In Vitro Synthesis of Transfer RNA

I. PURIFICATION OF REQUIRED COMPONENTS*

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SUMMARY

We have described an in vitro system in which active suTyr is synthesized from a φ80psuDNA template. Using this system, we have identified four essential components that are required for synthesis of tRNA. The first of these is DNA-dependent RNA polymerase. It has been shown that a crude preparation of DNA-dependent RNA polymerase synthesizes suTyr precursor similar to that which has been isolated in vivo, and that this preparation is capable of supporting high levels of tRNA synthesis. With purified DNA-dependent RNA polymerase, the suTyr precursor was not observed as a transcription product and tRNA synthesis was below detectable levels. On this basis, a second essential component for tRNA synthesis was identified. This fraction, designated Fraction V, in combination with purified RNA polymerase, catalyzes the synthesis of precursor tRNA. The third component is a ribonuclease (RNase P III), which specifically catalyzes the removal of the extra nucleotides present at the 3' terminus of the mature tRNA. In the absence of this fraction, the in vitro synthesized suTyr is slightly larger than 4 S and contains additional nucleotides beyond the normal —CA transitions. The fourth essential component required is a fraction containing RNase P I, a previously identified endonuclease which specifically catalyzes the removal of the 5' extra nucleotides present on tRNA precursors.

The biosynthesis of transfer RNA is a complex process. It has been shown both in mammalian cells (1, 2) and in Escherichia coli (3-5) that tRNAs are initially transcribed as larger precursor molecules, and that these precursors are subsequently cleaved and modified into the mature 4 S species. The sequences of several tRNA precursors from E. coli have been determined, and it has been shown that these molecules contain extra nucleotides at both their 3' and 5' ends (3-5). An endonuclease, RNase P, which catalyzes the release of nucleotides from the 5' ends of tRNA precursors, has been purified from the ribosomes of E. coli (6). The isolation and characterization of E. coli mutants harboring a lesion in the gene for RNase P that gives rise to temperature-sensitive enzyme have provided proof that RNase P plays an essential role in tRNA biosynthesis (7, 8). In contrast, identification of the enzyme that catalyzes the removal of the extra nucleotides present at the 3' end of tRNA precursors has not been reported.

In vivo, the biosynthesis of tRNA is a very rapid process, such that the events leading from transcription to mature tRNA are difficult to discern. For this reason, in vitro systems have been utilized in an attempt to dissect the processes of transcription and post-transcriptional maturation of tRNA precursors and to define the enzymatic mechanisms of these reactions. DNA from the bacteriophage φ80psu contains the genes for a single bacterial suppressor tRNA (9). Several laboratories have studied the process of tRNA transcription in an in vitro system consisting of φ80psu DNA and purified DNA-dependent RNA polymerase and ρ factor (10-12). Analysis of the transcription products by acrylamide gel electrophoresis and competitive hybridization demonstrated the presence of material containing the tRNA sequence which was larger than 4 S (11). However, direct sequence analysis of this material was not performed, and the ability of this material to give rise to mature suTyr was not clearly demonstrated. Thus, the relationship between this in vitro synthesized material and the suTyr tRNA precursor, which has been isolated in vivo, remains unclear.

An in vitro system for tRNA synthesis has been described that utilizes T4 DNA as template (13). DNA from this bacteriophage contains the genes for eight different phage-specified tRNAs (14, 15). Transcription of the DNA was carried out with purified DNA-dependent RNA polymerase. Then post-transcriptional cleavage and modification were subsequently performed, incubating the isolated RNA transcription products with extracts prepared from E. coli. Direct identification of specific tRNA precursors among the primary transcription products was not reported. However, the ability of this material to give rise to mature tRNA was demonstrated by the direct sequence analysis of one of the tRNA products.

A DNA-directed cell-free system for the synthesis of suTyr tRNA was recently described by Zubay et al. (16). As judged by sequence analysis and its capacity to suppress an amber muta-
tion in the gene for β-galactosidase, the in vitro-synthesized tRNA was identical with natural *su*~11~ tRNA^Tyr~77~ and had complete biochemical activity (16, 17). Thus, all the essential factors for correct transcription and post-transcriptional cleavage and modification must be present and active in this system.

This report describes the purification from these extracts of four components that are required for the synthesis of active tRNA. The first of these is DNA-dependent RNA polymerase. The second essential component is a fraction of unknown function, which allows purified DNA-dependent RNA polymerase to function in tRNA synthesis. The third essential fraction is a purified tRNA. The fourth component required for the in vitro synthesis of tRNA is a ribonuclease that specifically catalyzes the removal of the extra nucleotides present at the 3' end of tRNA precursors.

**EXPERIMENTAL PROCEDURE**

**Materials**

**Bacterial and Bacteriophage Strains—** The bacterial and bacteriophage strains used have been described elsewhere (16).

**Chemicals—** Phosphoenolpyruvic acid was purchased from Calbiochem as the triethylammonium salt. It was converted into the sodium salt by passing it over a column of Dowex 50 and neutralizing the free acid with 1 M NaOH as described by Nirenberg (18).

Ammonium sulfate was the ultrapure grade obtained from Schwarz/Mann. DEAE-cellulose, DE52, was obtained from Whatman. Sephadex G-200 was purchased from Pharmacia Fine Chemicals. Polyethylene glycol 6000 was from Union Carbide Corp. Adenosine 5'-triphosphate was purchased as the disodium salt from Sigma. Guanosine 5'-triphosphate, cytidine 5'-triphosphate, uridine 5'-triphosphate, and cyclic adenosine 3':5'-monophosphate were purchased from Calbiochem.

**tRNA—** Suppressor tRNA^Tyr~77~ used to standardize the in vitro suppression assay was purified from *ψ0*ψ*ψ*-infected cells as previously described (19). The *su*~11~ tRNA^Tyr~77~ precursor, uniformly labeled with 32P, was purified from *ψ0*ψ*ψ*-infected *E. coli* A49 by the procedure described by Altman and Smith (3).

**Enzymes—** Electrophoretically purified pancreatic DNase was obtained from Worthington Biochemical Corp. DNA-dependent RNA polymerase was purified from *E. coli* 514 by a slight modification of the procedure described by Burgess (20). The specific activity of the enzyme after the second glycerol gradient was 730 units/mg. Tyrosyl-tRNA synthetase was purified from *E. coli* 514 by a slight modification of the procedure described by Calendar and Berg (21). The final specific activity of the enzyme was 820 units/mg.

**Buffers—** Buffer II consisted of 10 mM Tris-acetate (pH 8.2), 140 mM potassium acetate, 25 mM NH_4Cl, 2 mM ATP, 0.18 mM GTP, 0.05 mM dithiothreitol, and 10% (v/v) glycerol.

Buffer IV was the same as Buffer II, but containing 5% (v/v) glycerol. Buffer III contained 20 mM Tris-Cl (pH 7.4), 10 mM KCl, 1 mM magnesium acetate, 2 mM dithiothreitol, and 10% (v/v) glycerol.

**Methods**

**Protein Determination—** In most cases, protein was determined by the method of Lowry et al. (22). However, when the protein concentration was less than 250 μg/ml, the protein was measured by the procedure of Lowry et al. (23).

**Acrylamide Gel Electrophoresis—** Electrophoresis was carried out on 10% polyacrylamide slab gels (20 × 20 × 0.2 cm) in a continuous buffer system containing, per liter: 10.8 g of Tris base, 0.93 g of disodium EDTA, and 5.5 g of boric acid (final pH, 8.3) for 12 hours at 400 volts and 25°C. The position of the radioactive RNA bands in the gels was determined by autoradiography.

**Enzyme Assays—** DNA-dependent RNA polymerase activity was determined under the conditions described by Burgess (20), utilizing calf thymus DNA as template and measuring the incorporation of [γ-32P]UTP into acid-precipitable material.

Tyrosine acceptance activity of *su*~11~ tRNA^Tyr~77~ was determined under the conditions described by Gefter and Russell (24), utilizing purified tyrosyl-tRNA synthetase and measuring the incorporation of 3H-tyrosine into acid-precipitable material.

**In Vitro Suppression Assay—** The suppression assay has been previously described in detail (16). Briefly, the assay utilizes a DNA-directed cell free system capable of synthesizing β-galactosidase. DNA from the transducing phage λdlac545 contains an amber mutation in the gene for β-galactosidase (16). When this DNA was incubated with the 30,000 X g supernatant (SS0) of a crude extract prepared from *E. coli* 514 and the cofactors and substrates necessary for RNA and protein synthesis, synthesis of β-galactosidase was completely dependent upon the addition of amber suppressor tRNA. Furthermore, as shown in Fig. 1, the amount of β-galactosidase synthesized under these conditions was directly proportional to the amount of *su*~11~ tRNA^Tyr~77~ added. The linear range of the assay was between 1 and 10 pmol of suppressor tRNA. The conditions for the synthesis and assay of β-galactosidase were the same as those previously reported (16), except that incubations were carried out for 2 hours at 35°C in the presence of 0.8% (w/v) polyethylene glycol. Bacterial extracts and DNA were prepared according to the previously described procedures (25).

The suppression assay provides a reproducible and accurate means of determining the amount of *su*~11~ tRNA^Tyr~77~ synthesized in *vitro*. However, the absolute amount of β-galactosidase synthesized could vary 2- to 3-fold for a given amount of *su*~11~ tRNA^Tyr~77~ in the reaction, depending upon the age of the particular λdlac545 DNA preparation and the over-all efficiency of the particular preparation of SS0 for DNA-directed protein synthesis. Due to this variability, each set of suppression assays included a set of standard reactions with known amounts of *su*~11~ tRNA^Tyr~77~ purified from *ψ0*ψ*ψ*-infected cells.

**Synthesis and Isolation of *su*~11~ tRNA^Tyr~77—** Reaction mixtures were incubated for 1 hour at 35°C with gentle agitation in a rotary shaking water bath. Each reaction contained in a final volume of 2 ml: 40 mM Tris-acetate (pH 8.2), 2 mM dithiothreitol, 50 mM potassium acetate, 25 mM NH_4Cl, 2 mM ATP, 0.18 mM GTP, 0.05 mM CTP, 0.05 mM UTP, 0.5 mM cyclic 3':5'-AMP, 21 mM phosphoenolpyruvate, 0.870 (w/v) polyethylene glycol. Bacterial extracts and DNA were prepared according to the previously described procedures (25).

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endoprophate, 29 mm magnesium acetate, 0.1 mm isopentenylpyrophosphate, 100 µg of E. coli RNA, and S30 (6 mg). In cases where radioactive labeling of the RNA for analysis by acrylamide gel electrophoresis was desired, reactions were scaled down to a total volume of 0.6 ml and [α-32P]CTP or [α-32P]GTP was also included at final specific activity of 100 mCi/ml. At the end of the incubation, pancreatic DNase was added (final concentration 50 µg/ml) and the reaction was continued for a further 15 min at 37°C. Then, an equal volume of freshly neutralized, redistilled phenol was added, and the mixture was vigorously agitated. After centrifugation for 10 min at 2500 rpm, the aqueous layer was carefully removed. The RNA was precipitated by the addition of 0.5 ml of 1 M sodium acetate (pH 5) and 5 ml of absolute ethanol. After 1 hour at -20°C, the precipitated RNA was rinsed with air-dried 70% ethanol, and the RNA was redissolved in a small volume of double-distilled water. In cases where the RNA was to be analyzed by acrylamide gel electrophoresis, sucrose and bromphenol blue were added, and the sample was layered directly onto the gel. With nonradioactive RNA products, the amount of sample was determined by absorbance at 260 nm, solid ammonium sulfate was added gradually to yield the nucleic acid-free S100 (volume, 112 ml). The S100 was collected by centrifugation, the pellet was carefully dried, and then the RNA was redissolved in a small volume of double-distilled water. In cases where the RNA was to be analyzed by acrylamide gel electrophoresis, sucrose and bromphenol blue were added, and the sample was layered directly onto the gel. With nonradioactive RNA products, the amount of sample was determined by absorbance at 260 nm.

Preparation of S30 for Synthesis of tRNA—Initially, extracts were prepared from E. coli 514 according to the method described by Zubay et al. (25). However, a 2- to 3-fold enhancement of activity that had been synthesized was determined by its ability in the in vitro suppression assay as previously described or by its ability to accept tyrosine in an amino acyl-tRNA synthetase reaction.

Preparation of S100 and Nucleic Acid-free S100—Unless otherwise stated, all operations were performed at 4°C. The preparation described is that for 20 g of cells.

The S30 (60 ml) was thawed in a 5°C water bath. Ribosomes were removed by centrifugation for 3 hours at 57,000 rpm in the SW 50.1 rotor of the Beckman model L ultracentrifuge. The clear supernatant was removed to yield the S100. The extract was adjusted to 0.1 M NaCl with Buffer I containing no KCl. The (2.2 ± 14) cm previously equilibrated with Buffer II containing 0.4 M KCl. The column was washed with Buffer II containing 0.4 M KCl, and fractions containing the yellow color were pooled to yield the nucleic acid-free S100 (volume, 112 ml).

Ammonium Sulfate Fractions of Nucleic Acid-free S100—Unless otherwise stated, all ammonium sulfate precipitations were carried out as follows. Solid ammonium sulfate was added gradually with constant stirring. The pH was maintained at pH 7.5 by the dropwise addition of 1 N NaOH. After all the ammonium sulfate had dissolved, the solution was stirred for 15 min to ensure quantitative precipitation. The resulting precipitate was removed by centrifugation for 20 min at 8000 rpm in the GS-3 rotor of a Sorvall RC2-B centrifuge.

Solid ammonium sulfate (10.4 g/100 ml of nucleic acid-free S100) was added, and the resulting precipitate (0 to 30%) was discarded. Then 11.4 g of solid ammonium sulfate per 100 ml of resulting supernatant were added to give a 50% saturated solution. The resulting precipitate (Fraction I) was dissolved in 12 ml of Buffer IV and dialyzed for 6 hours against 1 liter of Buffer IV. This fraction contained 90 to 95% of the DNA-dependent RNA polymerase activity as determined in the assay described above. Finally, 23 g of solid ammonium sulfate per 100 ml of resulting supernatant were added to give an 85% saturated solution. The precipitate (Fraction II) was dissolved in 12 ml of Buffer III and dialyzed for 6 hours against 1 liter of Buffer III. After dialysis, the specific conductivity of each fraction was determined to be less than 6 X 104 mho/cm at 25°C.

Purification of DNA Polymerase—Further purification of Fraction I was carried out by chromatography on DEAE-cellulose as follows. Fraction I was applied to a DEAE-column (1.7 X 27 cm) previously equilibrated with Buffer IV containing 0.35 M KCl. The column was washed with Buffer IV containing 0.4 M KCl. Fractions having an A260 of greater than 1.0 were pooled. The volume of the pooled fractions was generally between 70 and 100 ml. The activity was concentrated by ammonium sulfate precipitation as described above. The resulting precipitate, designated Fraction IIA, was dissolved in 8 ml of Buffer IV and dialyzed overnight against 1 liter of Buffer IV. In addition, fractions from the upper part of the gradient having an A260 of greater than 0.5 were pooled, and the protein was concentrated by ammonium sulfate precipitation as described above. The following procedure was repeated, designated Fraction V, was dissolved in 8 ml of Buffer IV and dialyzed overnight against 1 liter of Buffer IV.

Purification of Other Active Fractions—Fractions III and IV were purified from Fraction II as follows. Fraction II was applied to a DEAE-cellulose column (1.7 X 27 cm) previously equilibrated with Buffer III. The column was washed with Buffer III containing 0.35 M KCl. Fractions having an A260 greater than 1.0 were pooled. The volume of the pooled fractions was generally between 70 and 100 ml. The activity was concentrated by ammonium sulfate precipitation as described above. The resulting precipitate, designated Fraction IC, was dissolved in 8 ml of Buffer IV and dialyzed overnight against 1 liter of Buffer IV. In addition, fractions from the upper part of the gradient having an A260 of greater than 0.5 were pooled, and the protein was concentrated by ammonium sulfate precipitation as described above. The following procedure was repeated, designated Fraction V, was dissolved in 8 ml of Buffer IV and dialyzed overnight against 1 liter of Buffer IV.

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The 30,000 x g supernatant (S30) of extracts prepared from *Escherichia coli* 514 was capable of synthesizing active *su*II tRNA<sup>tyr</sup> (16, 17). By a process of separation and reconstitution, four essential components for tRNA synthesis have been purified from these extracts. The following scheme was employed.

**Purification Procedure**—The removal of ribosomes from the S30 was without effect; tRNA synthesizing activity was quantitatively retained in the S100. Removal of bulk nucleic acid was achieved by passage of the extract over a DEAE-cellulose column. Under these conditions, effective removal of greater than 99% of the endogenous tRNA from the extract (as determined by its amino acid acceptance activity) was achieved. Thus, after this procedure, the ability of the extract to support de novo synthesis of tRNA could be assayed directly by the appearance of amino acid acceptance activity.

Ammonium sulfate fractionation yielded two fractions, both of which were required for the achievement of maximal levels of tRNA synthesis. Fraction I contained 90 to 95% of the DNA-dependent RNA polymerase activity. As shown in Table III, this fraction synthesized tRNA at levels that were approximately 20% of that originally observed with the S100. Fraction II was completely devoid of measurable tRNA-synthesizing activity when assayed alone. However, optimal levels of tRNA synthesis, comparable to those observed in the S100, were achieved when both Fraction I and Fraction II were included in the *in vitro* synthesis reaction. When Fraction I was further purified by chromatography on DEAE-cellulose, the DNA-dependent RNA polymerase activity was quantitatively recovered in Fraction IA.

As shown in Table III, for any given fraction, the amount of tRNA synthesized as measured in the suppression assay was always twice that determined directly by its tyrosine acceptance activity. Thus, both assay methods yielded the same information as to the relative ability of a particular fraction to support the *in vitro* synthesis of tRNA. The further purification of Fraction IA and Fraction II was monitored employing their capacity to support tRNA synthesis as determined by the tyrosine acceptance activity.

Further purification of Fraction II by chromatography on DEAE-cellulose revealed that this fraction contained two dis...
distinct activities, both of which were required for the achievement of maximal levels of tRNA synthesis. Fraction III, when incubated with Fraction IA, was unable to support detectable levels of tRNA synthesis. Incubation of Fraction IV with Fraction IA allowed tRNA synthesis to occur at levels approaching 25% of that observed with Fraction II. However, maximal levels of tRNA synthesis were achieved when both Fraction III and Fraction IV were included with Fraction IA in the in vitro synthesis reactions. Thus, tRNA synthesis requires the combined activities of three components, Fraction IA, Fraction III, and Fraction IV. The data presented in Fig. 2 demonstrate that when in vitro synthesis reactions were carried out in the presence of saturating amounts of any of the two of these components, synthesis of su\textsubscript{II} tRNA was dependent upon the addition of the third component. The data further demonstrate that under these conditions, the amount of tRNA synthesized was proportional to the amount of the third component added to the system. On this basis, further purification of Fractions III and IV was achieved.

The further purification of the DNA-dependent RNA polymerase in Fraction IA on a second DEAE-cellulose column did not result in a significant increase in the specific activity of the enzyme. However, when Fraction IB replaced Fraction IA in the in vitro system, synthesis of tRNA was found to be completely dependent upon the addition of Fraction III. Thus, the 25% level of tRNA synthesis previously observed when Fraction IV was incubated with Fraction IA was probably the result of small amounts of Fraction III contaminating Fraction IA. This suggestion was confirmed by analysis of the in vitro synthesized products by acrylamide gel electrophoresis as described below.

**Enzymatic Activity of Fraction IV**—A precursor su\textsubscript{II} tRNA\textsuperscript{77} has been isolated from \$80ps\textsubscript{ssu}\textsuperscript{-}-infected E. coli (3). This molecule as isolated has been shown to contain 41 additional nucleotides at the 5' end and three additional nucleotides at the 3' end compared to mature su\textsubscript{II} tRNA\textsuperscript{77} (3). It has been demonstrated that RNase P specifically catalyzes the removal of the extra nucleotides present at the 5' end of this molecule, and, indeed, the purification and characterization of RNase P were based on its ability to perform this reaction (6). When assayed directly on \textsuperscript{32}P-labeled su\textsubscript{II} tRNA precursor, Fraction IV was found to contain high levels of RNase P activity. It was also determined that the RNase P activity was quantitatively recovered in Fractions IVA and IVB. These observations are consistent with the suggestion that the essential function provided for tRNA synthesis by Fraction IV in the in vitro system is the removal of the 3' extra nucleotides from tRNA precursor.

As determined by the DNA-dependent incorporation of radioactively labeled ribotriphosphates into acid-insoluble material, the amount of total transcription was inhibited 3- to 5-fold when Fraction IBV replaced Fraction IV or IVA in the in vitro synthesis reactions. This inhibition was not the result of contaminating ribonuclease activity for the following reasons. First, the inhibition was not observed when Fraction IVB was incubated in in vitro synthesis reactions that already contained saturating levels of Fraction IV. Second, the inhibitory effect was not observed when calf thymus DNA replaced \$80ps\textsubscript{ssu} DNA as the template for transcription. If, instead of gel filtration on Sephadex G-200, sucrose gradient centrifugation was utilized as an alternative method for the further purification of Fraction IVA, the enzyme activity recovered from these gradients exhibited the same inhibitory effect upon total transcription in the in vitro synthesis reactions that described from Fraction IVB. The basis for this observation is not clear.

**Enzymatic Activity of Fractions IA and III**—That the achievement of maximal levels of tRNA synthesis required the combined activities of Fractions IA, III, and IV is qualitatively demonstrated by the data shown in Fig. 3. When incubated alone, Fraction IA (crude RNA polymerase) synthesized several discrete RNA products (Column 1). Among these transcription products was a species, designated pre-tRNA\textsubscript{IA}, that is a mixture of two RNA species: su\textsubscript{II} tRNA\textsuperscript{77} precursor and a \$80-specific RNA of unknown function. These two RNA species were identified as follows. When \$80 DNA replaced \$80ps\textsubscript{ssu} DNA as the template DNA, one of the transcription products was found to comigrate with the pre-tRNA\textsubscript{IA} species synthesized when \$80ps\textsubscript{ssu} DNA was the template. The su\textsubscript{II} tRNA\textsuperscript{77} precursor species was identified by direct sequence analysis. It was shown to contain the complete nucleotide sequence of the mature su\textsubscript{II} tRNA\textsuperscript{77} and the 41 additional nucleotides present at the 3' end of the su\textsubscript{II} tRNA\textsuperscript{77} precursor isolated in vivo.

As shown in Column 2, the addition of Fraction II resulted in the disappearance of the pre-tRNA\textsubscript{IA} species and the appearance of 4 S su\textsubscript{II} tRNA\textsuperscript{77}. As shown in Columns 3 to 5, when Fraction IV, IVA, or IVB was incubated with Fraction IA, small amounts of mature 4 S su\textsubscript{II} tRNA\textsuperscript{77} were synthesized. However, the 4 S band was noticeably less intense than in Column 2, where Fraction II was incubated with Fraction IA. Furthermore, the appearance of an RNA species, designated pre-tRNA\textsubscript{IA}, that migrated slightly slower than the mature 4 S su\textsubscript{II} tRNA\textsuperscript{77}, was observed in Columns 3 to 5. By direct sequence analysis, this RNA species has been shown to be su\textsubscript{II} tRNA\textsuperscript{77} containing extra nucleotides at its 3' end. As shown in Column 6, when Fraction III was incubated with Fraction IA, there was no detectable change of 4 S su\textsubscript{II} tRNA\textsuperscript{77}, nor was there any detectable change in the mobility of the pre-tRNA\textsubscript{IA} transcription product of the Fraction IA polymerase. However, as shown in Columns 7 and 8, when Fraction III was incubated with Fractions IA and IV, pre-tRNA\textsubscript{IA} was converted into mature 4 S su\textsubscript{II} tRNA\textsuperscript{77}, and the amount of tRNA synthesized was comparable to that observed when Fraction II was incubated with Fraction IA.

The appearance of small amounts of mature 4 S su\textsubscript{II} tRNA\textsuperscript{77} in Columns 3 to 5 was the result of small amounts of Fraction III contaminating Fraction IA. This suggestion was confirmed by the observation that when Fraction IB replaced Fraction IA in
FIG. 3 (left). Three essential activities for in vitro synthesis of tRNA. In vitro synthesis reactions and acrylamide gel electrophoresis of the RNA products were carried out as described under “Experimental Procedure.” Reaction mixtures included [γ-32P]GTP at a final specific activity of 100 mCi/mmol. DNA-dependent RNA polymerase was purified according to the procedure described by Burgess (20). Reactions in Columns 1 and 2 contained 0.2 mg of Fraction IA. In addition, where indicated, Fraction IV (0.6 mg), Fraction IV (25 μg), Fraction IVA (2 μg), Fraction IVB (2 μg), and/or Fraction III (0.7 mg) was included.

FIG. 4 (right). In vitro synthesis of tRNA with purified RNA polymerase. In vitro synthesis reactions and acrylamide gel electrophoresis of the RNA products were carried out as described under “Experimental Procedure.” Reaction mixtures included [γ-32P]GTP at a final specific activity of 100 mCi/mmol. DNA-dependent RNA polymerase was purified according to the procedure described by Burgess (20). Reactions in Columns 1 and 2 contained 0.2 mg of Fraction IA (29 units of DNA-dependent RNA polymerase). Reactions in Columns 3 and 4 contained 25 μg of purified RNA polymerase (29 units). In addition, reactions in Columns 2 and 4 contained 0.6 mg of Fraction II.

The in vitro synthesis reactions, addition of Fraction IV resulted in the accumulation of partially matured s III tRNA^Tyr precursor at the pre-tRNA position (data not shown). Thus, in the complete absence of Fraction III, there was no detectable synthesis of the mature 4 S s III tRNA^Tyr species. We therefore conclude that the essential function for tRNA synthesis provided by Fraction III in this system is the removal of the 3' extra nucleotides from the s III tRNA^Tyr precursor. The results concerning this enzymatic activity are presented in the accompanying paper (27).

RNA Polymerase—As shown in Table IV, purified DNA-dependent RNA polymerase is capable of giving rise to transcripts that can be processed into biologically active s III tRNA^Tyr. When Fraction II was included in the in vitro synthesis reactions, the purified DNA-dependent RNA polymerase supported levels of tRNA synthesis comparable to those observed with Fraction IA as the source of DNA-dependent RNA polymerase activity (Table IV). However, when the purified polymerase was incubated with Fractions III and IVA, the levels of tRNA synthesis were 50% of those observed with Fraction IA as the source of DNA-dependent RNA polymerase. Finally, with Fraction IIIA and Fraction IVA, the purified DNA-dependent RNA polymerase was unable to support detectable levels of tRNA synthesis in the in vitro system. These results were confirmed by acrylamide gel analysis. As shown in Fig. 4, the pre-tRNA species, consistently observed as a transcription product of the Fraction IA polymerase, was not observed as a transcription product of the purified DNA-dependent RNA polymerase. Under these conditions, the RNA transcription products of the purified enzyme were sufficiently large such that 90 to 95% of the material did not enter the gel. Thus, purified DNA-dependent RNA polymerase is necessary but not sufficient for transcription of s III tRNA^Tyr precursor.

Fraction V—The results presented above are consistent with the possibility that there is at least one other protein besides RNA polymerase that is required for correct transcription of the s III tRNA^Tyr gene, and that this protein(s) is present in the crude preparation of RNA polymerase (Fraction IA) as well as...
in the crude preparation of RNase P III (Fraction III), but it is absent from the purified RNA polymerase as well as from the more purified RNase P III (Fraction IIIA). Evidence confirming this suggestion was provided by the further purification of Fraction IA polymerase by glycerol gradient centrifugation.

As shown in Table V, the RNA polymerase recovered from the glycerol gradient (Fraction IC) was unable to support detectable levels of tRNA synthesis. However, a fraction separable from DNA-dependent RNA polymerase by glycerol gradient centrifugation was discovered, designated Fraction V, which when added to reactions containing Fraction IC polymerase, was able to reconstitute tRNA synthetic activity. Furthermore, as shown in Table V, Fraction V was able to restore tRNA synthesis activity to reactions containing DNA-dependent RNA polymerase purified according to the procedure described by Burgess (20). We therefore conclude that Fraction V contains one or more proteins that are essential for tRNA synthesis. On the basis of the acrylamide gel analyses shown in Fig. 4, we further suggest that these protein(s) is involved in the synthesis, rather than the maturation, of suIrr tRNA Tyr precursor.

### DISCUSSION

We have described an in vitro system in which active suIrr tRNA Tyr is synthesized from a φ80psuIrr DNA template. Using this system, we have identified four essential components that are required for synthesis of tRNA: (a) DNA-dependent RNA polymerase, (b) a fraction (Fraction V) of unknown function that allows purified DNA-dependent RNA polymerase to function in tRNA synthesis, (c) RNase P, the endonuclease that specifically cleaves tRNATyr at the 3' end of tRNA precursors, and (d) a ribonuclease (RNase P III) that specifically catalyzes the removal of the extra nucleotides present at the 3' end of tRNA precursors. A detailed discussion of the roles of RNase P, RNase P III, and Fraction V in tRNA biosynthesis is presented in the accompanying paper (27).

Suppression assays were carried out in the presence of crude extracts known to contain all of the enzymes necessary for post-transcriptional processing of precursor molecules; therefore, primary transcription products potentially maturation into suIrr tRNA Tyr would be expected to have the same suppressor activity in these assays as the mature 4 S species. We have shown that RNA synthesized by the Fraction IA polymerase has no detectable activity in the in vitro suppression assay. However, we have also demonstrated by acrylamide gel analysis that among the transcription products of Fraction IA is a species of RNA (pre-tRNA) that by direct sequence analysis contains the suIrr tRNA Tyr precursor. The ability of this material to give rise to mature 4 S suIrr tRNA Tyr upon post-transcriptional cleavage has been demonstrated (27). So the question arises, why is there no detectable activity when this suIrr tRNA Tyr precursor is incubated in the in vitro suppression assay? One possible explanation is that the isolated precursor molecule is much more susceptible to degradation by nonspecific nucleases than is the mature 4 S species. If this suggestion were correct, then it would be expected that when the mature 4 S species was incubated in the in vitro suppression assay, effectively all of the material added would function in suppression. With isolated precursor molecules, however, it would be expected that only a small percentage of the added material would be specifically cleaved into the mature 4 S species, which would then function in suppression. This explanation is consistent with the observation that when 32P-labeled suIrr tRNA Tyr was incubated with crude extracts, 90% of the added material was recovered as 4 S RNA. However, when 32P-labeled suIrr tRNA Tyr precursor was incubated with crude extracts, less than 50% of the added material was recovered as 4 S RNA.

Another possible explanation is that efficient synthesis of tRNA in the in vitro system depends upon the ability of one or more components of the complete system to function repeatedly and that this recycling can only occur under conditions where the synthesis of mature suIrr tRNA Tyr can be achieved. This suggestion is consistent with the observation that the amount of suIrr tRNA Tyr that is synthesized in the presence of the complete
The suffix tRNA^{37}Tyr precursor molecule isolated \textit{in vivo} was shown to contain pppGp as the 5' terminal nucleotide (3). Thus, the initiation site for transcription of the suffix tRNA^{37}Tyr is known to be located 41 nucleotides to the right of the structural gene for the tRNA. However, the precise site for termination of transcription of the suffix tRNA^{37}Tyr gene has not yet been determined. The precursor molecule isolated \textit{in vivo} was shown to contain three extra nucleotides beyond the normal —CCA on 3' terminus of the mature 4 S species (3). Our results, obtained by direct sequence analysis of the \textit{in vitro} synthesized transcription product, indicate that the suffix tRNA^{37}Tyr precursor contains between 4 and 13 additional residues beyond the —CCA 3' terminus.

We have demonstrated the \textit{in vitro} synthesis of this suffix tRNA^{37}Tyr precursor molecule with a crude preparation of DNA-dependent RNA polymerase (Fraction IA). However, this molecule was not observed as a transcription product with DNA-dependent RNA polymerase purified according to the procedure described by Burgess (20). It has also been demonstrated that the purified RNA polymerase was capable of supporting levels of tRNA synthesis comparable to those observed with Fraction IA polymerase when crude preparations of RNase P and RNase P III were included in the \textit{in vitro} synthesis reactions. However, with purified preparations of RNase P and RNase P III, the purified DNA-dependent RNA polymerase was unable to support detectable levels of tRNA synthesis. Thus, we conclude that DNA-dependent RNA polymerase as described by Burgess (20) is necessary but not sufficient for synthesis of suffix tRNA^{37}Tyr precursor.

A fraction has been identified (Fraction V) which, when added to reactions containing purified DNA-dependent RNA polymerase, was able to reconstitute tRNA synthetic activity. On the basis of acrylamide gel analyses, we suggest that this fraction is involved in the synthesis rather than the maturation of suffix tRNA^{37}Tyr precursor. Direct proof of this suggestion is presented in the accompanying paper (27).

Several other laboratories have studied \textit{in vitro} transcription of φ80 suffix DNA with purified DNA-dependent RNA polymerase, (10–12). It has been reported (28) that the termination factor \( \rho \) (12) is required for the \textit{in vitro} synthesis of suffix tRNA^{37}Tyr precursor (10–12). Beckmann and Daniel (12) demonstrated that in the presence of \( \rho \) factor there was a 4-fold enhancement of the relative amount of tRNA^{37}Tyr-like sequences as determined by competitive hybridization of the \textit{in vitro} synthesized tRNA with \( ^{32}P \) tRNA. However, the size of the transcripts containing the tRNA^{37}Tyr sequence was not reported. Ikeda (11) reported that in the presence of \( \rho \) factor, purified DNA-dependent RNA polymerase synthesized one major and two minor species of RNA that contained the tRNA^{37}Tyr sequence (11). These species were not synthesized in the absence of \( \rho \) factor.

Thus, the possibility exists that the essential activity for tRNA synthesis provided by Fraction V is the termination factor \( \rho \). However, the fact that synthesis of suffix tRNA^{37}Tyr precursor of a discrete size identical with that isolated \textit{in vivo} has not been achieved in systems with purified DNA-dependent RNA polymerase and \( \rho \) factor suggests that there may be another protein that either instead of \( \rho \) or in addition to \( \rho \) is involved in the synthesis of suffix tRNA precursor. Further purification of this complex enzymatic system should resolve this and other questions regarding the mechanism of tRNA biosynthesis.

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