Total Synthesis of a Tyrosine Suppressor tRNA Gene

XV. SYNTHESIS OF THE PROMOTER REGION*

By use of polynucleotide kinase and polynucleotide ligase, the 10 deoxyoligonucleotide segments, whose syntheses have been described in accompanying papers, have been joined to form the 62-nucleotide-long DNA corresponding to the promoter region of an *Escherichia coli* suppressor tRNA gene. The following sequence in the joining reactions was used to obtain error-free and optimal yields of the products: 1) joining of Segment P-1 to P-3 in the presence of Segment P-2; 2) joining of Segments P-4 to P-7 to form Duplex [P_{4-7}]; 3) joining of Segments P-8 to P-10 to Duplex [P_{8-10}] to form Duplex [P_{4-10}]; and finally, 4) joining of P-(1 + 3) and P-2 to Duplex [P_{4-16}] to form the total promoter Duplex [P].

As a requirement for the total synthesis of the *Escherichia coli* tyrosine suppressor tRNA gene, the nucleotide sequence in its promoter region was determined (2, 3). In further work, a plan was formulated for the synthesis of this part of the gene (1, 4). This plan, which is shown in Fig. 1, included the first 51 nucleotides of the promoter sequence as well as the EcoRI endonuclease-specific sequence at the appropriate terminus. Thus, chemical syntheses of 10 deoxyoligonucleotide segments (P-1 to P-10) were required, and the preceding two papers (1, 4) have documented the successful conclusion of this phase of the work. The present paper reports on a systematic study of the polynucleotide ligase-catalyzed joining of the chemically synthesized segments; this has led to the successful synthesis of the total promoter duplex.

The plan shown in Fig. 1 for segmentation of the promoter duplex emerged as a compromise between the demands on chemical synthesis and possible errors or difficulties in enzymatic joinings. The palindromic features in the promoter structure (2) were anticipated to cause some difficulties in error-free joinings. Thus, Segment P-5 has a great deal of self-complementarity (Fig. 2). Similarly, there is considerable complementarity between Segments P-7 and P-10 as shown in Fig. 3. Self-complementarity to varying degrees within individual synthetic segments was found to be unavoidable on several occasions during previous work (e.g. 5-7), and reasonably satisfactory solutions were found in all cases. The most serious aspect in the present work seemed to be the homology at the 5'-end between Segments P-2 and P-5. Therefore, they could substitute for each other and, as a result, in joining reactions in which Segments P-2 to P-4 (Fig. 4A) or Segments P-3 to P-5 (Fig. 4B) are used, duplexes longer than the expected products could form. This was in fact found to be the case (see below). Therefore, it was clear that, in the planning of the enzymatic work, Segment P-2 could only be grouped with Segments P-1 and P-3, and that Segment P-5 could be grouped with Segments P-4, P-6, and additional segments beginning with P-7.

In initial studies on grouping of the segments for joining purposes, the joining of Segment P-1 to Segment P-3 in the presence of Segment P-2 proceeded well (3). Similarly, Segment P-5 joined well to Segment P-7 in the presence of Segment P-6. Further, although Segments P-4 and P-6 did not join significantly in the presence of Segment P-5, the four-component system consisting of Segments P-4 to P-7 gave an essentially quantitative yield of the expected duplex. In further work, the joining of Segment P-8 to Segment P-10 with Segment P-9 as the template was found not to proceed satisfactorily.1 This three-component system, no advantage accruing when a fourth component, Segment P-7, was added, presumably due to the complementarity between P-7 and P-10 (Fig. 3). In another attempt, one-step joining of the seven segments (P-4 to P-10) was investigated. However, the extent of total joinings was unexpectedly low. From these studies, it became clear that the joining of Segments P-4 to P-10 must be carried out in two steps. The first step would constitute the formation of Duplex [P_{4-7}]. The resulting duplex should then be extended by the addition of Segments P-8 to P-10.

* This work has been supported by Grant CA11981-07, awarded by the National Cancer Institute, Department of Health, Education and Welfare; Grant PCM73 06757, awarded by the National Science Foundation, Washington, D. C.; Grant NP-140 from the American Cancer Society, Inc.; and by funds made available to the Massachusetts Institute of Technology by the Sloan Foundation. This is Paper CL in the series, "Studies on Polynucleotides." The preceding paper, Paper 14 in this present series, is Ref. 1. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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*** Supported by National Institutes of Health Traineeship T32 CA09112.

1 Unsatisfactory joinings using three-component systems have been observed in previous work (6, 7). This is, presumably, because one or more of the segments can adopt alternative bihelical structures.
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**Fig. 2.** Self-complementarity in oligonucleotide Segment P-5 leading to duplex formation by antiparallel base-pairing.

\[
\begin{align*}
5\prime &-T-G-A-C-T-C-C-A-G-T (3\prime) \\
3\prime &-C-G-C-G-C-G-C-G-C (5\prime) \\
5\prime &-T-G-A-C-G-C-G-C (3\prime) \\
3\prime &-C-G-C-G-C-G-C (5\prime)
\end{align*}
\]

FIG. 3. Possible duplex formation between Segments P-7 and P-10.

\[
\begin{align*}
5\prime &-T-T-G-T-A-A-T-G-T (3\prime) \\
3\prime &-A-A-T-T-T-T-T-C (5\prime)
\end{align*}
\]

FIG. 4. Possible formation of undesired duplexes longer than expected from Segments P-2 to P-4 (A) or from Segments P-3 to P-5 (B). Additional joinings in both cases result from the homology between Segments P-2 and P-5.

Conditions were indeed found in which the two-step synthesis of Duplex [P_{110}] was highly satisfactory. In the final step, the latter duplex was joined to Segments P-(1 + 3) and P-2. The plan thus arrived at for the synthesis of the promoter is illustrated in Fig. 5.

**MATERIALS AND METHODS**

DNA segments were synthesized chemically as described in previous papers (1, 4). Phosphorylation and T4-ligase-catalyzed joining of segments were performed by the general method described previously (5). In some experiments, phosphorylated segments were not isolated free from the excess of \([\gamma-32P]ATP\); instead, the kinase...
longer duplexes in the joining of Segments P-2, P-3, and P-4 and of Segments P-3, P-4, and P-5. Appropriate segments were phosphorylated by using polynucleotide kinase and [γ-32P]ATP. Small portions (0.5 μl of 50 μM solutions) of the reaction mixtures were directly combined and the resulting mixture was heated at 97°C for 3 min, cooled to 18°C, and dithiothreitol, ATP, and T4-ligase (standard concentrations as described previously (5)) were added. The final mixtures (5 μl each) were incubated at 5°C overnight. The products were separated by electrophoresis using 15% polyacrylamide gel. Channel a shows the results obtained with the system containing Segments P-2 to P-4, while Channel b shows the system containing Segments P-3 to P-5. Arrows with XC or BPB indicate the positions of the dye markers xylene cyanol and bromphenol blue, respectively.

reaction mixtures were subjected directly to ligase reaction after heating at 100°C for 2 min. Gel electrophoresis and nucleotide analyses were performed as described elsewhere (5). Mononucleotides were separated by electrophoresis on Whatman No. 1 paper in 5% trichloroacetic acid (pH 3.5).

Polyadenylate ligase from T4-infected E. coli was prepared by a modification of the procedure of Weiss and co-workers (8). To remove residual nucleic acid, the ligase in 1 ml of Buffer A (20 mM potassium phosphate, pH 7.6, 0.1 M KCl, 0.01 M mercaptoethanol) + 50% glycerol, was applied to a bed of phosphocellulose (1.5 x 2.2 cm) equilibrated in Buffer A. The column was washed with 12 ml of Buffer A (Eluate I), then 12 ml of Buffer A containing 0.35 M KCl (Eluate II), and finally with 12 ml of Buffer A containing 0.35 M KCl and 0.1 mM ATP (Eluate III). Eluate III was diluted immediately by addition of an equal volume of Buffer A and applied to a second phosphocellulose column (1.5 x 1.3 cm) equilibrated in Buffer A. After adsorption of the enzyme to this column, the latter was washed with Buffer A (6 ml) and the enzyme was then eluted with 6 ml of Buffer A containing 0.5 M KCl. This eluate was dialyzed against Buffer A + 50% glycerol and stored at -20°C at a concentration of 8,000 units/ml (9). Recovery of ligase activity from these procedures has varied between 30% and 50%.

RESULTS

Joining Experiments using (a) Segments P-2 to P-4 and (b) Segments P-3 to P-5—As shown in Fig. 6a, the combination of Segments P-2, P-3, and P-4 gave a product containing four segments as the major product. Formation of this product would be expected to occur as in Fig. 4A. The system containing Segments P-3, P-4, and P-5 gave several products (Fig. 6b) which contained two, four, six, seven, and eight segments. A four-component duplex would form as shown in Fig. 4B. In this duplex, the two protruding regions (3' ends of Segment P-5) can pair with Segment P-5 in an antiparallel fashion and give a six-component system. Again, in the six-component duplex, the 5' halves of Segment P-5 would provide the two protruding regions which can pair with Segment P-3. This would explain the formation of products containing seven and eight oligonucleotide components.

Synthesis of the Polynucleotide P-(1 + 3)—Because of the impure* nature of synthetic Segment P-1 (4), it was used in excess and the desired product was purified after the joining reaction. Unphosphorylated P-1 (8.5 nmol of about 50% pure sample) and [γ-32P]ATP (4.5 nmol) were joined using T4-ligase in the presence of [γ-32P]ATP (4.9 nmol) as the template. As shown in Fig. 7, the product, P-(1 + 3), with a tailing impurity of somewhat shorter size was obtained in a yield of 53% as based on P-3. Pure P-(1 + 3) was obtained by preparative gel electrophoresis in an amount of 3 nmol from two reactions carried out on the scale described above. Characterization of P-(1 + 3) thus obtained is given in Table Ia. Thus, when the product was digested to 5' and 5' mononucleotides, the radioactivity was found only in dGp and dG, respectively.

Preparation of Duplex [P-(1 + 3)] Containing Segments P-4 to P-7—Formation of the self-complementary duplex structure from Segment P-5 (Fig. 2), appeared to be concentration-dependent and, therefore, it was desirable to keep the concentration of this segment at about 5 μM or less. There are analogies for this from previous experience (5) and from the present work; at 40 μM concentration of Segment P-5, the reaction mixture containing the above four segments did not give any joining. However, when a 5-nmol scale reaction was performed using the conditions shown in Fig. 8, the joining reaction occurred as shown, and 75% of the total radioactivity originally present was converted to the expected duplex. The result indicates a 100% utilization of Segment P-4, which was present in a limiting amount, in the formation of Duplex [P-(1 + 3)]. In repetitions of the reaction at the same scale, yields of about 85% of the duplex were obtained. The duplex was isolated by gel filtration through a Sephadex G-100 column (1.2 x 90 cm) (Fig. 9).

Characterization of Duplex [P-(1 + 3)] is given in Table Ic. When the duplex was first treated with bacterial alkaline phosphatase until recently, it has now been purified by high pressure liquid chromatography. As expected, it joins to Segment 3 in high yield in the experiment of Fig. 7.
Characterization of the joined deoxyribopolynucleotides belonging to the promoter region by degradation to radioactive 3'- and 5'-nucleotides

For degradation to 3'-nucleotides, the polynucleotides were first treated with bacterial alkaline phosphatase to remove the radioactive 5'-phosphate group. Mononucleotides were separated by paper electrophoresis at pH 3.5.

| Joined products | 3'-Mononucleotides | 5'-Mononucleotides |
|-----------------|--------------------|--------------------|
|                 | dCP                | dAP                | dGP                | dTP                | P    | pdC    | pdA    | pdG    | pdT    | P    |
| a. P-(1 + 3)    | 2,980              | 1.0                | 1                   | 0                   | 0     | 0      | 1      | 0      | 0      | 1.0  |
|                 | Molar ratio        | Found              | Theoretical         |                     |       |        |        |        |        |       |
| b. [5'-32P]P-(1 + 3) | 360                | 1.0                | 0.9                 | 1                   | 0     | 0      | 2      | 0      | 0      | 0.9  |
|                 | Molar ratio        | Found              | Theoretical         |                     |       |        |        |        |        |       |
| c. [P-1]       | 7,460              | 1.0                | 1.0                 | 1                   | 0     | 0      | 1      | 0      | 0      | 1.0  |
|                 | Molar ratio        | Found              | Theoretical         |                     |       |        |        |        |        |       |
| d. [P-10]      | 3,410              | 1.0                | 1.0                 | 1                   | 0     | 0      | 2      | 0      | 0      | 1.0  |
|                 | Molar ratio        | Found              | Theoretical         |                     |       |        |        |        |        |       |
| e. [P-10]      | 1,490              | 1.0                | 1.0                 | 1                   | 0     | 0      | 2      | 0      | 0      | 1.0  |

*The radioactive phosphate belonging to 5'-end of P-1 in this product (b and e) had 65 times higher specific activity than that of the other.

Synthesis of Duplex [P,4-7] by Addition of Segments P-8, P-9, and P-10 to Preformed Duplex [P,4-7]—For effective joining of the Segments P-8 to P-10 to the preformed Duplex [P,4-7], Segments P-8 and P-9 were used in excess (about 2 molar equivalents) over the latter. Furthermore, Segment P-10 was added in even larger excess (about 4.5 molar equivalents) since the joining of the latter to Duplex [P,4-7] was found to be very slow. The synthesis of Duplex [P,4-7] was carried out as described in Fig. 10 and the kinetic data of joining are shown. After 1 h the only new product detected was Duplex [P,4-10]. Since no [P,4-7] was detected, the joining of P-8 and P-10 was catalyzed by T4-kinase and then digested to 3'-mononucleotides, radioactivity, besides being in inorganic phosphate, was found only in dCP. When the duplex was digested to 5'-mononucleotides, the radioactivity was found in pdG and pdT in the expected ratio of 1:3.

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9 probably occurred concomitantly. The joining of P-10 was rate-limiting, but after 24 h, 60% of the starting duplex [P-7] had been converted to [P-10] and another 23% was present as [P-9]. Pure [P-10] was obtained by preparative electrophoresis on a 15% polyacrylamide gel. In more recent experiments, the conversion of Duplex [P-S] to [P-9] + [P-10] has been higher than that recorded above, only a trace of unreacted [P-7] being observed.

Characterization of Duplex [P-10] by degradation to 3'- and 5'-mononucleotides is given in Table I. Thus, when [P-10] was digested to 3'-mononucleotides, following bacterial alkaline phosphatase treatment, radioactivity was found in dCp and dTp in the expected ratio of 4:1. A higher molar ratio observed for inorganic phosphate was caused by some dephosphorylation of mononucleotides by a phosphatase which contaminated the spleen phosphodiesterase preparation used. Degradation of [P-10] to 5'-mononucleotides gave pdA, pdG, and pdT in the expected ratio of 1:4. Similar degaradations of [P-8] gave distribution of radioactivity in the expected mononucleotides in correct ratios.

The Total Promoter Duplex [P], Containing Segments P-1 to P-10—The 5'-end of Segment P-1, which later is to be joined to the structural gene (following paper), should carry a [32P]phosphate group of high specific activity, while the opposite terminus, which contains the complementary EcoRI endonuclease sequence, should not carry a phosphate group during any of the ligase reactions. Therefore, the single-stranded polynucleotide P-1 (4 + 3) as prepared above (Fig. 7) was phosphorylated using [γ-32P]ATP which had specific activity 65 times higher than that of the ATP which had been used for phosphorylation for the purpose of joinings at the internal sites. Phosphorylation (kinetics not shown) was complete under the standard conditions and the radioactivity, on degradation of the product to 5'-nucleotides, was found only in dpG (Table I).

For synthesis of the promoter duplex, 5'-32P-labeled P-1 (4 + 3) was joined to [P-10] in the presence of 32P-labeled P-2. In this reaction, while [P-10] and P-1 (4 + 3) were used in equivalent amounts, 32P-phosphorylated [P-10] was used in excess (3 molar equivalents). As is seen in Fig. 11, about 40% of [P-10] was converted to the promoter, [P]. Joining of [P-10] to P-1 (4 + 3) also occurred in the absence of P-2 to give, as expected, slightly shorter product (Fig. 11a). From a 550-pmol scale reaction of [P-10], 169 pmol of the duplex [P] was obtained as a final product.

Characterization of the isolated [P] is given in Table I. Thus, when Duplex [P] was first treated with bacterial alkaline phosphatase and then digested to 3'-mononucleotides, radioactivity was found in dCp, dAp, and dTp in the expected ratio of 5:2:1. Radioactive inorganic phosphate, which was produced from the 5'-dG end of [P], had the expected high specific activity and was recovered in the correct molar amount. Degradation of [P] to 5'-mononucleotides gave radioactive pdA, pdG, and pdT in a ratio of 1:3:5, which again was as expected.

Comments—The enzymatic joining of chemically synthesized deoxyribo-oligonucleotide segments still requires considerable empirical work in order to arrive at the optimal strategy for the synthesis of the final product. Economy in chemical synthesis will continue to be the more important aspect in determining the segments to be synthesized for two reasons: First, chemical synthesis still constitutes the more time-consuming and laborious part of the work; and secondly, because of the considerable choice in segment combination in joining reactions, reasonably satisfactory schemes for error-free joinings can be worked out. Once the duplexes with the comple-
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Fig. 11. Preparation of the total promoter Duplex [P]. The 5'-hydroxyl group in the joined single-stranded polynucleotide P-(1 + 3) (650 pmol) (Fig. 7) was phosphorylated using polynucleotide kinase, and the reaction mixture was heated at 97°C for 2 min. Duplex [P-(3)] (550 pmol) and [35P]P-(2) (1780 pmol) were added. The final reaction mixture (121 µl) contained other components as described under "Materials and Methods," including 83 µM ATP and 400 units/ml of T4-ligase. The reaction was performed at 5°C. At the time intervals indicated, 0.2 µl of aliquots were subjected to electrophoresis on 15% polyacrylamide gel. After 6.5 h, more [35P]P-(2) and T4-ligase (equal in amounts to those already present) were added. For analysis, gel bands were cut out and the radioactivity was determined by Cerenkov radiation. Kinetics obtained are shown. Channel e shows the experiment in which the reaction mixture containing Duplex [P-(3)] and Segment P-(1 + 3), as described above, was incubated at 5°C for 6.5 h in the absence of Segment P-2.

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