Total Synthesis of a Tyrosine Suppressor Transfer RNA Gene

XIV. CHEMICAL SYNTHESIS OF OLIGONUCLEOTIDE SEGMENTS CORRESPONDING TO THE TERMINAL REGIONS

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In the preceding paper (1), a plan was described for synthetic work in the promoter region of the Escherichia coli tyrosine suppressor tRNA gene. The DNA duplex (Fig. 1) to be synthesized included the first 51 nucleotides in the promoter region and the EcoRI restriction enzyme sequence at the appropriate 5'-end. As shown in Fig. 1, a total of 10 deoxyribonucleotide segments were to be synthesized and of these, syntheses of Segments P-1 to P-5 were documented in the preceding paper (1). The present paper describes the synthesis of the Segments P-6 to P-10 corresponding to the nucleotide sequence -30 to -56, thus completing the chemical work necessary for the total synthesis of the promoter region.

The present paper also describes a modification of the previously synthesized 23-unit-long DNA corresponding to the sequence adjoining the C-C-A end of the tyrosine tRNA gene. The previously synthesized DNA and the segments which were used for its synthesis (2) are shown in Fig. 2A. The modified duplex which now forms the terminus distal to the promoter region is shown in Fig. 2B. The decision to add the latter duplex to the C-C-A end of the structural gene previously synthesized (3) was arrived at from the following considerations.

Previous studies with the E. coli tyrosine suppressor tRNA gene, which has formed the topic of present investigations, have been carried out mainly after its integration into the transducing bacteriophage φ80. By transduction, the "doublet" strain (diagram, Fig. 3A) which contains the suppressor tRNA\textsuperscript{TY} (su\textsuperscript{+}) and the suppressor-negative tRNA\textsuperscript{TY} (su\textsuperscript{−}) genes in tandem has been isolated. Unequal recombination between the two tRNA\textsuperscript{TY} genes in this strain gives rise to a "singlet" strain φ80psw10 (diagram, Fig. 3B), in which only one of the two genes is present (4). Determination of the nucleotide sequences in the promoter region and at the ends adjoining the C-C-A sequence of the tRNA\textsuperscript{TY} genes using the doublet and the singlet strains gave the results which are shown in Fig. 3 (5). Work on the \textit{in vitro} and \textit{in vitro} transcription of the above genes shows that termination of transcription does not occur either at or near Sequence 3 or the region between the tandem tyrosine tRNA genes.\textsuperscript{1} Therefore, the primary transcript of the tyrosine tRNA genes must undergo processing and, clearly, the tRNA precursor discovered by Altman and Smith (6) must have undergone processing at the X'-end prior to isolation. In the present goal of total synthesis, it was a prerequisite to determine the length of the DNA region adjoining the C-C-A end which would contain the required processing signal. Therefore, transcription experi-

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\item \textsuperscript{1} Recent work on transcription in \textit{in vitro}, of tRNA\textsuperscript{TY} gene shows that termination may occur about 80, 225, or 375 nucleotides downstream from the end of the structural gene (Kupper, H., Sekiya, T., Rosenberg, M., Egan, J., and Landy, A. (1978) \textit{Nature} 272, 423–428; Kitamura, N., Ikeda, H., Yamada, Y., and Ishikura, H. (1977) \textit{Eur. J. Biochem.} 73, 297–306).
\end{itemize}
Fig. 2. A, the DNA duplex previously synthesized and corresponding to the 23-nucleotide-long sequence adjoining the C-C-A end of the structural gene of the E. coli tyrosine suppressor tRNA gene. The nucleotide sequence numbered 121 to 126 is the terminal hexanucleotide sequence of the tyrosine tRNA. The adjoining sequence begins with nucleotide 127. The segments chemically synthesized are shown in brackets. B, the modified duplex which is to constitute the terminus, distal to the promoter, of the totally synthetic suppressor tRNA gene. Segment 29' contains the single-stranded sequence specific for the EcoRI restriction endonuclease.

ments were carried out, in vitro, with the synthetic structural gene (3) to which the 23-nucleotide-long DNA (Fig. 2A) had been joined (7). This work showed that the processing signal for nucleolytic cleavages downstream from the C-C-A sequence is indeed contained within the relatively short synthetic DNA shown in Fig. 2. Further sequence work at the 3'-end was therefore unnecessary from the standpoint of the synthesis of a biologically functional gene.

With the previously synthesized Segments 25 to 29 (Fig. 2A) in hand, the main concern was to add the appropriate EcoRI restriction enzyme sequence at the terminus with minimal additional synthetic work. Clearly, the simplest possibility was to replace Segment 29 with d(A-A-T-T-C-T-T-C), which contains the required recognition sequence at the 5'-end and which had already been synthesized as the terminal segment (Segment P-10) for the promoter duplex (Fig. 1). Thus, in the modified plan shown in Fig. 2B the only additional synthetic work involved modification of the original Segment 28 (Fig. 2A) to Segment 28'. Since the protected pentanucleotide, d([MeOTr]bzA-mbG-T-anC-anC), was available from previous work, completion of the work involved only the two steps shown in Chart 6.

During the progress of the present work, the use of reverse phase high pressure liquid chromatography was being developed concurrently (8) and a distinctive and practically important feature of the syntheses herein described has been the use of this technique for the rapid and efficient separation of synthetic oligonucleotide reaction mixtures.

DISCUSSION OF METHODS

Synthesis of Segment P-6 (Fig. 1), d(T-T-T-A-C-A-G-C-G-G-C)—The plan followed for the synthesis of this undecanucleotide d(T-T-T-A-C-A-G-C-G-G-C) is shown in Chart 1. The synthetic scheme was primarily designed to make use of the already available dinucleotide d[pibG-anC(Ac)] and the trinucleotide d[pibG-bbG-anC(Ac)]. The tetranucleotide d([MeOTr]T-T-T) was synthesized by the stepwise addition of the mononucleotide, d[pT(Ac)], to the nucleoside d(MeOTr)T, and both the di- and trinucleotides were isolated by extraction with organic solvents. The trinucleotide was then reacted with d[pbxA(Ac)] to give the tetracotiducleotide d([MeOTr]T-T-T-bzA) which was isolated by DEAEC-cellulose ion exchange chromatography. The tetracotiducleotide was then condensed with the protected dinucleotide, d[panC-bzA(Ac)], and the resulting hexanucleotide was isolated by DEAEC-cellulose ion exchange chromatography (Fig. 4A).

All abbreviations used in this paper have been defined in Ref. 1.
Synthesis of Tyrosine Suppressor tRNA Gene

Sequence 1 as in Fig 1
Sequence 2 (5') G-C-T-T-C-C-C-G-A-T-A-A-G-G-G-A-G-C A-G-C-C-A-G-T-A ..(3')
Sequence 3 (5') T-C-A-C-T-T-T-C-A-A-A-A-G-C-T (3')
Sequence 4 (5') T-A-A-T-T-C-A-C-C-A-C-A-G-C ..(3')

FIG. 3. Schematic representation of the promoter, structural regions, and sequences adjoining the C-C-A end in the E. coli tRNA¹⁷⁵ genes. A represents the doublet strain, φ80ψsuI and carries the tRNA¹⁷⁵ (su') and tRNA¹⁷⁵ (su) genes in tandem. B, the singlet strain, φ80ψsu', which carries a single tRNA¹⁷⁵ (su) gene. The sequences 1 to 4 (taken from Ref. 5) and their positions in the genes are shown. Of note is Sequence 3, corresponding to duplex A (Fig. 2), which follows su' gene in A and su' gene in B.

CHART 1. Steps in the synthesis of the undecanucleotide d(T-T-T-A-C-A-G-C-G-G-C) (Segment P-6, Fig. 1).

It should be noted that the preparative purification of the deprotected undecanucleotide without purification was phosphorylated by polynucleotide kinase using [γ-32P]ATP. Purity and sequence of the resulting preparation was confirmed by the two-dimensional fingerprint (Fig. 7) of a partial snake venom phosphodiesterase digest of the labeled undecanucleotide (9).
Synthesis of Tyrosine Suppressor tRNA Gene

Peak IV contained the unreacted hexanucleotide, d[(MeOTr)T-T-bzA-anC-bzA], while Peak V contained the desired octanucleotide. B, analysis of the octanucleotide by hplc using the μBondapak C₁₈ column (0.4 × 30 cm). The eluant was 0.1 M ammonium acetate containing 35% acetonitrile and the flow rate was 2 ml/min.

Octa- and undecanucleotides was facilitated by an atypically longer retention time, in each case, of the product relative to that of its starting material. The synthesis of the trinucleotide d[pibG-bzA-anC(Ac)] has been described previously (10). For this work, it was further purified by preparative hplc (Fig. 8).

Synthesis of Segment P-9, d(A-C-G-T-T-G-A-G-A-A-A-G)—The plan followed in the synthesis of this dodecanucleotide is shown in Chart 2. The trinucleotide d[(MeOTr)bzA-anC-bzA] was synthesized by the stepwise addition of appropriate mononucleotides to the nucleoside d(MeOTr)bzA. The trinucleotide was next condensed with the dinucleotide d[pT(Ac)], and the product, d[(MeOTr)bzA-anC-piT(Ac)], was isolated by a combination of solvent extraction and reverse phase high pressure liquid chromatography (Fig. 9). Condensation of the pentanucleotide with the dinucleotide d[pibG-bzA(Ac)] gave the heptanucleotide d[(MeOTr)bzA-anC-pibG-bzA(Ac)], which was partially purified by extraction with a dichloromethane/1-butanol mixture. The aqueous phase from these extractions contained the unreacted dinucleotide d[pibG-bzA(Ac)], which was isolated pure in high recovery by preparative hplc. The partially purified heptanucleotide was further purified by hplc using the preparative reverse peak IV contained the unreacted octanucleotide, d[(MeOTr)T-T-bzA-anC-bzA-anC-bzA], while Peak V contained the desired undecanucleotide. B, analysis of the undecanucleotide on the analytical hplc column. The eluant was 0.1 M ammonium acetate containing 35% acetonitrile.

FIG. 7. Autoradiogram of a two-dimensional fingerprint of a partial snake venom phosphodiesterase digest of [5'-32P]d(T-T-A-C-A-G-C-G-G-C) (Segment P-6). The sequence is verified as shown in the reproduction pattern. The dashed circle indicates the position of the dye marker, xylene cyanol.

FIG. 5. A, separation of the products formed in the condensation of the hexanucleotide, d[(MeOTr)T-T-bzA-anC-bzA] with d[pibG-anC(Ac)] on the preparative hplc column. The eluant was 0.1 M TEAA (pH 7.0) with varying percentages of acetonitrile, as shown. Peak II contained the excess dinucleotide block, d[pibG-anC(Ac)].
Synthesis of Tyrosine Suppressor tRNA Gene

With TAA column (Fig. 10), but it could not be completely freed
from the unreacted pentanucleotide. Since the next step re-
quired the addition of the same dinucleotide, [pibG-
] to the heptanucleotide, addition of the dinucleotide
the contaminating pentanucleotide would give the same
heptanucleotide and, therefore, no new unwanted product
would be produced. Hence, the contaminated heptanucleotide
was used directly in the next condensation with the protected
dinucleotide [pibG-bzA(Ac)]. The resulting nonanucleotide,
[(MeOTr)bzA-anC-bzG-T-T-bzA-bzG-bzA] was purified
by anion exchange chromatography and the fractions which
contained only the nonanucleotide as shown by hplc were
pooled.

The nonanucleotide was finally condensed with the trinu-
clide [pibC-bzG-anC(Ac)] to give the protected dodeca-
nucleotide. The product along with the unreacted nonanu-
ucleotide was separated from the excess of trinucleotide and its
pyrophosphate by preparative hplc (Fig. 11). After complete

FIG. 8. Purification of the trinucleotide, [pibG-bzG-anC(Ac)]
on the preparative hplc column. The eluant was 0.1 M
TEAA (pH 7.0) with varying percentages of acetonitrile, as shown. Peak I contained the trinucleotide.

\[
d\text{(MeOTr)bzA}
\downarrow
\text{
[ppanC(Ac)]
}
\]
\[
d\text{[(MeOTr)bzA-anC]}
\downarrow
\text{
[pibC(Ac)]
}
\]
\[
d\text{[(MeOTr)bzA-anC-bzG]}
\downarrow
\text{
[pT-T(Ac)]
}
\]
\[
d\text{[(MeOTr)bzA-anC-bzG-T-T]}
\downarrow
\text{
[pibG-bzA(Ac)]
}
\]
\[
d\text{[(MeOTr)bzA-anC-bzG-T-T-ibG-bzA]}
\downarrow
\text{
[pibU-bzA(Ac)]
}
\]
\[
d\text{[(MeOTr)bzA-anC-bzG-T-T-ibG-bzA-bzG-bzA]}\]
\downarrow
\text{
[pbza-bzA-bzG(Ac)]
}
\]
\[
d\text{[(MeOTr)bzA-anC-bzG-T-T-ibG-bzA-bzG-bzA-bzG-bzA-bzG]}\]
\downarrow
\text{
[1. NH3]
}
\]
\[
\text{[2. H+]
}
\]
\[
d\text{[(A-C-G-T-T-A-A-A-G) (Segment P-9, Fig. 1)]
}
\]

CHART 2. Steps in the synthesis of the dodecanucleotide [d(A-C-
G-T-T-A-A-A-G)] (Segment P-9, Fig. 1).

deprotection, the dodecanucleotide, [d(A-C-G-T-T-A-A-A-G)]
was isolated by chromatography on a DE-52 column
in the presence of 7 M urea (Fig. 12). Its two-dimensional
fingerprint is shown in Fig. 13. The trinucleotide [pbza-bzA-
ibG(Ac)] was synthesized by condensing [TPS (TPS)pbza] with
[pbza-bzGibG(Ac)] as described under "Experimental Section."
A final purification of this trinucleotide was achieved by hplc
(Fig. 14).

Synthesis of Segment P-7, d(T-G-T-A-A-A-G-T-G-T-T)-
The steps used in this synthesis are shown in Chart 3.
Condensation of [pibG(Ac)] with [MeOTr]T gave
[d(MeOTr)T-bibG] and the latter was next condensed with
[pT-T(Ac)]. The protected di- and trinucleotides were isolated
rapidly by solvent extraction. The trinucleotide was con-
densed with the dinucleotide [pbza-bzA(Ac)] to give the
pentanucleotide [d(MeOTr)T-bibC-T-bzA-bzA] and the latter
was then condensed with the dinucleotide [pbza-bzAibG(Ac)] to
give the protected heptanucleotide (Chart 3). Both the penta-
and the heptanucleotides were purified by anion exchange
chromatography (Figs. 15 and 16).""3 The heptanucleotide was

\[3\text{Portions of this paper (including Figs. 15 to 31 and Table I) are}
\text{presented in miniprint at the end of this paper. Miniprint is easily}
\text{read with the aid of a standard magnifying glass. Full-size photocopies}
\text{are available from the Journal of Biological Chemistry, 9000 Rockville}
\text{Pike, Bethesda, Md. 20014. Request Document No. 78M-1431, cite}
\text{author(s), and include a check or money order for $4.65 per set of}
\text{photocopies.}"

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phy (Figs. 20 and 21). The octanucleotide was finally condensed with the tetracnucleotide [pbzA-anC-bzA-anC(Ac)] to give the desired protected dodecanucleotide. Because of the lipophilic 5'-O-monomethoxytrityl group on the 5'-termini of the octa- and the dodecanucleotides, these oligonucleotides were conveniently separated from the tetrancnucleotide block and its pyrophosphate by preparative hplc. However, it was not possible to separate the octa- and dodecanucleotides on this column. This mixture was fully deprotected and the required dodecanucleotide, d(T-C-A-A-C-G-T-A-C-A-C), was isolated by DE-52 column chromatography in the presence of 7 M urea (Fig. 22) and characterized by the two-dimensional fingerprinting method (Fig. 23).

The tetrancnucleotide [pbzA-anC-bzA-anC(Ac)] required above was prepared by the condensation of [pbzA-anC(Ac)] with the dimncnucleotide [p(SESe)pzbA-anC] or [CNBtpzba-anC], as described under "Experimental Section."

Synthesis of Segment P-10, d(A-A-T-T-C-T-T-T-C)—The sequence of synthetic steps is shown in Chart 5. Condensation of the protected nucleoside d(MeOTry)bzA with the protected nucleoside [pbzA(Ac)] followed by condensation of the resulting dimncnucleotide [p[pT(Ac)] gave the tetrancnucleotide d[MeOTry]bzA-bzA(bzA-A-T)]. The latter was next condensed with the dimncnucleotide [p[pT-anC(Ac)]] to give the pentanucleotide d[MeOTry]bzA-bzA(Ac-T-A] which was purified by anion exchange chromatography (Fig. 24). Condensation of the pentancnucleotide with [p[pT(Ac)] gave the required heptancnucleotide. This product was purified by hplc using the preparative reverse phase column (Fig. 25). Because of the small lipophilic contribution of the thymine residues relative to the standard hydrophilic contribution of the two-phosphate dissociations of the added dimncnucleotide [p[pT(Ac)]] there was a relatively large difference in retention time between the product and unreacted starting material. This permitted isolation of the heptancnucleotide completely free of the pentancnucleotide. The heptancnucleotide was finally condensed with [p[pT-anC(Ac)]], and the nonancnucleotide was isolated by anion exchange chromatography (Fig. 26). It was further purified after complete deprotection by chromatography in the presence of 7 M urea (Fig. 27). Analysis by the fingerprinting method (Fig.

Finally condensed with the tetrancnucleotide [p[pT-bzA(T)-T(Ac)] to give the protected undecancnucleotide, which was isolated by DEAE-cellulose anion exchange chromatography (Fig. 17). The tetrancnucleotide used was prepared by condensation of [p(SESe)pT-bzG] with [p[pT(Ac)].

The undecancnucleotide, after removal of the protecting groups, was finally chromatographed on an anion exchanger column in the presence of 7 M urea. The material pooled as shown was further characterized by the two-dimensional fingerprinting method following phosphorylation of the 5'-OH group by polynucleotide kinase using [γ-32P]ATP (Fig. 19).

Synthesis of Segment P-8, d(T-C-A-A-C-G-T-A-C-A-C)—The plan for the synthesis of this dodecanucleotide is shown in Chart 4. The protected tetrancnucleotide, d[MeOTry]T-anC-bzA-bzA] was prepared by condensation of the dimncnucleotide [pbzA-bzA(Ac)] with the dimncnucleotide d[MeOTry]T-anC], the latter being prepared from the protected thymidine and d[panC(Ac)]. The tetrancnucleotide was further condensed with the dimncnucleotide d[panC-bzG(Ac)] to give the hexancnucleotide and the latter then condensed with [p[pT-bzA(Ac)] to give the protected octancnucleotide d[MeOTry](T-anC-bzA-bzA-anC-ibbG-T-bzA]. The product after each of these condensations was isolated by DEAE-cellulose ion exchange chromatography (Fig. 20) and characterized by the two-dimensional fingerprinting method (Fig. 21).

The reaction products were fractionated after the dodecanucleotide with gradients of acetonitrile in 0.1 M TEAA as indicated. Peak I contained the excess trinucleotide d[p[pbA-bzA-ibG(Ac)], while Peak II contained both the unreacted nonancnucleotide and the desired dodecanucleotide. B, analysis on the analytical hplc column of the precipitated 5'-O-monomethoxytritylated material collected from the preparative column (Peak II). The eluant was 0.1 M ammonium acetate containing 35% acetonitrile.

The heptancnucleotide was finally condensed with d[p[pT-bzA(Ac)] and its pyrophosphate by preparative hplc. However, it was not possible to separate the octa- and dodecanucleotides on this column. This mixture was fully deprotected and the required dodecanucleotide, d(T-C-A-A-C-G-T-A-C-A-C), was isolated by DE-52 column chromatography in the presence of 7 M urea (Fig. 22) and characterized by the two-dimensional fingerprinting method (Fig. 23).

The tetrancnucleotide [pbzA-anC-bzA-anC(Ac)] required above was prepared by the condensation of [pbzA-anC(Ac)] with the dimncnucleotide [p(SESe)pzbA-anC] or [CNBtpzba-anC], as described under "Experimental Section."

Synthesis of Segment P-10, d(A-A-T-T-C-T-T-T-C)—The sequence of synthetic steps is shown in Chart 5. Condensation of the protected nucleoside d(MeOTry)bzA with the protected nucleoside [pbzA(Ac)] followed by condensation of the resulting dimncnucleotide [p[pT(Ac)] gave the tetrancnucleotide d[MeOTry]bzA-bzA(bzA-A-T)]. The latter was next condensed with the dimncnucleotide [p[pT-anC(Ac)]] to give the pentancnucleotide d[MeOTry]bzA-bzA(Ac-T-A] which was purified by anion exchange chromatography (Fig. 24). Condensation of the pentancnucleotide with [p[pT(Ac)] gave the required heptancnucleotide. This product was purified by hplc using the preparative reverse phase column (Fig. 25). Because of the small lipophilic contribution of the thymine residues relative to the standard hydrophilic contribution of the two-phosphate dissociations of the added dimncnucleotide [p[pT(Ac)]] there was a relatively large difference in retention time between the product and unreacted starting material. This permitted isolation of the heptancnucleotide completely free of the pentancnucleotide. The heptancnucleotide was finally condensed with [p[pT-anC(Ac)]], and the nonancnucleotide was isolated by anion exchange chromatography (Fig. 26). It was further purified after complete deprotection by chromatography in the presence of 7 M urea (Fig. 27). Analysis by the fingerprinting method (Fig.

FIG. 12. Chromatography of the nonancnucleotide, d(A-C-G-T-T-G-A-G-A), and the dodecanucleotide, d(A-C-G-T-T-G-A-G-A-G-A), on a DEAE-cellulose column (Whatman 52, chloride form) (1.0 × 113 cm) pre-equilibrated at room temperature with 0.05 M sodium chloride containing 7 M urea, 0.02 M Tris-HCl (pH 7.5). Fractions of 2.5 ml were collected. Peak I contained the dodecanucleotide.

FIG. 11. A, preparative hplc of the reaction products in the preparation of the dodecanucleotide d((MeOTry)bzA-anC-ibG-T-T-ibG-bzA-ibG-bzA-bzA-ibG(Ac)]. The reaction products were fractionated with gradients of acetonitrile in 0.1 M TEAA as indicated. Peak I contained the excess trinucleotide d[p[pbA-bzA-ibG(Ac)], while Peak II contained both the unreacted nonancnucleotide and the desired dodecanucleotide. B, analysis on the analytical hplc column of the precipitated 5'-O-monomethoxytritylated material collected from the preparative column (Peak II). The eluant was 0.1 M ammonium acetate containing 35% acetonitrile.
Synthesis of Tyrosine Suppressor tRNA Gene

CHART 3. Steps in the synthesis of the undecanucleotide d(T-G-T-A-A-A-G-T-G-T-T) (Segment P-7, Fig. 1).

28) after [32P]phosphorylation of the 5'-OH group and by hplc using an analytical column showed the product to be pure.

**Synthesis of Segment 28**, d(A-G-T-C-C-G-A-A-A-G)—Starting with the pentanucleotide, d(MeOTr)bzA-mbG-T-anC-anC] (2), the decanucleotide was synthesized as shown in Chart 6. Thus, condensation with the dinucleotide d[pibG-bzA(Ac)] gave the corresponding heptanucleotide, which was isolated by anion exchange chromatography on DEAE-cellulose (Fig. 29). The heptanucleotide was then condensed with the trinucleotide d[pbzA-bzA-ibG(Ac)], which was also available from the synthesis of Segment P-9, described above, to give the desired protected decanucleotide. The product was purified by anion exchange chromatography using DEAE-cellulose (Fig. 30). In both of the above condensations, appropriate fractions were pooled after checking them by hplc on the analytical column.

**Characterization of Synthetic Deoxyribo-oligonucleotides**—As described, the nucleotide sequences in the final synthetic compounds were confirmed by two-dimensional fingerprinting of the partial digests obtained upon degradation with snake venom phosphodiesterase. The other methods used for characterization of several protected and unprotected deoxyribo-oligonucleotides are described in earlier papers (10-13). In the present work they were analyzed by reverse phase high pressure liquid chromatography on the analytical hplc column. This method could be used in all phases of oligonucleotide synthesis and was especially suitable for detecting any loss of the amino protecting groups. The details of these procedures have been described elsewhere (8).

**Concluding Remarks on Synthetic Procedures**—Chemical synthesis of deoxyribonucleotides corresponding to the entire two strands constitutes the first and the most demanding phase of total synthesis of a DNA. The separation, purification, and characterization of the synthetic intermediates and of the final products are the most time-consuming aspects. The introduction of hydrophobic protecting groups permitted the use of solvent extraction procedures and this general technique is now used routinely for the isolation of protected short oligonucleotides (1, 10, 14). However, a main feature of the present work has been the use of reverse phase high pressure liquid chromatography in rapid and highly efficient separations of the synthetic intermediates. While the development of the method as an analytical tool in synthetic work has been described elsewhere (8), examples of its use on a preparative scale have been provided in the present work. Thus, in the preparation of the undecanucleotide d(MeOTr)T-T-T-bzA-anC-bzA-ibG-anC-ibG-ibG-anC], the condensation reaction mixture contained the desired product, the octanucleotide precursor, and the trinucleotide block. Using preparative high pressure liquid chromatography, these

Fig. 14. A, purification of the trinucleotide d[pbzA-bzA-ibG(Ac)] on the preparative hplc column. The eluant was 0.1 M TFIAA (pH 7.0) with varying percentages of acetonitrile, as shown. Peak I contained the required trinucleotide. B, analysis of d[pbzA-bzA-ibG(Ac)] on the analytical hplc column. The eluant was 0.1 M ammonium acetate containing 22% acetonitrile.

![Chart 3. Steps in the synthesis of the undecanucleotide d(T-G-T-A-A-A-G-T-G-T-T) (Segment P-7, Fig. 1).](http://www.jbc.org/)

![Chart 4. Steps in the synthesis of the dodecanucleotide d(T-C-A-A-C-G-T-A-A-C-A-C-G-T-T) (Segment P-8, Fig. 1).](http://www.jbc.org/)
5772

Synthesis of Tyrosine Suppressor tRNA Gene

All materials and methods used for the synthesis and isolation of oligonucleotides by anion exchange chromatography have been described in earlier papers (10-13). Reverse phase high pressure liquid chromatography (hplc) was performed on a system consisting of the following components available from Waters Associates: two M6000A solvent delivery systems, a 660 solvent programmer, a 168K injector, a 440 UV detector operating at wavelengths of 254 and 280 nm, a μBondapak C18 column (0.4 × 30 cm), and a Houston Instruments Omniscribe TM chart recorder. For preparative separations, the column and detector of the above system were replaced with a μBondapak C18/Porasil B column (0.7 × 183 cm) and an Altex model 151 UV detector equipped with a preparative flow cell operating at a wavelength of 254 or 280 nm. Pumps and solvent programmer were operated in such a way that one pump delivered aqueous buffer and the other acetonitrile. The resulting eluent is a mixture (volume/volume) of the two components which, for convenience, is expressed as a percentage of acetonitrile in 0.1 M ammonium acetate or 0.1 M TEAA. Other materials and methods used for hplc have been described earlier (8).

Whenever hplc was used for the separation of a condensation reaction mixture, the following general procedure was adopted. After quenching the condensation reaction mixture with water and DIEA, the mixture was evaporated to a gum and taken up in 0.2 M TEAB as usual. Non-nucleotidic components were partially removed by four manual extractions, two using ethyl acetate and two using ethyl acetate with 5 to 10% of 1-butanol. The contents of the aqueous phase were isolated by evaporation with pyridine and precipitation with dry ether. At this stage, two samples (100 to 300 μg each) were taken from the precipitate, one was subjected to deacetylation and the other to removal of acetyl. The sample was taken from the precipitate, one was subjected to deacetylation and the other to removal of acetyl, HCl (5 M) in the usual manner. The two samples were compared by hplc on the μBondapak C18 column using conditions that give good resolution of the tritylated oligonucleotides (30 to 35% acetonitrile in 0.1 M ammonium acetate at 0.1 M TEAA that elutes polar impurities and pyridine reasonably fast but strongly retains the acetylated di-, tri-, or tetranucleotides). In most cases, the product peak was easily identified by its shift to shorter retention time after alkaline treatment.

The main sample, precipitated as above, was dissolved in TEAA/ethanol (1:1, v/v) and centrifuged in an IEC clinical centrifuge for about 10 min to spin down all insoluble material. This solution was kept in an ice bath and applied to the preparative column as soon as possible. For preparative separations, the μBondapak C18/Porasil B column was used with a flow rate of 9.9 ml/min. A column "capacity of this column is approximately 300 mg of nucleotide material; however, poorly resolved mixtures are best chromatographed in 200-μg batches."
gradients (A to B and B to C) were used to fractionate the precipitated reaction mixture on the preparative column. After that, a step gradient to 20% more acetonitrile was applied and the column washed for 10 min.

When the fractions from preparative hplc were concentrated, special attention was paid to ensure that a sufficient amount of pyridine was present at all times during evaporation of solvents.

**Protected Dinucleotides Carrying 5'-Phosphate Groups**

All of the protected dinucleotides carrying 5'-phosphate groups used in this work were prepared by using the TPSE-protecting group, as described earlier (10). In a few instances, the compounds were further purified by hplc.

**The Trinucleotide, d(pbG-ibG-ibC)**

The synthesis of this trinucleotide has been described (10). For the present work, the acetylated trinucleotide was further purified by preparative hplc (Fig. 8).

**The Trinucleotide, d(pbA-bzA-ibG)**

An anhydrous pyridine solution (55 ml) of d[(TPSE)pdzA] (6.63 g, 7.1 mmol) and d[pbaZ-ibG(Ac)] (3.16 g, 2.48 mmol) was allowed to react with TPS (4.73 g, 15.6 mmol) for 5% h at room temperature. After quenching the reaction in the usual manner, the mixture was concentrated to a mobile oil and taken up in 0.2 ml TEAB. Extraction with ethyl acetate (four times) and ethyl acetate/1-butanol (9:1, v/v, three times; 8:2, v/v, two times) removed the unreacted d[(TPSE)pbaZ]. The trinucleotide, d[(TPSE)pbaZ-bzA-ibG(Ac)], was extracted into dichloromethane/1-butanol (9:1, v/v, two times). These extracts were combined and concentrated in vacuo, evaporated several times with pyridine and finally dissolved in pyridine/ethanol/water (4:3:3, v/v, 100 ml). This solution was cooled to 0°C and 100 ml of precooled 2 n sodium hydroxide was added. After 5 min at 0°C, pyridinium Dowex 50-X8 was added. The resulting mixture was filtered through a column of fresh Dowex. The eluant was concentrated in vacuo, extracted with diethyl ether (three times) to remove the elimination product of the TPSE protecting group, concentrated, and then precipitated into ether. The crude trinucleotide, d[pbaZ-bzA-ibC], (2.07 g, 1.32 mmol) was obtained in 46% yield. The precipitated material was dissolved in anhydrous pyridine (40 ml) and acetylated by the standard procedure. The crude product was isolated as a dry powder (2.44 g) and subjected to preparative hplc (six injections, approximately 410 mg for each injection). The tracing from one injection is shown in Fig. 14A. The effluent corresponding to Peak I contained the trinucleotide. After concentration and precipitation, pure d[pbaZ-bzA-ibG(Ac)] (0.5 mmol) was isolated in 18% overall yield. The purity of this product was checked by hplc analysis (Fig. 14B).

**Synthesis of d(T-T-T-A-C-A-G-C-G-C-G) (Segment P-6)**

**The Dinucleotide, d(MeOTr)T-T**—d(MeOTr)T (1.19 g, 2.27 mmol) and pyridinium d[pT(Ac)] (2.2 g, 5.0 mmol) were allowed to stand at room temperature in the presence of TPS (2.88 g, 9.5 mmol) in dry pyridine (20 ml) for 5% h. After the usual work-up and alkaline hydrolysis, the solution was concentrated to a gum and 200 ml of 0.2 M TEAB was added and extracted continuously with disopropyl ether at 4°C over night. The aqueous phase was extracted with 1-butanol/dichloromethane (1.9, 5 × 250 ml). The organic phases were combined and evaporated to an anhydrous pyridine solution (30 ml). The desired product was then precipitated by dropwise addition of this solution to an excess of anhydrous ether (1600 ml). Yield of the dinucleotide was 91% (2.06 mmol). The product was homogeneous on tlc in Solvents K and Q. The UV data of the protected and unprotected compounds are given in Table I.

**The Trinucleotide, d[(MeOTr)T-T-T]**—d[(MeOTr)T-T] (1.99 g, 2.03 mmol) and d[pT(Ac)] (2.49 g, 5.65 mmol) were allowed to stand at room temperature in the presence of TPS (3.70 g, 12.3 mmol) in pyridine (20 ml) for 5% h. After the usual work-up and alkaline hydrolysis, the solution was concentrated to 30 ml and 0.2 M TEAB (200 ml) was added. The resulting solution was extracted continuously with ethyl acetate overnight at 4°C. The aqueous layer was extracted with 250-ml portions of 7%, 10%, and, finally, 12% 1-butanol/ethyl acetate, each organic phase being backwashed with 0.2 M TEAB. The aqueous phase and backwashings (350 ml) were extracted with 1-butanol/dichloromethane (3.7, 5 × 250 ml) and the organic layers backwashed as before. These were combined and evaporated to a pyridine solution (50 ml). TLC in Solvents M and Q still showed a trace of contaminating dinucleotide. Therefore, after dilution with 0.2 M TEAB (250 ml), the solution was extracted with 1-butanol/ethyl acetate (15:85, 2 × 250 ml). The aqueous layer and backwashings were combined and evaporated to an anhydrous pyridine solution (50 ml). The product was then precipitated by dropwise addition of this solution to an excess of anhydrous ether (3 liters). The yield of trinucleotide was 70% (1.42 mmol). The product was homogeneous in Solvents M and Q. The UV data of the protected and unprotected compounds are given in Table I.

**The Tetranucleotide, d[(MeOTr)T-T-T-bzA]**—d[(MeOTr)T-T] (1.95 g, 1.38 mmol) and d[pbaZ(Ac)] (2.85 g, 5.77 mmol) were allowed to stand at room temperature in the presence of TPS (4.09 g, 13.5 mmol) in pyridine (15 ml) for 5% h. After the usual work-up and alkaline hydrolysis, the solution was concentrated to 40 ml of pyridine solution and then diluted to 250 ml with 0.2 M TEAB. The solution was extracted continuously at 4°C overnight with ethyl acetate. The aqueous layer was evaporated to a pyridine solution (30 ml) and diluted to 850 ml with 0.02 M Tris-HCl (pH 7.5), containing 5% ethanol. This was applied to a DEAE-cellulose column (column pattern not shown). The fractions which contained the desired tetranucleotide were pooled and desalted by membrane filtration using an Amicon 2000 apparatus fitted with a UM05 membrane. The retentate (approximately 200 ml) was passed slowly through a column of pyridinium Dowex 50-X8 (50 ml) in the presence of 20% pyridine. The eluate was evaporated to an anhydrous pyridine solution (30 ml) and product precipitated by dropwise addition to anhydrous ether (800 ml). The yield of tetranucleotide was 52% (0.72 mmol). The product showed only traces of impurity on tlc in Solvent Q. UV data of the protected and unprotected compounds are given in Table I.

**The Hexanucleotide, d[(MeOTr)T-T-bzA-ibC]**—d[(MeOTr)T-T-bzA] (0.72 g, 0.39 mmol) and d[panC-bzA(Ac)] (1.92 g, 1.6 mmol) were allowed to stand at room temperature in the presence of TPS (1.35 g, 4.47 mmol) in pyridine (7 ml) for 5% h. After the usual work-up and alkaline hydrolysis, the solution was extracted with ethyl acetate (250 ml). The organic layer was backwashed with 20% aqueous pyridine (30 ml). The aqueous layer and backwashings were combined and evaporated to a pyridine solution (20 ml). This was diluted with 250 ml of 0.02 M Tris-HCl (pH 7.5) containing 5% ethanol and applied to a DEAE-cellulose column. The details of chromatography and the elution profile are shown in Fig. 4A. Peak III which contained the desired hexanucleotide was pooled and desalted by membrane filtration using an Amicon 2000 apparatus fitted with a UM05 membrane. The retentate was made 20% with respect to TEAB.
spect to pyridine and passed slowly down a pyridinium Dowex 50-X8 column (100 ml), in the presence of 20% pyridine. The eluant was evaporated to an anhydrous pyridine solution (20 ml) and the product precipitated by dropwise addition to anhydrous ether (1 liter). The yield of hexanucleotide was 46% (0.17 mmol). Analysis of this compound by hplc indicated a significant amount of an impurity.

A portion of the above hexanucleotide was further purified by preparative hplc (Fig. 4B), as described under "Discussion of Methods." Twenty micromoles (1500 Å2 of pure hexanucleotide and the product precipitated by dropwise addition to pyridine and passed slowly down a pyridinium Dowex 5774 Synthesis of 1 stand with TPS (0.7 mmol) for 6 h at room temperature. The reaction was terminated by the standard method using 1.4 ml of TEAB and precipitated into anhydrous pyridine solution (75 ml) of d[MeOTr]bzA-anC-bzA-[bG-anC-anG]-An anhydrous pyridine solution (10 ml) and precipitated into anhydrous ether by dropwise addition. The mixture, as a dry powder, was dissolved in 4 ml of ethanol 0.1 M TEAA (1:1) and separated by hplc using the preparative reverse phase column (in 2 portions). The details of this chromatography and the elution profile are shown in Fig. 5A. Peak V which contained the desired octanucleotide was collected. The yield of this compound after precipitation was 30% (6 Åmol, 620 Å2). The product was homogeneous on hplc (Fig. 5B) and on tlc in Solvent Q. Its spectral properties are given in Table I.

The Undecanucleotide, d[MEOTr]T-T-bzA-anC-bzA-[bG-anC-anG]An anhydrous pyridine solution (5 ml, 530 Å2) and pyridinium d[piG-anC-Ac] (114 Åmol) was allowed to react at room temperature for 5% h. The reaction was terminated by the standard method using 1.4 ml of water (1.4 ml). After concentrating to a thick gum, the mixture was evaporated to a gum and 0.2 ml of the octanucleotide (5 pmol, 530 Åzo and pyridinium) was treated with TPS (0.2 ml) of the undecanucleotide (3.1 g, 2.74 mmol) and precipitated into anhydrous pyridine solution (40 ml) of d[(MeOTr)bzA-anC-anG] (1.66 g, 1.03 mmol), d[pT-T(Ac)] (3.33 g, 3.68 mmol) and TPS (7.17 g, 23.7 mmol) was allowed to react at room temperature for 5% h. After quenching the reaction in the usual manner, the mixture was concentrated to a mobile oil and taken up in 0.2 M TEAB (120 ml). This solution was extracted with ethyl acetate (4 times) and ethyl acetate/1-butanol (95:5, v/v, one time). The product was extracted into dichloromethane/1-butanol (95:5, v/v, three times). These extracts were combined and concentrated in the presence of pyridine. After the undecanucleotide was precipitated by dropwise addition to anhydrous ether and deacetylated by alkaline hydrolysis, it was freed from contaminating dinucleotide by the following extraction procedure. The mixture was taken up in 0.2 M TEAB (50 ml) and extracted with ethyl acetate/1-butanol (92.5:7.5, v/v, one time, and 9:1, v/v, one time). Finally, the pure undecanucleotide was extracted into dichloromethane/1-butanol (82:2, v/v, two times); evaporation and precipitation of this material as usual afforded d[MEOTr]bzA-anC-[bG] (2.1 g, 1.3 mmol) in 48% yield. Its spectral and chromatographic properties are given in Table I.

The Pentanucleotide, d[MEOTr]T-T-BzA-anC-[bG-T-T]-An anhydrous pyridine solution (40 ml) of d[MEOTr]bzA-anC-[ibG] (1.66 g, 1.03 mmol), d[pT-T-Ac] (3.33 g, 3.68 mmol) and TPS (3.9 g, 13.10 mmol) was allowed to react at room temperature for 5% h. After quenching the reaction in the usual manner, the mixture was concentrated to a mobile oil and taken up in 0.2 M TEAB and extracted with ethyl acetate (three times). This solution was extracted successively with dichloromethane/1-butanol (9:1, v/v, one time, 85:5, v/v, one time, 82:2, v/v, one time, and 7:3, v/v, one time). Each fraction was precipitated separately and yielded 0.23, 0.47, 0.73, and 0.58 g, respectively, of product. The 0.23- and 0.47-g fractions, contaminated with traces of the trinucleotide, were 1) combined, 2) deacetylated by alkaline hydrolysis, and 3) precipitated in the usual manner to give 0.60 g of compound; and 4) subjected to hplc purification (see below). A portion of the above undecanucleotide was phosphorylated enzymatically and then digested with snake venom phosphodiesterase and analyzed by two-dimensional homochromatography. The fingerprint (Fig. 7) was consistent with the nucleotide sequence, as expected.

Synthesis of Dodecanucleotide, d(A-C-G-T-T-G-A-G-A-A-G) (Segment P-9)
was further purified by the following extraction procedure. The precipitated material was then taken up in 0.2 M TEAB and extracted with dichloromethane/1-butanol (91:1, v/v, one time; and 85:15, v/v, one time); evaporation and precipitation of this material afforded 0.13 g of pentanucleotide. This portion along with the 0.73-g portion from above was 1) combined; 2) deacetylated; 3) precipitated to give 0.76 g of product; and 4) subjected to hplc purification. A portion of the pentanucleotide (1.00 g) was dissolved in 0.1 M TEAA (pH 7.0)/ethanol (1:1, v/v) and separated in seven injections using a Bondapak C₁₈/Porasil B column (183 × 0.7 cm). The tracing from one injection is shown in Fig. 9A. The effluent corresponding to Peak I contained the desired d[(MeOTr)bzA-anC-ibG-T-T-ibG-bzA]. Concentrations of this effluent followed by precipitation afforded the pentanucleotide (0.68 g, 0.28 mmol). Its spectral and chromatographic properties are given in Table I. Fig. 9B shows a tracing from the hplc analysis of this compound.

The Heptanucleotide, d[(MeOTr)bzA-anC-ibG-T-T-ibG-bzA]—An anhydrous pyridine solution (10 μl) of d[(MeOTr)bzA-anC-ibG-T-T-ibG-bzA] (0.48 g, 0.2 mmol), d[pibG-bzA(Ac)] (0.84 g, 0.75 mmol), and TPS (0.92 g, 3.05 mmol) was allowed to react at room temperature for 5½ h. After the reaction was quenched by the standard procedure, the mixture was concentrated in the presence of pyridine and precipitated in the usual manner to yield 0.97 g of dinucleotide, d[pibG-bzA-bzA] (46 mg, 0.67 μmol), d[pibG-bzA-bzA(Ac)] (160 mg, 130 μmol) and TPS (182 mg, 600 pmol) was allowed to react at room temperature for 5½ h. After the reaction was quenched by the standard method, the mixture was 1) subjected to the general extraction procedure; 2) precipitated; and 3) subjected to hplc separation (two injections). The tracing from one injection is shown in Fig. 11A. The effluent corresponding to Peak I contained the trimetide, while that corresponding to Peak II contained the nona- and dodecanucleotide. After concentration and precipitation, the pure trimetide, d[pbzA-bzA-bzA(Ac)] (90 mg, 73 μmol), was isolated in 59% of theoretical recovery. The fraction containing the 5'-O-methoxymethoxylated material (41 mg) was further characterized by analytical hplc (Fig. 11B).

The Nonanucleotide, d[(MeOTr)bzA-anC-ibG-T-T-ibG-bzA-bzA]—An anhydrous pyridine solution (5 μl) of d[(MeOTr)bzA-anC-ibG-T-T-ibG-bzA] (207 mg, 62 pmol, see above), d[pibG-bzA(Ac)] (441 mg, 400 μmol), and TPS (485 mg, 1.6 mmol) was allowed to react at room temperature for 5½ h. After the reaction was quenched the mixture was subjected to the general extraction procedure (see “Materials and Methods”) to afford a precipitate (726 mg) which was a mixture of nucleotidic material and chloride salts. After hydrolysis, the dinucleotide was separated from the pentanucleotide (Fig. 10B). Each fraction of the partially purified heptanucleotide was separately deacetylated and precipitated to afford 45 mg, 207 mg, and 89 mg, respectively, of product. The 207 mg fraction had the ratio of ε₂₆₀/ε₂₄₀ = 1.00 and ε₂₄₀/ε₂₀₀ = 1.95.

The Unprotected Dodecanucleotide, d(A-C-G-T-T-G-A-G-A-A-G)—A portion (7 mg) of the mixture of nona- and dodecanucleotide was completely deprotected as described under “Discussion of Methods” and then chromatographed on DEAE-cellulose in the presence of 7 M urea (Fig. 12). Fracions 231 to 244 (Peak I) which contained the desired dodecanucleotide were pooled and then desalted on a short DEAE-cellulose column. The eluant of this column was lyophilized to afford the unprotected dodecanucleotide (14.6 A₂₆₀, 98 mmol). Its spectral properties are given in Table I. The dodecanucleotide was phosphorylated with [γ-³²P]ATP and T₄-polynucleotide kinase, partially digested with snake venom phosphodiesterase and analyzed by two-dimensional homochromatography. The fingerprint was consistent with the nucleotide sequence (Fig. 13).

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