Total Synthesis of the Structural Gene for the Precursor of a Tyrosine Suppressor Transfer RNA from *Escherichia coli*

10. ENZYMATIC JOINING OF CHEMICALLY SYNTHESIZED SEGMENTS TO FORM THE DNA DUPLEX CORRESPONDING TO THE NUCLEOTIDE SEQUENCE 86-126*

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The polynucleotide ligase-catalyzed joining of the eight chemically synthesized deoxypolynucleotides (segments 19 to 26), comprising the nucleotide sequence 86-126 of the DNA corresponding to the *Escherichia coli* tyrosine tRNA precursor has been investigated. Joining was studied using various combinations of 3, 4, or larger number of segments at a time. The extent of joining was in general low (0 to 40%) for the three-component as well as for the four-component systems. Joining of the five- and six-component systems was more satisfactory with yields from 25 to about 60%. The three duplexes [IVa] to [IVc] were prepared in single step reactions in yields of about 50% and were characterized. Duplex [IVd] could not be prepared in a single step reaction because of the failure of 5’-phosphorylated segment 26 to join to the rest of the duplex. Using a carefully annealed mixture of segments 24, 25, and phosphorylated segment 26, the joining of the latter to segment 24 could be realized in about 25% yield, much activated intermediate being concurrently present.

The total synthesis of the DNA corresponding to the tyrosine tRNA precursor has involved (a) the chemical synthesis of 26 deoxyribopolynucleotide segments (2-7) and (b) investigation of the polynucleotide ligase-catalyzed joining of segments to form DNA duplexes. Systematic studies led to the division of the above polynucleotides into four groups. The synthesis and characterization of three DNA duplexes representing between them the nucleotide sequence 1 (numbered from the 3’-end of the tyrosine tRNA)-94 have been described in the preceding three papers (1, 8, 9). The present paper describes enzymatic work on the joining of segments 19 to 26 (Fig. 1), representing nucleotide sequence 86-126 of the tRNA precursor.

In systematic investigations of the joining of different segments, combinations of three, four, five, or more segments were examined. As often found in the earlier work, joining was more efficient when four or more segments were used than with fewer segments. In early preparative work, because of sequence homology between segments 24 and 25, interference from structures such as those shown in Fig. 2 was considered. Therefore, segments 25 and 26 were deliberately omitted from the one-step synthesis and the preparation of duplex [IVa] containing segments 19 to 24 was carried out. However, it was found that using nonphosphorylated segment 25, the preparation of the duplex [IVb] containing the seven segments (19 to 25) could also be realized in high yield.

The above plan for the synthesis of this part of the gene was modified, however, when difficulties were encountered in quantitative phosphorylation of the 5’-OH end groups in duplex [IVa]. Segment 19 was therefore transferred to duplex [III] (1) and duplex [IVc] (Fig. 1) containing both 5’-OH groups in protruding single-stranded ends was prepared. In an alternative synthesis of [IVc] for subsequent joining to duplex [III], prephosphorylated [33P] segment 20 was used.

A surprising result during the present work was the failure of 5’-phosphorylated segment 26 (Fig. 1) to join to duplex [IVc] under the standard conditions used for the preparation of the duplex. Similarly, attempts to join segment 26 to a preformed duplex [IVa] also failed. However, careful annealing of a mixture of [33P] segment 20, segment 24, and segment 25, lead to the joining of segment 24 to segment 26 in moderate yield with comparable amounts of the activated (pyrophosphate) derivative of segment 26 being concurrently formed.

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FIG. 1. DNA duplexes corresponding to nucleotide sequence 86-126 (numbering from the 3' end to tRNA) of the gene for a tyrosine tRNA precursor. Segments 19 to 26 (sequences between caret) were all chemically synthesized. Duplexes [IVa] to [IVc] were synthesized in one-step enzymatic joining reactions. Duplex [IVd] could not be synthesized because of failure of segment 26 to join the remainder of the duplex. An alternative workable approach to the synthesis of this duplex is discussed in the text.

EXPERIMENTAL PROCEDURE

Materials—Chemical syntheses of the deoxyribopolynucleotide segments used in the present work (Fig. 1) have been described in accompanying papers. Unfortunately, a contamination was discovered in the 5'-end group of segment 20. When the latter was phosphorylated with [γ-32P]ATP and polynucleotide kinase and the product analyzed for radioactive 5'-nucleotides, about 10% of radioactivity was found in pdA, the remainder being in pdG as expected. The implications of this in the experimental work are described below.

Methods—These were as described in an accompanying paper (8).

RESULTS

Joining Experiments Using Three Deoxyribopolynucleotide Segments—The results obtained using different permissible combinations of three deoxyribopolynucleotides are shown in Table I. System 1 gave 12% joining, whereas systems 2 and 3 exhibited no joining. The results with system 4 were much better (34%). This system was examined more thoroughly by varying temperature and magnesium ion concentration. No increase in yield was observed up to 10° using 20 mM magnesium ion concentration. Preincubation with the ligase in the absence of ATP did not affect the yield (10). In one experiment, the yield at 15 mM magnesium ion and 0° did increase from 33% in 24 hours to 40% after a 5-day reaction time.

The product from system 6 was separated from the starting material on a Bio-Gel A-0.5m column. The included symmetrical product peak (Peak I, Fig. 3) containing the expected duplex was homogeneous. Enzymatic characterization of Peak I is recorded in Table II. Degradation to 3'-mononucleotides gave the expected equivalent molar amounts of [32P]dTp and [32P]dCp. The 5'-mononucleotide analysis gave [32P]pdA and [32P]pdT the molar ratio being 0.87; the expected ratio was 1.0.

Joining Experiments Using Four Deoxyribopolynucleotide Segments—All of the four-component systems derivable from segments 19 to 24 were examined (Table I). Systems 5 and 7 gave low yields of the joined product (3 and 14%, respectively). However, system 6 gave 34% joining. The extent of joining was 30 to 35% at 10 to 20 mM magnesium chloride and 0-10°. The yield decreased to 22% at 5 mM magnesium ion concentration. Preincubation with the ligase in the absence of ATP did not affect the yield (10). In one experiment, the yield at 15 mM magnesium ion and 0° did increase from 33% in 24 hours to 40% after a 2-day reaction time.

The product from system 6 was separated from the starting material on a Bio-Gel A-0.5m column. The included symmetrical product peak (Peak I, Fig. 3) containing the expected duplex was homogeneous. Enzymatic characterization of Peak I is recorded in Table II. Degradation to 3'-mononucleotides gave the expected equivalent molar amounts of [32P]dTp and [32P]dCp. The 5'-mononucleotide analysis gave [32P]pdA and [32P]pdT the molar ratio being 0.87; the expected ratio was 1.0.

Joining Experiments Using Five Deoxyribopolynucleotide Segments—The two five-component systems shown in Table I give markedly different results. The five-component system...
corresponded to the duplex expected from the five components. Phosphatase assay indicated 96% of the 3aP was resistant to the enzyme, and, therefore, present in internal positions oligonucleotides. The characterization is in Table III. The groups and segments 20 and 23 being unphosphorylated at found, as well as the peak corresponding to the unreacted main peak which corresponded to the joined product was the 5'-ends. The elution pattern is shown in Fig. 4. Only one reaction mixtures (22 pl each) were set up containing Tris-HCl (pH 7.6) (20 mM), dithiothreitol (10 mM), ATP (66 mM), and each of the four segments of 1 mM concentration. The concentration of MgCl2 was 10 and 20 mM respectively and ligase was added at 1000 units/ml. When the reactions (0') had leveled off (at 30 to 35% joining), excess EDTA was added and the pooled mixtures were fractionated at 4° on a Bio-Gel A-0.5m column (105 x 1.2 cm) using 0.1 M triethylenediamine bicarbonate at 4°. Fractions of 300 pl were collected and analyzed for 32P radioactivity.

Table II

Characterization of duplex from segments 20, 21, 22, and 23 (Peak I of Fig. 3)

| 3'-Nucleotide analysis (cpm) | dAP | dGP | dTP | dCP |
|-----------------------------|-----|-----|-----|-----|
| 5                           | 9   | 438 (1.0) | 449 (1.0) |

| 5'-Nucleotide analysis (cpm) | pdA | pdG | pdT | pdC |
|-----------------------------|-----|-----|-----|-----|
| 71 (0.87)                   | 67  | 822 (1.00) | 4   |

consisting of segments 19 to 23 gave an optimal yield of 39% at 20 mM Mg2+ ion concentration. At lower Mg2+ ion concentrations (5 or 10 mM) the yield was reduced to 25%. System 9 (segments 20 to 24) gave a much higher yield (53%), the concentration of Mg2+ ions being 10 mM. At 20 mM Mg2+ ion concentration, there seemed to be a further increase (to 57%) in yield, but there was a sharp decrease (to 38%) at 5 mM concentration.

The five-component system (system 9), consisting of segments 20 to 24 was studied further by isolation and characterization of the joined product. The reaction was carried out with segments 21, 22, and 24 carrying 5'-P-phosphate groups and segments 20 and 23 being unphosphorylated at the 5'-ends. The elution pattern is shown in Fig. 4. Only one main peak which corresponded to the joined product was found, as well as the peak corresponding to the unreacted oligonucleotides. The characterization is in Table III. The phosphatase assay indicated 96% of the 32P was resistant to the enzyme, and, therefore, present in internal positions as expected. The 5'-analysis verified that the main product corresponded to the duplex expected from the five components. Equivalent counts were found in pdA, pdG, and pdT.

Table III

Characterization of duplex from segments 20, 21, 22, 23, and 24 (Peak I of Fig. 4)

| Phosphatase assay (cpm) | Resistant | Sensitive |
|------------------------|-----------|-----------|
| 32P                    | 11,350    | 515       |

| 5'-Nucleotide analysis (cpm) | pdA | pdG | pdT | pdC |
|-----------------------------|-----|-----|-----|-----|
| 4,000 (1.05)                | 3,800 (1.00) | 3,470 (0.91) | 39 |

19 to 24 was obtained in 42% yield at 10 mM magnesium ion and at 0° (Table I). The yield could be improved slightly (48 to 50%) when the magnesium chloride concentration was increased to 20 mM. At 5 mM magnesium ion, the yield was only 30%. These results were not affected by the method of annealing. Segments at the appropriate magnesium concentration were either heated to 100° or taken to 37° followed by slow cooling at 0° without a significant difference in the yield of the duplex. The products of reactions carried out at 10 and 20 mM magnesium ion were isolated from small scale reaction mixtures. The elution patterns were similar to that shown below in Fig. 5 for the large scale reaction. Analyses of the isolated duplexes were all in agreement with the expected values. Therefore, the duplex [IVa] could be prepared in a reasonably satisfactory yield in a one-step joining reaction containing the six segments 19 to 24.

Preparative Synthesis of Duplex [IVa] (Fig. 1) Three separate preparations at 5 mmol scale each were carried out. Segments 19, 21, 22, and 24 carried 5'-P-phosphate groups whereas segments 20 and 23 were used with free 5'-hydroxyl groups. The segments were mixed in approximately equimolar amounts and annealed before reaction. Although in small
scale experiments, the concentrations of the segments were 1 μM, the segment concentration was increased to 44 μM for the preparative scale syntheses with no apparent effect on the rate or extent of joining. The kinetics of joining and the elution profile of the reaction products are shown in Fig. 5. The characterization of the product in Peak I is given in Table IV. Degradation to 3'-mononucleotides gave radioactivity in dTp and dCp in a molar ratio of 1:1. Analysis by digestion to 5'-mononucleotides gave radioactivity in pdA, pdG, and pdT, the molar ratio being 2:1:1, respectively. All these results are as expected for the correct duplex. Further, the duplex (Peak I, Fig. 5) was analyzed on polyacrylamide gels under denaturing conditions (Fig. 6). At 2.8 μM concentration, the duplex [IV] gave a diffuse pattern as if denaturation was partial. However, after phosphorylation of the external 5'-hydroxyl groups with [γ-32P]ATP of high specific activity, it was necessary to use only a low concentration (20 nM). Under these conditions, the duplex was completely denatured and the separated strands were clearly visible. No faster traveling, partially joined bands were visible after phosphorylation under denaturing conditions. Polyacrylamide gel electrophoresis also indicated that Fractions II and III (Fig. 5) were only slightly contaminated (5 to 10%) with faster migrating intermediate size joined products. The isolated yield of the duplex [IV], including Fractions I, II, and III, was 38%.

**Phosphorylation of Duplex [IVA] (Segments 19 to 24) with [γ-32P]ATP and Polynucleotide Kinase—**In a typical experiment the duplex (15.7 pmol) in a 25-μl reaction mixture (50 mM Tris, pH 8.2, 2 mM MgCl2) was heated at 100° for 2.5 min. After chilling in ice and then 15 min at 37° [γ-32P]ATP (70 pmol), T4-polynucleotide kinase (1.5 units), and dithiothreitol (1 mM) were added. The incubation was carried out at 37°. Aliquots (about 0.5 μl) were directly spotted on DE81 paper strips (chromatography in 0.35 M ammonium formate). After 60 min, the reaction mixture was heated to 100°, quick cooled, and fresh kinase and dithiothreitol were added. Incorporation of 32P into polynucleotide material had leveled off after about 35 min and corresponded to 77% of the theoretical value, the concentration of the duplex [IV] being calculated from the specific activity of the 32P-segments used in the preparation. The product was isolated by gel filtration (Sephadex G-50) in the presence of 50 mM triethylammonium bicarbonate. This sample was loaded at 20 nM in 100% formamide at 54°.

**Preparation of Duplex [IVB] (Fig. 5)—**Several experiments carried out on the single step joining of the seven components...
(segments 19 to 25) showed that the formation of the expected duplex [IVb] proceeded quite rapidly and in high yield. The kinetics of a typical reaction are shown in the inset in Fig. 7 and the conditions of the experiment are shown in the legend. The joined product was separated satisfactorily as shown in the figure and the pooled peak was characterized as in Table V. All of the results of nearest neighbor analysis and the 5'-nucleotide analysis were as expected for the specific joinings. Thus, the phosphatase assay showed the joined product to be 96% resistant. On degradation to 3'-nucleotides, radioactivity was found mostly in dGp, dTp, and dCp in the ratio of 1.07:2:1.9, the expected ratio being 2:2:1. Analysis of 5'-nucleotides gave radioactivity in pdA, pdG, and pdT and the molar ratio was 2.26:0.83, the expected ratio being 2:2:1. The yield of the duplex [IVb] (72%) was satisfactory.

Joining of Segment 25 and Attempting Joining of Segment 26 to Preformed Duplex [IVa] Consisting of Segments 19 to 24 —The above-described duplex [IVa] (segments 19 to 24) was phosphorylated with \([\gamma-^{32}P]ATP\) and polynucleotide kinase. As found above, phosphorylation went to 77% of theoretical value. The phosphorylated duplex (3.3 pmol) and segment 25 (7.5 pmol) were brought together in a 10-μl reaction mixture (Tris buffer, pH 8, 50 mM and MgCl\(_2\), 10 mM). A second identical reaction mixture was set up, and this in addition to 8.5 pmol of \([\gamma-^{32}P]\)segment 26 was added. (Specific activity of \([\gamma-^{32}P]\)segment 26 was very low compared to that in \([\gamma-^{32}P]\) at the 5'-ends of duplex [IVa].) Both reaction mixtures were incubated at 37° for 10 min, then cooled slowly (about 1 hour) to 4°. ATP (40 μM), dithiothreitol (2 mM), and ligase (600 units/ml) were added. Approximately 80% of the radioactivity had become resistant to the phosphatase by the time the reaction was followed by the alkaline phosphatase assay. The resistance of \([\gamma-^{32}P]\) radioactivity increased from 4.5% at zero time to 51.2% at 1.5 hours, 55% at 7 hours, and 58% at 26 hours. Thus, the kinetics were similar to those described below in the experiment of Fig. 10. The total reaction mixture was applied to a 15% polyacrylamide gel and the pattern obtained is shown in Fig. 8. The band corresponding to the duplex [IVa] was eluted from the gel with 2 m triethylammonium bicarbonate. The product had mobility identical on the 15% gel with that prepared below using \([\gamma-^{32}P]\)segment 25. Further, phosphorylation with \([\gamma-^{32}P]ATP\) as described below proceeded as expected.

Phosphorylation of Duplex [IVc] (Segments 20 to 25) Containing 5'-OH Groups with \([\gamma-^{32}P]ATP\) and Polynucleotide Kinase —The reaction mixture in 18 μl contained 30 pmol of the duplex prepared above, 130 pmol of \([\gamma-^{32}P]ATP\), and the standard components for the kinase reaction including 5'-mononucleotides. All of the \([\gamma-^{32}P]\) radioactivity was found in pdG.

Formation and Characterization of Duplex [IVc] (Segments 20 to 25) Using \([\gamma-^{32}P]\)Segment 20 —The duplex [IVc] prepared

| TABLE V |
| --- |
| Characterization of duplex [IVc] (Fig. 1) containing free 5'-OH end groups (Peak I of Fig. 7) (segments 19 to 25) |
| Phosphatase-sensitive radioactivity (cpm) |
| Resistant | Sensitive | % Resistant |
| 21,890 | 1,040 | 96% |

| Radioactivity in 3'-nucleotides (cpm) |
| --- |
| dAp | dGp | dTp | dCp |
| 585 | 6,360 | 11,890 | 11,330 |

| Ratio |
| --- |
| Found | Expected |
| 1.9 | 1 |

Radioactivity in 5'-nucleotides

| pdA | pdG | pdT | pdC |
| --- |
| 3,850 | 4,310 | 1,660 | 108 |

| Ratio |
| --- |
| Found | Expected |
| 0.83 | 1 |

Fig. 7 (left). Synthesis and isolation of the duplex [IVb] (Fig. 1). The reaction mixture in 40 μl contained the standard Tris buffer, MgCl\(_2\) (10 mM), dithiothreitol (2 mM), ATP (60 μM), and approximately 100 to 110 pmol of the seven segments (except for segments 20 and 22, all the segments had 5'-phosphate end groups). Ligase (20 units) was added and after 7½ hours 10 units more of the ligase were added. Approximately 80% of the radioactivity had become resistant to the alkaline phosphatase. After addition of EDTA (920 μM), the mixture was loaded onto an Agarose 0.5 m column (200 x 1 cm). Elution was with 0.05 M triethylammonium bicarbonate.

Fig. 8 (right). Preparation and polyacrylamide gel electrophoresis (18%) of duplex [IVc] with free 5'-OH end groups. Band I is the desired duplex, II contains the duplex lacking segment 25 and III is the unreacted phosphorylated segments.
above with free 5'-OH end groups would require phosphorylation before it can be used for joining to duplex [III] (1). For certain purposes it would be an advantage to have the flexibility of a 5'-OH group at the terminus of segment 25, but have only the labeled phosphate group at the 5'-end of segment 20 which would actually be required for joining to duplex [III]. Therefore, preparation of duplex [IVc] using prephosphorylated [32P]segment 20 was undertaken.

Preliminary experiments using equimolar amounts of the five phosphorylated segments ([32P]segment 20, [32P]-phosphorylated segments 21 to 24) and of unphosphorylated segment 25 showed that, (a) the joining of segments 20 to 24 to form the duplex was rapid but that the joining of segment 25 to segment 23 was slower; (b) some of the [32P]-end group in segment 20 was converted to a form resistant to the bacterial alkaline phosphatase; and (c) electrophoresis on polyacrylamide gels showed the formation of a higher molecular weight product, which appeared to be the dimer (Fig. 9). (The characterization of this dimer is given below in Fig. 10 and Table VI.)

The formation of the dimer could be inhibited by including an excess of nonphosphorylated segment 19, which itself was unable to undergo covalent joining. Further, the joining of segment 25 to segment 23 in the duplex could be accelerated by using an excess of segment 25 relative to the other segments. Following these observations, the synthesis of duplex [IVc] containing segments 20 to 25 ([32P]segment 20) was performed as shown in Fig. 10.

5'-33P-phosphorylated segments 21 to 24 (4000 pmol each), [32P]segment 20 (4100 pmol), and 2- to 2.5-fold excess of each of segments 25 and 19 were used in a joining reaction as described in the legend to Fig. 10. The kinetics of the joining are shown in the inset. The products were separated by gel filtration as shown. Before pooling the peaks, especially of the desired material, small amounts of selected fractions were examined by polyacrylamide gel electrophoresis (Fig. 11). This analysis helped delineate the dimer, the required duplex containing all the six segments and the duplex lacking segment 25.

Characterization of Peak A—This was concluded to be the dimer with the structure shown in Fig. 9. Thus, its molecular weight, as estimated from electrophoretic mobility on a polyacrylamide gel was consistent with the structure. All of the analysis (Table VI) including [32P]/[33P] ratio (4.11) and the 3'-nucleotide analysis (80% of [33P] in dCp) were all in agreement with this conclusion. Further, it has been mentioned above that the 5'-end nucleotide (33P)guanosine) in segment 20 was contaminated by [32P]pdA in dCp. This contamination was annealed by heating to 80° and then slowly cooling to 4° (8 hours). The joining reaction was started by adding ATP, dithiothreitol, and ligase to final concentrations of 0.1 mM, 2 mM, and 600 units/ml, respectively. Joining was followed by the phosphatase assay. The kinetics of the reaction are given in the inset. The reaction was stopped by adding EDTA to 40 mM and the mixture was fractionated on an Agarose 0.5m column (150 x 1 cm) by elution with 50 mM triethylammonium bicarbonate (pH 8.0) with 10 mM DTT, 5% glycerol, and 50 mM triethanolamine (pH 8.0). The elution pattern is shown in the inset. Characterization of Peaks A and B is in the text and Tables VI and VII. The unreacted segments were eluted in the last peak.

Characterization of Peak B—This contained the required duplex [IVc]. Its electrophoretic mobility was consistent with its size (Fig. 11) and all of the analyses (Table VII) were in agreement with those expected. The molar ratio of [32P]/[33P] (terminal label on segment 20) was found to be 3.97, theoretical being 4. On degradation to 5'-nucleotides, [32P] was found in pdA, pdG, and pdT, the molar ratios being 1.0:2.08:0.92; those expected were 1:2:1. [32P] was found mostly in pdG but about 4% was also present in pdA. This reduced "contamination" by pdA was evidently due to the preferential participation of the duplex [IVc] carrying 5'-pdA end group in dimer formation. On degradation of [IVc] to 3'-nucleotides, dCp, dGp, and dTp contained [32P] in the ratio of 1.0:0.99:2.18, the theoretical ratio being 1:1:2. These analyses taken together showed a single product with mobility similar to that of duplex [IVc] (Peak B of Fig. 10). This result is interpreted to mean that the strands of the dimer after coming apart simply form hairpins on themselves and the product, therefore, having one-half the molecular weight has the mobility close to that of duplex [IVc].
with those of Peak A of Fig. 10 as well as the analyses found for related systems in this part of the gene leave no doubt regarding the accuracy of the joinings.

Inhibition of Dimer (Fig. 9) Formation in Presence of Segment 19—In the above-described experiment of Fig. 10 on the preparation of duplex [IVc], an excess of unphosphorylated segment 19 was added. The following experiments showed that the addition of the latter segment inhibited the formation of the dimer (Fig. 9) derived from the duplex [IVc]. In two parallel experiments performed under standard conditions, 66 pmol of [5'-32P]segments 21 to 24, [5'-33P]segment 20, and unphosphorylated segment 25 were added and the mixture annealed. To one reaction mixture were added 66 pmol of segment 19. The joining reaction was performed as usual. After 12 hours, the two mixtures were compared by electrophoresis on 15% polyacrylamide gel, by the phosphatase assay and by 3' nucleotide analysis. All the 3'-nucleotide results were in agreement with those expected for the joining reactions shown in Tables VI and VII (Fig. 10). In the phosphatase assay, in the experiment in which segment 19 was absent, 33P radioactivity resistant to the phosphatase was reduced to 16.5%. In the experiment of Fig. 10, a 2- to 3-fold excess of segment 19 had been used.

Joining of [32P]Segment 26 [32P]d(G-G-A-A-G-C) to Segment 24 in Presence of Segment 25—Duplexes [IVa], [IVb], and [IVc] (Fig. 1) permit the attachment of another DNA duplex with a complementary single-stranded protruding end. This would be necessary for later studies of the promoter function in transcription. However, the terminus of duplex IV containing segments 25 and 26 corresponds to the initiation site for the transcription of the tyrosine tRNA precursor (2). It was, therefore, desirable to prepare duplex [IVd] with evenly base-paired end for possible studies of the mechanism of initiation of transcription. Surprisingly, attempts to prepare this duplex (a) by including segment 26 in the remainder of the segments (20 to 25) as well as (b) by adding segments 25 and 26 to preformed duplex [IVA] failed (see above). Therefore, further attempts were made to join segment 24 to [32P]segment 26 in the presence of segment 25. If this reaction were successful, then the octadecanucleotide (segment 24 + segment 26) could be isolated and be used in a second step for the construction of duplex [IVd].
The results of two parallel experiments on the joining of \[^{32P}\]segment 26 to segment 24 in the presence of segment 25 are shown in Table VIII. As is seen, (a) joining was slow, (b) it occurred only when the components had been carefully annealed, (c) the activated intermediate AMP-\(\cdot\)\(\^\prime\)-G-G-A-A-G-C (\(\^\prime\) represents \[^{32P}\]) (10, 11) was formed in an amount comparable to the joined product itself and it persisted even after prolonged reaction periods and (d) the joining reaction never proceeded in high yield despite repeated annealing of the components and further additions of the ligase.

Using the reaction conditions of the experiment in Table VIII, the influence of Mg\(^{2+}\) ion concentration, monovalent salt, and thiol reagents was studied. At 5 mM Mg\(^{2+}\) ion concentration, after 14 hours, 18% of the joined product was formed together with 10% of the activated intermediate; at 10 mM Mg\(^{2+}\) ions, the yield of the product was 13; and 18% of the activated intermediate was formed while 20 mM Mg\(^{2+}\) was the poorest, the joined product being present in 5% and the intermediate in 7%. Ammonium chloride (100 mM) inhibited the reaction and 10 mM \(\beta\)-mercaptoethanol could substitute 2 mM dithiothreitol. Finally, the reaction proceeded better at 4\(^\circ\) than at room temperature.

**Influence of ATP Concentration on Joining of Segment 26 to Segment 24**—The mechanism of the ligase-catalyzed joining reaction involves at least two main steps (12). In the first step, the appropriate 5'-phosphate is converted to the AMP-pyrophosphate by a molecule of the AMP-enzyme. In the second step, the phosphodiester bond is formed. However, it has never been established whether the second step is mediated by the "free" form of the ligase or whether AMP-enzyme is also capable of catalyzing this step. If, as is likely, the free form of the ligase is required for the second step, then in reactions in which the activated intermediates tend to accumulate (present experiments, and Refs. 10 and 11) a delicate concentration of ATP would be required for optimal joining. Therefore, as in an earlier paper (11), the influence of ATP concentration on the formation of the joined product and the activated intermediate was studied. The results obtained using two extreme concentrations are given in Fig. 12.

The formation of the joined product was maximal between 20 to 40 \(\mu\)M ATP concentration. As expected, with increasing ATP concentration, the intermediate increased at the expense of the joined product.

The joined product (G-C-T-C-C-T-T-T-A-T-C-G-\(\cdot\)G-G-G-A-A-G-C) was isolated by polyacrylamide gel electrophoresis. On degradation to 3'-nucleotides, radioactivity was found mostly in dGp (807 cpm) with little radioactivity being elsewhere (dCp, 2 cpm; dAp, 59; dTp, 13).

### DISCUSSION

Many of the experimental findings recorded in the present work are consistent with the previous experience. Thus, in the testing of different combinations of segments for enzymatic joining, reaction mixtures containing three to four segments gave, in general, poor joinings, whereas reaction mixtures containing five or more components gave better joining. Further, joining reactions containing segments at the left end of the duplex (22 to 25) went better and more rapidly than those which included the segments to the right only. The reactions in general plateaued at 50 to 70% and it was not possible to increase the extent of joining further. The same was true for many of the joining reactions previously studied.

As also observed in an accompanying paper (9), the segments at the ends may join slower than those in the middle of the duplex and care has to be exercised in completing such joining as far as possible in order to avoid contamination of the required duplex by duplexes lacking one or more terminal segments. In the present work, the joining of segment 25 was slow relative to the other joinings in the system containing segments 20 to 25. The joining of segment 25 was completed by using an excess of the segment and by giving a longer time (there was careful monitoring of the joining by polyacrylamide gel electrophoresis). In the large scale preparation of the required duplex, a delicate concentration of ATP would be required for optimal joining. However, in reactions in which the activated intermediates tend to accumulate, the free form of the ligase is required for the second step, the phosphodiester bond is formed. However, the second step, the phosphodiester bond is formed. However, it has never been established whether the second step is mediated by the "free" form of the ligase or whether AMP-enzyme is also capable of catalyzing this step. If, as is likely, the free form of the ligase is required for the second step, then in reactions in which the activated intermediates tend to accumulate (present experiments, and Refs. 10 and 11) a delicate concentration of ATP would be required for optimal joining. Therefore, as in an earlier paper (11), the influence of ATP concentration on the formation of the joined product and the activated intermediate was studied. The results obtained using two extreme concentrations are given in Fig. 12.

The formation of the joined product was maximal between 20 to 40 \(\mu\)M ATP concentration. As expected, with increasing ATP concentration, the intermediate increased at the expense of the joined product.

The joined product (G-C-T-C-C-T-T-A-T-C-G-\(\cdot\)G-G-G-A-A-G-C) was isolated by polyacrylamide gel electrophoresis. On degradation to 3'-nucleotides, radioactivity was found mostly in dGp (807 cpm) with little radioactivity being elsewhere (dCp, 2 cpm; dAp, 59; dTp, 13).

### TABLE VIII

| Time (hr) | Heat-quick cool | Heat-slow cool |
|----------|----------------|---------------|
|          | % | Total cpm | Activated inter- | % | Total cpm | Activated inter- |
| 0        | 1.0 | 3740 | 17.5 | 2550 | 10.8 |
| 5        | 1.7 | 7790 | 13.7 | 2340 | 13.9 |
| 11       | 1.8 | 6940 | 16.3 | 6980 | 16.5 |
| 23       | 2.1 | 3320 | 18.7 | 4900 | 17.8 |
| 31       | 1.6 | 4990 | 20.9 | 4980 | 17.8 |

**FIG. 12** The influence of ATP concentration on the joining of segment 24 to segment 26. A bulk reaction mixture was set up with \[^{32P}\]segment 26 (18 \(\mu\)M), segment 24 (0.75 \(\mu\)M), segment 25 (0.75 \(\mu\)M), Tris (50 mM), pH 8.0, and MgCl\(_2\) (5 mM). After heating and slow cooling as in Table VIII, the mixture was divided into seven portions. ATP was then added, varying in concentration from 5 to 200 \(\mu\)M. Dithiothreitol (2 mM) and ligase (400 units/ml) were added. The joining reactions and the formation of the intermediate were followed by chromatography on DEAE-chromatography with 0.3 M ammonium formate + 7 M urea after the standard phosphate treatment. The rates and yields of the joined product and of the activated intermediate are compared for 20 \(\mu\)M ATP and 200 \(\mu\)M ATP concentrations.
of the duplex [IVa], an analysis of the different parts of the product peak (Fig. 5) was similarly made to separate contaminants lacking terminal segments.

The use of prephosphorylated segment 20 in the synthesis of the duplex [IVc] gave interesting results. First, the formation of the dimer (Fig. 9) confirmed what was already concluded in the preceding paper (1) that the ligase can with facility bring “head to head” dimer formation even if the base-pairing of the protruding tetranucleotide sequence is not perfect. Second, it was interesting and useful to be able to inhibit the dimer formation by “tying up” the single-stranded region with segment 19 containing the complementary sequence. Although prephosphorylated segment 20 could be used reasonably well in the synthesis of duplex [IVc], the use of prephosphorylated segments at termini cannot be recommended. Instead, the aim should be to so arrange the joining reactions that the 5′-OH groups, which would be available for subsequent phosphorylation, are carried by those terminal segments which have protruding single-stranded ends.

While, as discussed above, the segments at termini may have a slower rate of joining, the experience with the joining of segment 26 was a surprise. Indeed, the results in these experiments emphasized again the lack of basic understanding in this area of the ligase-catalyzed joining of short deoxyoligonucleotide segments. The hexanucleotide segment (segment 26, Fig. 1) failed to join to form the duplex [IVd] in all the many attempts that were carried out. This failure was surprising in view of the past successful joining of hexanucleotides at termini. At least two successful examples are: (a) the joining of p·C·C·A·C·C·A (segment 2) to the remainder of duplex [A] in the synthesis of the gene for yeast alanine tRNA (11), and (b) the joining of segment 1 (3′-G·G·T·G·G·T-5′) to segment 3 in duplex [I] described in a preceding paper (8). Further, it is striking that in the alternative approach, in which prior joining of segment 24 to segment 26 was attempted, careful annealing of the three components (segments 24 to 26) was essential. This is one of the few joining reactions studied so far in which the necessity for slow annealing has been demonstrated.

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