Purification and In Vitro Transcription of a Transfer RNA Gene

(E. coli/bacteriophage/Neurospora endonuclease/tyrosine/electron microscopy)

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ABSTRACT A gene specifying tyrosine transfer RNA has been purified and transcribed in vitro. The purification procedure made use of two specialized transducing phages carrying the tRNA tyr gene of Escherichia coli inserted into their DNA in opposite orientations. The separated heavy strands of the two phages were annealed and the single-stranded tails of the resulting hybrid were removed by digestion with Neurospora endonuclease. The size of the purified double-stranded structures was determined by electron microscopy. These isolated duplexes served as template for the in vitro transcription of tRNA tyr-like molecules.

Recently there have been many attempts to purify DNA that contains individual genes. Several techniques have been used for this purpose. In a limited number of cases where the base composition of the gene to be isolated differed considerably from that of the bulk DNA, it could be enriched by shearing the DNA molecules to an appropriate size. The fragments of interest were then isolated by density gradient centrifugation (1, 2) or hydroxylapatite column chromatography (3). More commonly, genes have been purified by hybridizing denatured DNA fragments with the specific RNA product of the gene in question. This technique of DNA–RNA hybridization has been used to enrich for ribosomal RNA (4, 5) and transfer RNA (6) cistrons. In these studies, however, DNA was fragmented by a random process of mechanical shearing. As a result, the desired gene might have been fragmented as well. In other cases, specific nucleases were used to remove nonhybridized, single-stranded DNA, a well as single-stranded DNA ends from the DNA–RNA hybrid (7–9). However, since the initiation and regulation sites of the isolated genes were not protected by hybridization with RNA, they were subjected to digestion during the nuclease treatment. Transducing phages carrying bacterial genes inserted in their DNA in opposite orientations have been used to isolate a gene with its promoter–operator region extant; for example, Shapiro et al. (10) hybridized the isolated heavy DNA strands from two transducing phages carrying the lac operon of Escherichia coli. Subsequent removal of nonhybridized DNA regions by endonuclease treatment yielded a duplex DNA fragment which was, presumably, the lac operon. However, the identity of the isolated fragment was not established unequivocally. In the present report, two φ80psu+ phages carrying the E. coli structural gene for tyrosine-specific transfer RNA (11, 12) in opposite orientations were used to isolate the DNA sequence containing this gene. The heavy strands of both phage DNAs were hybridized and the single-stranded tails of the heteroduplex formed were then removed by digestion with Neurospora endonuclease. The purity and size of the isolated duplexes were determined by electron microscopy. The duplex molecules were shown to serve as templates for in vitro tRNA tyr transcription, thus establishing their identity as E. coli DNA fragments containing the su3 gene.

MATERIALS AND METHODS

Phage and bacterial strains

Phage φ80psu+(O) was received from Dr. H. Ozeki and was grown on cells of E. coli CA 274 (Hfr C lac−Amber−Amber−su3) on λ-broth agar (13) containing 0.25% NaCl. This phage contains the sense strand for the tRNA tyr gene su3+ on the light DNA chain. Phage φ80psu+(−)(S) was received from Dr. J. D. Smith and contains the sense strand for the tRNA tyr genes, su3− and su2− on the heavy DNA strand. This phage was grown on cells of E. coli CA 274 in liquid medium containing 1% Bacto Tryptone and 0.5% NaCl. Both phages were isolated and purified by equilibrium density centrifugation (14) [32P]trRNA was prepared from E. coli B cells grown on Tris–glucose medium as described (15).

Preparation of Neurospora crassa endonuclease

Neurospora crassa, ATC 9279 was grown in 1000-ml Fernbach flasks in Vogel’s synthetic medium (16) supplemented with 1% glycerol and 1% sucrose. The endonuclease was prepared from the conidia by chromatography on phosphocellulose followed by purification through a hydroxylapatite column (17). The enzyme obtained was chromatographed on a second hydroxyapatite column. This treatment helped to remove traces of activity toward double-stranded DNA. The assay for enzyme activity was performed as described by Rabin et al. (18). A unit of activity is defined as the amount of enzyme required to release one A260 unit of acid-soluble material in 30 min at 37°C. The specificity of the enzyme toward single-stranded DNA was determined by the use of [3P]-labeled E. coli native or denatured DNA as substrate. The reaction mixture contained in 0.3 ml: 10 mM Tris-HCl buffer (pH 7.5); 0.3 M NaCl; 2 mM MgCl2; 2 units of enzyme (8000 units/mg protein) and 3 μg of E. coli [3P]DNA. Incubation was for 3 hr at 37°C. Under these conditions, 90% of the denatured [3P]DNA was rendered acid-soluble. On the other hand, double-stranded [3P]DNA remained completely stable (100% acid-precipitable) even after 5 hr of incubation at 37°C.

Isolation of the E. coli DNA fragment containing the su3 gene

A heteroduplex was formed by annealing the heavy strand of φ80psu+(−)(S) DNA with the heavy strand of φ80psu+(O)
DNA at a concentration of 18 µg/ml (each) in 3.0 ml of 10 mM Tris-HCl (pH 7.5)–50 mM NaCl–1 mM EDTA. The mixture was incubated for 18 hrs at 30°C. For the digestion with Neurospora endonuclease, the heteroduplex was first dialyzed against 5 mM Tris-HCl buffer (pH 7.5)–0.15 M NaCl to eliminate EDTA. The solution was then brought by flash evaporation to a final concentration of 0.3 M NaCl and 0.01 M Tris-HCl (pH 7.5). MgCl₂ (final concentration, 2 mM) and 16 units/ml of Neurospora endonuclease were added and the mixture was incubated for 150 min at 37°C. The reaction was stopped by the addition of EDTA to a final concentration of 0.01 M. The E. coli DNA fragment containing the su₁ gene was extracted with water-saturated phenol, dialyzed against 10 mM Tris-HCl (pH 7.9)–0.3 M NaCl–1 mM EDTA, and then against 2 mM Tris-HCl (pH 7.9)–2 mM NaCl. The solution was dried by flash evaporation and the isolated DNA fragment was dissolved in 50 µl of water.

**In vitro transcription of the E. coli DNA fragment carrying the su₁ gene**

DNA-dependent RNA polymerase was purified from E. coli MRE-600 cells by the procedure of Chamberlin and Berg (20). The enzyme preparation was then subjected to low salt glycerol gradient centrifugation (21) and the core enzyme was separated from the σ subunit by phosphocellulose chromatography (22). Transcription was done in a reaction mixture containing in 0.1 ml: 50 mM Tris-HCl (pH 7.9); 10 mM MgCl₂; 1 mM dithiothreitol; 0.1 M KCl; 1 mM (each) of ATP, CTP, and GTP; and 0.16 mM [3H]UTP (3 × 10⁶ cpm/nmol); 1 µg of core RNA polymerase; 1.5 µg of σ factor; and the E. coli DNA fragment containing the su₁ gene. The mixture was incubated for 60 min at 37°C, after which 1 µl of electro-photically purified DNase was added and the incubation was continued for an additional 15 min at 37°C. The mixture was then made 0.25% with respect to sodium dodecyl sulfate, chilled, and extracted with water-saturated phenol. Aliquots were precipitated with trichloroacetic acid and the amount of [3H]UMP polymerized was determined. The amount of RNA synthesized was calculated on the assumption of an equimolar base composition for the isolated E. coli DNA fragment.

**Electron microscopy**

DNA samples were adjusted to a concentration of 3 µg/ml in a buffer containing 0.01 M Tris-HCl (pH 7.5)–1 M KCl–0.01 M EDTA.

**Table 1. Hybridization of ³²P-labeled E. coli tRNA with the separated strands of φ80psu⁺(O) and φ80psu⁺⁺(S) DNA**

<table>
<thead>
<tr>
<th>DNA</th>
<th>Strand</th>
<th>Hybridized E. coli [³²P]tRNA (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>φ80psu⁺(O)</td>
<td>Light</td>
<td>1970</td>
</tr>
<tr>
<td>φ80psu⁺⁺(S)</td>
<td>Heavy</td>
<td>50</td>
</tr>
<tr>
<td>φ80psu⁺⁺(S)</td>
<td>Light</td>
<td>40</td>
</tr>
<tr>
<td>φ80psu⁺⁺(S)</td>
<td>Heavy</td>
<td>2180</td>
</tr>
</tbody>
</table>

The hybridization mixture contained in a total volume of 0.3 ml: 0.3 M NaCl, 0.03 M sodium citrate (2 × SSC), 2 µg of the separated single-stranded DNA and 0.22 µg of E. coli [³²P] tRNA. The mixtures were incubated at 68°C for 120 min, diluted with 2 ml of 2 × SSC, loaded on nitrocellulose filters (Schleicher and Schuell B6, 27 mm), and washed with 50 ml of 2 × SSC on each side. The filters were treated with pancreatic RNase (25 µg/ml in 2 × SSC) for 60 min at room temperature, washed again with 50 ml of 2 × SSC on each side, dried, and counted.

**RESULTS**

**Separation of the DNA strands of φ80psu⁺(O) and φ80psu⁺⁺(S)**

The complementary strands of the two φ80psu⁺ phage DNAs were separated and purified in a CsCl gradient (Fig. 1a and b). The main fractions of each band (shaded areas) were pooled separately and then hybridized with [³²P]-labeled E. coli tRNA. In this way, the transcribed strand of each of the phage DNAs was identified (Table 1). In the case of φ80psu⁺(O), the tRNA Tyr chains are transcribed from the light strand (also refs. 14, 25), while in the case of φ80psu⁺⁺(S) DNA there is an inversion of the E. coli DNA fragment carrying the su₁ gene (26) and transcription of the tRNA Tyr chains takes place on the heavy strand. Under the electron microscope, the isolated light strands of both phage DNAs appeared intact, while the heavy strands appeared fragmented. The reason for the preferential fragmentation of one of the strands is not known. In CsCl density gradients, the heavy-strand peaks of both phage DNAs were repeatedly observed to be smaller than those of the eight strands (Fig. 1). It may be that pieces of heavy strand with lower buoyant density sediment at the same position on the gradient as does the light strand. If so, the light strand may
Table 2. Digestion of φ80psu*+(O) DNA strands by Neurospora endonuclease

<table>
<thead>
<tr>
<th>DNA strands</th>
<th>% Resistance to endonuclease digestion</th>
<th>0.3 M NaCl</th>
<th>0.1 M NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Light strand</td>
<td>38</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Heavy strand</td>
<td>13</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>(b) Light strand incubated for 120 min at 70°C</td>
<td>37</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Heavy strand incubated for 120 min at 70°C</td>
<td>10</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>(c) Light and Heavy strands annealed for 12 h at 30°C</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

(a) The reaction mixture contained in 0.3 ml: 3 μg of the separated strands of φ80psu*+(O) [32P] DNA (5000 cpm/μg); 0.3 M NaCl containing 0.01 M Tris-HCl (pH 7.5); 2 mM MgCl₂ or 0.1 M NaCl containing 0.1 M Tris-HCl (pH 7.5); 0.01 M MgCl₂; and 2.1 units of Neurospora endonuclease. The mixture was incubated for 5 hr at 37°C, aliquot samples of 40 μl were removed, and acid-insoluble radioactivity was measured. Resistance to digestion of an aliquot removed at 0-time was taken as 100%.

(b) Reaction mixtures containing light or heavy strands of [32P]DNA from φ80psu*+, but without the endonuclease were kept at 70°C for 120 min. After cooling, Neurospora endonuclease was added, the mixture was incubated, and acid-insoluble radioactivity was determined.

(c) A mixture of 5 μg (each) of [32P]-labeled light and heavy strands of φ80psu*+(O) DNA was annealed in 0.05 M NaCl-0.01 M Tris-HCl (pH 7.5)-1 mM EDTA by incubation for 12 hr at 30°C. The mixture was then dialyzed against 0.3 M NaCl-0.01 M Tris (pH 7.5), adjusted to a final concentration of 2 mM MgCl₂ and incubated with 7 units/ml of Neurospora endonuclease. Aliquots were removed and the resistance to digestion determined.

Table 3. tRNA<sup> Thy </sup>-Polymerase chains synthesized in vitro expressed as a percentage of the total RNA transcribed

<table>
<thead>
<tr>
<th>DNA transcribed</th>
<th>Relative amounts of tRNA&lt;sup&gt; Thy &lt;/sup&gt; in the RNA synthesized in vitro (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) φ80psu*+(O)</td>
<td>1.2-2.0</td>
</tr>
<tr>
<td>(b) φ80psu*+-(S)</td>
<td>3.6</td>
</tr>
<tr>
<td>(c) Light-strand φ80psu*+(O)-</td>
<td>0.6</td>
</tr>
<tr>
<td>Light-strand φ80psu*+-(S) heteroduplex</td>
<td></td>
</tr>
<tr>
<td>Heavy-strand φ80psu*+(O)-</td>
<td>0.2</td>
</tr>
<tr>
<td>Heavy-strand φ80psu*+-(S) heteroduplex</td>
<td></td>
</tr>
<tr>
<td>(e) E. coli DNA fragment carrying the su&lt;sup&gt;3&lt;/sup&gt; gene</td>
<td>25-80</td>
</tr>
</tbody>
</table>

* Heteroduplex d) after treatment with Neurospora endonuclease.

form some double-stranded regions by hybridizing with the heavy-strand pieces. That this is indeed the case is suggested by the partial resistance of the light strand to digestion by Neurospora endonuclease specific for single-stranded DNA. Table 2 demonstrates that in the presence of 0.3 M NaCl, 37% of the light strand remains resistant to endonuclease digestion after incubation for 120 min at 70°C. On the other hand, only 10% of the heavy strand remains acid-insoluble when incubated with the Neurospora enzyme. When these experiments were repeated under conditions more favorable for endonuclease digestion, i.e. in 0.1 M NaCl, 30% of the light-strand radioactivity was still acid-precipitable, while over 99% of the heavy-strand radioactivity was rendered acid soluble*. In a control experiment where heavy strands were annealed to light strands and then incubated with the enzyme, no degradation was observed. The small pieces of heavy-strand DNA contaminating the light-strand preparations seem to be derived from the φ80 phage DNA and not from the transduced E. coli DNA fragments. This is suggested by the lack of hybridization of tRNA<sup> Thy </sup> with the φ80psu*+-(S) light-strand DNA (Table 1).

These studies were complemented by electron microscopic observations. A considerable number of single-stranded molecules (ss) with double-stranded regions (ds) were observed in a preparation of self-annealed light strands (Plate 1a). When this preparation was treated with Neurospora endonuclease, the double-stranded structures remained, showing that they were true duplexes. Heteroduplexes formed by annealing the light strands of φ80psu*+-(S) and φ80psu*+(O) DNAAs were also visualized. An increase in the amount of double-stranded structures was indeed observed in this preparation (Plate 1b); however, it was impossible to differentiate between duplexes resulting from hybridization of the E. coli DNA complementary regions and the double-stranded structures already existing in each of the isolated light strands.

With preparations of purified heavy-strand DNA, the amount of double-stranded structures seen after self-annealing

* The digestion mixture routinely contained 0.3 M NaCl to confer greater resistance to endonuclease digestion upon double-stranded DNA.
was low (less than 8% of the total number of the molecules counted). From these studies, it was concluded that the isolated heavy strands of both phage mutants constitute suitable material for the isolation of the E. coli DNA fragment containing the 8u4 gene.

**Formation of heteroduplexes between heavy strands of 080psu−+(O) and 080psu−−(S) DNA**

Hybridization of the heavy strand of 080psu+−(S) DNA and the heavy strand of 080psu+−(O) DNA resulted in the formation of a duplex showing short, double-stranded structures with single-stranded bush-like tails (Plate 2a). The length of the duplex segment was measured, its size distribution is presented in Fig. 2. A mean length of 0.89 μm (corresponding to 1.7 × 10^5 daltons) and a mode of 0.855 μm (1.3 × 10^5 daltons) [47% of the molecules] was found for the double-stranded regions. This is in agreement with the size of the double-stranded region reported recently by Miller et al. (26), for a heteroduplex formed between two other transducing 8ps0 phages carrying the 8u4 gene. In order to isolate the pure E. coli fragment containing the 8u4 gene, the single-stranded tails of the heavy-strand heteroduplex were digested with *Neurospora* endonuclease. Electron microscopic examination of the digest revealed a pure population of short, duplex molecules (Plate 2b). The size of the endonuclease-resistant duplex molecules was close to that of the double-stranded region of the heteroduplex before endonuclease treatment (Fig. 2). The mean length of the duplex shifted to a value of about 0.79 μm (1.5 × 10^5 daltons) with a mode of about 0.57 μm (1.08 × 10^5 daltons) [80% of the molecules].

**Transcription of the isolated DNA fragment carrying the su3 gene**

The isolated duplex containing the E. coli su3 gene was transcribed by E. coli RNA polymerase. We examined the ability of the product synthesized in vitro to compete with E. coli [32P]tRNA for hybridization sites on the light strand of 080psu+−(O) DNA. As described (14, 25), this experiment is performed with constant amounts of light-strand 080psu+−(O) DNA and E. coli [32P]tRNA in the presence of increasing amounts of RNA synthesized in vitro. The results, presented in Fig. 3, clearly show that the RNA synthesized in vitro contains polynucleotide chains homologous to tRNA_\text{TYr} synthesized in vivo. From the amounts of the RNA synthesized in vitro, 50% competition with a given amount of E. coli [32P]tRNA, the relative amounts of tRNA_\text{TYr} in the in vitro product may be calculated (assuming that tRNA_\text{TYr} represents 5% of the whole tRNA mixture). In a similar way, 080psu+−(O) and 080psu−−(S) DNAs and heavy-strand or light-strand heteroduplexes not treated with endonuclease were transcribed by E. coli RNA polymerase. The tRNA_\text{TYr}-like polynucleotide content of the synthesized RNA was then determined. The results, presented in Table 3 show that transcribed 080psu+−(O) and 080psu−−(S) RNAs contained between 1.2 and 3.6% tRNA_\text{TYr}-like chains, while transcription of the isolated E. coli DNA fragment containing the 8u3 gene.

**Fig. 2.** Length distribution of heteroduplexes formed by annealing the heavy strands of 080psu+−(O) and 080psu−−(S) DNA. Length determinations were made with a map measurer from prints magnified 30,000–40,000 times. Heteroduplex molecules with single-strand tails (100 molecules) (solid line). Heteroduplex molecules after digestion with *Neurospora* endonuclease (85 molecules, broken line).

**Fig. 3.** Competition of the RNA transcribed in vitro on the isolated E. coli DNA fragment containing the su3+ gene, with E. coli [32P]tRNA for hybridization sites on the light strand of 080psu+−(O) DNA. The heteroduplex formed by annealing the heavy strands of 080psu+−−(S) and 080psu+−(O) DNAs was digested by endonuclease and transcribed. The hybridization mixture contained: 1.2 μg of the light strand of 080psu+−(O) DNA, 0.11 μg of [32P]tRNA (8.2 × 10^4 cpm/μg), and different amounts of RNA synthesized in vitro, in a total volume of 0.3 ml of 2 x SSC. Mixtures were incubated for 2 hr at 68°C, loaded on filters, washed, treated with pancreatic RNase (25 μg/ml), washed again, and counted. The radioactivity remaining on the filter was expressed as a percentage of the control, which contained no competing RNA.
gene gave a product in which 25–80% of the total RNA synthesized was tRNA\(^{\text{Tyr}}\)-like molecules. Transcription of heteroduplexes not digested with the endonuclease gave lower amounts of tRNA\(^{\text{Tyr}}\)-like chains, probably due to nonspecific RNA transcription along the single-stranded tails.

**DISCUSSION**

\(80\text{psu}^+\)-(S) DNA reportedly contains two tRNA\(^{\text{Tyr}}\) genes, \(80\text{psu}^+\) and \(80\text{psu}^-\) (ref. 12), while \(80\text{psu}^+(O)\) DNA contains one \(80\text{psu}^+\) gene (11). However, DNA–RNA hybridization experiments show that these phage DNAs are saturated with tRNA\(^{\text{Tyr}}\) at a level of only about 1.0 and 0.5 molecules per genome, respectively (unpublished results). These saturation concentrations are 50% lower than would be expected from genetic criteria, and confirm previous observations by Russell et al. (12). In any event, both in hybridization and transcription studies, we constantly observed the \(80\text{psu}^+\) gene dosage to be at a ratio of 2:1 for \(80\text{psu}^+\)-(S), as compared to \(80\text{psu}^+(O)\) phage DNA. As a consequence of this difference, one should expect to find within the duplex structure short, single-stranded loops. Such loops were visualized under the electron microscope by Miller et al. (28) in a similar heteroduplex structure by mounting the DNA in the presence of formamide. The aqueous mounting method used in our studies for the visualization of DNA did not allow us to determine with certainty the presence of such loops. However, the formation of such single-stranded regions could also result from noncomplementarity in other regions of the E. coli DNA fragment. Indeed, the two \(80\text{psu}^+\) phages used by us were obtained through independent recombination processes; thus their transduced E. coli DNA segments need not be identical.

The method of gene isolation used in this study is based on complementary DNA–DNA hybridization, followed by endonuclease digestion. It has the advantage that an entire unit of transcription containing its regulatory sites is isolated. However, introduction of nicks by endonuclease in some of the isolated genes cannot be ruled out. Proof that the double-stranded DNA fragment isolated from the heteroduplex indeed contained the \(80\text{psu}^+\) gene was obtained from transcription experiments. In these experiments 25–80% of the synthesized RNA chains were tRNA\(^{\text{Tyr}}\)-like.

The mass of the E. coli DNA fragment carrying the tRNA\(^{\text{Tyr}}\) gene was about 1.08 \(\times\) 10^6 daltons. This represents a purification of about 2000-fold, compared to the E. coli genome. The use of this DNA fragment will greatly facilitate the study of tRNA transcription and maturation into a functional tRNA molecule.

The competent assistance of Mr. Y. Tichauer is gratefully acknowledged. We thank Drs. H. Ozeki and J. D. Smith for kindly providing us with \(80\text{psu}^+\) phage mutants. This work was supported in part by U.S. Public Health Service agreement No. 45514.