# CODING BY T4 PHAGE DNA OF SOLUBLE RNA CONTAINING PSEUDOURIDYLIC ACID

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Earlier studies on RNA synthesis in *E. coli* showed that after infection with a T-even bacteriophage there was a rapid incorporation of $P^{32}$ orthophosphate into an RNA whose base composition was similar to that of the phage DNA.[1] While the bulk of the labeled RNA synthesized after infection was of a high molecular weight and functioned as phage-specific mRNA,[2, 3] some 8 per cent was found as a low-molecular-weight sRNA fraction.[4] Recently the base composition and the hybridization properties of such sRNA have been reported.[5, 6]

In addition, several examples of virus-induced modification of tRNA have been observed. Thus, MAK column chromatography[7] and reverse phase chromatography[8] revealed a change in the elution profile of several leucyl-tRNA's isolated after infection of *E. coli* with a T-even bacteriophage; and such an infection was also shown to cause methylation[9] and thiolation[10] of sRNA. However, these observations could not distinguish between *de novo* phage-induced sRNA synthesis and modifications of pre-existing host sRNA. This report presents evidence for the induction by T4 of the synthesis of a novel sRNA containing pseudouridylic acid.

*Experimental Methods.—$P^{32}$ labeling of E. coli cells infected with T4 bacteriophage:* *E. coli* B cells were grown in TG medium[11] containing $1 \times 10^{-4}$ *M* inorganic phosphate ($P_i$). The culture was grown with shaking at 37°C to a concentration of $5 \times 10^8$ cells/ml. L-Tryptophan (10 $\mu$g/ml) was added, and the culture was infected with phage T4D at a multiplicity of 10. The suspension was incubated for 2 min without shaking to allow phage adsorption, $^{32}P_i$ was added (0.01–0.06 mc/ml), and shaking was resumed for an additional 10 min. The cell suspension was then superinfected with additional phage (final multiplicity of 20) to cause lysis inhibition and thus prolong the latent period. Nonradioactive $KPO_4$ buffer, pH 7.0, was added to a final concentration of $1 \times 10^{-2}$ *M*, and the suspension was shaken for 60 min, rapidly cooled, and harvested by centrifugation. Analysis of an aliquot removed at 10 min indicated that over 99% of the cells were infected with phage.

*Extraction of sRNA and MAK chromatography:* The pelleted cells obtained from a 50–100-ml culture were suspended in 2 ml of a solution (containing 10 mM Tris-HCl buffer, pH 7.4, 10 mM $MgCl_2$, and 10 mM NaCl) and extracted three times with 2 ml of freshly distilled phenol. To the aqueous phase 0.2 vol of 5 *M* NaCl and 2.2 vol of ethanol were added at 4°C, and DNA was removed by spooling on a glass rod. The RNA was collected by centrifugation, washed three times with ethanol-NaCl, and dissolved in 1 ml of $10^{-2}$ *M* Tris-HCl, pH 7.4 containing $1 \times 10^{-2}$ *M* $MgCl_2$; 40 $\mu$g of electrophoretically purified DNase (Worthington Biochemicals) was added and the mixture was incubated at room temperature for 60 min. Then 100 $\mu$g Pronase was added and the incubation was continued for 4 hr at 37°C. To reduce possible traces of nucleases, the Pronase solution was preincubated for 90 min at 37°C (2 mg/ml in 0.01 *M* Tris-HCl buffer, pH 7.4) before use.[12] The RNA solution was diluted to 20 $\mu$g/ml with 0.1 *M* $NaPO_4$ buffer, pH 6.7, applied on a $1.5 \times 18$ cm MAK column,[7] washed with 20 ml of the diluting buffer, and eluted at room temperature with a linear salt gradient consisting of 160 ml of 0.2 *M* NaCl and 160 ml of 1.2 *M* NaCl, both containing 0.05 *M* $NaPO_4$ buffer, pH 6.7. Fractions of

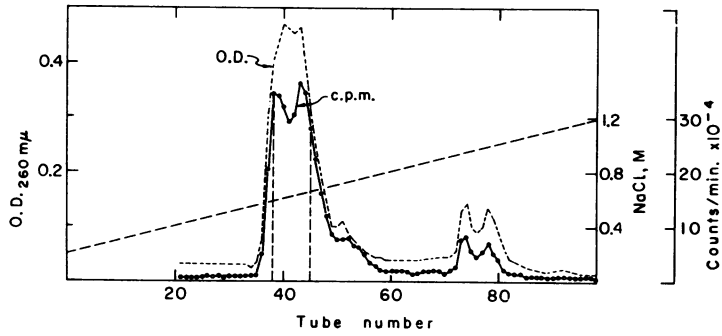


FIG. 1.—MAK column chromatography of T4 sRNA labeled with $P^{32}$. The cellular sRNA in this preparation provides most of the optical density.

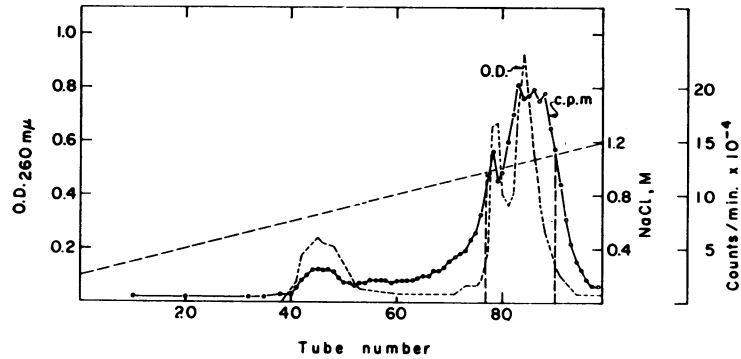


FIG. 2.—MAK column chromatography of bulk $P^{32}$-RNA isolated from T4-infected *E. coli* cells. The cellular bulk RNA in this preparation provides most of the optical density.

3 ml were collected. The fractions between the vertical lines in Figure 1 were pooled and stored at −15°C. This purified sRNA was free of DNA (i.e., it contained less than 0.10% alkali-resistant material).

*Isolation of high-molecular-weight T4 mRNA:* The cells were infected and were incubated with $^{32}P_i$ as described above, but they were cooled and harvested without superinfection after the 10-min incubation. Bulk RNA was extracted by the method of Bautz and Hall.[13] After ethanol precipitation it was treated with DNase and Pronase and then subjected to MAK column chromatography, as described above. The fractions between the vertical lines in Figure 2, eluting at 0.9–1.1 *M* NaCl, were pooled and stored at −15°C.

*Isolation of T4 phage DNA:* T4D bacteriophage was obtained from Dr. R. H. Epstein and purified by differential centrifugation as described by Thomas and Abelson.[14] The phage was incubated for 60 min at room temperature in a solution containing 0.01 *M* Tris-HCl, pH 7.4, 0.01 *M* $MgCl_2$, RNase (10 μg/ml), and DNase (10 μg/ml). After recovery by centrifugation for 60 min at 105,000 × *g*, the phage was suspended in 0.01 *M* Tris-HCl, pH 7.4 (5–7 OD at 260 mμ/ml), an equal volume of water-saturated phenol was added, and the suspension was shaken for 15 min at room temperature. The aqueous phase was separated by centrifugation and was re-extracted twice by phenol. The DNA was then precipitated from the aqueous phase by 2 vol of ethanol, removed by spooling on a glass rod, and washed with ethanol. The DNA was dissolved in 1/100 SSC (1.5 mM NaCl and 0.15 mM Na-citrate) and dialyzed against the same buffer.

*DNA-RNA hybridization:* DNA was denatured by alkali[15] and immobilized on cellu-

lose nitrate filters (type B-6, coarse, 27 mm, Schleicher and Schuell). Hybridization of RNA to the fixed DNA was carried out according to the procedure of Gillespie and Spiegelman.[15]

*Results.*—(a) *Isolation of T4 phage sRNA:* To obtain labeled T4 sRNA, *E. coli* cells were infected with phage and incubated for ten minutes in the presence of $^{32}P_i$. To minimize labeling in mRNA, the cells were further incubated with superinfection in the presence of excess nonradioactive phosphate. This procedure made it possible to prolong the "chase" period with nonradioactive $P_i$ without causing appreciable lysis of the cells. The whole cells were treated with phenol in order to extract the sRNA selectively and to minimize contamination with high-molecular-weight mRNA. The sRNA thus obtained was further purified by chromatography on a MAK column.

Figure 1 demonstrates that the $P^{32}$-labeled T4 sRNA eluted from the column in the same region as the *E. coli* sRNA. Fractions 38–44 (Fig. 1) were pooled and used for further studies. A small shoulder of labeled material was eluted after the main sRNA peak; this material was not further examined. Figure 3 shows

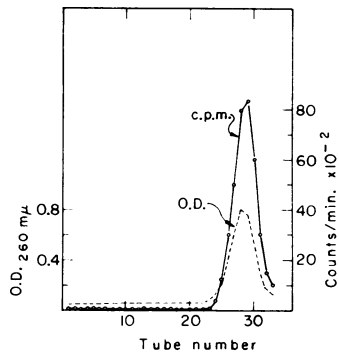


FIG. 3.—Sucrose gradient centrifugation of *E. coli* sRNA (optical density) and T4 $P^{32}$-sRNA. *E. coli* sRNA (0.14 mg) solution was mixed with $P^{32}$-labeled T4 sRNA in 0.2 ml of 0.03 *M* Tris-HCl (pH 7.4) and layered onto a linear 5–20% sucrose gradient containing 0.03 *M* Tris-HCl. The gradient was centrifuged at 4° for 11 hr at 39,000 rpm in a Spinco rotor SW-39.

that the T4 $P^{32}$-sRNA (cpm) and *E. coli* sRNA (OD) have identical sedimentation profiles and consequently the same sedimentation constants, when centrifuged in a linear sucrose gradient.

(b) *Hybridization properties of T4 phage sRNA:* The capacity of the T4 sRNA to hybridize with T4 DNA was compared to that of T4 mRNA in order to establish the origin and the size of the corresponding genome. The properties of an RNase-resistant hybrid formed by incubating a fixed amount of T4 DNA with increasing amounts of T4 $P^{32}$-sRNA, or of high-molecular-weight T4 $P^{32}$-mRNA, were examined. The saturation curve obtained (Fig. 4*A*) with the sRNA reached a plateau at a lower concentration than that found for mRNA. The marked difference in the saturation curves for the two RNA species supports the conclusion that the sRNA is quite distinct from mRNA. Different T4 sRNA preparations hybridized with 0.05–0.2 per cent of the T4 DNA, probably because of variable contamination with degraded high-molecular-weight RNA.

The ability of the $P^{32}$-sRNA to hybridize with T4 DNA is specific, since *E. coli* sRNA isolated from uninfected cells was unable to compete in this reaction (Fig. 4*B*). In addition, it was found that not more than 0.001 per cent of the T4 DNA was able to hybridize with *E. coli* $P^{32}$-sRNA.

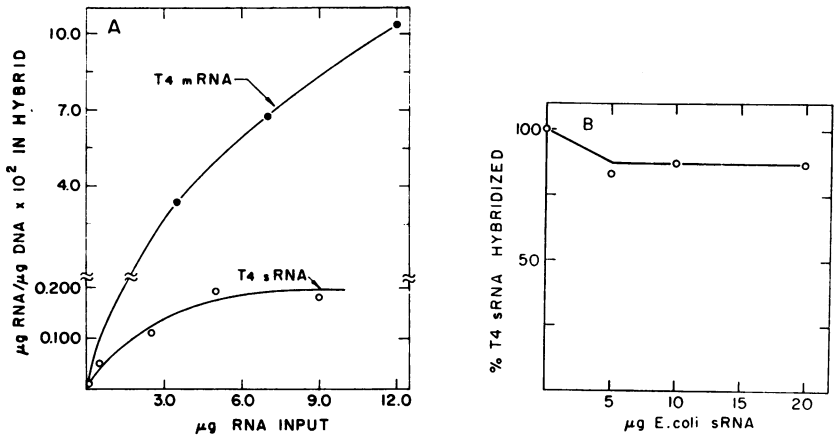


FIG. 4.—(*A*) Hybridization of T4 sRNA with T4 DNA: Various amounts of T4 $P^{32}$-sRNA ($5.6 \times 10^4$ cpm/μg) were hybridized to denatured T4 DNA immobilized on nitrocellulose filters (9 μg per filter). Hybridization of T4 mRNA with T4 DNA: Various amounts of T4 $P^{32}$-mRNA ($3.5 \times 10^3$ cpm/μg) were hybridized with T4 DNA (11 μg DNA per filter).

(*B*) Competition of *E. coli* sRNA with T4 sRNA for the T4 DNA: Various amounts of *E. coli* sRNA were added to a hybridization reaction of 5.6 μg T4 $P^{32}$-sRNA ($1.27 \times 10^5$ cpm/μg) with T4 DNA (11 μg DNA per filter).

(c) *Nucleotide composition of the T4 sRNA:* $P^{32}$-labeled T4 sRNA was hydrolyzed with alkali in the presence of carrier *E. coli* sRNA, and the nucleotide composition was analyzed by Dowex-1 formate column chromatography. The elution profile was similar to that of a more purified preparation, described below, which is presented in Figure 5. In addition to the four main nucleotide peaks, there was a distinct peak of radioactivity in the ψUMP region coinciding with an $A^{260}$ peak. It should be noted that the cpm were derived from the T4 $P^{32}$-sRNA, while the $A^{260}$ originated from the carrier *E. coli* sRNA. Moreover, a small amount of label was also detected in at least two minor peaks (designated *X* and *Y*).

The nucleotide composition of the T4 RNA's, calculated from the amount of label found in each of the isolated peaks, is presented in Table 1. It is seen that the T4 sRNA has a relatively low A + U content and contains significant amounts of ψUMP. In contrast, the T4 mRNA, as previously observed by Bautz and Hall,[13] had a high A + U content (Table 1); moreover, it did not contain ψUMP. Some variations were found in the base composition of different batches of T4 sRNA, probably due to variable contamination with degraded high-molecular-weight RNA. The inconsistent values reported by several investigators[1, 4–6] may thus be accounted for.

(d) *Analysis of T4 sRNA recovered from hybrid with T4 DNA:* Since infection of *E. coli* with T4 phage might not result in complete shutdown of host nucleic acid synthesis, the presence of $P^{32}$-ψUMP in the T4 sRNA preparations that we obtained may have arisen by incorporation of the label into contaminating host sRNA. To exclude this possibility, labeled T4 sRNA was first hybridized with T4 DNA. After filtration, the hybrid was treated with RNase to

ensure the digestion of any nonspecific-bound RNA. The T4 $P^{32}$-sRNA was then eluted from the filter, hydrolyzed, and analyzed. The $P^{32}$ profile of the fractionated nucleotides (Fig. 5) clearly shows distinct peaks in the region of the

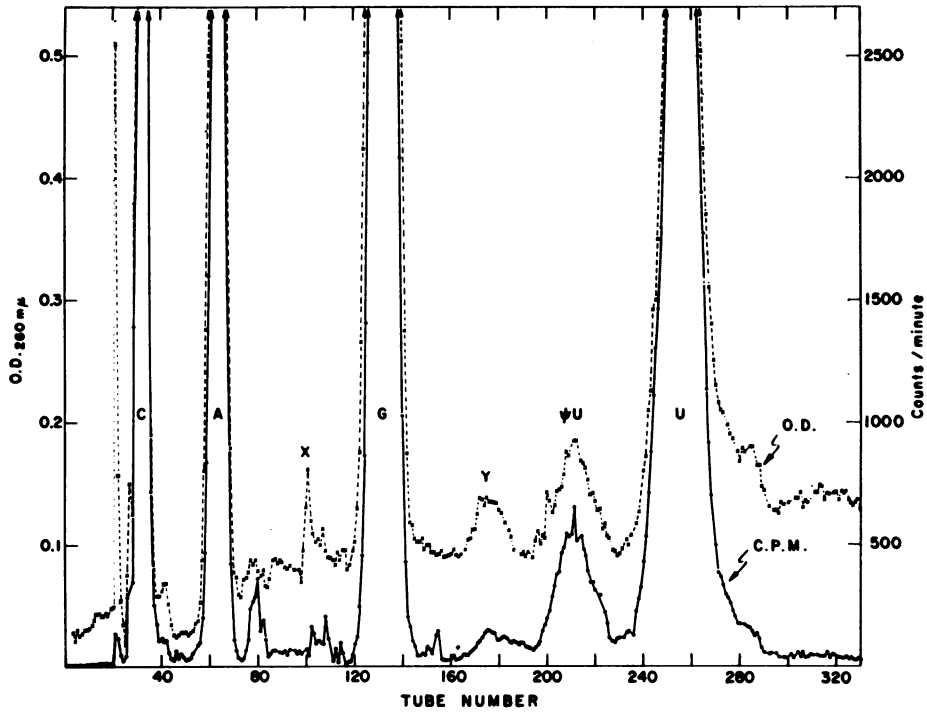


FIG. 5.—Chromatography of an hydrolysate of T4 sRNA isolated from a hybrid with T4 DNA. Denatured T4 DNA solution containing 2 mg DNA was mixed with 80 $\mu$g T4 $P^{32}$-sRNA in a total volume of 50 ml. Solid NaCl and Na-citrate were added to bring the mixture to 0.9 *M* and 0.09 *M*, respectively (6 × SSC). The mixture was incubated at 70° for 60 min and then cooled slowly at room temperature. A nitrocellulose filter (150 mm, type B6 coarse, Schleicher and Schuell) was presoaked and washed in 6 × SSC, after which the hybridization mixture was applied with a low vacuum. The binding of the DNA was monitored by reading the $A^{260}$ of the filtrate. Over 90–95% of the T4 DNA was bound. The filter was then washed with suction on each side with 100 ml of 6 × SSC and incubated for 60 min at room temperature in 50 ml of 2 × SSC containing 28 $\mu$g/ml RNase (preheated for 10 min at 80° to inactivate any contaminating DNase). The filter was then washed on each side with 100 ml of 6 × SSC. The hybrid was eluted by suspending the filters in 40 ml of 0.01 *N* NaOH at 37° for 10 min; about 90–95% of the $P^{32}$-RNA was recovered. After concentration to 4.0 ml by flash evaporation, 5 mg of *E. coli* sRNA and 10 *N* NaOH (0.4 *N* final concentration) were added. After hydrolysis at 37° for 17 hr, the solution was then acidified to pH 2.5 with HCl and any precipitated DNA removed by filtration through a nitrocellulose filter (27 mm). The solution was then diluted 50 times with $H_2O$, adjusted to pH 10, and applied on a 0.8 × 40-cm Dowex-1 formate ×2 column, 200–400 mesh.[16] The column was washed with 100 ml of water and 2′,3′-CMP was eluted with a nonlinear gradient by using 50 ml of water in the mixing flask and 30 ml of 1.2 *N* HCOOH. The next gradient, consisting of 100 ml of 0.56 *N* HCOOH in the mixing flask and 300 ml of 4.2 *N* HCOOH, eluted the 2′ and 3′ isomers of AMP, GMP, and $\psi$UMP. The 2′,3′-UMP was eluted with 5 *N* HCOOH. Fractions of 2.0 ml were collected, and the optical density at 260 and 280 m$\mu$ was measured. Each sample was transferred to a scintillation vial and dried at 80°, and 10 ml of a scintillation solution was added[17] before counting.

TABLE 1. *Nucleotide composition of RNA isolated from T4 phage-infected E. coli.*

| Sample | Total cpm | Percentage of $P^{32}$-Labeled Nucleotide | | | | | | | (A + U)/ (G + C)† |
|---|---|---|---|---|---|---|---|---|---|
| | | C | A | G | U | ψU | X | Y | |
| High-molecular-weight T4 mRNA* | $5.5 \times 10^5$ | 15.8 | 32.1 | 20.3 | 29.8 | — | — | — | 1.71 |
| Crude T4 sRNA | $7.1 \times 10^5$ | 21.2 | 19.8 | 31.1 | 23.7 | 2.5 | 0.2 | 1.3 | 0.89 |
| T4 sRNA isolated from hybrid with T4 DNA | $3.5 \times 10^5$ | 21.2 | 26.6 | 21.8 | 24.8 | 3.2 | 0.49 | 0.66 | 1.20 |

* Between the GMP and UMP peaks, two small peaks containing 0.6 and 0.43% of the total label were found. However, they did not correspond to either peak Y or ψUMP.
† Including ψUMP.

minor nucleotides, particularly in the ψUMP region; the calculated nucleotide composition is presented in Table 1. The ψUMP thus obtained was further identified by paper chromatography (Fig. 6) and by its highly characteristic acid and alkali absorption spectra.

*Discussion.*—The present report shows that ψUMP and two other unidentified nucleotides (possibly methylated nucleotides) are present in the sRNA synthesized after infection of *E. coli* cells with T4 phage.

This sRNA could conceivably be synthesized under the direction of the phage DNA, or it could be host sRNA still synthesized after infection, or the ψUMP could be inserted into pre-existing host sRNA by a phage-induced enzyme. The last two possibilities were eliminated by showing that sRNA synthesized after infection could be specifically hybridized with T4 DNA and that this T4 sRNA contains considerable amounts of ψUMP (Fig. 5).

Thus, at least a significant portion of the ψUMP-containing sRNA is transcribed by the T4 genome. Moreover, there seems to be very little homology between T4 sRNA and *E. coli* sRNA. This conclusion is supported by the observation that *E. coli* sRNA is unable to compete with T4 sRNA for hybridization with T4 DNA (Fig. 4*B*). These results, of course, do not exclude the presence of very short regions of homology in the sRNA species, which are unable

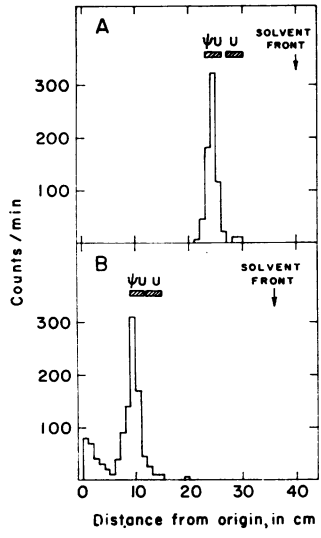


FIG. 6.—Paper chromatograms of the ψUMP peak isolated from the Dowex-1 formate column.
(*A*) Isopropanol-$H_2O$ (7:3 v/v), with $NH_3$ in the gas phase.
(*B*) Isobutyric acid-0.5 *N* $NH_4OH$ (7:3, v/v).

to form a stable hybrid with heterologous DNA under the conditions employed.

Since $\psi$UMP is believed to be present predominantly in transfer RNA, its presence in an sRNA fraction coded for by the T4 genome suggests that at least some of this RNA may have amino acid acceptor function. Experiments are now under way to examine this possibility.

*Summary.*—*E. coli* cells infected with T4 phage in the presence of $P^{32}$-orthophosphate yielded $P^{32}$-sRNA which hybridized specifically with T4 DNA; *E. coli* sRNA did not compete with the T4 sRNA in this hybridization. The fraction of T4 DNA hybridizable to T4 sRNA (0.05–0.2%) was considerably lower than that hybridizable to high-molecular-weight T4 mRNA. Moreover, the T4 sRNA and T4 mRNA differed in base composition. The T4 sRNA preparation was found to contain pseudouridylic acid. In addition, pseudouridylic acid was also isolated from a preparation that had been purified by hybridization with T4 DNA.

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Abbreviations used: MAK, methylated albumin kieselguhr; $\psi$UMP, pseudouridylic acid; sRNA, soluble RNA; tRNA, transfer RNA; mRNA, messenger RNA; Tris, tris(hydroxymethyl)aminomethane; SSC, standard saline citrate.

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