The Physical Mapping of Bacteriophage T5 Transfer tRNAs*

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Transfer RNAs, isolated from Escherichia coli F cells infected with T5 bacteriophage, were charged with radioactive amino acids and used in RNA-DNA hybridization studies to detect and locate T5 tRNA cistrons in the T5 DNA chromosome. Hybridization of 14 \textsuperscript{3}H-aminoaacyl-tRNA species, including purified T5 [\textsuperscript{3}H]Met-tRNA\textsubscript{M} and [\textsuperscript{3}H]Met-tRNA\textsubscript{R}, to the separated strands of T5\textsuperscript{+} DNA indicates that most, if not all, of the T5 tRNAs are transcribed from the continuous heavy strand of T5 DNA.

Heteroduplex mapping of eight mutant T5 DNA deletions has enabled us to locate and determine the size of these deleted segments. By correlating this information with the presence and absence of specific tDNA sequences in these mutants, as determined by tRNA-DNA hybridization, we were able to define the physical limits of four tDNA-containing loci along the T5 DNA molecule. A physical map for 15 tRNA genes was constructed from the maps of Hayward and recently, this same laboratory demonstrated that two isoacceptor T5 methionine tRNA species, tRNA\textsubscript{M} and tRNA\textsubscript{R}, are present in T5 infected cells (21). Unpublished results obtained by our group indicate that the synthesis of T5 tRNA falls into the time-class category of an early phage DNA transcript, similar to that reported for T4 tRNA synthesis (22).

Some of the physical and transcriptional features of T5 DNA have been incorporated in the T5 DNA map illustrated in Fig. 1, which was constructed from the maps of Hayward and Smith (13), Hendrickson and Dujard (14), and Hayward (3).

In this laboratory (15), regions B, C, D, and E of the T5 chromosome code for early phage genes in T5 DNA for 15 tRNA species by use of the techniques of aminoacyl-tRNA-DNA hybridization and heteroduplex DNA mapping. The results show that almost all of the phage tRNA genes are clustered in the C segment of T5 DNA. According to the physical transcription map of T5 DNA (Fig. 1), regions B, C, D, and E of the T5 chromosome code for early RNA synthesis. One would expect, therefore, that T5 tRNA genes are located in one or several of these DNA segments.

In this report, we describe the physical location of tRNA genes in T5 DNA for 15 tRNA species by use of the techniques of aminoacyl-tRNA-DNA hybridization and heteroduplex DNA mapping. The results show that almost all of the phage tRNA genes are distributed within a segment of T5 DNA. In agreement with the transcript map shown in Fig. 1, our data indicate that T5 tRNAs are transcribed from the heavy continuous strand of T5 DNA. A preliminary report on a part of this work has been presented (23).

MATERIALS AND METHODS

Growth and Purification of Phage—Wild type T5\textsuperscript{+} and all deletion mutants of T5 were grown at 37\textdegree C in NCG medium (8 g nutrient broth (Difco)/10 g casamino acids (Difco)/20 g glycerol/2.5 ml of 1 M Tris-HCl, pH 7.8, in a total volume of 1 liter) (9), with E. coli F as host. Phage was purified essentially as reported by Jacquemin-Sablon and

* The Franklin McLean Memorial Research Institute is operated by The University of Chicago for the United States Energy Research and Development Administration. This study was partly supported by the National Institutes of Health Grant AI-12479 (formerly CA-12561), and recently, this same laboratory demonstrated that two isoacceptor T5 methionine tRNA species, tRNA\textsubscript{M} and tRNA\textsubscript{R}, are present in T5 infected cells (21). Unpublished results obtained by our group indicate that the synthesis of T5 tRNA falls into the time-class category of an early phage DNA transcript, similar to that reported for T4 tRNA synthesis (22). According to the physical transcription map of T5 DNA (Fig. 1), regions B, C, D, and E of the T5 chromosome code for early RNA synthesis. One would expect, therefore, that T5 tRNA genes are located in one or several of these DNA segments.

In this report, we describe the physical location of tRNA genes in T5 DNA for 15 tRNA species by use of the techniques of aminoacyl-tRNA-DNA hybridization and heteroduplex DNA mapping. The results show that almost all of the phage tRNA genes are clustered in the C segment of T5 DNA. In agreement with the transcript map shown in Fig. 1, our data indicate that T5 tRNAs are transcribed from the heavy continuous strand of T5 DNA. A preliminary report on a part of this work has been presented (23).

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FIG. 1. The physical and transcriptional map of bacteriophage T5+ DNA. This model was constructed from other reported models (13, 14). The heavy lines represent the heavy and light DNA strands of T5+ wild type DNA with the polarities of the chains indicated by 3' and 5' designations. The molecular weight values shown (×10^6) are for the light chain segments created by the single strand interruptions as reported by Hayward (3). However, measurements for some of the light chain segments in this report differ slightly, e.g. A = 3.1 ± 0.5, and C = 6.0 ± 0.1 × 10^6 which in turn gives the percentage of total length for these same segments as 7.7, 9.0, and 14.7, respectively, using a single strand molecular weight of 40.6 × 10^6 for T5+ DNA (see "Results"). The broken and solid lines with arrows show the region and direction of T5+ pre-early, early, and late RNA transcription.

Preparation of Aminoacyl Synthetase and 3H-aminoacyl-tRNAs—Aminoacyl synthetase was prepared from soluble cell extracts of E. coli MRE-600 by the method of Keilmers et al. (26) and further purified by DEAE-chromatography and (NH4)2SO4 precipitation, as described elsewhere (21). The final enzyme preparation had an absorbance ratio of A280/A650 of 1.7 or greater; it was adjusted to contain 50% glycerol and stored at –80°C. No loss of activity was observed over a 1-year period.

All aminoacylations were done in reaction mixtures containing the following (per milliliter): 100 mM Tris-HCl (pH 7.5), 10 mM Mg(OAc)2, 2.5 mM ATP, 5 mM KCl, 100 to 250,000 cpm of a single 3H-amino acid, 200 μg of T5+ DNA, and 500 μg of enzyme. Incubation was carried out at 37°C for 10 min, after which the mixture was subjected to three extractions with phenol, previously equilibrated with 1 × SSC (pH 6.0); the charged tRNA was isolated by ethanol precipitation after addition of 0.1 volume of 3 M KAc (pH 6.4). After standing at –20°C for 1 hour, the precipitate was collected by centrifugation, washed once with 100 μg of T5+ DNA, and 500 μg of enzyme. Incubation was carried out at 37°C for 10 min, after which the mixture was subjected to three extractions with phenol, previously equilibrated with 1 × SSC (pH 6.0); the charged tRNA was isolated by ethanol precipitation after addition of 0.1 volume of 3 M KAc (pH 6.4). After standing at –20°C for 1 hour, the precipitate was collected by centrifugation, washed once with 100 μg of T5+ DNA, and stored at –80°C. The specific activity of radioactive 3H-aminoacyl tRNAs (purchased from Schwarz BioResearch, Inc. and New England Nuclear Corp.) ranged from 2.6 to 59 Ci per μmol.

Preparation of Purified T5 tRNA$^{3H}$ and tRNA$^{3H}$ for Hybridization—The isolation of T5-specific tRNAs, free of host tRNA, was accomplished by hybridization of T5-infected cell tRNAs to T5 DNA and recovery of the hybrid RNA as described previously (21). The charging of T5 tRNAs with L-$^{3H}$methionine, 100 to 200 Ci per mmol (New England Nuclear Corp.), formylation, and separation of the two charged isoacceptor methionine tRNA species by reversed-phase chromatography were also done as previously reported (21). The peak RPC-5 fractions containing the individual $^{3H}$-labeled 15 Met-tRNA and fMet-tRNA species were used directly for hybridization to T5 DNA filters in annealing mixtures containing 50% formamide, without the addition of 2 × SSC. The radioactivity retained by the DNA filters was determined as described below.

Aminoacyl-tRNA Hybridization with DNA—Hybridizations of DNA and tRNA were carried out on membrane filters (type B-3, Schleicher and Schuell, Inc.) which were impregnated with approximately 40 μg of DNA per filter. Filters were spotted with E. coli MRE-600 by the method of Kelmers et al. (26). The DNA preparations (2.5 μ1 each) were mixed with 25 μ1 of 0.2 M EDTA (pH 8.5), 12.5 μ1 of 2 mM NaOH, and water to a final volume of 225 μ1. After 10 min of gentle shaking at room temperature, the solution was subjected to dialysis against 1 × SSC (pH 7.0) for 4 hours in 2 × SSC containing 50% formamide (pH 5.5). The exact annealing conditions and determination of the radioactivity "fixed" to membranes, including a wash procedure with T1 RNase treatment, were as reported before (15).

Heteroduplex DNA Formation and Electron Microscopic Analysis—The procedure of Davis et al. (28) was used for heteroduplex DNA analysis. T5 bacteriophage were gently extracted with phenol that had been equilibrated with a solution containing 0.05 M NaCl/0.1 M Tris-HCl (pH 8.5)/0.001 M EDTA, and the aqueous phase was subjected to dialysis against the same buffer. Two bacteriophage DNA preparations (2.5 μ1 each) were mixed with 25 μ1 of 0.2 M EDTA (pH 8.5), 12.5 μ1 of 2 mM NaOH, and water to a final volume of 225 μ1. After 10 min of gentle shaking at room temperature, the solution was subjected to dialysis against T1 RNase and stored at –70°C. A mixture of 40 to 50 μ1 containing 0.25 μg of renatured T5 heteroduplex DNA, 0.1 μg of mouse mitochondrial DNA, 0.05 μg of eX74 DNA, 40 μg per ml of yeast tRNA, and 0.01 M EDTA was spread on a hypophase of 22% formamide, 0.01 M Tris-HCl (pH 8.5), and 0.001 M EDTA. After the basic protein...
monolayer was relaxed for 2 min, parlodion-coated grids were touched to the surface, stained in alcoholic uranyl acetate, dehydrated, and shadowed with Pt/Pd (80/20). Native T5+ DNA was co-spread with χX174 RF DNA under the same spreading conditions for measurements of the intact molecule. These two DNAs were also measured against a replica grating of 2158 lines per mm which served as an external standard.

Molecules of DNA were photographed at 12,000 to 18,000 diameters on an RCA EMU-4 electron microscope. Tracings of contour lengths from a negative at 100,000 to 200,000 diameters were measured with a Keuffel and Esser 620-315 map measure. As internal standards, single-stranded DNA was measured relative to the length of χX174 DNA, the length of which was taken as 0.112 ± 0.002 times the length of ADNA (29); double-stranded DNA was calibrated relative to mouse mitochondrial DNA the length of which was taken as 0.323 ± 0.013 times ADNA. The measurement of 5 to 20 heteroduplex molecules and of at least five standard DNA molecules per heteroduplex measurement were used in the calculation of the size of a single deletion. The following conversion factors were also used: ADNA (sodium salt) = 30.8 ± 1.0 x 10^6 daltons = 46,500 nucleotide pairs (29); this corresponds to 3.019 kilobases per 1 x 10^6 daltons of single-stranded DNA. In this paper, for convenience, DNA length is measured in daltons of single-stranded DNA calculated as a multiple of the χX174 single strand (1.7 x 10^6 daltons (29)).

RESULTS

When T5+ DNA is denatured, the linear duplex molecule dissociates into multiple single-stranded fragments of different molecular sizes, which can be partially separated by velocity centrifugation in sucrose density gradients (6, 9), as shown in Fig. 2. The material designated as Peak I represents the continuous heavy T5 DNA strand, whereas Peaks III and IV represent the smaller DNA fragments derived from the opposing light strand which contains the multiple single strand interruptions. Peak II material, which was usually small and not always apparent, is believed to derive from partially degraded heavy chains. Hence, an increase in Peak II material was usually associated with a decrease in Peak I A₂₆₀ absorbance.

To determine whether tRNAs were coded from the heavy or light T5 DNA fragments, or perhaps from both, we charged T5+ infected tRNAs with a mixture of labeled amino acids and annealed them with the Peak A₂₆₀ fractions obtained by subjecting denatured T5+ DNA to velocity gradient centrifugation.

R. Morimoto and J. Locker, unpublished results.

Fig. 3. Hybridization of T5+ charged tRNAs to denatured T5+ DNA subjected to gradient centrifugation. T5+ DNA was denatured, subjected to sucrose gradient (5 to 20%) centrifugation at 25,000 rpm for 3.5 hours and fractions collected as described under "Materials and Methods." Peak fractions were combined, dialyzed against 6 x SSC (pH 7.0) and impregnated onto nitrocellulose filters. The DNA-containing filters and a blank filter (no DNA) were incubated with charged tRNAs isolated from T5+ infected cells in an annealing mixture (0.6 ml) containing 50% formamide/2 x SSC, and the hybridized radioactivity determined after washing and T1 RNase treatment as reported elsewhere (15). All filters containing radioactivity were dissolved in 10 ml of toluene-Triton X-100 (3:1) scintillation mixture and counted in a Mark I Nuclear Chicago scintillation spectrometer. A, T5+ infected tRNAs were charged simultaneously with a mixture of amino acids including [3H]alanine (52 Ci/mmol); [3H]arginine (35 Ci/mmol); [3H]glycine (30 Ci/mmol); and [3H]phenylalanine (40 Ci/mmol). tRNA (19 µg) charged with this mixture containing 1 x 10⁶ cpm (total) was used in separate annealing reactions with approximately 10 ng of DNA from the peak regions of I, II, III, and IV as shown above. B, same as in A except that the amino acid mixture used for charging T5 tRNAs contained [3H]isoleucine (30 Ci/mmol); [3H]proline (17.5 Ci/mmol); [3H]valine (16.4 Ci/mmol); and [3H]methionine (2.6 Ci/mol). This mixedly charged tRNA (20 µg) containing 4.4 x 10⁶ cpm was used in separate annealing reactions with DNA (10 ng) from the peak regions of I, III, and IV.
tion. As shown in Fig. 3, A and B, tRNAs from T5+-infected cells charged with two different mixtures of tryptophane amino acids hybridized more efficiently with Peak I material (the heavy continuous strand) than with any of the DNA fragments associated with the other peak fractions, especially Peaks III and IV. Since some hybridization was found in those gradient fractions in which light chain segments are known to be located (Peaks III and IV), and not exclusively with the intact heavy chain, it was necessary to carry out a more extensive hybridization analysis by use of individually charged tRNAs. Table I gives the results for the hybridization of 16 individually charged tRNA species when a crude preparation of T5+-infected cell tRNAs and the purified T5 methionine tRNA species were used. For each labeled aminoacyl-tRNA examined, hybridization to Peak I DNA was uniformly observed, though not to the same extent. Furthermore, in each case, hybridization was significantly higher with Peak I DNA than with DNA from either Peak III or IV. Although significant hybridization was observed for many of the tRNA species with all three DNA peak fractions, the amount of radioactivity fixed to Peak I DNA, relative to that for Peak III and IV DNA, clearly suggests that tRNA genes for almost all T5 tRNA species are present in the heavy continuous strand and few, if any, are present in the opposing light strand. The low hybridization values obtained for [3H]tyrosyl-tRNA do not allow us to draw any conclusions as to DNA strand preference for this particular tRNA species.

In Fig. 4 are shown the physical DNA maps for the phage deletion mutant T5 st(0) and for wild type T5 phage. The genome size of T5 st(0) is significantly shorter than that of wild type DNA since it contains a deletion of approximately 3 to 4 x 10^6 daltons. Hayward and Smith (30) have indicated that the tRNAs used for charging and hybridization were isolated from T5+-infected cells and not further purified except for T5 tRNA, and tRNA, which were purified by hybridization to T5 st(0) DNA was positive, but amounted only to approximately one-half that observed for hybridization to T5+ DNA. In view of these findings, we attempted the construction of an approximate map for the physical location of the tRNA genes in the T5 chromosome with DNA isolated from several different T5 phage deletion mutants.

Two types of deletion mutants were obtained; their genome construction is illustrated schematically in Fig. 7. Group A mutants contain a DNA deletion within the C and D regions of the T5 map which includes the single strand interruption, whereas the deletion in Group B mutants is entirely within the C region, so that the C-D nick is preserved. The molecular sizes indicated in Fig. 7 for the single strand light segments represent values obtained by Hayward (3). The deletion sizes for the T5 mutants b3 and b4 were indistinguishable by the gel electrophoresis technique used by Hayward.

Transfer RNAs from T5+-infected E. coli F cells were

* G. S. Hayward, personal communications.

### Table I: Hybridization of T5+ [3H]aminoacyl-tRNAs to denatured T5+ DNA fractionated by sucrose gradient centrifugation

| Labeled aminoacyl-tRNA | Specific activity | Input x 10^-3 | Control DNA (in ng) | DNA Total fixed | Hybrid DNA Total | Hybrid DNA Total | Hybrid DNA Total |
|------------------------|------------------|---------------|---------------------|-----------------|-----------------|-----------------|-----------------|
|                        | C/mmol | cpm | cpm | µg | cpm | µg | cpm | µg | cpm | µg | cpm | µg | cpm | µg | cpm | µg | cpm | µg | cpm | µg | cpm | µg |
| Tyr                    | 52.0   | 9.3  | 326 | 9  | 3784 | 384 | 10  | 656 | 33 | 11  | 351 | 2  |
| Arg                    | 35.0   | 11.0 | 552 | 10 | 2632 | 208 | 11  | 594 | 4  | 10  | 562 | 1  |
| Gly                    | 30.0   | 6.8  | 80  | 11 | 1683 | 148 | 11  | 555 | 18 | 9   | 91  | 3  |
| Phe                    | 59.0   | 5.4  | 667 | 9  | 3706 | 258 | 10  | 910 | 24 | 9   | 596 | 0  |
| Leu                    | 30.0   | 1.3  | 33  | 17 | 1692 | 96  | 10  | 67  | 3  | 11  | 63  | 3  |
| Val                    | 16.4   | 2.5  | 32  | 19 | 119  | 5   | 10  | 60  | 3  | 10  | 65  | 3  |
| Pro                    | 17.8   | 0.3  | 6   | 19 | 223  | 11  | 21  | 5   | 0  | 10  | 10  | 0  |
| Asp                    | 13.0   | 2.9  | 35  | 7  | 624  | 81  | 5   | 61  | 5  | 8   | 46  | 1  |
| Leu                    | 41.0   | 17.9 | 457 | 7  | 1499 | 149 | 9   | 876 | 47 | 8   | 780 | 46 |
| Gly                    | 16.2   | 3.9  | 55  | 6  | 145  | 15  | 9   | 76  | 2  | 8   | 100 | 6  |
| Ala                    | 52.0   | 3.3  | 98  | 11 | 347  | 23  | 11  | 212 | 10 | 10  | 82  | 0  |
| Ser                    | 3.4    | 0.6  | 32  | 7  | 427  | 56  | 9   | 49  | 2  | 8   | 31  | 0  |
| His                    | 10.2   | 2.3  | 82  | 8  | 280  | 25  | 9   | 150 | 8  | 7   | 91  | 1  |
| Lys                    | 33.0   | 21.2 | 553 | 8  | 1006 | 57  | 11  | 734 | 17 | 8   | 500 | 0  |
| Met_                   | 367.8  | 1.0  | 205 | 7  | 1168 | 1640| 9   | 886 | 87 | 9   | 417 | 24 |
| Met_                   | 367.8  | 0.5  | 77  | 7  | 711  | 90  | 9   | 134 | 6  | 8   | 104 | 3  |

* Met_ = purified T5 Met-tRNA.

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individually charged with different radioactive amino acids and separately annealed to wild type and mutant DNAs. In Fig. 8, an example of the type of hybridization data obtained, is illustrated the results observed with 4 of the 15 aminoacyl-tRNAs used in this study. For [³H]alanine- and [³H]arginyl-tRNAs (Fig. 8, A and B), the results clearly indicate that certain T5 mutant DNAs form hybrid complexes with these tRNAs species at levels nearly equivalent to those for wild type T5+ DNA (within the limits of experimental variations), whereas other mutant DNAs failed to show significant hybrid formation (less than 10% of wild type DNA). Positive hybridization for [³H]phenylalanyl-tRNA (Fig. 8C) was found for only two mutant DNAs, st(20) and st(14); however, the extent of hybridization to st(14) DNA was approximately one-fourth of that observed for wild type DNA. Repeat annealings gave similar results and, since the level of hybridization to st(14) DNA was significantly higher than for control filters containing no DNA, the hybridization was scored as positive. Several possible explanations for these results will be discussed later.

In Table II are summarized the hybridization data obtained for 15 T5 tRNA species. Hybrid values less than 10% of those for the wild type were considered not to be significant and were
scoring as negative, whereas values more than 10% were scored as positive. The positive and negative scoring implies the leucine, lysine, and methionine (formyl species); isoleucine, follows: tyrosine and phenylalanine; alanine, proline, valine, hence, it falls into a group by itself. Other tRNA species show similar hybridization patterns for the one reviews this summary, it becomes apparent that certain hybridization pattern is different from all other tRNA species; gene sequences) in the T5 DNA chromosomes examined. As presence or absence of tRNA genes (single, multiple, or partial formation of hybrid radioactivity were as described in Fig. 3. Blank filters at the two levels of tRNA concentrations used contained, on the average, 34 and 81 cpm, respectively. B, hybridization with T5+ [3H]tyrosyl-tRNA. The specific activity of [3H]tyrosine was 28 Ci/mmol and the charged tRNA preparation (0.133 mg/ml) contained 2.5 x 10^6 cpm/ml. The conditions of annealing were the same as in A. At the two levels of tRNA concentration used, blank filters contained 105 and 104 cpm, respectively. C, hybridization with T5+ [3H]phenylalanine-tRNA. The specific activity of [3H]phenylalanine was 59 Ci/mmol and the charged tRNA preparation (0.133 mg/ml) contained 2.5 x 10^6 cpm/ml. The conditions of annealing were the same as in A. At the two levels of tRNA concentration used, blank filters contained 134 and 121 cpm, respectively. D, hybridization with T5+ [3H]isoalleucyl-tRNA. The specific activity of [3H]isoalleucine was 30 Ci/mmol and the charged tRNA preparation (0.133 mg/ml) contained 2.1 x 10^6 cpm/ml. The conditions of annealing were the same as in A. At the two levels of tRNA concentration used, blank filters contained 90 and 47 cpm, respectively.

### Table II

| Amino- | DNA  | T5+ | b1  | b2  | b3  | st(0) | st(8) | st(14) | st(20) |
|--------|------|-----|-----|-----|-----|-------|-------|--------|--------|
| Arg    |      | +   | +   | +   | +   | +     | -     | -      | -      |
| Tyr    |      | +   | +   | -   | +   | +     | +     | +      | +      |
| Phe    |      | +   | +   | -   | +   | +     | -     | -      | +      |
| Ala    |      | +   | +   | -   | +   | +     | +     | +      | -      |
| Pro    |      | +   | +   | -   | +   | +     | +     | +      | -      |
| Val    |      | +   | +   | -   | +   | +     | +     | -      | +      |
| Leu    |      | -   | -   | -   | -   | -     | -     | -      | -      |
| Lys    |      | +   | +   | -   | +   | +     | +     | -      | +      |
| Metα   |      | +   | +   | -   | +   | +     | +     | +      | +      |
| Ileu   |      | +   | +   | -   | +   | +     | +     | +      | +      |
| Gly    |      | +   | +   | -   | +   | +     | +     | +      | +      |
| Asp    |      | +   | +   | -   | +   | +     | +     | +      | +      |
| Metα   |      | +   | +   | -   | +   | +     | +     | +      | +      |
| Ser    |      | +   | +   | -   | +   | +     | +     | +      | +      |
| His    |      | +   | +   | -   | +   | +     | +     | +      | +      |

* Metα, purified T5 Met-tRNA.
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DNAs, therefore, no grouping assignment could be made. It should be noted that T5 st(20), which contains the smallest deleted DNA segment, shows no hybridization for tRNA*met, while T5 b1, which contains the largest DNA deletion, showed positive hybridization only for tRNA*met. Hence, the gene locations of the tRNAs listed in Table II must map between the right end of the st(20) deletion and the left end of the b1 deletion.

Since the exact size and location of the individual mutant DNA deletions were not precisely known, it was necessary to obtain this information before we could attempt to construct a physical tRNA gene map for T5 DNA. It was first necessary to measure the intact genome of T5. Such measurements were made by electron microscopy, as described under "Materials and Methods." Native T5 DNA was measured as 23.86 ± 0.32 x 10^6 daltons. Measurements were also made against an external standard (a replica grating), yielding values of 1.63 ± 0.04 μm for φX174 RF DNA and 38.9 ± 0.5 μm for T5 DNA. Thus, for T5 DNA in 50% formamide, the mass to length ratio is 2.08. Heteroduplexes were then measured relative to the length of T5 DNA.

In Fig. 9 is shown a schematic representation of the heteroduplex forms which one might expect to obtain by cross-hybridization of the DNA strands from T5+ wild type (Form 1) and a Group A deletion mutant (Form 2). It should be possible to obtain two different DNA heteroduplex structures by the renaturation of opposing heavy (H) and light (L) DNA strands (Forms 3 and 4). Form 3 shows a conventional loop...
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FIG. 9. Heteroduplex forms expected between T5+ DNA and a group A T5 mutant DNA. Forms 1 and 2 represent the native DNA structures for T5+ wild type and a group A T5 deletion mutant (Fig. 7). Form 3 shows the expected heteroduplex loop structure between the heavy continuous T5+ strand and the T5 mutant light chain segments. Since a group A mutant has a DNA deletion which includes the C-D single strand interruption, one would expect a branched heteroduplex structure (Form 4) if annealing occurs between the mutant heavy DNA strand and wild type light chain segments.

FIG. 10. Electron micrograph of a complete T5+:T5 st(0) heteroduplex DNA molecule. The heteroduplex molecule was formed by annealing denatured DNAs prepared from T5+ wild type and st(0) mutant phages. The procedures used for forming such structures and for observation by electron microscopy were as described under "Materials and Methods." The letters A and H designate the left and right end of the heteroduplex chain. The lettered segments A-B, C-D, E-F, and G-H designate double-stranded regions which arise from incomplete annealing. The single-stranded loop structure, D-E, represents the st(0) deletion segment.
structure in the T5+ H-strand since the equivalent complementary DNA segment is missing in the mutant L-strand. A reciprocal loop structure should also form from the annealing of wild type L-strand segments and mutant H-strand if the deletion does not include a single strand interruption as in the case of the Group B T5 mutants. Another type of heteroduplex structure, Form 4, can also be predicted if hybridization occurs between the H-strand of a Group A mutant DNA and the L-strand segments of wild type DNA. In this case, a double-branched single strand structure should appear since the deletion includes the C-D single strand interruption which preempts the formation of a DNA loop. For either of the heteroduplex forms, the size and position of the DNA deletion can be ascertained by measurement of the lengths of the DNA loop or the branched structures as well as the distance of their branch points to either end of the heteroduplex chain.

In Fig. 10 is shown a DNA heteroduplex molecule as seen under the electron microscope and formed by annealing of T5+ DNA with T5 st(0) DNA. The entire T5 chromosome is shown in this micrograph; the letters A and H indicate the left and right ends of the heteroduplex chain, respectively. The letters B through G indicate positions where native and denatured regions are located along the DNA molecule. The single stranded regions in the linear portion of the chain derive from incomplete hybridization of the heavy T5+ DNA strand with the complementary DNA segments of the opposing light strand. Fig. 11 represents a tracing of this heteroduplex molecule, with the double- and single-stranded regions drawn as heavy and light lines and their measured lengths recorded next to the appropriate lettered segments. It may be seen that, for the T5+st(0) heteroduplex molecule, there is one single-stranded loop structure, segment DE, located 10.4 x 10^6 daltons from the left end of the chain (point A) and measuring 3.0 x 10^6 daltons in length. This single-stranded loop represents the st(0) deletion segment. As described under "Materials and Methods," φX174 bacteriophage DNA and mouse mitochondrial DNA served as standards for the measurement of single- and double-stranded heteroduplex regions, respectively.

The electron micrographs in Fig. 12 show two heteroduplex structures which formed after annealing of denatured T5+ DNA and T5 st(20) DNA. The loop structure on the right corresponds to Form 3 (Fig. 9), whereas the branched structure on the left with its two single-stranded segments corresponds to Form 4. The branched segments labeled C and D represent that portion of T5 DNA (to the left and right of the C-D interruption) which is missing or deleted in st(20) DNA. The small circular structures are single-stranded φX174 DNA.

Table III summarizes our measurements of the different heteroduplex forms obtained between wild type T5+ DNA and the various DNAs isolated from the T5 mutant phages. For each of the mutant DNAs examined, the data offer length measurements from the left end and right end of the deletion loop to the left end of the T5 chromosome. The difference between these two measurements is the size of the deletion indicated in the last column. The position of the C-D single strand interruption was ascertained from similar measurements of the double-branched structure seen in the heteroduplex between T5+ and st(0) DNAs. In addition, we have measured the A, B, and C segments of T5+ DNA (not shown in Table III) as A = 3.10 ± 0.07; B = 3.68 ± 0.49; and C = 5.97 ± 0.48, x 10^6 daltons.

As shown in Fig. 13, a physical map of the C segment containing the approximate locations of T5 tRNA genes was constructed from the hybridization data of Table I and the size and location of the DNA deletions shown in Table III. Deletion sizes and the size of the tRNA groupings, regions I, II, III, and IV, are reported in kilobase lengths for a single strand molecule. The map was constructed as follows: Since the cistron for T5 arginine tRNA is missing in the st(20), st(8), and st(0) mutants, but is present in all other deletion strains, the tDNA" sequence must be present between the right end of the st(20) deletion and the right end of the b2 deletion. The other

![Fig. 11. A schematic drawing of the T5+T5 st(0) heteroduplex DNA molecule shown in Fig. 10. The thick line represents double-stranded DNA and the thin line represents single-stranded DNA. The contour lengths of each lettered segment are given as daltons x 10^6. The method used for converting linear length into units of molecular weight is described under "Materials and Methods."](http://www.jbc.org/)

![Fig. 12. Electron micrographs of T5+T5 st(20) heteroduplex DNA structures. The conditions for heteroduplex DNA formation with T5+ and T5 st(20) DNAs were as described under "Materials and Methods." The loop structure shown on the right corresponds to Form 3 (Fig. 9). The branched structure shown on the left with branches C and D correspond to Form 4 (Fig. 9). The small circular structures in the lower right section of the photograph are single-stranded φX174 DNA molecules.](http://www.jbc.org/)

| Segment | Single Strand Length (kb) |
|---------|---------------------------|
| AB      | 3.1                       |
| BC      | 4.0                       |
| CD      | 3.3                       |
| DE      | 3.0st(0)DeletionLoop      |
| EF      | 2.6                       |
| FG      | 1.6                       |
| GH      | 1.6                       |
| A-H     | 40.1 Total               |
Mapping of T5 tRNAs

TABLE III

Measurements of mutant T5 DNA deletions

All measurements were made relative to the left end of the T5 DNA strand except for st(8) and st(20) which were made relative to the C-D nick as measured in the st(0)/T5+ heteroduplex structure. The columns designated left end and right end refer to the left and right end of the heteroduplex loop structure. Percentages were calculated using a single-stranded molecular weight length for T5+ DNA equal to $40.6 \times 10^{6}$ daltons.

| DNA  | Left end | C-D nick | Right end | Deletion size |
|------|----------|----------|-----------|--------------|
| st(0)| 10.29±0.12 | 25.3     | 32.6      | 7.3          |
| st(8)| 10.84±0.24 | 26.7     | 32.5      | 5.9          |
| st(20)| 11.96±0.05 | 29.5     | 32.3      | 2.9          |
| b1   | 8.56±0.16  | 21.1     | 29.3      | 8.2          |
| b2   | 9.54±0.10  | 23.5     | 29.9      | 6.4          |
| b3   | 9.95±0.01  | 24.0     | 29.6      | 5.1          |
| b4   | 9.54±0.12  | 23.5     | 28.5      | 5.0          |
| st(14)| 9.94±0.14 | 24.5     | 28.2      | 3.7          |

Fig. 13. A physical map of mutant T5 DNA deletions and T5 tRNA genes. The top bar represents the complete C segment of T5+ DNA with a small portion of the B and D segments included showing the position of the B-C and C-D single strand interruptions. The position of the DNA segments deleted in the eight mutant phages listed on the left are designated by the lower bars. The sizes of the deletions (kilobases and percentage of total genome assuming a single strand length of 40.6 $\times 10^{6}$ daltons for T5+ DNA) are listed on the right. The vertical broken lines define the four regions of T5+ DNA containing tRNA genes as specified by the amino acids listed under each tRNA gene grouping in the C segment. DNA sequences for tRNA Phe and tRNA Tyr were located between the right end of the b4 deletion and the right end of the st(14) deletion. The tentative assignment of a second isoleucine tRNA gene (Ileu,) in this same region will be discussed later. Genes for tRNA Arg, Metf, Leu, Lys, Met, Lys, and Leu, were positioned between the left end of the st(0) deletion and the left end of the b3 deletion; the genes for tRNA His, Ser, Ileu, Gly, Asp, Met, and His, Ser, Ileu, Gly, Asp, Met, were located between the left end of the b4 deletion (which coincides with the left end of b2) and the left end of the b1 deletion. The single strand lengths of the three spaces between the four tDNA-containing regions were measured as 1.24 (I-II), 3.44 (II-III), and 1.69 (III-IV) kilobases.

In Fig. 14 are shown the sizes and locations of the four tDNA-containing regions (I, II, III, and IV) relative to the whole T5+ DNA molecule. The single strand values for the DNA lengths and molecular weights occupied by these regions are shown in the table included in this figure. The total nucleotide span for the 15 T5 tRNAs contained within the segment of T5 DNA between the right end of the st(20) deletion and the left end of the b1 deletion is approximately 13.8 kilobases long and represents 11.2% of the wild type genome using a value of 40.6 $\times 10^{6}$ daltons as the single strand length for T5+ DNA.

DISCUSSION

The hybridization of charged tRNAs from T5-infected cells to the separated strands of T5 DNA, following denaturation
and gradient centrifugation, suggests that the heavy continuous strand contains the nucleotide sequences from which T5 tRNAs are transcribed. In support of this suggestion, our results show that the genes which code for T5 tRNAs map almost exclusively in the C segment of T5 DNA, and RNA transcription in this region of the T5 chromosome has been reported to occur only from the heavy strand (13, 14). Although the hybridization data of Table I do not completely eliminate the possibility that the light DNA segments contain some information for tRNA synthesis, it seems unlikely that the same tDNA sequences would be present in both heavy and light chains for any single tRNA species.

Several heat-stable (st) mutants of T5 phage carry a deletion in the D and C regions of T5’ DNA (Figs. 4 and 7). We had previously reported that the st(0) T5 deletion mutant lacked the tRNA genes for arginine, tyrosine, and phenylalanine, and that the length and location of the st(0) deleted DNA segment could be determined accurately by electron microscopic examination of heteroduplex molecules formed between wild type and mutant DNAs (22). In subsequent studies by Scheibl and Rhoades (31), heteroduplex mapping of heat-stable mutants of T5 bacteriophage has also been described. By correlating the physical locations and the sizes of the mutant deletions with the presence or absence of detectable tRNA genes, we have defined the positions and the maximal sizes of four tDNA-containing loci in T5’ DNA (Figs. 13 and 14).

The hybridization technique for locating tDNA sequences by use of labeled aminoacyl-tRNAs depends on several critical factors, which include the presence of functional tRNA molecules capable of accepting amino acids; the efficiency of enzyme charging; the specific activity of the individual radioactive amino acids; the stability of aminoacyl-tRNAs under the annealing conditions employed; the stability of the charged tRNA-DNA complex following hybridization, which includes treatment with T1 RNase; as well as the presence of a sufficient number of tRNA gene copies to permit detection. Variations in the levels of hybridization observed for some of the T5 mutant DNAs could be related to one or several of these factors. A negative hybridization result does not necessarily exclude the possible existence of the corresponding tRNA gene in the DNA genome. However, positive results do indicate the presence of tRNA complementary sequences in T5 DNA which, in turn, imply the presence of tRNA cistrons and transcription into active tRNA molecules.

A molecule of T5 DNA should not need to possess an entire tRNA sequence in order to retain detectable radioactivity from hybridization with a labeled aminoacyl-tRNA. Partial DNA sequences of a tRNA molecule could give rise to a branched tRNA-DNA hybrid structure which, if stable, would be sufficient for its detection by the method used here. If the 3’ aminoacyl end of the tRNA molecule were part of the branched unannealed portion of the hybrid, and if a guanine residue were present in this branch, the hybrid complex would go undetected since our procedure includes T1 RNase treatment following hybridization. If the guanine residue were close to the point of branching of the partial hybrid structure, it might not be readily accessible to the action of T1 RNase, thus causing incomplete digestion. It is possible therefore, since various deletion mutant DNAs are being used, that the number of imperfect hybrid structures formed would vary and that expected levels of hybridization relative to wild type DNA would not be observed. It is not clear whether this problem is related to some of the lower levels of hybridization detected for certain mutant DNAs. On the other hand, if T5 wild type DNA contains multiple tRNA cistrons for a single tRNA species (isoacceptor or repeated tRNA genes), and if the mutant DNA contains a smaller number of gene copies for the same tRNA species, one would expect to find lower levels of aminoacyl-tRNA hybridization to the mutant DNA. It is, therefore, important that when low hybridization values relative to wild type DNA are encountered to distinguish (if possible) between intrinsic variations due to the procedure itself and meaningful values which reflect the size, location, and number of tRNA cistrons in the mutant DNAs.

The hybridization curves shown in Fig. 8 typify the results obtained for the 15 T5 aminoacyl-tRNA species. Those hybridization values for the mutant DNAs which differ slightly from the results obtained with T5’ DNA probably can be attributed to intrinsic variations in the assay procedure. However, the very low values found for phenylalanyl-tRNA hybridization to st(14) DNA (25% of that of wild type DNA), and the approximate half-values found for isoleucyl-tRNA hybridization to five of the mutant DNAs, cannot readily be ascribed to some intrinsic error, but are more probably due to the deletion of tDNA sequences (either partial or complete tDNA cistrons). This laboratory has reported on the presence of two isoacceptor T5 methionine tRNAs, tRNA<sup>Met</sup> and tRNA<sup>Met</sup> (21). In addition, we have recently been able to demonstrate that there are two T5 isoacceptor tRNAs for isoleucine, one of which is missing in the T5 mutant b2. The T5 tRNA cistron map (Fig. 13) derived from the hybridization and deletion measurements in this study shows four groups of tRNAs located close to one another at four separate regions in the C segment of T5 DNA. Region IV, which contains only the cistron(s) for tRNA<sup>Met</sup> encompasses the C-D single strand interruption; hence, the tDNA<sup>Met</sup> sequences could be positioned on either side of the C-D nick within the 3020-nucleotide length that comprises this region.

**Fig. 14.** A schematic representation of the location and size of T5 tRNA-containing regions relative to the total size of the T5’ genome. The four tDNA-containing loci listed above are shown in their approximate position along the heavy continuous strand of T5’ DNA. The chart (lower right) lists the single strand molecular sizes for each of the four tRNA regions in daltons and kilobase lengths as well as their percentage of total length relative to wild type DNA.

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* C. Hunt and S. B. Weiss, unpublished results.
which is 390 nucleotides in length, contains the sequences for tRNA"^{\text{Phe}}", tRNA"^{\text{Arg}}", and tRNA"^{\text{Met}}" (tentative) and is located 1690 nucleotides from the left end of region IV. For reasons discussed above, we believe that there are two separate isoleucine tRNA genes, and that the positioning of one of these genes (Ileu"_i") in region III would satisfy the hybridization data obtained in Fig. 8D. If the low hybridization values for phenylalanyl tRNA to st(14) DNA are due to the partial deletion of tRNA"^{\text{Met}}" sequences, then it is likely that the tRNA"^{\text{Met}}" gene boundary would extend farther to the left of region III, making this region slightly larger than estimated. If multiple tRNA"^{\text{Met}}" cistrons are involved, extension to the left of region III could be greater, but no farther than the left end of the st(8) deletion, since hybridization to this mutant DNA was not observed. Region II contains the T5 tRNA cistrons for Ala, Pro, Val, Leu, Lys, and Met. It is approximately 1030 nucleotides long and is displaced to the left of region III by some 3440 nucleotides. The cistrons for His, Ser, Ileu, Gly, Asp, and Met tRNAs were located in region I, which comprises a length of 2960 nucleotides and is separated from region II by 1240 nucleotides. Unequal hybridizations relative to wild type DNA were also observed for some of the tRNAs coded in regions I and II. An attempt to explain such phenomena on a basis similar to that used for tRNA"^{\text{Met}}" and tRNA"^{\text{Phe}}" is not possible at this time.

In the mapping of T5 tRNA genes, different T5 deletion mutants were used, the DNA deletions of which varied in size and extended to various degrees into the T5 cistron-containing region of the T5"^+" genome. Heat-stable mutants of T5 phage were first described by Adams and Lark (32) and Adams (33). Hertel et al. (34) later isolated several heat-stable mutants, including the b series of deletion mutants used in this work, and found that their relative stability was correlated with their density; the greater their stability, the lower their density. Rubenstein (35) demonstrated that the st mutants of T5 phage contain less DNA than wild type T5. By sedimentation studies, it was determined that the fractional content of T5 wild type DNA (82.7 x 10^6 daltons) for the T5 mutants, in decreasing order for st(20) > st(14) > st(8) > st(0) > st(26), was 0.983, 0.966, 0.941, 0.938, and 0.930, respectively. Using agarose gel electrophoresis for analysis of the single-stranded DNA segments of T5 DNAs, Hayward (Fig. 7) found that the fractional content of T5 wild type DNA (80.6 x 10^6 daltons), in decreasing order for st(20) > st(14) > st(8) > st(0) > b3 > b2 > b1, was 0.995, 0.970, 0.961, 0.955, 0.953, 0.938, and 0.926, respectively. The order of DNA content correlated exceedingly well with the order of phage stability and phage density (from 1.550 for st(20) to 1.529 for b1). The agarose gel technique indicated little difference in the deletion size of the b3 and b4 DNAs and Hertel et al. (34) showed that the b4 and b3 phage densities (1.534 and 1.535) were almost identical. Our results from heteroduplex measurements confirm these observations and give the order of DNA content as st(20) > st(14) > b4 > b3 > st(8) > b2 > st(0) > b1, which is the reverse order of the deletion sizes (Table III). In general, the deletion sizes relative to the total T5"^+" DNA obtained in this study were similar but slightly larger than those obtained by Hayward (3), particularly for those mutant DNAs the deletion sizes of which include the C-D single strand interruption, namely, st(20), st(8), and st(0). The measurement of contour length, under the conditions employed here, may have yielded a better estimate of the deletion sizes than gel electrophoresis relative to total DNA, even though the accuracy of length measurements in this study were limited by such factors as the physical variations of DNA length in the basic protein monolayer, the ability to discriminate between single- and double-stranded regions, and the arbitrary assignments of molecular weights of reference DNAs.

In this work, the T5"^+" genome was measured as 81.1 x 10^6 daltons, a slightly greater length than, e.g. the 74.9 x 10^6 daltons reported by Lang (2), but in good agreement with the values reported by Hayward (3) and Rubenstein (35). As discussed by Freifelder (36), the value of 74.9 x 10^6 gave the unusually low mass to length ratio of 1.88 by aqueous DNA spreading, which he suggested to be a function of the base composition of this DNA. It is likely that the DNA is somewhat more extended in 50% formamide, resulting in the increased length reported here and in the higher mass to length ratio of 2.08. In particular, we measured the single strand C segment as 6.0 x 10^6 daltons compared to the value of 5.1 x 10^6 daltons reported by Hayward and Smith (39). However, when duplex fragments corresponding to the C segment were isolated by Hayward (3) from sheared T5"^+" DNA, denatured, and subjected to gel electrophoresis, two bands were observed corresponding to a molecular size of 5.1 and 6.0 x 10^6 daltons, respectively. Since the two bands represent complementary DNA strands and must be the same size, the different electrophoretic migrations must reflect differences in molecular configuration resulting from differences in base sequence and composition. The strand exhibiting the higher molecular weight value probably has a more extended configuration suggesting that the 6.0 x 10^6 value may be a closer estimate of the true molecular length for the single strand C segment.

The segment of T5"^+" DNA containing all of the tRNA sequences examined thus far lies between the left end of the T5 b1 deletion and the right end of the T5 st(20) deletion. This segment of DNA is estimated to be 11.2% of the total T5"^+" genome, or approximately 13,800 nucleotides long. Therefore, the T5 tDNA cistrons appear to be more loosely clustered than their T4 counterparts in which all eight tRNA cistrons are confined to a T4 DNA segment of approximately 2,500 nucleotides (37), representing only 1.3% of the total T4 genome (120 x 10^6 daltons). If one takes into consideration the difference in the number of tRNA species for T4 and T5, as well as the 50% larger genome size for T4, the difference in tRNA clustering for these two phages becomes less significant. However, T5 tRNA genes are more highly clustered than the tRNAs mapped in E. coli (38).

In the T5 map of Fig. 13, regions I and IV are less well defined than regions II and III, relative to the number of tRNA genes contained in these regions. There is an average of 172 and 130 bases per tRNA gene in regions II and III, respectively, compared to 493 and 3020 bases per tRNA gene for regions I and IV, respectively, assuming no multiple genes for a given tRNA species. It is possible, however, that T5"^+" DNA contains multiple tRNA cistrons for some of the existing tRNA species. Stoichiometric titration of T5"^+" DNA with T5"^+" [H]seryl-tRNA indicated that, at saturation, approximately 1.6 molecules of tRNA"^{\text{Met}}" were hybridized per molecule of T5"^+" DNA (data not shown). If we consider the very likely presence of multiple cistrons for a single tRNA species, as well as the tRNA genes for amino acid species not examined in this report, the clustering of tRNA genes in the T5 genome, per unit length of DNA, may be more compact than presently shown by our map.

* G. S. Hayward, personal communications.
In addition, our present map does not permit an estimation of spacer nucleotides between tRNA genes since we do not know the number and distribution of tRNA cistrons within each tDNA-containing region. It is obvious, therefore, that our tRNA map requires further refinement for a precise understanding of the tRNA gene arrangement in the T5 phage genome.

Although phage tRNAs are nonessential for phage growth under laboratory conditions, as indicated by the viability of tRNA deletion mutants, they may be essential under some conditions encountered in the wild state. The isolation of phage tRNA nonsense suppressors indicates that at least some phage tRNAs can function, in vivo, for the synthesis of proteins (39, 40). The continued presence of viral tRNAs may reflect the consequence of natural selection for a tRNA population optimal for the translation of viral messenger RNA. Scherberg and Weiss (20) obtained results supporting this hypothesis from studies on in vitro protein synthesis. In addition, mutations in the structural gene of a single T4 phage tRNA become lethal for phage viability if E. coli strain CT439 serves as the host (41). The physiological role of phage tRNAs is not yet understood. Knowledge of the tRNA gene arrangement in chromosomes is important for an understanding of tRNA biosynthesis which, in turn, may play an important role in the regulation of protein synthesis.

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