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Eight Transfer RNAs Induced by Infection of *Escherichia coli* with Bacteriophage T4

(RNA sequence analysis/gel electrophoresis/biosynthesis of tRNA/deletion mutation)

WILLIAM H. McCLAIN*†, CHRISTINE GUTHRIE†, AND B. G. BARRELL*

*MRC Laboratory of Molecular Biology, Cambridge, England; and †Department of Bacteriology, The University of Wisconsin, Madison, Wis. 53706

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ABSTRACT **Bacteriophage T4 induces the synthesis of eight transfer RNAs upon infection of *E. coli*. The tRNAs are easily detected and resolved into pure species by polyacrylamide gel electrophoresis of RNA labeled with ^{32}P after T4 infection. Two-dimensional fingerprints of RNase T_1 products derived from individual gel bands give patterns characteristic of single tRNAs. Furthermore, the T_1 digest of each gel band has a single oligonucleotide that contains the minor nucleotides Tp and ψp, a characteristic feature of all known tRNAs.**

Four larger RNAs are also seen in the polyacrylamide gels. Fingerprint and genetic analyses demonstrate that these molecules are related to the tRNAs, but the exact nature of this relationship is not known.

RNA synthesized during infection of *Escherichia coli* by bacteriophage T4 is predominantly messenger RNA transcribed from T4 DNA. Littauer (1) and Weiss (2) have demonstrated by enzymatic acylation with labeled amino acids, followed by DNA–RNA hybridization, that a minor fraction of the T4 transcript is tRNA. They have identified five T4-specific tRNAs among 18 aminoacid acceptor activities examined. While this method is reliable when a positive result is obtained, some caution must be exercised in the interpretation of a negative result because there are several difficulties inherent in the method (1, 2). The total number of T4 tRNAs, therefore, remains an open question.

The present work provides a different approach to this problem. Total RNA made after infection was labeled with ^{32}P and subsequently analyzed by two successive steps of polyacrylamide gel electrophoresis. Eight RNA components with electrophoretic mobilities characteristic of tRNA were found. Fingerprint and sequence analyses indicate that each component is a single, unique species of tRNA. Four components larger than tRNA were also identified and analyzed. The relationship of these molecules to the tRNAs is discussed.

RESULTS

Resolution of bacteriophage T4-induced RNAs by electrophoresis on polyacrylamide gels

When *E. coli* is infected with T4 and pulsed with [^{32}P]orthophosphate, and the low-molecular-weight RNA is extracted and analyzed on a 10% polyacrylamide gel, eight components are seen (Fig. 1). Of these eight, only components *1–4* have appropriate mobilities for tRNAs, as seen by comparison with control RNA from uninfected cells, where 4 S marks the characteristic position of tRNA (3). The gel pattern for components *1–4* is extremely reproducible, and is the same when cells are labeled early or late (4–8 min or 12–16 min at 37°) after infection, or when lysis-inhibited cells are labeled continuously for periods up to 1.5 hr after infection. The amount of label that accumulates in the components appears to be proportional to the length of the labeling period, a finding that indicates that the components are not unstable and rapidly turning over. These results are in general agreement with the findings of other workers (4, 5) who also examined labeled RNA made after T4 infection by gel electrophoresis.

Eight labeled components in samples from T4-infected cells are in contrast to the profile obtained from uninfected cells (Fig. 1). The synthesis of host RNA is thus effectively terminated upon T4 infection (1, 2, 4), a fact that allows us to label and examine T4 RNA exclusively. All eight components induced by T4 were shown to be RNA by their sensitivity to digestion with RNase, but not with DNase. Further analysis of the components revealed that each behaved as a single entity upon isolation and re-electrophoresis on a 10% gel. This result makes unlikely the possibility that the distinctive banding pattern of Fig. 1 is due to the presence of aggregates.

While seven of the T4-induced components appear in sharp bands that may be characteristic of a single molecular

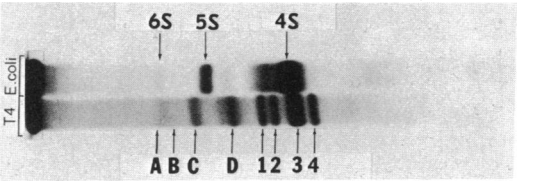

FIG. 1. Autoradiograph of ^{32}P-labeled RNA from T4-infected and uninfected cells fractionated by electrophoresis on a 10% polyacrylamide gel. This method separates RNA primarily according to size. Transfer RNAs from uninfected cells migrate in the region of 4 S. *E. coli* was grown to 3×10^8 cells per ml at 37° in LP medium (3) and infected at time 0 with a multiplicity of 10 T4 per cell, [^{32}P]orthophosphate was added at 4 min, and the cells were harvested at 15 min. RNA was extracted, prepared as "stage 1 tRNA" (3), and electrophoresed in a slab of 10% polyacrylamide gel. Component *4* had migrated 21 cm from the origin (*left*). Methods are described elsewhere (3).

Abbreviation: ψ, pseudouridine.

† To whom reprint requests should be sent.

TABLE 1. *Properties of bacteriophage T4 RNAs*

Gel component	Chain length[a]	Yield[b]	Molar yield[c]	No. of TpψpCp[d]	Comments
A	190	39	0.3	2	"Precursor" of *1* and *3β*[e]
B	180	23	0.2	2	"Precursor" of *3α* and *3δ*[e]
C	140	64	0.7	1	No known tRNA counterpart[e]
D	100	29	0.4	0	No known tRNA counterpart[e]
1	90	59	1.0	1	Serine tRNA: psu_1^+ suppressor[e]
2	85	54	1.0	1	Leucine tRNA (5)
3	80	197	3.8	1	
		3α(1.0;1.0)[f]		1	
		3β(1.5;0.9)		1	42% is *3γ*[g]
		3γ(0.5;1.0)		1	27% is *3δ*[g]
		3δ(0.25;0.4)		1	
		3ε(1.0;1.0)		1	
4	70	84	1.8	1	Glycine tRNA[e]

[a] Calculated from gel mobility (6).
[b] cpm $\times 10^{-6}$ in each component (after elution from the gel, ref. 3) from a 500-ml culture infected for 1.5 hr labeled with 50 mCi of ^{32}P.
[c] Ratio of yield/chain length, normalized to component *1*.
[d] Number of oligonucleotides containing the sequence TpψpCp, identified as indicated in the legend of Fig. 3.
[e] Conclusions from this article and our unpublished observations.
[f] Values obtained in an independent experiment after purification of component *3* on a 20% gel. The first value is total activity relative to *3α*; the second value is corrected for the indicated cross-contamination.
[g] Estimated from RNase T_1 fingerprint; see text.

species, the width of the band of component *3* suggests the presence of several different RNAs. These speculations were confirmed by further fractionation of the components on a 20% polyacrylamide gel. Only component *3* was resolved into subspecies (Fig. 2); these five components (*3α*, *3β*, *3γ*, *3δ*, and *3ε*) behaved as unique entities upon re-electrophoresis on a 20% gel. (The fact that component *3* could be further resolved into five components on a 20% gel had been previously communicated to us by Drs. T. Ikemura and J. E. Dahlberg.)

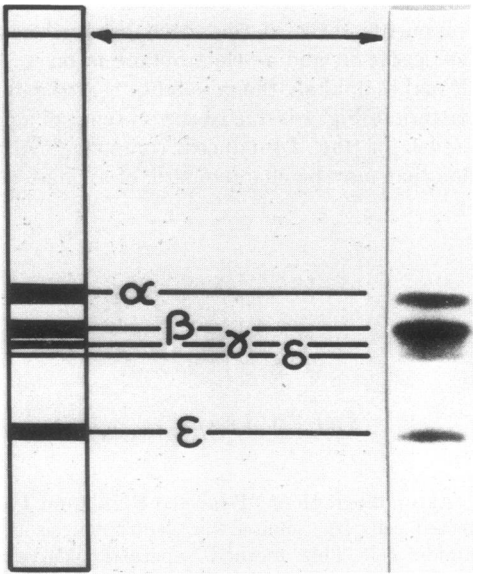

FIG. 2. 20% gel of component *3*. A band of gel corresponding to component *3* in Fig. 1 was cut out, eluted by homogenization (3), and fractionated on a slab of 20% polyacrylamide gel. Component *3ε* had migrated 9 cm from the origin (*top*). The gel recipe of (3) was modified as follows: the concentrations of acrylamide and bisacrylamide were doubled and the concentrations of the buffer and ammonium persulfate were halved.

Molecular weights and molar ratios of the RNAs

Table 1 gives the chain lengths of the eight components found in a 10% gel. These estimates are based on the measured electrophoretic mobilities and known chain lengths of 6S, 5S, and 4.5S RNAs, and on the empirical observation that electrophoretic mobility is inversely proportional to the logarithm of the molecular weight (6). Only components *1–4* are in the size range of tRNA molecules. Component *D* is about 10–30 nucleotides longer than tRNA, while components *A–C* are about twice the size of tRNA.

The amount of ^{32}P label incorporated into each of the eight components has been quantitated by scintillation counting of the eluted gel bands (Table 1). These values, together with the chain lengths, can be used to estimate the relative molar yields in the components (Table 1, column *4*); the yields in components *A–D* are significantly lower than those of the smaller components *1*, *2*, *3α–3ε*, and *4*. Independently labeled preparations show 1- to 4-fold variations in the yields of components *A* and *B*. These properties may reflect greater metabolic instability of the larger molecules, and/or their inefficient extraction from the cell during RNA isolation.

Fingerprint and sequence analyses of the RNAs

The two-dimensional nucleotide fractionation procedure of Sanger and his colleagues (7) can be used to judge the relatedness of two RNAs. Furthermore, if the chain length of an RNA is known, this fingerprint analysis can be used as a criterion of purity. RNase T_1 cleaves only after Gp nucleotides (7) and will, therefore, generate about 80/4 or 20 products upon complete digestion of a tRNA 80 nucleotides in length.

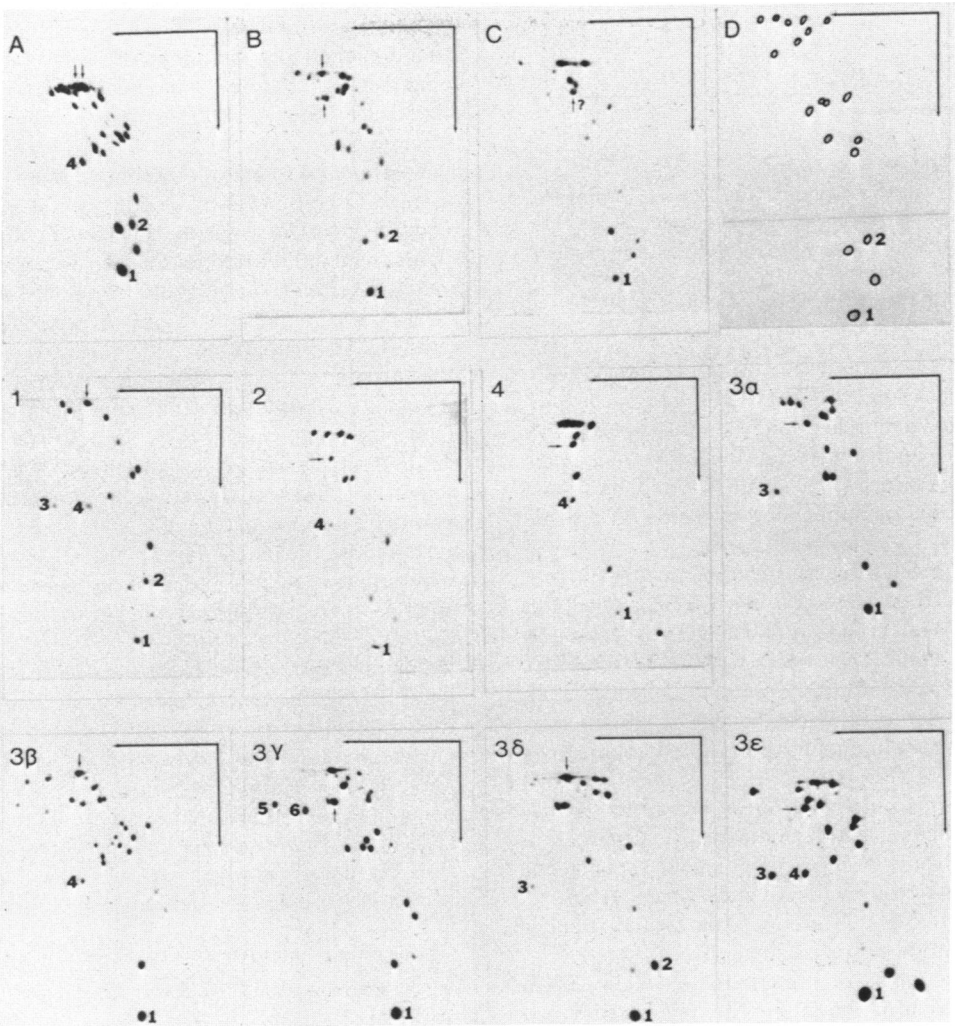

FIG. 3. Autoradiographs of two-dimensional fractionations of the products of digestion with RNase T_1. This figure is a composite of twelve components identified in Figs. 1 and 2. The positions of component *D* oligonucleotides are circled to facilitate reproduction. RNA samples were recovered from the gels as in Fig. 2, digested with RNase T_1 (which cleaves RNA after Gp residues), and electrophoresed: first, on cellulose acetate in pyridine acetate–7 M urea (pH 3.5) from *right* to *left;* and second, on DEAE paper in 7% formic acid (v/v) from *top* to *bottom*. More details are given elsewhere (7). The numbered oligonucleotides indicate the characteristic position in the two-dimensional fingerprint of: **1**, Gp; **2**, (Cp, Ap) Gp; **4**, UpGp; **6**, UpUpGp; and the two 5′-end groups **3**, pGp and **5**, pUpGp. Oligonucleotides that contained both Tp and ψp residues are indicated with *arrows*. In all 11 components, the arrowed spots satisfied the following criteria for identification of these two minor nucleotides: (*i*) hydrolysis of the eluted spot with alkali, followed by electrophoresis at pH 3.5 on Whatman 540 paper, gave a nucleotide (a mixture of Tp and ψp) with an R_u of about 0.95; and (*ii*) digestion with pancreatic RNase A, followed by electrophoresis at pH 3.5 on Whatman DE81 paper, gave a spot (a mixture of Tp and ψp) with an R_u of about 0.95. The minor nucleotides of components *A*, *B*, *1*, *4*, and *3α–3ε* were analyzed more extensively as follows. The ribonuclease T_1 products found in and above the graticule containing two Up residues (7), where all Tpψp containing products are located, were treated as in (*i*) above, and the Up bands that would contain Tp and ψp were analyzed by isopropanol–2.8 M HCl (3.9:1) chromatography, where Tp and ψp had R_u values of 1·1 and 0·8, respectively (7).

We have prepared T_1 fingerprints of the twelve T4-induced components (Figs. 1 and 2) to obtain information on the relatedness and purity of the components and, in conjunction with further sequence analyses, to permit the identification of tRNAs by their possession of certain minor nucleotides.

tRNAs. Components *1*, *2*, and *4* are pure, unique species of tRNA, as indicated by their simple and distinctive fingerprints (Fig. 3). The primary sequence of component *1* is known, and identifies it as a serine tRNA capable of recognizing one or more of the four serine codons UC.‡ (Barrell and McClain, manuscript in preparation). Abelson and his colleagues (5) have determined the complete sequence of component *2;* it is a leucine tRNA capable of recognizing the codons UUA and/or UUG. The primary sequence of component *4* is not yet completely determined, but sufficient information is available to identify it tentatively as a glycine tRNA that recognizes the codon GG.‡ (Barrell, Coulson, and McClain, unpublished observations; Stahl, Paddock, and Abelson, personal communication).

The sequence T-ψ-C is common to all known tRNAs, and is reliably present in one and only one copy per molecule (8). This structural feature is also seen in the three phage-induced

‡ · indicates any nucleotide is possible in this position.

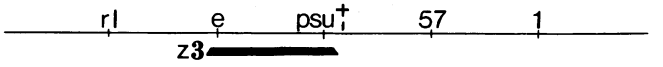

FIG. 4. Genetic order in the region of the T4 psu_1^+ suppressor gene. The order of genes around psu_1^+ is taken from Edgar (11). The deletion mutation, strain *Z3*, was isolated by selection for simultaneous loss of e^+ and psu_1^+ markers (to be reported in detail elsewhere). Growth media for strain *Z3* were supplemented with egg-white lysozyme (12).

tRNAs that have been sequenced (Fig. 3; the arrows indicate the oligonucleotides that contain T-ψ-C). For analysis of component *3* RNAs, we have relied on the presence of this characteristic sequence in molar yield as diagnostic of a single tRNA species. The presence of a single 3'- and/or 5'-end group was also accepted as supporting evidence of a single RNA species.

The complexity of the T_1 fingerprint of component *3* (not shown), which included at least two T-ψ-C oligonucleotides and at least four 3'-end groups, indicated that it was an impure mixture. This result is consistent with the heterogenity observed for component *3* on the 20% acrylamide gel (Fig. 2). However, each member of the component *3* subset (i.e., *3α*–*3ε*) appears, with certain qualifications noted below, to be a pure and unique tRNA (Fig. 3).

3α. 16 RNase T_1 products, including one 5'-end group (spot **3**) and one T-ψ-C oligonucleotide, indicate a single tRNA.

3δ. 15 T_1 products, one 5'-end group (spot **3**) and one T-ψ-C oligonucleotide are indicative of a single tRNA.

3ε. 19 T_1 products, one 5'-end group (spot **3**) and a single T-ψ-C oligonucleotide indicate a single tRNA.

3β. Too many nucleotides are present for a single tRNA. Careful examination of the fingerprint reveals that it is specifically contaminated with the entire set of T_1 products derived from component *3γ*. Quantitative analysis of the T_1 products, together with analyses of minor nucleotides (data not given), confirm the above conclusion and fix the level of cross-contamination at 42% (i.e., fraction *3β* is 58% pure, 42% of the mixture is *3γ*). When the background of *3γ* oligonucleotides is excluded, the remaining 16 T_1 products, with a single T-ψ-C, indicate a single tRNA.

3γ. By similar analyses as with *3β*, this component was shown to be cross-contaminated with 27% *3δ*. The 15 oligonucleotides unique to *3γ* include one 5'-end group (spot **5**) and one T-ψ-C oligonucleotide; this result is characteristic of a single tRNA species.

Further purification of components *3β* and *3γ* would obviously facilitate the fingerprint analyses. Cross-contamination of discrete components in 10% gels can often be eliminated by fractionation on a second gel of the same composition. We are presently examining the feasibility of this method for purification of components *3β* and *3γ*.

Larger RNAs. RNase T_1 fingerprints of components *A*–*D* (Fig. 3) are of the approximate complexity expected for pure RNAs of the indicated sizes (Table 1, column *2*). Component *A*, for example, is twice the size of tRNA (190 nucleotides) and gives about twice as many T_1 products [30]. These results strongly suggest a high degree of homogeneity for each of the larger RNA species.

The fingerprint of component *A* is not unique; it contains a collection of oligonucleotides that are also seen in T_1 digests of components *1* and *3β*. This collection includes both T-ψ-C oligonucleotides, as well as other minor nucleotides found in components *1* and *3β*. The end groups of the tRNAs, however, cannot be found in component *A*. Sequence analysis (to be reported elsewhere) has directly established extensive sequence identities between the three RNAs, *A*, *1*, and *3β*.

Another familial relationship exists between component *B* and tRNAs *3α* and *3δ* (Fig. 3). These results imply, but do not prove, that components *A* and *B* are precursors in the biosynthesis of tRNA (9). We are presently examining the role of components *A* and *B* in the production of mature tRNAs.

We have less information about components *C* and *D*. They give unique fingerprints (Fig. 3), and further analysis of the T_1 products has not revealed identities with any other component we have analyzed. In a preliminary analysis, the arrowed spot of component *C* in Fig. 3 appeared to contain Tp and ψp, although component *C* has no known tRNA counterpart. Paddock and Abelson (personal communication) have recently established the primary sequence of component *C*.

Component *D* coelectrophoreses with host 4.5S RNA, but Dr. B. E. Griffin has informed us (personal communication) that the two molecules are not related in sequence. No minor nucleotides have been found in component *D*.

While it is clear from these sequence studies that components *A*–*D* do not all bear a direct connection with the tRNAs, the following genetic experiment indicates a relationship, the details of which are yet to be elucidated, among all the T4 RNAs identified in Fig. 1.

Clustering of genetic information for all RNAs

In a separate study we have looked for and found genetic variants of several T4 tRNA genes (ref. 10, and unpublished observations by W.H.M.). One of the identified genes, psu_1^+, was shown by appropriate genetic crosses to map near the lysozyme gene, *e* (Fig. 4). Since no known essential genes are situated between *e* and psu_1^+ (11), it seemed reasonable to look for deletion-type mutations that encompassed this region. Such mutant strains of T4 have been isolated (unpublished observations of W.H.M.).

Analysis on a 10% gel of RNA induced by an *e*-psu_1 deletion mutation, strain *Z3*, showed clearly that all components induced by the wild-type T4 phage were absent. The twelve low-molecular-weight RNAs are thus shown to be related in that their appearance is controlled by one small region of the chromosome. DNA-RNA hybridization studies by Wilson, Kim, and Abelson (14) have shown that these RNAs are in fact transcribed from the *e*-psu_1 region.

Mutant strain *Z3* possesses no overt physiological defects (aside from a defective *e* gene). This result compels us to conclude that none of the RNAs described in this work are essential for T4 reproduction under the prevailing laboratory conditions.

DISCUSSION

The eight tRNAs of bacteriophage T4 identified in this study are unique, as judged by their RNase T_1 fingerprints. Nucleotide sequence analyses have identified anticodons in three tRNAs as corresponding to serine, leucine (5), and glycine. The remaining tRNAs probably correspond, at least in part,

to the molecules identified by previous workers, tRNAs corresponding to arginine, isoleucine, and proline (1, 2).

Does T4 code for more than eight tRNAs? Clearly, our preliminary sequence analyses of component-*3* tRNAs require confirmation. For example, the possibility that one or more of these species actually includes two tRNAs sharing extensive sequence homologies, as is often observed for isoacceptor tRNAs (8), has not been eliminated by our preliminary analyses. Only three preparations of component *3* have been analyzed on 20% gels; they gave reproducible banding patterns. We estimate that the lower limit of detection of a new component is about 25% of the amount of *3δ*. About 250 independent preparations of RNA have been analyzed on 10% gels during the course of these studies. On two occasions we observed a novel band labeled to the same extent as component *4* that migrated midway between components *3* and *4*. We do not know what conditions favor the appearance of this component; therefore, it has not been analyzed. In summary, with these reservations, we have evidence for only eight tRNAs.

No compelling experimental results are given in this paper that demonstrate that the twelve low-molecular-weight RNAs produced after infection are transcribed from T4 DNA. In forthcoming publications, however, we prove this point unequivocally by correlating genetic alterations with nucleotide changes in the RNAs. The T4 origin of these RNAs has been established in the related but independent work of Wilson, Kim, and Abelson (14).

What is the function of these viral tRNAs? Two of them can function as suppressors of nonsense mutations (Table 1, last column) and are, therefore, competent to participate in protein synthesis. *In vitro* studies with a protein-synthesizing system directed with T4 messenger RNAs have also demonstrated that four of the T4 tRNAs can donate amino acids (2). The viability of the tRNA-less mutant, strain *Z3*, and similar strains isolated and studied by Wilson (13, 14), clearly indicates that the tRNAs are dispensable under the prevailing laboratory conditions. Wilson (13) has speculated on their physiological role in nature.

The ability to isolate what appear to be tRNA precursor molecules and the ease with which the tRNA genes can be genetically manipulated make the bacteriophage T4 system attractive for the study of tRNA biosynthesis.

Dr. J. E. Dahlberg made these studies possible by permitting us the generous and extensive use of his RNA sequencing facilities. The contribution by Dr. David Pratt of equipment and supplies is gratefully acknowledged by W. H. M. Dr. T. Ikemura provided invaluable guidance with the 20% gel system. We are indebted to Dr. J. D. Smith for his interest and for his numerous suggestions. We thank Dr. John Abelson and Dr. John H. Wilson for sharing with us their results before publication. These studies were supported in part by grants from The Jane Coffin Childs Memorial Fund for Medical Research, The Merck Company Foundation, The University of Wisconsin Graduate School Research Committee, USPHS Training Grant GM 00686, and USPHS Research Grant AI 10257.

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