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Transfer RNA Synthesis *In Vitro*

(T4 DNA/RNA transcription/RNA processing/sequence analysis)

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ABSTRACT **During infection of *Escherichia coli*, bacteriophage T4 directs the synthesis of at least eight transfer RNAs and of two stable RNA species of low molecular weight, of unknown function. When T4 DNA is incubated with purified RNA polymerase and the appropriate substrates, a high molecular weight RNA is produced. This RNA, on further incubation with a supernatant fraction prepared from *E. coli*, is cleaved to several species of RNA. These cleaved RNAs were analyzed by gel electrophoresis and fingerprint techniques, and were found to be similar or identical to those made *in vivo*. The fingerprint analysis of one of these, a $tRNA^{Gly}$, is presented. The molecule made *in vitro*, except for the absence of modified bases, appears to be identical to the RNA made by T4-infected cells. Therefore, in this system the tRNA genes are transcribed with fidelity, the transcript is cleaved correctly, and the tRNAs are made in good yield.**

Since transfer RNAs play a central role in cellular metabolism, it is important to understand the details of their synthesis. In the case of the tyrosine suppressor tRNA encoded by bacteriophage ϕ80pSu$^+$III, the Su$^+$III $tRNA^{Tyr}$ is transcribed with additional residues at the 3′ and 5′ ends of the molecule. This unmodified precursor RNA must be further cleaved and modified to yield the functional tRNA (1, 2). Several enzymes have been described that catalyze these modifications (3). However, it is not completely understood how the tRNA genes are arranged on the chromosome, how such genes are transcribed, or what special features allow for their regulation.

Clearly much more could be learned about the synthesis of tRNA and its control by study of tRNA synthesis *in vitro*. Several groups have reported the *in vitro* synthesis of tRNA with the Su$^+$III system (4–6). We have developed an *in vitro* system that synthesizes several of the tRNAs coded by bacteriophage T4, and we have shown the fidelity of their transcription by fingerprint analysis.

After infection of its host, *Escherichia coli*, bacteriophage T4 directs the synthesis of some ten stable RNA species, eight of which are tRNAs (7–10, 23). The genes for these stable RNA species are all clustered together in a region of about 3000 base pairs on the T4 genome (11). Our working hypothesis is that these tRNA genes are part of a single transcription unit, and may constitute a tRNA operon. Thus, the T4 system provides us with the opportunity to study in detail the processing and modification of several tRNAs from a large precursor.

When T4 DNA is transcribed *in vitro*, RNA molecules of tRNA size are not made (12–14). However, it has been observed (*i*) that mature tRNAs are stable to nucleases present in a crude extract of *E. coli* (2), whereas other RNAs are degraded, and (*ii*) that the incubation of a large ϕ80pSu$^+$III transcript with an *E. coli* supernatant yields molecules of about tRNA size (6). Our experiments have thus been directed toward testing the possibility that tRNA sequences might be revealed after the incubation of such T4 transcripts with an *E. coli* extract.

In this communication, we show that the T4 tRNAs can be accurately transcribed *in vitro* from T4 DNA and processed correctly. We have analyzed one of the *in vitro* products, a T4 $tRNA^{Gly}$, and have concluded that the *in vitro* product has the same nucleotide sequence as that made *in vivo*.

MATERIALS AND METHODS

Phage T4D and phage T4 $pSu_b^-\Delta 33$ (11) were purified by equilibrium density centrifugation in cesium chloride. The DNA was extracted with phenol (15) and dialyzed against a solution containing 0.01 M Tris·HCl (pH 7.4)–0.05 M NaCl.

The supernatant fraction was prepared from *E. coli* Q13 (16). Cells were grown in broth to late-log phase and harvested by centrifugation. The cells were washed and resuspended in a buffer containing 0.01 M Tris·HCl (pH 8.0)–0.01 M $Mg(OAc)_2$–0.06 M KCl–6 mM 2-mercaptoethanol. After disruption of the cells in a French press at 15,000 lb/in^2, the extract was centrifuged for 30 min at 31,000 × *g* and the upper $^1/_3$ volume was collected. This fraction was extensively dialyzed against a solution containing 0.01 M Tris·HCl (pH 8.0)–0.01 M $Mg(OAc)_2$–0.06 M NH_4Cl–6 mM 2-mercaptoethanol, quick frozen, and stored at −20°.

RNA polymerase was isolated according to the method of Berg, Barrett, and Chamberlin (17). Fraction V enzyme had a protein concentration of 2.5 mg/ml.

The reaction mixture for RNA synthesis (18) contained in a volume of 0.5 ml: 0.04 M Tris·HCl (pH 7.9), 0.01 M $MgCl_2$, 0.16 M KCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.4 mM potassium phosphate (pH 7.5), 0.16 mM each of ATP, CTP, and UTP, 0.12 mM [α-^{32}P]GTP (6 Ci/mmol; New England Nuclear Corp., Boston, Mass.), 100 μg/ml of DNA, and 50 μg/ml of RNA polymerase. The reaction was incubated for 20 min at 37° and terminated by the addition of 10 μg of actinomycin D and 50 μg of DNase I (RNase-free, Worthington, Freehold, N.J.), immediately followed by the addition of 20 μl of supernatant fraction from *E. coli* Q13 and 90 μg of carrier *E. coli* tRNA. The incubation was continued for 30 min at 37°, and terminated by the addition of 0.75 ml of 0.25 M NaOAc (pH 5.0) and 0.5 ml of 0.01 N HCl.

The incorporation of [^{32}P]GTP was followed by the precipi-

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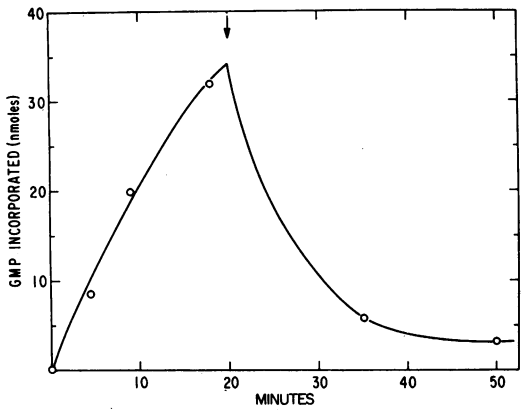

FIG. 1. Rate of [α-^{32}P]GTP incorporation by RNA polymerase with T4 DNA as template. At 20 min (*arrow*), *E. coli* Q13 supernatant fraction was added to hydrolyze the unstable RNA species and to cleave the tRNA precursor.

tation of 2-μl aliquots from the reaction mixture with 5% Cl_3CCOOH. Radioactivity was measured in a liquid scintillation counter.

The RNA was extracted by shaking the reaction mixture with an equal volume of H_2O-saturated phenol for 30 min at room temperature. The aqueous phase was fractionated on a column of Sephadex G-25 (1.8 × 25 cm) that had been equilibrated with 0.01 M NaOAc (pH 5.0). The column was eluted with the same buffer. Fractions comprising the excluded peak of radioactivity were pooled and precipitated with ethanol.

RNA was fractionated on acrylamide gels by the method described by DeWachter and Fiers (19). The 40-cm 10% gels (39:1, acrylamide–bisacrylamide) were prepared and run in 0.1 M Tris acetate (pH 8.3) at about 30 mA for 16–18 hr. Marker tRNAs were labeled *in vivo* with ^{32}P as described by Wilson and Abelson (20). Radioactive species on the gel were located by autoradiography.

The RNA species that were to be fingerprinted were recovered from the gel by homogenization of cut-out gel bands in 0.3 M NaCl. Acrylamide was removed by centrifugation and filtration through a Millipore filter.

The fingerprint analysis was performed according to the procedure of Sanger *et al.* (21), and involved T1 RNase digestion followed by two-dimensional electrophoresis on cellulose acetate and DEAE-paper.

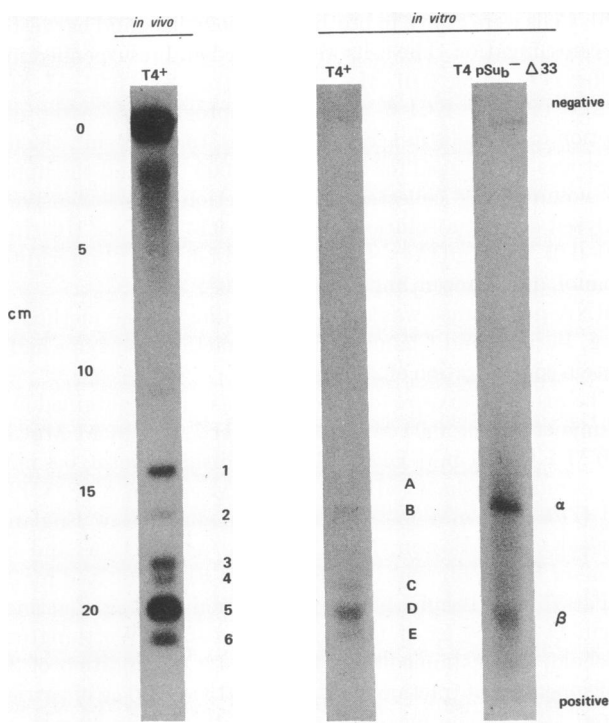

FIG. 2. Autoradiogram of acrylamide gel separations. Comparison of stable RNA species made *in vivo* and made *in vitro* with T4 or T4 pSu$_b^-$Δ33 DNA as template, as described in the legend to Fig. 1.

RESULTS AND DISCUSSION

Fig. 1 shows the incorporation of [α-^{32}P]GTP into RNA in an experiment in which T4D DNA served as template. After 20 min of incubation at 37°, RNA synthesis was terminated by the addition of actinomycin D and DNase I. A crude S-30 supernatant fraction from *E. coli* was added and incubation was continued for 30 min. At the end of the incubation, only 10% of the labeled RNA that was acid-precipitable at 20 min remained. This RNA was purified by passage through Sephadex G-25 (exclusion molecular weight about 10,000) and precipitated with ethanol. The RNA was subjected to electrophoresis in a 10% polyacrylamide gel. As a control, RNA prepared similarly with T4 pSu$_b^-$Δ33 DNA was used. All ten stable RNAs are deleted in this mutant. T4 [^{32}P]tRNA made *in vivo* served as a reference marker. Fig. 2 shows an autoradiogram of the gel separation.

The ten stable RNA species produced during T4 infection give rise to six bands in the gel separation. Bands *1* and *2*

TABLE 1. *Nearest-neighbor analysis of RNase T1 oligonucleotides from band E*

RNase T1 product	Sequence	Nearest neighbor predicted	Nearest neighbor found
1	G(A) G(D)	None	G
2	CG(G)	C,G	C,G
3	AG(T), AG(A)	A	A
4	UG(A) UG(A)	U	U
5	pG(C)	pGp	pGp
6	AUG(A) AUG(U)	U	U
7	TΨCG(A)	C	C
8	AUAUCG(U)	C	C
9	UAUAAUG(G)	U,G	U,G
10	UAUAAUGmeG(D)	—	Not present
11	AUUCUCAUUAUCCG(C)	C	C
12	AUUUACCUCAG(A)	A	A*
13	ACUNCCAAΨCUG(A)	U	U*
15	CUCCA$_{OH}$	None	Not present

The oligonucleotides were eluted from DEAE-paper and hydrolyzed by treatment with 0.2 N NaOH. The 3′ nucleotides were separated by electrophoresis at pH 3.5 on Whatman no. 52 paper.

* Oligonucleotides 12 and 13 were not resolved in Fig. 3*B*.

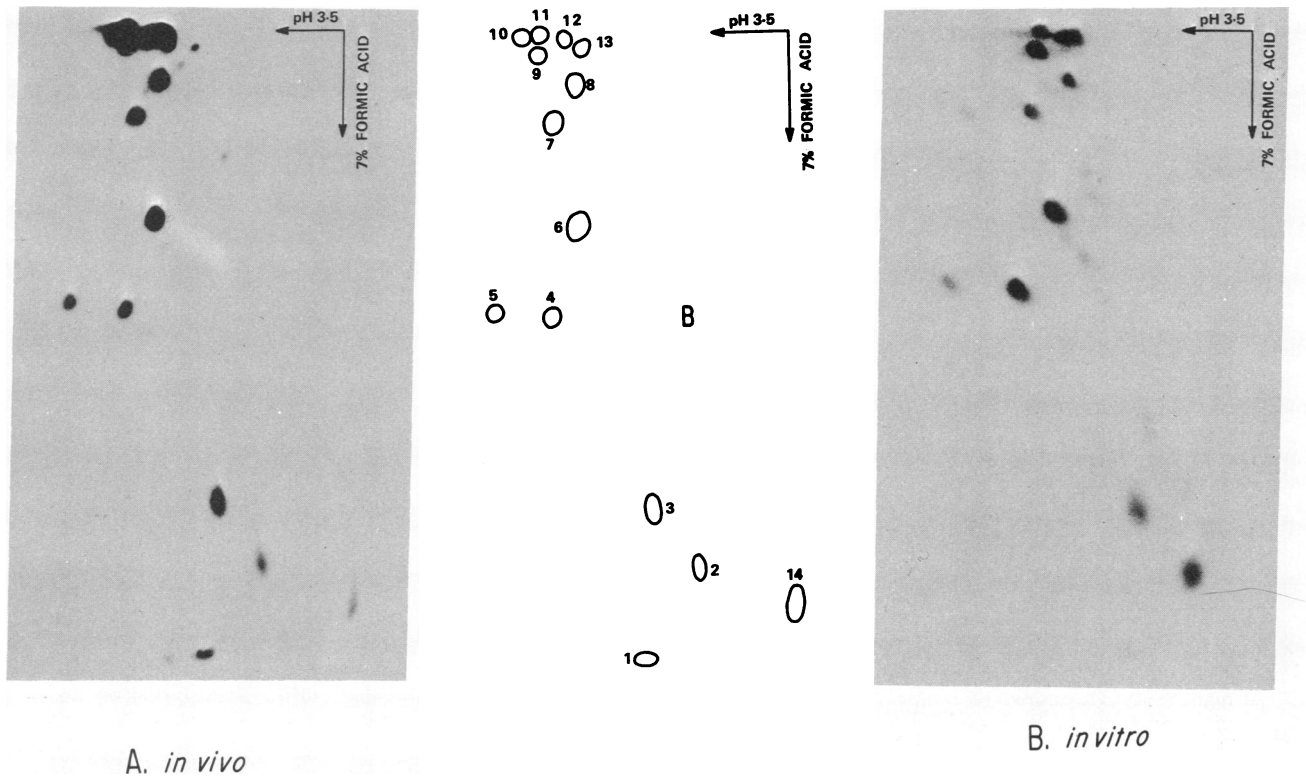

FIG. 3. Autoradiograms of fingerprints of $tRNA^{Gly}$ made *in vivo* (Band *6*, Fig. 1) and made *in vitro* (Band *E*, Fig. 1).

contain stable RNAs of unknown function. Band *1* RNA is 140 nucleotides long; it does not contain modified nucleosides, and its sequence is known (Paddock and Abelson, manuscript in preparation). Band *2* is about 100 nucleotides long. Band *3* is a $tRNA^{Ser}$ whose sequence has been determined by McClain and Barrell (personal communication). Band *4* contains a $tRNA^{Leu}$ whose sequence is known (22). Band *5* contains at least five tRNA species (23). Band *6* is a $tRNA^{Gly}$ whose sequence has been determined (Stahl, Paddock, and Abelson, manuscript in preparation). Studies of these T4 tRNAs by sequence analysis and by DNA–RNA hybridization indicate that all of them have distinctly different sequences from those of the host tRNAs (refs. 7, 9, 11, 22, 23, and unpublished results).

The electrophoretic gel pattern of stable T4 RNA made *in vitro* strongly resembles the RNA made *in vivo*, and is quite distinct from that produced when T4 $pSu_b^-\Delta 33$ DNA is used as a template. Fingerprint analysis of these RNAs by the methods of Sanger, Brownlee, and Barrell (21) confirm the identity of the *in vitro* and *in vivo* bands. By these criteria, band *A* is similar or identical to band *1*, band *C* to band *4*, and band *E* to band *6*. Band *D* is similar to band *5*, but may be contaminated by the material in band β. Apparently, neither band *3*, the $tRNA^{Ser}$, nor band *2* are made *in vitro*. (Band *B* does not have the same fingerprint as band *2*, and appears to correspond to band α.)

RNase T1 fingerprints of band *E* (*in vitro*) and band *6* (*in vivo*) are shown in Fig. 3. The *in vitro* labeling was performed with [α-^{32}P]GTP. Since RNase T1 cleaves adjacent to GMP residues, each fragment contains a labeled 3′-terminal GMP (except the 3′-terminal fragment of the molecule, which contains no GMP). Thus, differences in the fingerprint patterns will arise primarily from differences in sequences or from the presence of modified bases in the tRNA made *in vivo*, but possibly not formed *in vitro*. Table 1 lists the sequences of the *in vivo* RNase T1 fragments (Stahl, Paddock, and Abelson, in preparation). In the *in vivo* tRNA fingerprint there are two oligonucleotides (*9* and *10*) derived from the same original sequence. Fragment *9* is shorter than *10* by one residue, due to incomplete methylation *in vivo* (methylation forms the RNase T1-resistant sequence 2′-*O*-methylguanosyl GMP). Only the shorter fragment appears in the *in vitro* fingerprint, indicating the absence of this modification. The nucleotide representing the 5′-end of the tRNA molecule, pGp (guanosine 3; 5′-diphosphate), is found in the fingerprint of the *in vitro* product. In other experiments, we have synthesized T4 tRNA labeled with [α-^{32}P]CTP. RNase T1 fingerprints of band *E* from this preparation show a spot with a mobility identical to spot *14*, the 3′-terminal oligonucleotide. Thus, the cleavages at both ends of the molecule are identical to those occurring *in vivo*.

To further examine the fidelity of this transcriptional system, we have determined the nearest neighbor distribution of labeled GMP in the fragments isolated in the fingerprint shown in Fig. 2*B*. Alkaline hydrolysis of the RNase T1 fragments should lead to the transfer of ^{32}P to the nucleotide adjacent to the terminal G, while in a fragment where a terminal G is followed by another G in the tRNA sequence, the terminal G will also be labeled. Table 1 shows the results of this analysis. The distribution of ^{32}P in each fragment derived from the RNA made *in vitro* is as predicted by the sequence of $tRNA^{Gly}$. One exception is the presence of some free GMP in the analysis; this GMP probably arises from a slight contamination of the *in vitro* product.

These results allow and encourage further studies of tRNA transcription, processing, and modification. Since we suspect

that the tRNA genes are part of a large transcription unit, we are investigating the nature of the primary transcript. We have already determined that it is quite large (our unpublished results).

The absence in the *in vitro* product of bands *2* and *3* ($tRNA^{Ser}$) suggests that some factor, specified by the phage genome, is required for the synthesis or maturation of these RNAs. At least one bacteriophage T4 function, the product of the *mb* gene, appears to be necessary for the complete synthesis of T4 tRNA *in vivo*, since *mb* mutants fail to make bands *2*, *3*, and one or two of the band *5* species (20). In order to achieve a complete *in vitro* synthesis of the T4 tRNAs, it will presumably be necessary to supplement the system with the product of the T4 *mb* gene.

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