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

11. ENZYMATIC JOINING TO FORM THE TOTAL DNA DUPLEX*

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The DNA duplex corresponding to the entire length (126 nucleotides) of the precursor for an *Escherichia coli* tyrosine tRNA has been synthesized. Duplex [I] (Sekiya, T., Besmer, P., Takeya, T., and Khorana, H. G. (1976) *J. Biol. Chem.* **251,** 634–641), corresponding to the nucleotide sequence 1–26, containing single-stranded ends and carrying one appropriately labeled 5′-phosphate group, was joined to duplex [II] (Loewen, P. C., Miller, R. C., Panet, A., Sekiya, T., and Khorana, H. G. (1976) *J. Biol. Chem.* **251,** 642–650) (nucleotide sequence 23–66 or 23–60) containing also one labeled phosphate end group (nucleotide 27). The duplex [I + II] (nucleotide sequence 1–60) was phosphorylated with [γ-^{33}P]ATP at the 5′-OH ends. Duplex [III] (Panet, A., Kleppe, R., Kleppe, K., and Khorana, H. G. (1976) *J. Biol. Chem.* **251,** 651–657) (nucleotide sequence 57–94 (Fig. 2)) was also phosphorylated at 5′-ends with [γ-^{33}P]ATP and was joined to duplex [IV] (Caruthers, M. H., Kleppe, R., Kleppe, K., and Khorana, H. G. (1976) *J. Biol. Chem.* **251,** 658–666) (nucleotide sequence 90–126) which carried a ^{33}P-labeled phosphate group on nucleotide 90. The joined product, duplex [III + IV] (nucleotide sequence 57–126) was characterized. The latter duplex was joined to the duplex [I + II] to give the total duplex. The latter contains single-stranded ends (nucleotides 1 to 6 and 121 to 126) which can either be "filled in" to produce the completely base-paired duplex or may be used to add the promoter and terminator regions at the appropriate ends.

The plan for the total synthesis of the DNA duplex corresponding to the entire length (126 nucleotide units) of an *Escherichia coli* tyrosine tRNA precursor has been described (2). Chemical syntheses of all of the necessary deoxyribo-

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nucleotide segments have been documented in accompanying papers (3–7). Subsequent experiments on the enzymatic joining of the segments led to the grouping of the segments into four parts and the successful joining and characterization of the resulting duplexes [I], [II], [III], and [IV], have also been accomplished (1, 8–10). Joining of these duplexes in a stepwise fashion to form the duplexes [I + II] (Fig. 1) and [III + IV] (Fig. 2) and subsequent joining and characterization of the total duplex (Fig. 3) are the subject of this paper.

Duplex [I] (Fig. 1), which consisted of segments 2 to 5, contained, by design, a single 5′-^{32}P-phosphate group at the terminus of segment 5 (8). Similarly, duplex [II], the first preparation of which contained segments 6 to 13 (9), also contained, by design, a 5′-^{32}P-phosphate group at the terminus of segment 7. Joining of these duplexes proceeded rapidly and efficiently (Fig. 4) and the duplex [I + II] (segments 2 to 13) (Fig. 1) was characterized. As described also in the accompanying papers, the enzymatic phosphorylation of the duplexes in which the 5′-OH end groups are present at the shorter strands may proceed sluggishly or incompletely. In such events interference from even an extremely low contamination of the polynucleotide kinase with a nuclease may become a possibility. Experiments on phosphorylation of the end groups in duplex [I + II] (segments 2 to 13) gave variable results. Phosphorylation of the duplex as exemplified by the experiment of Fig. 6 was incomplete. However, in other experiments, phosphorylation was evidently more successful. To ensure rapid and complete phosphorylation, the plan for the joining reactions was modified (see accompanying papers) and the duplex [II], which now contained segments 6 to 12, was prepared. Joining of this duplex with duplex [I] now gave the product, duplex [I + II], containing segments 2 to 12. No difficulty was encountered in quantitative and rapid phosphorylation of the 5′-OH groups present at the ends of segments 3 and 12 in this product. As an important part of the strategy, the same preparation of [γ-^{33}P]ATP of suitable specific activity was used in the phosphorylation of the terminal groups in duplex [I + II], and in duplexes [III] and [IV] described below.

Duplex [III], the plan for which had also been modified (9), consisted of segments 13 to 19 and was phosphorylated at its 5′-OH ends. Duplex [IV], consisting of segments 20 to 25, contained, by design, a single ^{33}P-phosphate end group at the terminal segment 20. The joining of duplex [III] and duplex [IV] again went rapidly and in high yield and the product,

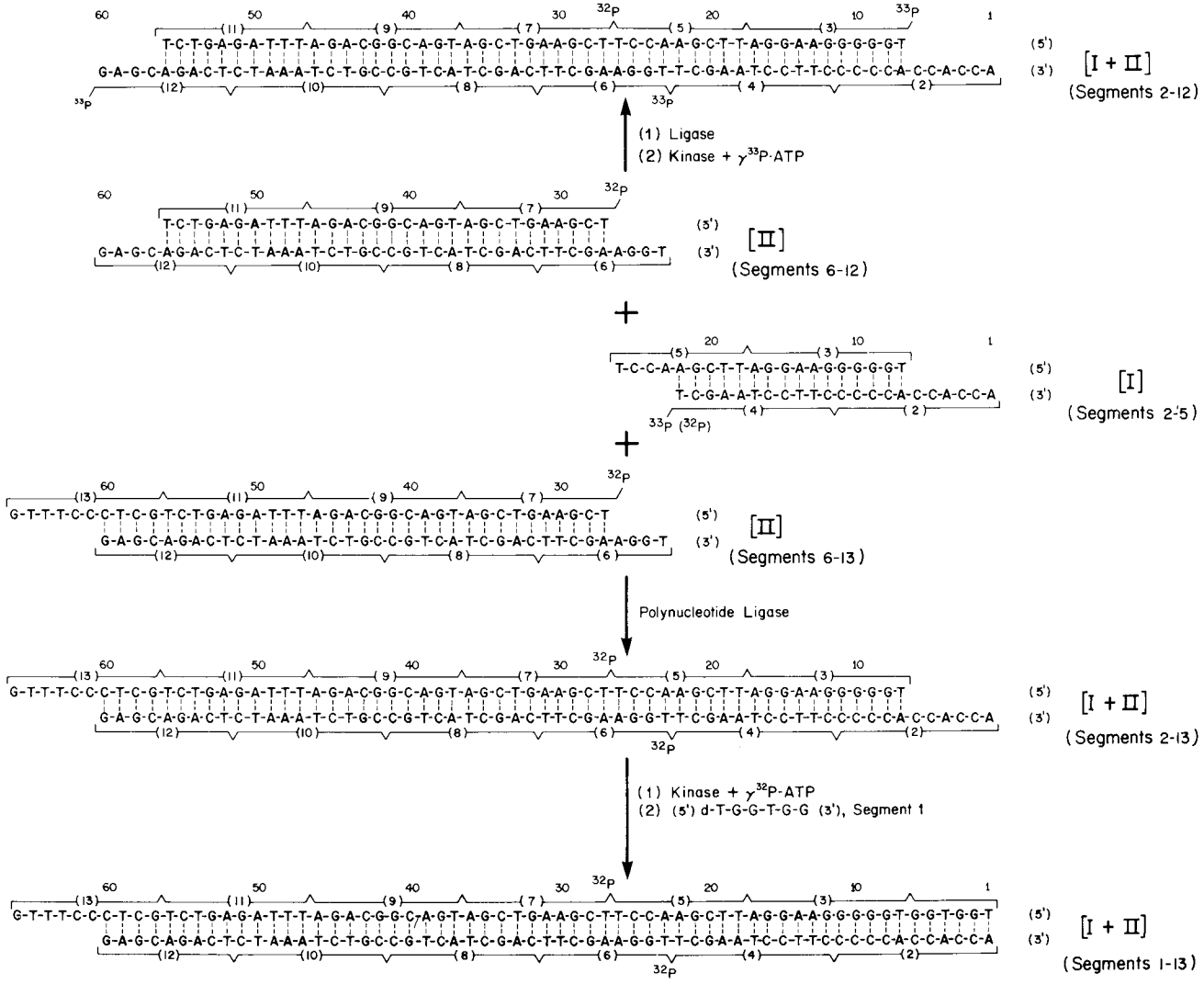

FIG. 1. The structure and synthesis of duplex [I + II] (nucleotides 1 to 60 or 1 to 66) from duplex [I] and duplex [II]. The ends carrying ^{32}P- or ^{33}P-groups and 3′-OH groups at the sites of joining reactions are shown.

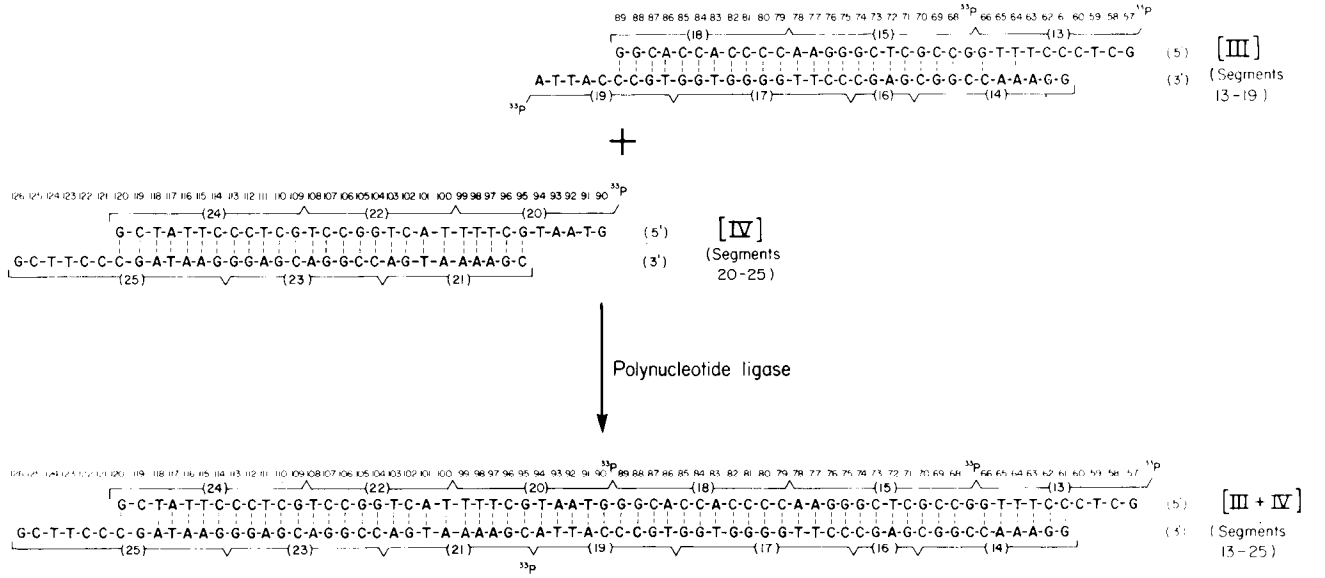

FIG. 2. The structure and synthesis of duplex [III + IV] (nucleotides 57 to 126) from duplex [III] and duplex [IV]. The structures and 5′-^{33}P-groups at the sites of joining reactions are shown.

duplex [III + IV], was characterized. The preparations of duplex [I + II] and duplex [III + IV] now contained only the ^{33}P-phosphate end groups at the sites of joining. This final joining proceeded well and the distinctive ^{33}P label aided the characterization of the resulting total duplex (Fig. 3).

The present work completes the total synthesis of the DNA duplex corresponding to the precursor for the tyrosine tRNA. The single-stranded portions at the two ends of the duplex are intended for use later in the extension of the duplex in the direction of the promoter or the terminator signals for transcription. Work on the sequence and the synthesis of these regions is in progress.

MATERIALS AND METHODS

These were as discussed in an accompanying paper (8).

EXPERIMENTAL RESULTS

Joining of Duplex [I] to Duplex [II] (Segments 6 to 13)—Several large (nanomole) scale reactions were carried out using duplex [I] which contained a 5′-^{32}P-phosphate group on segment 5 and duplex [II] (segments 6 to 13) which contained a 5′-^{32}P-phosphate end group on segment 7. The conditions used are shown in Fig. 4. Prior to these large scale reactions, a small scale reaction was performed under identical conditions. The products were examined by 12% polyacrylamide gel electrophoresis and the pattern is shown in Fig. 5 (*Channel a*). Two bands corresponding to the joined products are seen. As described in the accompanying paper on the synthesis of the duplex [II] (segments 6 to 13), the duplex was contaminated with two seven-component duplexes: one of the contaminants lacked segment 6 and the second lacked segment 13. Clearly, the contaminant lacking segment 6 would not join to duplex [I] and the contaminant lacking segment 13 would produce a duplex slightly shorter than the expected duplex [I + II] (segments 2 to 13). Therefore, the preparation of duplex [I + II] was modified. Duplex [I] and duplex [II] were allowed to join for 1 hour at which time 1 eq of segment 13 was added (9 μM). An additional amount of the ligase was added to bring its concentration to 400 units/ml and the reaction allowed to proceed for another 1.5 hours. An aliquot of this reaction mixture

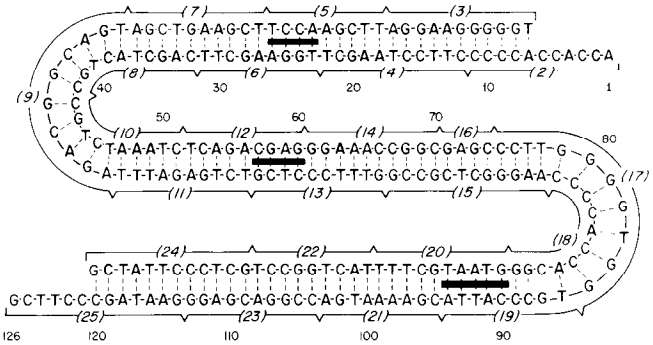

FIG. 3. The totally synthetic DNA duplex corresponding to the entire sequence (126 nucleotides) of the precursor for an *Escherichia coli* tyrosine suppressor tRNA. The numbers and distances between the *carets* show the oligonucleotides which were synthesized chemically. The *carets* indicate the sites where joining was accomplished by the use of polynucleotide ligase. The *three thick strips* between the duplex indicate the sites where (*a*) duplex [I] and duplex [II] (between nucleotides 23 and 26), (*b*) duplex [III] and duplex [IV] (between nucleotides 90 and 94) and (*c*) duplex [I + II] and duplex [III + IV] (between nucleotides 57 and 60) were joined.

showed only one band corresponding to the joined duplex (Fig. 5*b*). The kinetics of joining of the first part of this two-step reaction are shown in the *inset* to Fig. 4. The actual phosphatase resistance increased only from 80.3% to 92.5% because of the large number (eight) of internal ^{32}P-phosphate groups. The reaction mixture was then fractionated by flow through an Agarose 0.5m column giving the effluent pattern shown in Fig. 4. The large first peak contained the joined duplex [I + II] and was characterized as shown in Table I. Thus, it was 99.0% phosphatase-resistant and the nearest neighbor analyses showed radioactivity in dAp, dTp, and dCp in the expected molar ratios of 3:4:3. The 5′-nucleotide analysis was not completely satisfactory, giving slightly lower radioactivity in pdT and somewhat higher radioactivity in pdC than expected. However, this could have been due to the solvent system used for the separation of the mononucleotides.

The second peak in Fig. 4 contained unreacted duplex [II], and gel electrophoresis showed that this fraction con-

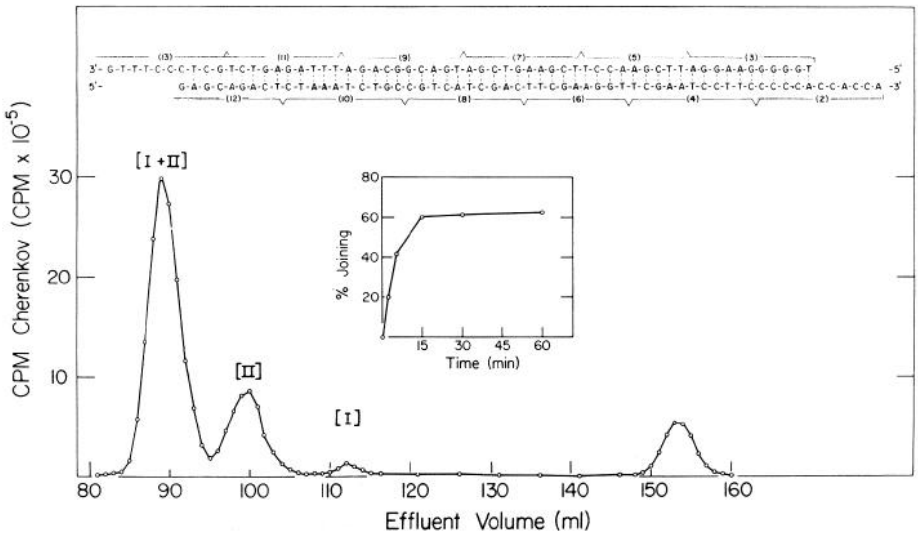

FIG. 4. The joining and purification of the duplex [I + II] (segments 2 to 13). The reaction mixture (400 μl) contained: duplex [I] and duplex [II] (9 μM), $MgCl_2$ (10 mM), Tris buffer, pH 7.6 (100 mM), dithiothreitol (10 mM), ATP (200 μM), and polynucleotide ligase (250 units/ml). After annealing at 37° for 15 min, the reaction mixture was kept at 5° for 15 min before addition of the ligase. Development of phosphatase resistance was followed by the DEAE-paper assay (kinetics in *inset*). After 1 hour, segment 13 (9 μM) and ligase (total concentration, 400 units/ml) were added and the reaction allowed to proceed for a further 1.5 hours. EDTA was added to 20 mM and the reaction mixture fractionated by passage through a column (1 × 150 cm) of Bio-Gel A-0.5m (200- to 400-mesh). The column was eluted at 4° with 0.1 M triethylammonium bicarbonate. Fractions of 350 μl were collected every 15 min.

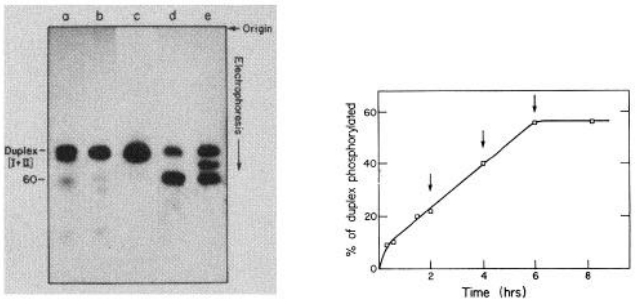

FIG. 5 (*left*). Gel electrophoresis of selected fractions of duplex [I + II]. Aliquots of various samples were denatured in 100% formamide at 37° and then subjected to electrophoresis on a 12% polyacrylamide gel in the presence of 7 M urea. *Channel a* contained the small scale reaction mixture before it was supplemented with segment 13 (see text). *Channel b* contained the reaction mixture after segment 13 had been added. *Channel c* contained the product duplex [I + II] as isolated in Fig. 4. *Channel d* contained duplex [I + II] (Fig. 4) after phosphorylation at the 5'-ends with [γ-^{32}P]ATP. *Channel e* contained duplex [I + II] after joining to segment 1, d(T-G-G-T-G-G). The *numbers* beside the autoradiograms denote the sizes of the various deoxypolynucleotide markers.

FIG. 6 (*right*). Time course of the phosphorylation of the duplex [I + II] (segments 2 to 13). The reaction mixture contained: 320 pmol of the duplex, 1300 pmol of [γ-^{32}P]ATP, 10 mM Tris-HCl, pH 8.0, 10 mM dithiothreitol, and 2 mM $MgCl_2$ in a total volume of 200 μl. Polynucleotide kinase (5 units) was added at the start of the reaction and further additions of the same amount of enzyme and 2 μl of 0.2 M dithiothreitol were made at times indicated by the *arrows*. Incubation was at 37°. The kinetics were followed as described under "Materials and Methods." After 8 hours the reaction mixture was made 20 mM in EDTA and 0.4 M in KCl, heated to 100° then cooled to room temperature over a period of approximately 30 min, loaded on a column of Bio-Gel A-0.5m (40 × 1 cm) equilibrated with 50 mM triethylammonium bicarbonate. The phosphorylated duplex came off as a single symmetrical peak, well separated from [γ-^{32}P]ATP.

TABLE I

Characterization of duplex [I + II] (segments 2 to 13) (Peak I of Fig. 4)

	Phosphatase assay (cpm)			
	Resistant		Sensitive	
	10,040		58	
	3'-Nucleotide analysis (cpm)			
	dAp	dGp	dTp	dCp
	5920	170	8080	6360
Molar ratios				
Found	3.0		4.1	3.2
Theoretical	3		4	3
	5'-Nucleotide analysis (cpm)			
	pdA	pdG	pdT	pdC
		1610	7130	4760
Molar ratios				
Found		1.2	5.4	3.5
Theoretical		1	6	3

tained almost 75% of the duplex containing seven segments. Thus, the bulk of the correct duplexes had joined, but the contaminant lacking segment 6 had remained. There was also a very small amount of duplex [I] remaining, but most of it had joined. The joined material [I + II] was further characterized by electrophoresis on 12% polyacrylamide gels. *Channel c* of Fig. 5 shows the isolated [I + II] (Fig. 4). There is a major band of undenatured duplex and a second band composed, presumably, of the two denatured single strands, both of which are 60 nucleotides in length.

^{32}P-Phosphorylation of 5'-OH End Groups in Duplex [I + II]

(*Segments 2 to 13*)—Experiments at phosphorylation of the 5′-ends in duplex [I + II] gave variable results and, in general, phosphorylation of the 5′-OH of the terminal deoxyguanosine (segment 12) was far from quantitative.

One experiment on phosphorylation is shown in Fig. 6. As seen in the figure, several additions of polynucleotide kinase were made. After a total of 8 hours at 37°, the reaction was terminated by the addition of EDTA (to 20 mM) and KCl (to 0.4 M), heated to 100° for 2 min, cooled slowly to room temperature, and the product separated from excess ATP by passage through a column of Bio-Gel A-0.5 (40 × 1 cm). The duplex was analyzed by degradation to 5′-nucleotides and showed radioactivity distribution as follows: pdA, 293 cpm; pdG, 6093; pdT, 24110; pdC, 254; the molar ratios were, respectively, 0.01:0.25:1.00:0.01.

In separate experiments, the duplex was heated to 100° for 2 min prior to the addition of the enzyme; no significant difference in the rate of phosphorylation was observed.

Evidently, there were differences in the rate and extent of phosphorylation in different experiments with different preparations of the kinase. For example, one kinase preparation brought about phosphorylation of duplex [I + II] to a very high extent as shown by the analysis after subsequent joining of segment 1 (following experiment).

Joining of Segment 1 d(T-G-G-T-G-G) to Phosphorylated (^{32}P) Duplex [I + II] (Segments 2 to 13)—Another sample (50 pmol) of the duplex [I + II] (*Peak I* of Fig. 4) was phosphorylated with [γ-^{32}P]ATP and polynucleotide kinase. Analysis of radioactivity at the 5′-ends was again not as expected. However, gel electrophoresis under denaturing conditions (Fig. 5) showed only two major bands (Fig. 5*d*) corresponding to the native and denatured duplex. This result confirmed the absence of nicks or the presence of incompletely joined portions in the duplex [I + II].

Phosphorylated [I + II] was joined to segment 1 in the experiment shown in Fig. 7. Thus, an excess (10-fold) of d(T-G-G-T-G-G) was used to ensure complete joining (7). The kinetics of development of phosphatase resistance are shown in Fig. 7. The maximum phosphatase resistance to be expected would be 50%. The actual resistance observed was 45.5% indicating 91% joining. However, quantitation of the result would be difficult if, as concluded above (Fig. 6), the phosphorylation of the two 5′-ends proceeded to different extents. The joined material was characterized by electrophoresis on a 12% polyacrylamide gel and it gave the pattern shown in Fig. 5*e*. Characterization of this product by phosphatase resistance, 5′-nucleotide analysis, and 3′-nucleotide analysis is given in Table II. Thus, after degradation to 3′-nucleotides, most of the radioactivity was present in dGp, while on degradation to 5′-nucleotide, pdT and pdG contained the bulk of the radioactivity and in equal amounts, while a similar degradation after the phosphatase treatment gave the radioactivity mostly in pdT. These results show, in addition, that the phosphorylation of 5′-OH groups in duplex [I + II] was not far from quantitative.

Synthesis of Duplex [I + II] (Segments 2 to 12)—In the plan finally used for the total synthesis, duplex [II] containing segments 6 to 12 was used for joining with duplex [I]. The conditions for the reaction and the kinetics of joining as monitored in 15% polyacrylamide gel electrophoresis are shown in Fig. 8. As is seen, the reaction proceeded well; it was rapid and was essentially complete in less than 30 min. The kinetics as obtained after radioactive counting of the different bands are shown in the inset to Fig. 9. Thus, the joining went to about 80% as based on duplex [II]. The products were separated by gel filtration in an Agarose 0.5m column (Fig. 9) and the pattern of elution obtained is shown. The joined product [I + II] was characterized as shown in Table III. Thus, all of the radioactivity (^{32}P and ^{33}P) was resistant to the alkaline phosphatase (^{33}P label is present on the 5′-end segment 4, while all the other phosphate groups contain ^{32}P label). On degradation to 3′-nucleotides, essentially all of the ^{33}P radioactivity was found in dTp, while ^{32}P radioactivity was present in dAp, dTp, and dCp. The molar ratios of the radioactivity (^{32}P and ^{33}P) in different nucleotides were close to those expected. Degradation to 5′-nucleotides, gave ^{33}P in pdT only and ^{32}P in pdT and pdC. The molar ratios found for radioactivity in pdT (^{33}P), pdT (^{32}P), and pdC (^{32}P) showed discrepancy in the case of pdC from the values expected. The cause of this is not known, but there is little doubt about the purity or the identity of the duplex [I + II].

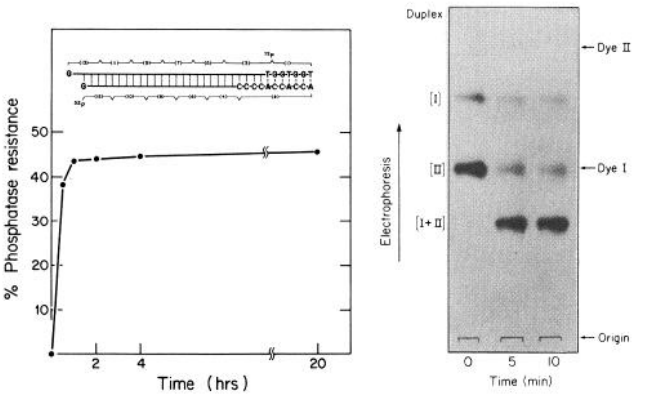

FIG. 7 (*left*). Kinetics of the joining of segment 1 to duplex [I + II] (Fig. 4). After the phosphorylation of the duplex (see text), a reaction mixture was prepared with 2 μM duplex [I + II], 20 μM segment 1, 100 mM Tris, pH 7.6, 10 mM dithiothreitol, 10 mM $MgCl_2$, and 100 μM ATP. The mixture was annealed at 37° for 15 min and at 5° for 15 min before the addition of the ligase (500 units/ml). Samples were taken and assayed for phosphatase-resistant (joined) product as described under "Materials and Methods."

FIG. 8 (*right*). Kinetics of the joining of duplex [I] and duplex [II] (segments 6 to 12). The reaction mixture (305 μl) contained 20 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 680 pmol of duplex [I], and 917 pmol of duplex [II]. The mixture was heated at 100° for 2 min and then kept at 4° for 60 min. Dithiothreitol (10 mM), ATP (50 μM), and T_4-ligase (465 units/ml) were added to start the reaction. Aliquots of 1 μl were removed and analyzed by electrophoresis on 15% polyacrylamide. The pattern obtained is shown. Radioactive bands corresponding to the joined product, duplex [I], and duplex [II] were cut out and counted by Cerenkov counting. The results are shown in the *inset* in Fig. 9.

Phosphorylation of 5′-OH End Groups in Duplex [I + II] (Segments 2 to 12)—Phosphorylation using [γ-^{33}P]ATP and polynucleotide kinase was performed as described in Fig. 10. The reaction went to completion in about 60 min. The product after isolation was analyzed for ^{33}P radioactivity in 5′-nucleotides. The counts per min found were as follows: dAp, 65; pdG, 1198; pdT, 1090; and pdC, 4. Thus, the ^{33}P radioactivity was mostly in pdG and pdT, the 5′-end groups in duplex [I + II] and the molar ratio for pdG/pdT was 1.1/1.0.

Joining of Duplex [III] (Segments 13 to 19) to Duplex [IV] (Segments 20 to 25)—Duplex [III] (Segments 13 to 19) was phosphorylated at its 5′-OH ends using T_4-polynucleotide

TABLE II

Characterization of product from joining (Fig. 7) of segment 1 to duplex [I + II] (segments 2 to 13) (Fig. 1)

[γ-^{32}P]ATP used for the phosphorylation of 5′-ends in duplex [I + II] has very high specific activity compared to the specific activity of internal ^{32}P-linkages.

Phosphatase assay (cpm)	Resistant	Sensitive
	12,140	16,230
Molar ratio		
Found	42.8%	57.2%
Expected	50%	50%

3′-Nucleotide analysis after phosphatase (cpm)	dAp	dGp	dTp	dCp
	246	4,345	345	139
Molar ratio				
Found	0.05	0.86	0.07	0.02
Expected	0	1	0	0

5′-Nucleotide analysis after phosphatase (cpm)	pdA	pdG	pdT	pdC
	37	208	1,660	42
Molar ratio				
Found	0.02	0.10	0.85	0.02
Expected	0	0	1	0

5′-Nucleotide analysis before phosphatase (cpm)	pdA	pdG	pdT	pdC
	416	15,090	14,020	244
Molar ratio				
Found		1.00	0.94	
Expected	0	1	1	0

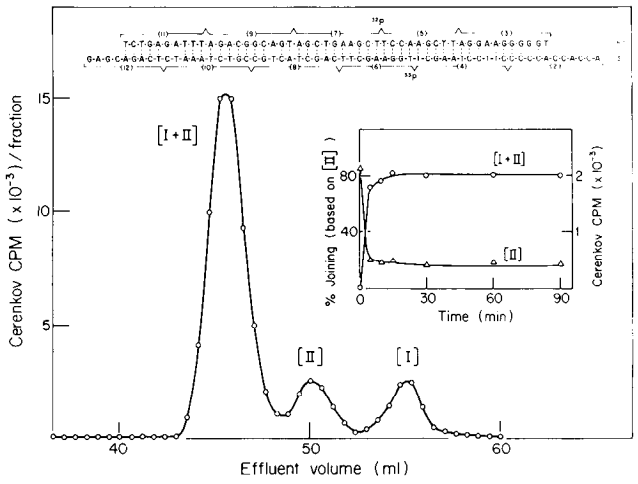

FIG. 9. Purification of the duplex [I + II] (segments 2 to 12). The reaction mixture and joining was as in Fig. 8. The reaction mixture was made 20 mM in EDTA and then applied on top of a column (150 × 1 cm) of Agarose 0.5m. Elution was at 4° using 0.05 M triethylammonium bicarbonate. Fractions of 0.6-ml volume were collected.

kinase and [γ-^{33}P]ATP. The same preparation of [γ-^{33}P]ATP had been used (*a*), in phosphorylation of segment 15, which was used in the above preparation of duplex [III] (see Ref. 10) and (*b*), in phosphorylation of segment 20, which was used in

TABLE III

Characterization of duplex [I + II] (segments 2 to 12) (Peak I of Fig. 9)

Alkaline phosphatase resistance (cpm)	Resistant	Sensitive
^{32}P	832	0
^{33}P	753	6

3′-Nucleotides (cpm)[a]	dAp	dGp	dTp	dCp
^{33}P (cpm)	76	6	1810	25
Molar ratio			1.0	
^{32}P (cpm)	1580	0	1080	1390
Molar ratio	2.9		2.0	2.6

[a] Molar ratio: dA^{32}p:dT^{33}p:dT^{32}p:dC^{33}p = 3.0:0.8:2.1:2.6; expected = 3:1:2:3.

5′-Nucleotides (cpm)[b]	pdA	pdG	pdT	pdC
^{32}P (cpm)	0	0	2050	1610
Molar ratio			5.0	3.9
^{33}P (cpm)	0	0	1830	0
Molar ratio			1.0	

[b] Molar ratio: ^{33}pdT:^{32}pdT:^{32}pdC, found: 1.1:5.0:4.0; expected: 1:5:3.

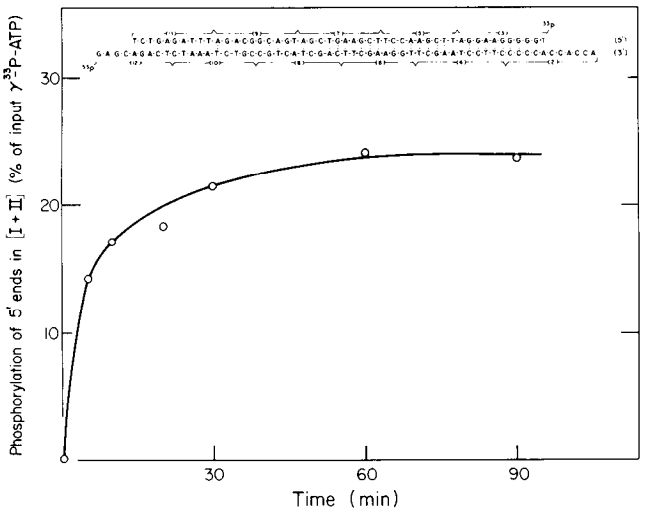

FIG. 10. Kinetics of the phosphorylation of the duplex [I + II] (segments 2 to 12). The reaction mixture (200 μl) contained 266 pmol of the duplex, 20 mM Tris-HCl (pH 7.6), 4 mM $MgCl_2$, 10 mM dithiothreitol, 1 mg/ml of spermidine, 5.2 nmol of [γ-^{33}P]ATP, and 50 units/ml of T_4-kinase. The reaction was carried out at 37°. The kinetics were measured by directly spotting aliquots (approximately ½ μl) on DEAE-paper strips and separating unused ATP by irrigation with 0.3 M ammonium formate.

the preparation of duplex [IV], which, in turn, is used in the experiments described in this paper.

The two duplexes (190 pmol each) were brought together in a 100-μl reaction mixture and treated with polynucleotide ligase as described in the legend to Fig. 11. The joining reaction performed at 4°, was followed by the phosphatase assay. The kinetics are shown in the *inset* (Fig. 11). As seen, the reac-

tion was rapid, being virtually complete within the first several minutes. The reaction was stopped by adding EDTA to 40 mM and the products were separated using an Agarose 0.5m column (1 × 150 cm); the elution buffer was 50 mM triethylammonium bicarbonate, fractions of 0.6-ml volume being collected. The effluent profile is shown in Fig. 11. The reaction was also analyzed by electrophoresis using 18% polyacrylamide gel and gave the pattern shown in Fig. 12. The joined product, duplex [III + IV], was characterized by the standard analyses as shown in Table IV. As is seen, all the results were consistent with theoretical expectations. Thus, 70% of the total ^{33}P radioactivity was resistant to the alkaline phosphatase, expected resistance being 75%. On degradation to 5′-nucleotides, most of the ^{33}P radioactivity was in pdA and pdG and this was distributed in the molar ratio (1:3) expected. Finally, on degradation to 3′-nucleotides, ^{33}P was again in the expected nucleotides, dCp and dGp, and was found in the expected molar ratio of 1:2.

This product was used in the synthesis of the total DNA duplex as described below.

Joining of Duplex [I + II] (Segments 2 to 12) to Duplex [III + IV] (Segments 13 to 25)—Duplex [I + II] was phosphorylated using [γ-^{32}P]ATP and polynucleotide kinase as described above (Fig. 10). This product and the above-described preparation of duplex [III + IV], carrying a single ^{33}P-end group on segment 20 (100 pmol each), were annealed at 37° and reacted with polynucleotide ligase as in Fig. 13. The joining was rapid as assayed by the phosphatase assay (*inset* to Fig. 13). After 30 min, the mixture was separated using an Agarose 0.5m column. Peak I was extensively analyzed as shown in Table V. Furthermore, the reaction mixture was also analyzed by electrophoresis on a polyacrylamide gel (Fig. 14). The extent of reaction shown by this method was similar to that indicated by the separation in Fig. 13. The results in Table V were all in general agreement with the expected values. Thus, five out of a total of six ^{33}P-labeled phosphate groups (Fig. 13) should be resistant to the phosphatase and the result

TABLE IV

Analysis of joined product, duplex [III + IV] (segments 13 to 25) (Peak I of Fig. 11)

	Bacterial alkaline phosphatase			
	Resistant	Sensitive	% Resistant	Theoretical %
Peak 1 (III + IV) ^{33}P	1994	849	70.1	75

Radioactivity in 5′-nucleotides				
	pdC	pdA	pdG	pdT
^{33}P (cpm)	154	896	2805	99
Molar ratio				
Found		1.0	3.1	
Expected		1	3	

Radioactivity in 3′-nucleotides				
	dCp	dAp	dGp	dTp
^{33}P (cpm)	2460	30	5010	118
Molar ratio				
Found	1.0		2.03	
Expected	1		2	

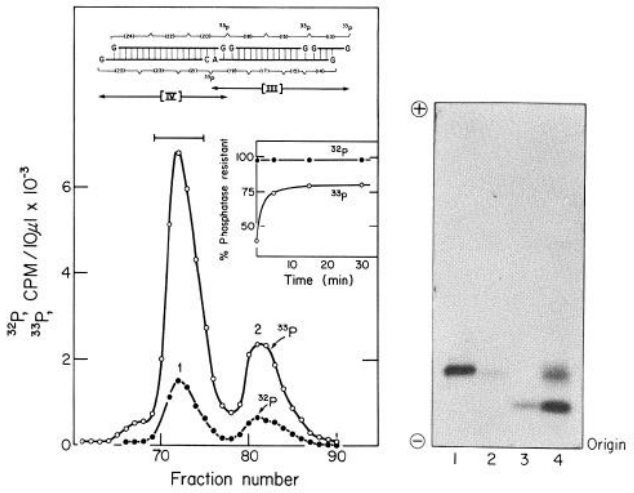

FIG. 11 (*left*). Joining of duplex [III] (segments 13 to 19) to duplex [IV] (segments 20 to 25). The duplexes as prepared in accompanying papers (190 pmol of each) were annealed at 37° in the presence of the standard components (Tris buffer, pH 8.0, 50 mM and $MgCl_2$, 10 mM). After cooling to 4°, ATP (0.1 mM), dithiothreitol (2 mM), and polynucleotide ligase (60 units/ml) were added, the total volume being 100 μl. The joining was followed by the phosphatase assay (see *inset*) and after 30 min, the reaction was stopped with EDTA and the mixture separated by flow through an Agarose 0.5m column as described in the text.

FIG. 12 (*right*). Polyacrylamide gel electrohpresis of the reaction mixture in the joining of duplex [III] and duplex [IV] (*4*). Polyacrylamide gel (18%) was used, the markers used being duplex [III] (*1*), duplex [IV] (2), and duplex [III + IV] (*3*) isolated from a previous reaction mixture.

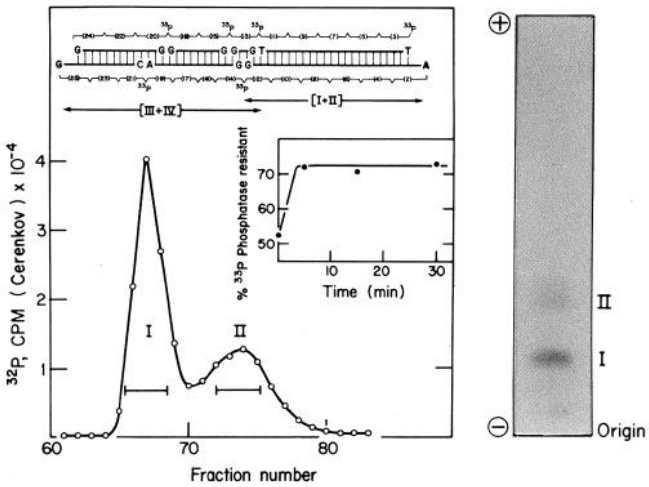

FIG. 13 (*left*). The joining of duplex [I + II] to duplex [III + IV]: synthesis of the total duplex [I + II + III + IV]. The reaction mixture, set up exactly as in the experiment of Fig. 11, contained in a reaction volume of 100 μl, the following components: duplex [I + II] (100 pmol), duplex [III + IV] (100 pmol), Tris, pH 8 (50 mM), $MgCl_2$ (10 mM), ATP (0.1 mM), dithiothreitol (1 ,M), and ligase (400 units/ml). The reaction was performed at 4° and about 0.5 μl or less aliquots were removed for phosphatase sensitivity as analyzed by DEAE-cellulose paper assay. The kinetics are shown in the *inset*. Conditions for separation by flow through an Agarose column were as described in the experiment of Fig. 11. The fractions were examined for radioactivity by Cerenkov counting.

FIG. 14 (*right*). Polyacrylamide gel (18%) electrophoresis of the reaction mixture obtained using duplex [I + II] and duplex [III + IV]. *Band I* corresponds to the joined product, duplex [I + II + III + IV], while *Band II* represents unreacted material.

TABLE V

Characterization of total DNA duplex [I + II + III + IV] (Peak 1 of Fig. 3)

Sensitivity to alkaline phosphatase				
	Resistant	Sensitive	Resistant (%)	
			Found	Expected
^{33}P (cpm)	5620	1175	82.7	83.3

3′-Nucleotides: distribution of radioactivity					
	dAp	dGp	dTp	dCp	pdTp
Before treatment with phosphatase					
^{33}P (cpm)	118	2967	1124	1109	735
Molar ratio					
Found		2.6	1.0	1.0	0.7[a]
Expected		2	1	1	1
After treatment with phosphatase					
^{33}P (cpm)	294	7110	2600	2140	
Molar ratio					
Found		3.0	1.1	0.9	
Expected		3	1	1	

5′-Nucleotides: distribution of radioactivity				
	pdA	pdG	pdT	pdC
^{33}P (cpm)	1030	3890	889	8
Molar ratio				
Found	1.0	3.8	0.9	
Expected	1	4	1	

[a] Degradation by the micrococcal nuclease (+ spleen phosphodiesterase) would be expected to give ^{33}pTp, ^{33}pTpGp, and smaller amounts of higher homologs. The molar amount of ^{33}pTp would easily be less than 100%.

(82.7%) was close to the 83.3% which was expected. The ^{33}P radioactivity in 3′-nucleotides both before and, in particular, after the phosphatase treatment was distributed in the molar ratios expected. Finally, the results on degradation to 5′-nucleotides were in excellent agreement with the expected values (Table V).

DISCUSSION

The work reported in this paper completes the task of the total synthesis of a double-stranded DNA corresponding to the entire sequence of 126 ribonucleotides present in the precursor to the tyrosine suppressor tRNA discovered by Altman and Smith (11). The present series of papers together with the work reported earlier (12) on the total synthesis of the 77-nucleotide-long DNA corresponding to a yeast alanine tRNA demonstrate that reasonably satisfactory methodology now exists for the laboratory synthesis of the genetic material. Further, the general synthetic strategy is such that the controlled synthesis of DNA duplexes much longer than those hitherto synthesized is feasible. The following comments based on the experience gained so far may be offered on the different chemical and enzymatic steps which comprise the methodology. (*a*) The first phase involves the chemical synthesis of the entire two strands of the desired DNA in the form of short deoxyribooligonucleotide segments. Chemical synthesis is easily the progress-determining factor. While it continues to improve steadily, there is still great room for ingenuity, rapidity, and efficiency. (*b*) The next step is the quantitative phosphorylation of the 5′-OH groups of the segments. This usually presents no difficulty and in fact the resulting ^{32}P-(^{33}P-)labeled oligonucleotides offer another advantage in that they can be subjected to very sensitive tests (fingerprinting) for additional checks of purity of the segments. (*c*) The third step is the division of the total segments into groups which will eventually be used to form duplexes of different parts of the total DNA. The yields in the different joining systems have varied widely and the results have generally been unpredictable. Therefore, a very large amount of empirical but systematic work is usually necessary before the subgrouping can be determined. In addition to the efficiency and accuracy in joining, a major consideration is the orientation of the terminal 5′-OH groups in the joined duplexes. From the present experience, it is clearly advantageous to have the 5′-OH groups in the duplexes corresponding to different parts of the DNA at the ends of protruding single-stranded regions. If this condition is met, then phosphorylation of the terminal 5′-OH groups in preparation for the last step presents no difficulty. (*d*) The final step is the end to end joining of the preformed duplexes containing suitably labeled terminal phosphate groups. This step invariably proceeds rapidly and in essentially quantitative yields. This is particularly satisfactory because losses at final stages in any multi-step synthesis are very costly.

It should be noted that the synthesis has been so designed that single-stranded hexanucleotide stretches (segments 1 and 26) are available for extension of the DNA to the adjacent regions, which presumably correspond to the promoter and terminator regions. However, the two single-stranded ends may be filled in by enzymatic repair (nucleotides 121 to 126) or by enzymatic joining (segment 1). As described in an accompanying paper (1), difficulty was experienced in joining the terminal segment 26 to segment 24 in duplex [IV]. The hexanucleotide sequence at this end may, however, be readily "repaired" by the DNA polymerase-catalyzed nucleotide additions. This procedure has been worked out satisfactorily for short polynucleotide chains (13). The addition of segment 1 at the opposite single-stranded end of the duplex [I + II] has been demonstrated in the present work. Thus, as described above, the synthetic plan included the phosphorylation of the duplex [I + II] at its ends. The experiment shown in Fig. 7 was performed to selectively add segment 1 to the phosphorylated duplex and this joining was satisfactorily characterized.

The principle of end to end joining of preformed DNA duplexes will undoubtedly be used in the insertion of the synthetic tRNA gene into a temperate bacteriophage such as ϕ80 or into a plasmid DNA. Lysogenization of a suitable amber mutant of *E. coli* with the resultant bacteriophage should enable the testing of the biological functioning of the synthetic gene. The technology for this kind of experiment is now straightforward (14).

As already stated (2), the next goal is the controlled transcription of the synthetic gene. If this can be realized, then the way would be open for a variety of studies. Before all else, it should be pointed out, however, that transcription would provide an additional highly sensitive means of characterization of the synthetic gene. Second, studies of the processing (15–17) of the primary transcript to a functional tRNA would be the next step. It should also be emphasized that the transcription product would provide excellent substrate for studies of the base modifications and the base-modifying enzymes. Suitable substrates for these studies have largely been lacking, because

in the precursors frequently isolated modifications have already occurred (18). Third, there should be the very large opportunity of studying structure-function relationships by studying the tRNAs containing predetermined structural changes.

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