesis was measured by precipitating a portion of the reaction mixture with 5% trichloroacetic acid and collecting the acid-insoluble material on glass fiber or cellulose nitrate filters (Millipore Corp.). After drying at 100 °C, the extent of [^{3}H]UTP incorporation was determined by liquid scintillation counting in toluene–Liquifluor (24:1, New England Nuclear Corp.) in a Beckman LS-250 scintillation spectrometer. Counting efficiency was 10%.

Isolation of in Vitro Synthesized RNA. Transcription was terminated by the addition of 20 μL of 25% sodium dodecyl sulfate, and 1 mL of 0.4 M ammonium acetate, pH 4.9, per mL of reaction mixture. The RNA was extracted twice with an equal volume of 90% phenol, dialyzed against water and, if necessary, concentrated by flash evaporation. Where indicated, 2.5 A_{260} of rat liver tRNA was added to the reaction mixture before phenol extraction and the RNA was recovered after extraction by precipitation with 2–3 volumes of cold (−20 °C) 95% ethanol. Samples were treated with DNase (50 μg/mL) either at the end of the incubation or after recovery of the RNA.

RNA:DNA hybridization assays were done in solution with DNA filters essentially according to the method described by Gillespie and Gillespie (1971). When preparing DNA filters, 600 μg of DNA was first diluted to a volume of 2.5 mL with water and then denatured by the addition of 1.5 mL 1 M KOH. After incubation at 37 °C for 1 h, the DNA was quickly chilled to 4 °C and the following added in quick succession: 0.2 mL of 1 M Tris-HCl, pH, pH 7.0, 22.3 mL of H_2O, 1.5 mL of 1 M HCl (added dropwise while stirring); and 12 mL of 10 × SSC. Final volume was 40 mL. The denatured DNA solution was then passed through a 47-mm membrane filter (Schleicher and Schuell) at atmospheric pressure. The filters were presoaked in 3 × SSC and supported in a Millipore filtration device. The filters were then washed with 15 to 20 mL of 3 × SSC, air-dried overnight, and then dried under vacuum at 80 °C for at least 3 h. Filters (6 mm) were cut from the large filters with a paper punch. The amount of DNA bound per filter was first determined chemically (Meijs and Schilperoort, 1971) and then later routinely by spectrophotometric analysis of the initial solution of DNA and resulting filtrate. Usually, 9–12 μg of DNA was bound per 6-mm filter.

In the saturation hybridization assays, DNA filters were incubated in 9 × 30 mm shell vials (A. H. Thomas) for 16–18 h at 37 °C in 250 μL of 3 × SSC–50% formamide with graded amounts of [^{32}P]- or [^{125}I]tRNA. The filters were then washed in 2 × SSC for 20 min, incubated with pancreatic ribonuclease (50 μg/mL) at room temperature for 20 min, and washed again with 2 × SSC for 20 min. The filters were dried and the bound radioactivity was measured by scintillation spectrometry.

In competition hybridization assays, graded amounts of transcription product were mixed directly with a saturating amount of [^{32}P]- or [^{125}I]tRNA (usually 2.5 μg) and the effect of the test RNA on the binding of authentic, labeled tRNA was determined.

Polyacrylamide Gel Electrophoresis. In vitro synthesized RNA was fractionated by acrylamide gel electrophoresis under both denaturing and nondenaturing conditions. When analyzed under nondenaturing conditions, the RNA was fractionated in 5% gels (0.6 × 8 cm) according to the method of Loening (1967). Where indicated, denaturing conditions were realized

by using 8% acrylamide gels that were 98% formamide (0.6 × 10 cm). Fifty to seventy micrograms of RNA in 25 μL of the electrophoresis buffer that was also 5% sucrose (or 85% formamide in the case of the formamide gels) was applied per gel and separated by electrophoresis (5 mA/gel) in buffer that was 0.04 M Tris-acetate, 0.02 M sodium acetate, 2 mM sodium EDTA, pH 7.8, at 5 °C. Bromophenol blue was used as a marker. When sizing the transcription products, the RNA standards used included: 16S, 6S, 5S, and 4.5S RNAs, leucine $tRNA_1$, and bulk 4S RNA. Following electrophoresis, the gels were cut into 1-mm slices with a Mickle gel slicer. The RNA was recovered from the gel slices by overnight extraction in 250 μL of 6 × SSC at 37 °C. The fractionation pattern was determined by precipitating 50 μL of extract from each gel with trichloroacetic acid and measuring the acid-insoluble radioactivity by scintillation counting as described above. When assaying for tRNA-containing transcription products, 150 μL of extract was analyzed by competition hybridization analysis.

Methylation and Processing of RNA. RNA methylation assays were performed in reaction mixtures that contained per 250 μL: 50 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 10 mM β-mercaptoethanol, 10 mM ATP, 20 M [*methyl*-^{3}H]-*S*-adenosylmethionine (12.3 Ci/mmol), 2.2 mg of methylase, and 0.86 A_{260} of methyl-deficient tRNA from methionine-starved *E. coli* W-1305 U^- (Fournier and Peterkofsky, 1975) or 70 μg of in vitro synthesized $\phi80su_3^+$ or $\phi80$ RNA. Incubations were carried out at 37 °C for 20 min. The extent of methylation was determined by precipitating a portion of the reaction mixture with trichloroacetic acid and measuring the acid-insoluble activity as described above for the transcription assays. When the RNA was to be analyzed further, the reaction was stopped by the addition of 0.1 volume of 20% potassium acetate, pH 4.5, and the RNA extracted with phenol and recovered as described above for the preparation of in vitro synthesized RNA. Nucleolytic processing of pre-tRNA was done by incubating 20–50 μg of unfractionated su_3^+ RNA in 250 μL of a reaction mixture that contained per mL: 20 μmol of Tris-HCl, pH 7.5, 2 μmol of ATP, 6 μmol of $MgCl_2$, and 3.4 mg of crude processing enzyme. After incubation for 20 min at 37 °C the RNA was recovered by ethanol precipitation and centrifugation as described above.

Aminoacylation Procedure. $tRNA^{Tyr}$ was aminoacylated in 500 μL of a reaction mixture that was 50 mM Tris-HCl, pH 7.5, 18 mM $MgCl_2$, 4 mM ATP, 10 mM β-mercaptoethanol, 5 mM NH_4Cl, 0.6–0.8 A_{260} su_3^+ RNA, 2 μM [^{3}H]tyrosine (38.3 Ci/mM), and sufficient aminoacyl-tRNA synthetase to effect complete acylation in 15 min at 37 °C, as determined in a preliminary experiment with bulk tRNA. The extent of aminoacylation was determined by measuring the incorporation of label into acid-insoluble material as described before for the transcription and methylation assays.

Compositional Analysis of su_3^+ RNA. Identification of [^{3}H]methyl-labeled nucleotides in the in vitro modified RNA was done by two-dimensional thin-layer chromatography on cellulose as described by Ohashi et al. (1974). Aminoacylated tRNA was analyzed by electrophoretic separation of the products released by treatment with a mixture of RNase CB and A. Following aminoacylation, 0.1 volume of 20% potassium acetate, pH 4.5, was added to the reaction mixture and the RNA recovered by chromatography on a small DEAE column followed by alcohol precipitation. The RNA was then solubilized in 12 μL of 20 mM ammonium acetate, pH 4.5, containing 12 μg of RNase CB and 1.25 μg of RNase A. After incubating overnight at room temperature, the reaction mixture was applied at the center of a 3 × 50 cm strip of cellulose acetate which had previously been soaked in 0.5% acetic acid–6 M urea–8 mM Na_2EDTA. The digestion products were separated by electrophoresis at 3000 V for 1.5 h in pyridine-acetate buffer (0.5% pyridine–5% acetic acid), pH 3.5. Radiolabeled adenosine, tyrosine, AMP, and tyrosyladenosine (prepared by RNase treatment of aminoacylated tyrosine-tRNA) were used as standards. After air-drying, the strips were cut into 1-cm portions and the radiolabeled products located by scintillation spectrometry.

Results

Detection of su_3^+ tRNA. su_3^+ $tRNA^{Tyr}$ can be detected in the in vitro transcription products derived from $\phi80su_3^+$ DNA by: RNA:DNA competition hybridization analysis (Daniel et al., 1970; Ikeda, 1971), fingerprinting (Manley et al., 1973; Daniel et al., 1975), and in vitro suppression assays (Zubay et al., 1971). Because the fingerprinting and biological activity assays require highly enriched and functionally competent tRNA, respectively, they are less useful than a competition hybridization method in identifying and quantitating the occurrence of tRNA in a mixture of unfractionated primary transcripts. For this reason, we elected to analyze the in vitro transcription products for tRNA by a hybridization assay. Here, the ability of transcripts to compete with authentic tRNA in hybridizing to complementary DNA sequences (tDNA) is determined. To obtain maximal sensitivity, an indirect, simultaneous, hybridization–competition assay procedure was used. In this procedure, the extent of hybridization of a saturating amount of pure [^{32}P]tRNA was determined in the presence of increasing amounts of RNA synthesized in vitro. At 50% competition, it is assumed that approximately equivalent amounts of in vitro synthesized tRNA and [^{32}P]-tRNA are present in the reaction mixture. While this assumption probably holds for competing species of about the same size, results from recent experiments by Beckmann and Daniel (1974a) indicate that hybrids formed with larger, precursor RNAs can be more stable than those composed of the corresponding smaller, mature species. In such cases, estimates of the content of a particular RNA species will be somewhat inflated. In spite of this deficiency, the method is still useful in detecting the presence of $tRNA^{Tyr}$ sequences in a mixture of transcription products and in evaluating the relative effects of different reaction conditions on tRNA gene expression.

The first step in establishing the assay conditions for a competition hybridization analysis is to determine the amount of radiolabeled tRNA required to saturate all complementary DNA sequences. The results of such a saturation hybridization analysis are shown in panel A of Figure 1. All tDNA sequences in the $\phi80su_3^+$ DNA were saturated when the input of tRNA was in excess of 1.5 μg. From direct aminoacylation assays of the [^{32}P]tRNA and competition hybridization experiments with unlabeled, unfractionated tRNA of known tyrosine acceptor activity, and partially purified tyrosine tRNA, it was determined that at saturation 0.59 ng of $tRNA^{Tyr}$ was bound per μg of DNA. In a series of such assays, from 0.06 to 0.085% of the su_3^+ DNA was hybridized with tRNA on a weight-weight basis. This value corresponds to one tRNA gene per phage chromosome and is in good agreement with the hybridization results reported by Landy et al. (1967) who first demonstrated the occurrence of a single $tRNA^{Tyr}$ gene in this particular $\phi80$-derived su_3^+ transducing phage. As expected, no tRNA was bound to DNA from the parent phage, $\phi80$, or to calf thymus DNA (latter result not shown).

In the competition hybridization experiments, a saturating amount of [^{32}P]tRNA was mixed with graded amounts of test

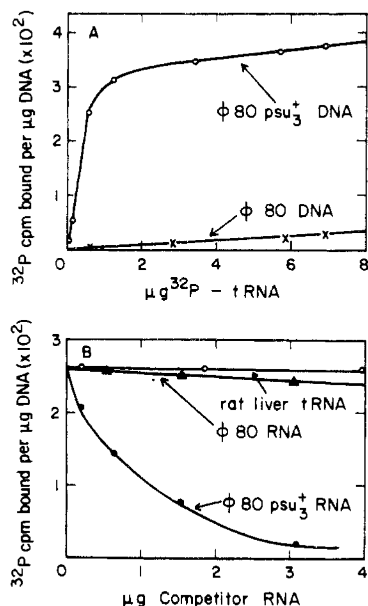

FIGURE 1: Competition hybridization analysis of in vitro synthesized ϕ80psu$_3^+$ RNA. Panel (A) Saturation hybridization of ϕ80psu$_3^+$ DNA with ^{32}P-labeled *E. coli* tRNA. Filters containing immobilized DNA (9.5 µg of ϕ80su$_3^+$ DNA or 13.2 µg of ϕ80 DNA) were incubated with the indicated amounts of uniformly labeled, chased [^{32}P]tRNA (6.1×10^5 cpm/µg) prepared from noninfected *E. coli*. After removing the unbound tRNA by washing and treating the filters with RNase, the amount of tRNA hybridized was determined by scintillation spectrometry. (—O—) tRNA bound to ϕ80su$_3^+$ DNA; (—X—) tRNA bound to ϕ80 DNA. The tRNA^{Tyr} gene dosage was calculated using 30.0×10^6 as the molecular weight of ϕ80 su$_3^+$ DNA and 28×10^3 as the molecular weight of tRNA^{Tyr}. (B) Competition by in vitro synthesized ϕ80su$_3^+$ RNA for [^{32}P]tRNA^{Tyr} binding sites on ϕ80su$_3^+$ DNA. Graded amounts of test RNA transcribed in vitro from ϕ80su$_3^+$ or ϕ80 DNA or rat liver tRNA were mixed with a saturating amount of ^{32}P-labeled *E. coli* tRNA (2.5 µg of bulk tRNA equivalent to 20 ng of tRNA^{Tyr}) and the effect on binding of tRNA^{Tyr} to ϕ80su$_3^+$ DNA was determined. Filters contained from 9 to 12 µg of DNA; the specific activity of the [^{32}P]tRNA was 4.8×10^5 cpm/µg. The results show the competition by RNA transcribed in vitro from ϕ80su$_3^+$ DNA (—●—), ϕ80 DNA (—▲—), and with rat liver tRNA (—O—). The conditions used for transcribing the phage DNAs were as given in Methods except that 24 µg of DNA and 6 units of RNA polymerase were used and the specific activity of the [^{3}H]UTP was 100 mCi/mmol. Full details of the procedures used in the preparation and analysis of the RNAs are given in Methods.

RNA and the effect of the test RNA on binding of [^{32}P]tRNA to the DNA determined. When unfractionated tRNA or tyrosine-specific tRNA was the competing species, hybridization of the [^{32}P]tRNA was reduced by 50% when equivalent amounts of labeled and unlabeled tRNA were present in the reaction mixture (data not shown). Panel B of Figure 1 shows the results obtained in a competition hybridization analysis of RNA synthesized in vitro from ϕ80 and ϕ80 su$_3^+$DNAs. An assay with rat liver tRNA was also performed as a control for nonspecific competition. It can be seen that hybridization of the [^{32}P]tRNA was effectively competed by the su$_3^+$ RNA, but, not with ϕ80 RNA or rat liver tRNA. Fifty percent competition was obtained with 800 ng of ϕ80su$_3^+$ RNA which corresponds to 20 ng of su$_3^+$ tRNA. Thus, in this preparation, the su$_3^+$ tRNA-like sequences constituted about 2.5% of the total RNA synthesized in vitro.

In Vitro Transcription of ϕ80su$_3^+$ DNA and the su$_3^+$ tRNA Gene. In the course of characterizing the in vitro transcription system, the effects on RNA synthesis of preincubation, incubation time, ionic strength, and age of the RNA polymerase were evaluated. When the effect of KCl on the synthesis of total ϕ80su$_3^+$ RNA and su$_3^+$ tRNA was examined, it was observed that, under all ionic conditions tested, the synthesis of both species proceeded rapidly for about 20 min, after which time the rate decreased markedly. Synthesis continued at the lower rate for at least another hour—the longest incubation time examined. The results in Table I show the levels of total RNA and tRNA-like RNA obtained with reaction mixtures which contained 0.06 M KCl, 0.15 M KCl, or no added KCl. More total RNA and tRNA were formed in 0.06 M KCl than under either of the other ionic conditions. For this reason 0.06 M KCl was used in all subsequent analyses of su$_3^+$ tRNA synthesis. The level of KCl determined to be optimal agrees with that observed in the su$_3^+$ transcription system developed by Beckmann and Daniel (1974b).

TABLE I: Effect of KCl on the in Vitro Transcription of ϕ80psu$_3^+$ DNA.[a]

KCl (M)	Yield of RNA (µg)	% tRNA
	21.7	3.8
0.06	23.8	6.0
0.15	16.2	3.8

[a] ϕ80psu$_3^+$ DNA was transcribed with σ-containing RNA polymerase (minus ρ) under the assay conditions described in Methods, except that the concentration of KCl was varied as indicated. After 30 min of synthesis, the reactions were terminated and the extent of RNA synthesis was determined by precipitating a portion of the reaction mixture with trichloroacetic acid and measuring the amount of [^{3}H]UTP incorporated into acid-insoluble material. The RNA was purified by phenol extraction and alcohol precipitation and the relative content of tRNA-like RNA determined by competition hybridization analysis with ^{32}P-labeled *E. coli* tRNA and ϕ80psu$_3^+$ DNA as described in Figure 1 and Methods. Details of the transcription conditions and procedures used to measure total RNA and tRNA synthesis are provided in Methods.

The activity of the RNA polymerase decreased with storage time. The loss of activity was most pronounced when transcription was performed at high ionic strength, to a lesser extent at 0.06 M KCl, and least of all in the absence of added KCl. After 2 and 4 months of storage, the specific activity of the enzyme with ϕ80su$_3^+$ DNA at 0.15 M KCl was 53% and 28% of the original activity. Over the same period of time there was a selective loss of tRNA synthesizing capability. Whereas the tRNA-like RNA usually comprised 10–15% of the total RNA synthesized with freshly prepared polymerase, the level dropped to 2–5% after several months of storage. The template activity of the DNA decreased with storage time too, although very gradually; it is not known if selective inactivation of the su$_3^+$ tDNA occurs.

Because it has been reported that preincubation of *E. coli* DNA at temperatures above 34 °C results in a significant increase in the level of ribosomal RNA and tRNA synthesized in vitro (Travers et al., 1973; Beckmann and Daniel, 1974b), the effect of preincubating the phage DNA on tRNA synthesis was also investigated with our system. Incubation mixtures complete except for polymerase were preincubated at 37 °C for 0, 5, 10, 15, and 30 min. At the end of the preincubation period, transcription was initiated by the addition of polymerase and synthesis allowed to proceed for 10 min. The levels of total RNA and su$_3^+$ tRNA were then determined. The results (not shown here) indicated that preincubation had little effect on the synthesis of either class of RNA. No significant effect was detected in two analyses while a small (20–30%) increase in the levels of both total and tRNA synthesis was observed in a third assay.

Size of the su$_3^+$ Gene Product and Effect of ρ. The size and heterogeneity of the in vitro synthesized pre-tRNA^{Tyr} was investigated to evaluate the accuracy of transcription in the

system, and, if good accuracy was indicated, to determine the size of the putative primary product of the su_3^+ gene. ^3H-labeled $\phi 80su_3^+$ RNA synthesized in the presence and absence of the termination factor ρ was fractionated by acrylamide gel electrophoresis and the tRNA-containing transcripts located by competition hybridization analysis with ^{32}P- or ^{125}I-labeled tRNA. The occurrence of a tRNA-containing transcript is reflected by a depression in the hybridization value. The results from one such set of experiments are shown in Figure 2.

The upper panel of Figure 2 shows the distribution of RNA products obtained in the absence of ρ. It can be seen that the products varied in size from 4S to species larger than 16S RNA. While much of the RNA migrated in the range of 4S to about 10S, as much as 40% of the material was larger than 16S RNA, consistent with the results expected with transcriptional read-through. The spectrum of products obtained is similar to that observed by others for in vitro transcription of $\phi 80$ and $\phi 80su_3^+$ RNA (Okamoto et al., 1970; Ikeda, 1971).

The hybridization results revealed that the bulk of the tRNA-like transcripts migrated between the 5S and 6S RNA markers and were estimated to have a molecular weight of about 55 000. Minor depressions were also observed in other regions of this and other profiles. Although most were variable and probably the result of nonspecific interactions and experimental "noise", some of the depressions were rather constant. By itself, this result suggests the possibility that the larger tRNA-containing species may arise from transcriptional inaccuracy. However, it will be shown below that most, perhaps all, of the tRNA species which are considerably larger than the major 5–6S pre-tRNA occur as a result of aggregation. In several preparations, a significant depression corresponding to 25–35% of the total tRNA-containing RNA was observed near the top of the gel and probably reflects transcriptional read-through.

The finding that the RNA synthesized in vitro contains multiple $tRNA^{Tyr}$ transcripts which differ in size suggests the possibility that transcription may not be accurate in this system and that improper initiation or termination may occur. Alternatively, the tRNA transcription unit may contain multiple initiation or termination sites. The transcription factor, ρ, is known to be required for proper termination in a variety of transcriptional units (Roberts, 1969) and has been reported to effect the yield (Beckmann and Daniel, 1974b; Ikeda, 1971) of su_3^+ tRNA synthesized in vitro. In one report (Ikeda, 1971), no tRNA could be detected in the RNA synthesized in the absence of ρ. The effect of ρ on su_3^+ RNA synthesis was also examined in our system.

The distribution of tRNA-containing products synthesized in the presence of ρ is shown in the lower panel of Figure 2. The results reveal that ρ caused a significant change in the profile of total RNA. In other experiments there was also an apparent increase in yield of low-molecular-weight pre-tRNA. There was a marked reduction in the amount of high-molecular-weight RNA which migrated near the top of the gel and a corresponding increase in the number of discrete products which migrated between 4S and 16S. This result indicates that most of the large RNAs synthesized in the absence of ρ were "read-through" transcripts resulting from improper termination by the RNA polymerase. The hybridization results indicated the occurrence of one major and possibly several minor species which contained the su_3^+ tRNA sequence. Little tRNA-like RNA occurred near the top of the gel. As was the case with RNA formed in the absence of ρ, the bulk of the tRNA-containing species were still between 5 and 6 S in size. In other experiments, there was an apparent small increase in

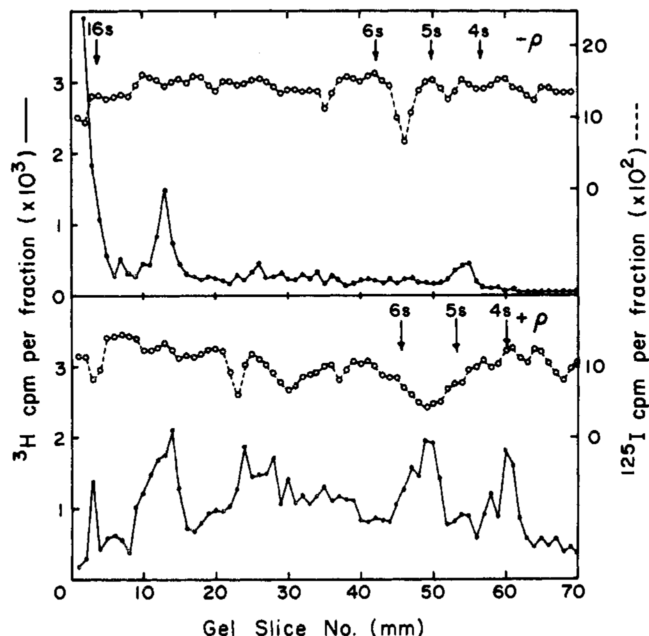

FIGURE 2: Analysis of the $\phi 80psu_3^+$ transcription products by polyacrylamide gel electrophoresis. [^3H]RNA synthesized in the presence or absence of ρ was fractionated by electrophoresis through a 5% acrylamide gel as described in Methods. The gels were cut into 1-mm slices and extracted with 250 μL of 6 × SSC (saline-sodium citrate) buffer, and the content of total RNA and tRNA-like RNA in each fraction was estimated. The profile for total RNA was determined by measuring acid-insoluble radioactivity; the tRNA-containing transcripts were identified by competition hybridization analysis. In these assays, 150 μL from each fraction was mixed with 2 μg of [^{125}I]*E. coli* tRNA (6 × 10^5 cpm/μg) and incubated with a filter containing $\phi 80su_3^+$ DNA in a final volume of 300 μL of 3 × SSC–50% formamide. The occurrence of tRNA-like RNA is indicated by a depression in the level of [^{125}I]tRNA hybridized. Gels containing marker RNAs were analyzed at the same time. (—●—) Total RNA; (—○—) [^{125}I]tRNA hybridized to $\phi 80su_3^+$ DNA. (Upper panel) Gel profiles of total RNA and tRNA-containing RNA from a sample of $\phi 80su_3^+$ RNA synthesized in the absence of ρ. Sixty-five micrograms of ^3H-labeled RNA (1.06 × 10^6 cpm) was analyzed. (Lower panel) Gel patterns of total RNA and tRNA-like RNA for a sample of su_3^+ RNA synthesized in the presence of ρ. The profiles were obtained with 48 μg of [^3H]RNA (7.5 × 10^5 cpm of ^3H). The assay procedures are described in detail in Methods.

the relative yield of low-molecular-weight pre-tRNA. The increase was difficult to quantitate, however, owing to the precision of the hybridization assay.

To rule on the possibility that some of the tRNA-like RNAs detected may arise through aggregation of smaller transcripts, the tRNA species formed in the presence and absence of ρ were analyzed under denaturing conditions. Figure 3 shows the hybridization results obtained from RNA synthesized in the presence of ρ and fractionated by electrophoresis in an acrylamide gel that was 98% formamide. The majority, possibly all, of the tRNA transcripts migrated in the region between the 5S and 6S marker RNAs. The formamide gel profile of tRNA synthesized in the absence of ρ was essentially like that of RNA made with ρ; only one species of pre-tRNA could be detected and this species also migrated between 5S and 6S markers. These results indicate that the tRNA species of higher molecular weight observed with RNA fractionated under nondenaturing conditions were most likely aggregates of the smaller pre-tRNA.

The observation that the bulk of the tRNA-like RNA formed occurs as a single species with a molecular weight of about 55 000 suggested that initiation and termination of synthesis occur in a site-specific manner and, accordingly, that transcription of the tDNA may be accurate; a broader distri-

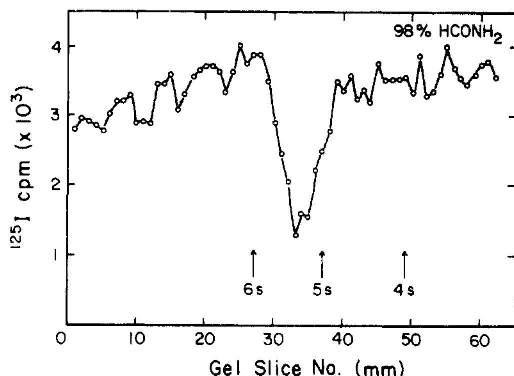

FIGURE 3: Electrophoretic analysis of in vitro synthesized su_3^+ tRNA under denaturing conditions. Seventy micrograms of RNA transcribed from $\phi 80su_3^+$ DNA in the presence of ρ was fractionated by electrophoresis through a gel that was 8% acrylamide:98% formamide. After slicing the gel and extracting the RNA, the tRNA-containing fractions were identified by assaying a portion of each fraction in a competition hybridization assay with 2 μg of ^{125}I-labeled *E. coli* tRNA (4.1×10^5 cpm/μg). The occurrence of transcripts containing a tRNA sequence is indicated by a decrease in binding of [^{125}I] $tRNA^{Tyr}$ to $\phi 80su_3^+$ DNA. Marker RNAs were analyzed in parallel in separate gels. Details are provided in Figure 2 and Methods.

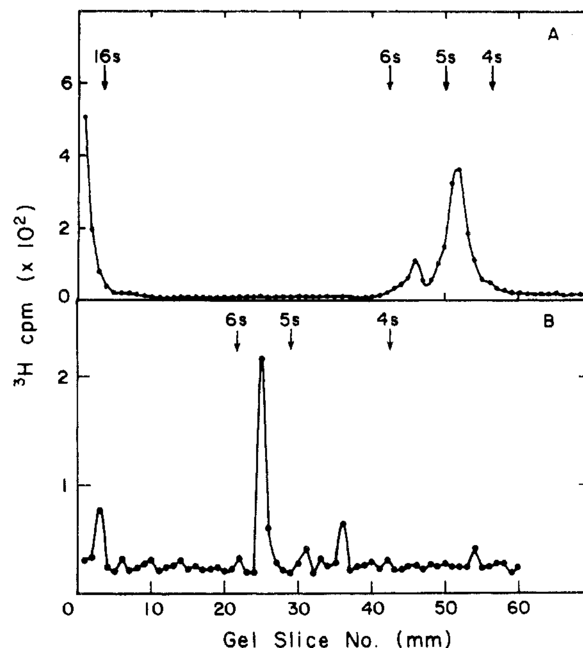

FIGURE 4: Electrophoretic analysis of $\phi 80su_3^+$ RNA methylated in vitro with fresh and aged preparations of *E. coli* tRNA methylase. In vitro synthesized $\phi 80su_3^+$ RNA was methylated in vitro by incubation with [*methyl*-^{3}H]-*S*-adenosylmethionine and a preparation of fresh or aged tRNA methylase. The [^{3}H]methyl-labeled RNA was recovered by phenol extraction and alcohol precipitation and analyzed by electrophoresis through gels of 5% or 10% acrylamide under nondenaturing conditions. The gels were sliced and extracted and the distribution of [^{3}H]methyl-labeled transcripts was determined by precipitating a portion of each sample with trichloroacetic acid and measuring the acid-insoluble radioactivity by scintillation counting. Control incubations lacking RNA or containing in vitro synthesized $\phi 80$ RNA were also performed and analyzed in the same way. A small amount of radioactivity (60–80 cpm) was usually observed in the $\phi 80$ RNA gels at a position slightly ahead of the 4S RNA marker. When observed, this activity was subtracted from the results obtained with the su_3^+ RNA. (Panel A) Pattern of methyl-labeled su_3^+ RNA obtained with a fresh preparation of tRNA methylase. Sixty-five micrograms of methylated RNA was analyzed in a 5% gel and 80% of the RNA from each fraction counted. (Panel B) Profile of methyl-labeled RNA obtained with the same preparation of methylase after storage of the enzyme at −20 °C for 5 months. The analysis was performed with 40 μg of RNA in a 10% gel; 50% of the RNA recovered from each slice was counted. Details of the methylation and fractionation procedures are provided in Methods.

bution of products would be expected if initiation or termination were not site-specific. However, because the pre-tRNA formed was about 50 nucleotides larger than the largest precursor su_3^+ $tRNA^{Tyr}$ to be detected in vivo thus far (molecular weight 43 000; Altman and Smith, 1971), we must consider the possibility that initiation and termination may be site-specific, but, yet artifactual in this system. Thus, while the results suggest that transcription may be accurate, proof of this must come from sequence analysis of the tRNA transcript and identification of the sequence of nucleotides in the DNA which constitute the natural termination site in vivo.

Methylation and Processing of Pre-tRNA. One purpose in establishing an in vitro biosynthetic system for tRNA is to obtain the primary gene product for use in in vitro studies of the maturation process. While it is clear that this process includes nucleolytic processing by specific RNases and the formation of a variety of modified bases by site-specific modifying enzymes, it is not known if the maturation events occur in a specific sequence, in random fashion, or by some combination of sequential and random events. One approach to the problem is to determine the effects of base modification in precursor tRNA on processing and on the formation of other modified bases. Similarly, the effect of specific nucleolytic processing on other pre-tRNA processing reactions and on modified base formation should be investigated. To this end, the putative precursor tRNA was treated with preparations of *E. coli* tRNA methylase and a crude preparation of processing RNase to determine whether this species could be used as a substrate by tRNA maturation enzymes.

Results from the methylation assays showed that the su_3^+ RNA could, in fact, be methylated by *E. coli* tRNA methylase. The methyl acceptor activity of several preparations of su_3^+ RNA was estimated to be between 0.1 and 0.5 methyl groups per chain of pre-tRNA. In control reactions no incorporation of [^{3}H]methyl groups was detected with equivalent amounts (100 μg) of $\phi 80$ RNA transcribed in vitro. Of course, the methylation values obtained for the su_3^+ RNA will be higher if the content of pre-tRNA in the in vitro synthesized RNA has been overestimated. As noted earlier, the competition hybridization technique may give an inflated estimate owing to the possibility that the larger, pre-tRNA species may hybridize to tDNA with greater efficiency than mature tRNA.

One- and two-dimensional chromatographic analysis of the methylated products revealed that both T and Gm were formed. The relative amounts and molar yields of each varied from preparation to preparation of methylase. However, the yield of T was usually at least twice that of Gm.

Fully modified, mature su_3^+ $tRNA^{Tyr}$ contains three methylated nucleosides: 2′-*O*-methylguanosine (Gm) at the 17th position from the 5′ terminus, 2-thiomethyl-Δ^6-isopentenyladenosine (ms^2i^6A) adjacent to the anticodon at position 38, and ribothymidine (T) at position 63 in the -GTΨC- sequence of the TΨC loop. Because the methyl group of ms^2i^6A is added after the incorporation of sulfur at position 2 of the adenosine moiety (Agris et al., 1975), it is unlikely that methylation of this base would occur in vitro in the presumed absence of the factors required for the thiolation reaction. Thus, it would be anticipated that only Gm and T could be formed in our in vitro methylation reaction.

Analysis of the methyl-labeled transcription products by acrylamide gel electrophoresis revealed that fresh preparations of methylase contained a processing-like RNase activity. This nuclease activity, reflected in the appearance of a second, smaller tRNA species, was reduced significantly upon storage

of the enzyme. After 2 months at −20 °C, the RNase activity was substantially lower, but still readily detectable; after 5 months the bulk of the nuclease activity had been lost. The gel profiles of methyl-labeled RNAs obtained with fresh and aged methylase are shown in panels A and B, respectively, of Figure 4.

When pre-tRNA were methylated with enzyme prepared the same day, two species of methyl-labeled RNA were obtained in the gel (panel A)—a minor species with the same electrophoretic behavior as the pre-tRNA of 55 000 daltons and a second, major species which migrated between the 4S and 5S marker RNAs. The smaller species was about the same size as the precursor described by Altman and Smith (1971) suggesting that it may correspond to that material. If indeed the Altman–Smith precursor was formed, then processing may have occurred at the 3′ terminus only. Hybridization assays showed that both methylated species corresponded to tRNA-containing species. Because the RNA used in this experiment was synthesized in the absence of ρ, a considerable amount of methyl-labeled RNA was also found at the top of the gel.

When preparations of pre-tRNA were treated with methylase which had been stored at −20 °C for 5 months, the bulk of the methyl-label remained associated with the 55 000-dalton precursor (panel B, Figure 4) and only that species was readily apparent in hybridization assays. The "processing" of pre-tRNA by preparations of methylase appears not be be dependent on methylation as the smaller tRNA species was formed with about the same efficiency in the absence of *S*-adenosylmethionine. The possibility that some methylation did occur owing to the occurrence of enzyme-bound *S*-adenosylmethionine cannot be discounted, however. Both T and Gm could be detected in the larger precursor tRNA, indicating that processing was not required to gain methyl acceptor activity.

Using a freshly prepared DEAE-cellulose treated high-speed supernatant (S100) from *E. coli* MRE 600 (RNase I^-) as a source of crude processing RNase, it was determined that the primary precursor could be converted almost completely to 4S RNA with apparent good yield. This result (not shown) implies that the pre-tRNA is a suitable substrate for the tRNA processing enzymes as well as the methylases. The possibility that the apparent processing resulted from the action of nonspecific RNases was not discounted, however, until it was later shown that pre-tRNA processed in this way is biologically active.

Aminoacylation of in Vitro Synthesized su_3^+ tRNA. Evidence that the 4S tRNA formed in vitro was biologically active came from an experiment in which it was shown that su_3^+ tRNA synthesized in the presence of ρ and processed (separately) with enriched, supernatant enzymes could be aminoacylated with tyrosine. Proof that the radiolabeled tyrosine was incorporated into the in vitro synthesized su_3^+ tRNA and not merely into residual, endogenous tRNA in the synthetase preparation was provided by the demonstration that doubly labeled tyrosyladenosine could be derived from the product of the aminoacylation reaction when [^{3}H]tyrosine and su_3^+ RNA labeled with [^{14}C]AMP were used. Following aminoacylation, the RNA was recovered and hydrolyzed with RNases CB and A, and the digestion products were fractionated by high-voltage electrophoresis. The results of this analysis are shown in Figure 5. It is clear that coincident peaks of ^{14}C and ^{3}H activity occur which are electrophoretically indistinguishable from tyrosyladenosine. In a control experiment, no ^{3}H activity was found in the tyrosyladenosine region when "RNA" recovered from an aminoacylation reaction mixture lacking su_3^+ RNA was analyzed. In the analysis depicted, 1% of the ^{14}C activity

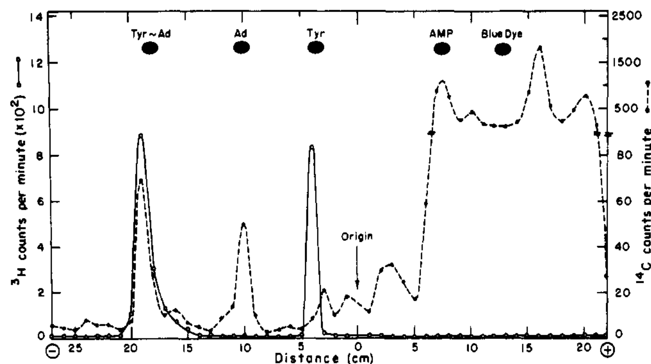

FIGURE 5: Electrophoretic analysis of the RNase CB and A products derived from in vitro synthesized, processed, and aminoacylated su_3^+ tyrosyl-tRNA. Twenty-one micrograms of [^{14}C]ATP-labeled su_3^+ RNA transcribed in vitro was treated with an enriched preparation of tRNA precursor processing enzymes as described in Methods. The processed tRNA was recovered by phenol extraction and alcohol precipitation and aminoacylated with [^{3}H]tyrosine under standard assay conditions. The [^{3}H]tyrosyl-[^{14}C]RNA was repurified by adsorption onto a small (0.5 mL) DEAE-cellulose column equilibrated in 0.01 M sodium acetate (pH 4.5)–0.3 M NaCl and, after washing with the same buffer, eluted with buffer containing 1 M NaCl. Twenty-five micrograms of carrier rat liver tRNA was added and the RNA recovered by alcohol precipitation. The RNA was then partially hydrolyzed by incubation with a mixture of RNases CB and A and the digestion products were analyzed by high-voltage electrophoresis on cellulose acetate at pH 3.5. Radiolabeled adenosine, tyrosine, AMP, and tyrosyladenosine (from authentic tyrosyl-tRNA) were applied to a separate strip analyzed in parallel to serve as standards in the analysis. After electrophoresis, the paper was cut into 1-cm strips and the distribution of ^{3}H and ^{14}C products determined by scintillation counting. The migration position of the standards is indicated in the upper portion of the figure. In this experiment the RNA was not completely hydrolyzed as indicated by the occurrence of ^{14}C-labeled oligonucleotides which migrated in the region of the blue marker dye just ahead of the [^{14}C]AMP. Recoveries of ^{14}C and ^{3}H were ~100% and 74%, respectively. Complete details of the conditions used for processing, aminoacylation, and hydrolysis of the in vitro synthesized tRNA are provided in Methods.

recovered was associated with tyrosyladenosine while 61% of the ^{3}H-label migrated with that material. The free tyrosine and at least some of the adenosine were presumably derived from deacylation of tyrosyl-tRNA during preparation of the sample for electrophoretic analysis. The relative content of functional tyrosine tRNA can be estimated from the content of tyrosyladenosine. In this preparation the active tRNA corresponded to 0.7% of the su_3^+RNA.

From results of various aminoacylation assays, it was estimated that 4–15 pmol of tyrosine was incorporated per A_{260} of processed, but unfractionated RNA. As the tRNA content of these preparations was estimated by hybridization measurements to be 2–3%, the incorporation values correspond to acceptor activities of 130–750 pmol of amino acid per A_{260}; this result suggests that 7–42% of the tyrosine tRNA was active. However, such an estimate must be considered imprecise owing to the precision of the hybridization method (±15%) and the aforementioned possibility that estimates of tRNA content based on hybridization data may be high because of possible differences in the stabilities of hybrids formed with precursor and mature tRNA. Nevertheless, the results do indicate that a significant proportion of the in vitro synthesized pre-tRNA was converted to biologically active tRNA.

Discussion

The results show that the su_3^+ $tRNA^{Tyr}$ gene contained in the DNA of the phage $\phi 80su_3^+$ can be transcribed in vitro with apparent good efficiency using a highly enriched preparation of *E. coli* RNA polymerase. Based on competition hybrid-

ization assays, the tRNA content of the in vitro synthesized RNA was found to range from 2 to 15%. While the best yields were realized with fresh preparations of RNA polymerase and DNA, even the lowest value suggests that preferential expression of the tDNA sequence occurs. The DNA which corresponds to mature $tRNA^{Tyr}$ is only 0.2% of the phage chromosome indicating that the amount of tRNA synthesized is 10–70-fold greater than would be expected if all of the DNA sequences were transcribed equally well. High yields of su_3^+ tRNA have also been observed by others using similar transcription systems and competition hybridization measurements (Daniel et al., 1970; Ikeda, 1971; Beckmann and Daniel, 1974b). In the last work cited, yields as high as 40–60% were reported.

The basis for the selective transcription observed is not known in any of the studies reported. However, at least a portion of the apparent high yield could result from inflated estimates owing to possible differences in the stability of hybrids formed with the precursor and mature species. Beckmann and Daniel (1974a) reported that the stability of RNA:DNA hybrids is related to the size of the RNA fragment and that large fragments can apparently displace smaller RNA chains from a pre-formed hybrid. Hybrids containing intact ribosomal RNA were more stable than those formed with shorter, homologous fragments. In hybridization assays with in vitro synthesized su_3^+ RNA and mature tRNA, these workers found that the su_3^+ transcripts could efficiently displace mature $tRNA^{Tyr}$ hybridized to $\phi 80su_3^+$ DNA. tRNA sequences could be detected in the in vitro produced RNA by an indirect competition assay, but not in a direct competition assay with labeled transcripts and unlabeled *E. coli* tRNA. Unfortunately, the size of the tRNA transcripts used in those particular experiments was not determined. If the tRNA-containing species used were the 7–8S transcripts described by these investigators in an earlier report (Daniel et al., 1970), the stability problem is probably much greater than for a smaller precursor such as that observed by us. Nonetheless, the results from that study show that the mechanics of the hybridization process are more complicated than first believed and that results from competition experiments may be useful only in comparing the relative yields of an RNA species formed under different transcription reaction conditions. While there may well be preferential expression of the su_3^+ tRNA gene in the in vitro system described here and in those characterized by others, final proof and quantitation will have to come either from hybridization assays done with processed 4S transcripts or direct sequence analyses.

Only one tRNA-containing transcript could be detected when the transcription products were analyzed under denaturing conditions. For this reason it seems likely that the apparent multiplicity of tDNA transcripts observed under nondenaturing conditions reflects the occurrence of pre-tRNA aggregates rather than the occurrence of multiple initiation or termination sites in the su_3^+ transcription unit or transcriptional read-through from or into an adjacent unit of transcription. Aggregation may be the basis for the polydispersity and seemingly very high molecular weights of the su_3^+ transcripts observed by Daniel et al. (1970) and Ikeda (1971). Daniel and co-workers reported that the tRNA-like RNA synthesized in their system sedimented through a sucrose gradient as a heterogeneous band with a broad peak at about 7–8 S. Using acrylamide gel electrophoresis to analyze the size and heterogeneity of his su_3^+ tRNA, Ikeda detected three tRNA-containing RNA chains with apparent molecular weights of 2.8×10^5, 1.7×10^5, and 8×10^4. All of these species are considerably larger than the Altman–Smith precursor (mol wt 4.3×10^4) or the precursor observed by us (mol wt 5.5×10^4). Because the tRNA products were not analyzed under denaturing conditions in either study, it is not possible to decide if the heterogeneity observed was due to aggregation of smaller monomeric precursors or heterogeneity of the primary transcripts. The discovery that aggregation of the pre-tRNA can occur provides an alternative to the proposal that the su_3^+ transcription unit may contain three promoters (Ikeda, 1971).

Using highly purified RNA polymerase, Bikoff et al. (1975) observed that the tRNA transcripts derived from $\phi 80su_3^+$ DNA were longer than the Altman–Smith precursor by at least 100 nucleotides and, thus, more than 50 nucleotides larger than the transcript characterized by us. This large transcript could be converted to the Altman–Smith precursor by treatment with a particular protein fraction.

The fact that the precursor formed in our system is intermediate in size to the transcript observed by Bikoff and her colleagues and the Altman–Smith precursor suggests the possibility that the polymerase preparations used in the two studies may differ in composition. It is possible that our preparation contains a factor which promotes termination at an earlier site 50 nucleotides beyond the normal 3′ terminus of mature $tRNA^{Tyr}$. Alternatively, our preparation may contain a maturation activity which cleaves a larger primary transcript to create the 55 000-dalton precursor. Additional work, perhaps with polymerase reconstituted from purified subunits, will be required to resolve this issue.

Küpper et al. (1975) have reported that transcription of a tDNA fragment prepared from $\phi 80su_3^+$ DNA with restriction enzymes yields a precursor tRNA which is the same size as that formed in our system. However, as their product is precisely the same length as the sequence of DNA between the putative promoter and the end of the template, it was not possible to obtain a larger tRNA transcript. If transcription in our system is accurate and the 175 nucleotide precursor is the primary transcript, then the *Hin*dIII endonuclease used by Küpper et al. may cleave the su_3^+ DNA at a site close to or at the termination sequence. The finding that the su_3^+ tRNAs produced in these in vitro systems are larger than the 120-nucleotide precursor described by Altman and Smith suggests that the in vivo precursor characterized by those workers may be an intermediate pre-tRNA which has already undergone some processing at the 3′ terminus.

Including the termination factor ρ in the transcription reaction improved the relative yield of pre-tRNA in our system without affecting its size. Others have also observed an increase in the yield of tRNA (Beckmann and Daniel, 1974b) when ρ is used and without apparent effect on the size, although the species produced both in the presence and absence of ρ were 7–8 S in size (Daniel et al., 1970). Similarly, ρ had no effect on the size of the pre-tRNA derived from the su_3^+ tDNA restriction fragments in the study described by Küpper et al. (1975). Our results suggest the possibility that transcription of the su_3^+ tRNA gene terminates at a ρ-independent site. The improved yield of monomeric pre-tRNA may result from reduced read-through from the preceding transcriptional unit, although these results do not preclude the possibility of a ρ-stimulated termination site which occurs very close to or at the site utilized in the absence of ρ.

Evidence has been presented by others which indicates that the two normally closely linked su_3^+ and su_3^- $tRNA^{Tyr}{}_I$ genes are transcribed together in vivo (Ghysen and Celis, 1974) and in vitro (Daniel et al., 1975). In view of this result, we must consider the possibility that the putative termination site used when only the su_3^+ gene is present may be part of a different

transcriptional unit. A termination sequence could have been inserted into the su_3^+ $tRNA^{Tyr}$ transcription unit in the $\phi80su_3^+$ phage by the recombinational event which resulted in the loss of the su_3^- gene.

These comments about the termination process are valid only if initiation is accurate and occurs at only one site. That initiation in this in vitro system is accurate is suggested by results from sequence analyses of the pre-tRNA produced in vitro in the reconstituted transcription system of Bikoff and co-workers (Bikoff and Gefter, 1975; Bikoff et al., 1975) and from the su_3^+ restriction fragment (Küpper et al., 1975). The sequencing results demonstrated that initiation in these systems occurs at the same site as initiation in vivo and with high specificity. While it seems likely that the same specificity is realized in the system described here, proof of this must come from an analysis of the sequence of the tDNA transcript. This work is currently in progress.

The finding that the 55 000-dalton precursor can be methylated indicates that nucleolytic processing is not required for the formation of 5-methyluracil (ribothymidine) or 2′-*O*-methylguanosine. Others have shown that ribothymidine, pseudouridine, and isopentenyladenosine can be formed both in vivo and in vitro in the su_3^+ tRNA precursor of Altman and Smith (Altman and Smith, 1971; Schäfer et al., 1973) and that these modifications as well as dihydrouridine can also occur in precursors to T4 phage and cellular tRNAs (Guthrie et al., 1973; Sakano et al., 1974a). Although Gm can be formed in the precursor produced in this system, its absence in the in vivo su_3^+ tRNA precursor (Schäfer et al., 1973) and in in vivo derived T4 and bacterial tRNA precursors (Guthrie et al., 1973) argues that this base is usually formed at the level of a 4S RNA intermediate.

Although others have also been able to obtain active tRNA from transcripts synthesized in different fractionated, cell-free systems (Küpper et al., 1975; Bikoff et al., 1975; Zeevi and Daniel, 1976), the demonstration that the precursor produced in our system can be converted to functional tRNA is of obvious importance in evaluating the accuracy of transcription in this system. The finding that the precursor can be converted to active tRNA by treatment with a crude preparation of processing nucleases indicates that the transcripts: (1) contain a complete tRNA sequence; (2) can be cleaved specifically by the tRNA processing nucleases; and (3) that the mature-size tRNA produced is a substrate for tyrosyl-tRNA synthetase. In addition, this result supports the view that only one and possibly none of the modified bases in mature fully modified $tRNA^{Tyr}$ are required for acceptor activity. This suggestion is based on the supposition that only ψ could be formed during the transcription, processing, and aminoacylation reactions. The preparations of polymerase, processing nucleases, and aminoacyl-tRNA synthetase used in this work should have been free of the low-molecular-weight cofactors and substrates required for the formation of the other modified bases which occur in mature $tRNA^{Tyr}$. However, because pseudouridine formation appears not to require dialyzable cofactors (Cortese et al., 1974) and because others have determined that ψ can be formed in in vitro synthesized su_3^+ tRNA treated with dialyzed supernatant (Zeevi and Daniel, 1976), it seems likely that the final product may have contained some pseudouridine.

Taken together, the results demonstrate that much information about the process and products of tRNA biosynthesis can be derived from in vitro transcription–maturation experiments and that good progress is being made toward the goal of establishing a completely defined cell-free system capable of producing fully mature, functional tRNA.

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Transfer RNA in Posterior Silk Gland of *Bombyx mori*: Polyacrylamide Gel Mapping of Mature Transfer RNA, Identification and Partial Structural Characterization of Major Isoacceptor Species†

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ABSTRACT: Transfer RNAs (tRNAs) from the posterior silk gland and carcass tissues of the silkworm *Bombyx mori* L. were fractionated by high resolution polyacrylamide gel electrophoresis. tRNAs from each source resolved into 50 distinct spots, many of which represented pure tRNA species. Nonlabeled tRNA of the posterior silk gland, purified by benzoylated diethylaminoethyl-cellulose column chromatography and by counter current distribution, were used to aid in identification of $tRNA^{Ala}$, $tRNA^{Gly}$, and $tRNA^{Ser}$ isoacceptor species. These tRNA species constituted about 70% of total tRNA population in the posterior silk gland. The high resolution of tRNA separation on polyacrylamide gels thus provided a quantitative estimate of the posterior silk gland isoacceptor tRNA distribution which is adapted to produce large amounts of the protein, silk fibroin, during the fifth larval instar. Several of the major ^{32}P-labeled tRNA species of the posterior silk gland, isolated by two-dimensional polyacrylamide gel electrophoresis, were subjected to structural analysis by oligonucleotide fingerprinting of RNase T_1 digests and nucleotide separation analysis of RNase T_2 hydrolysates. The results indicated that a specific $tRNA^{Ala}$ species ($tRNA^{Ala}{}_{2a}$), which is present in the posterior silk gland but absent from the carcass tissue [and in the middle silk gland; see Meza, L., et al. (1977), *FEBS Lett. 77*, 255-260], differs structurally by substitution of a single base in one hexanucleotide indicated in the T_1-oligonucleotide fingerprint map, from the other major $tRNA^{Ala}$ species ($tRNA^{Ala}{}_{2b}$) found in both tissues. The main $tRNA^{Gly}$ and $tRNA^{Ser}$ species are present in both silk glands and in carcass tissues. The structural properties of these tRNA species are also described.

Significant progress was made during recent years on understanding the mechanism of synthesis and processing of tRNAs encoded by bacteriophage and *Escherichia coli* genes, but little is known about biosynthesis of tRNA in eukaryotes (for reviews, see Burdon, 1971; Schäfer and Söll, 1974; Altman, 1975; Smith, 1976). We have chosen the posterior silk gland of the silkworm, *Bombyx mori* L., as a model eukaryotic system for studies on biosynthesis of tRNA and its regulation. A unique feature of the developing silk gland is the appearance of four preponderant tRNAs specific for glycine, alanine, serine, and tyrosine during the terminal differentiation of the gland in the fifth larval instar (silk fibroin secretion phase). The four tRNAs constitute almost 80% of the total tRNA population of the posterior silk gland (Garel et al., 1970; Chavancy et al., 1971; Delaney and Siddiqui, 1975; Majima et al., 1975). It was proposed (Garel, 1974, 1976) that such an adaptation of tRNA population which results from quantitative changes in specific isoacceptor tRNA species (Garel et al., 1970; Delaney and Siddiqui, 1975; Araya et al., 1975) could ensure rapid and efficient decoding of fibroin mRNA. Silk fibroin is composed mainly of the four amino acids mentioned above and is synthesized exclusively in the posterior part of the silk gland (Tashiro et al., 1968; Daillie et al., 1971). The presence of a relatively small number of predominant tRNA species in the posterior silk gland thus presents an opportunity for the isolation of pure precursor tRNA molecules and for the investigation of the mechanism involved in the accumulation of specific tRNA species.

We (Chen and Siddiqui, 1975) and others (Tsutsumi et al., 1974, 1976) have recently described the isolation and characterization of low molecular weight RNA species in the posterior silk gland of *B. mori* that have properties expected of a precursor to tRNA. In continuation of our studies on biosynthesis of tRNA and to elucidate the correlation between the compositions of mature tRNA and the corresponding precursor tRNA population in the silk gland, we looked for a tRNA separation system with high resolution capacity. The polyacrylamide gel electrophoretic procedures described below were designed (i) to establish a gel map that can be used to identify the mature tRNA species or the precursor tRNA species present during distinct physiological stages (growth

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