glycosyl conformation in solution. However, bulky groups such as methyl at the 6 position shift the torsional angle into the syn range, as does a 5,6-fused benzene ring. Vicinal H–H and ^{13}C–H coupling constants are supportive of the assigned glycosyl preferences. The relative proportion of syn and anti conformers appears to be about the same in DMSO as in water, although the torsional angle may be altered. The population distribution of conformers is relatively unchanged by temperature alteration.

Acknowledgment. We thank Dr. George Kreishman for stimulating discussions and several ^{13}C spectra.

Synthesis of Phage Specific Deoxyribonucleic Acid Fragments. I. Synthesis of Four Undecanucleotides Complementary to a Mutated Region of the Coat Protein Cistron of fd Phage Deoxyribonucleic Acid

Herbert Schott* and Hans Kössel

Contribution from the Institut für Biologie III (Genetik, Molekularbiologie und Biophysik), University of Freiburg, Freiburg, West Germany.
Received December 4, 1972

Abstract: A general approach for the production of synthetic mutations in known sequences of circular single-stranded DNA by chemically prepared mutated oligonucleotides, used as primers, is proposed. In order to test this approach, synthesis of the four undecanucleotides dA-C-C-A-T-T-C-A-N-G-C (N = A, C, G, or T), corresponding to mutated minus strand fragments of a region of the coat protein cistron of fd phage DNA has been undertaken. In this sequence thymidylic acid in the sixth position replaces a cytidylic acid residue of the corresponding wild type sequence (N refers only to the degenerate position of an alanine codon). The octanucleotide sequence dA-C-C-A-T-T-C-A, common to all four undecanucleotides, was prepared in the protected form using protected dinucleotide blocks as intermediates for condensation reactions in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride. Individual reactions of the protected octanucleotide with one of the protected trinucleotides, dpbzA-ibuG-anC(Ac), dpanC-ibuG-anC(Ac), dpibuG-ibuG-anC(Ac), and dpT-ibuG-anC(Ac), yielded the four desired undecanucleotides. A new technique is described for the column chromatographic separation of trityl-containing nucleotide blocks or intermediates from compounds carrying N-protecting groups only: trityl-free compounds are eluted from DEAE-cellulose by using salt gradients in the presence of 20% methanol, whereas subsequent elution of the more lipophilic tritylated blocks or reaction products is achieved by salt gradients in the presence of 50% ethanol. Application of this technique is essential for the separation of protected tetra- and hexanucleotides from unutilized dinucleotides applied in excess and from the respective symmetric pyrophosphates. Unutilized blocks recovered by this method can also be reutilized for subsequent condensation steps without further purification.

Induction of mutations by chemical reagents or by irradiation in general is based on random processes in respect to the DNA or RNA regions involved, whereby the populations to be mutagenized usually become diminished by several orders of magnitudes. It seems therefore desirable to search for techniques, by which mutagenesis is achieved exclusively at small defined regions or even at distinct single base pairs without inactivation of the rest of the respective genoms.

In order to test the possibility of inducing specific mutations *in vitro* in circular single-stranded DNA by the use of synthetic mutated oligonucleotides acting as primers, synthesis of the four fd phage specific undecanucleotides dA-C-C-A-T-T-C-A-N-G-C (N = A, C, G, or T[1]) was undertaken. The selection of this sequence is based on the following considerations: from the amino acid sequence Ala-Trp-Met-Val, observed in the coat protein of the fd phage,[2] four possible undecanucleotide sequences of the corresponding mRNA and of the minus strand DNA can be deduced[3] (Chart I; the ambiguity in one position designated by N and M is due to the degeneracy of the alanine codons[3]). Conversion of the tryptophan codon UGG to the terminator codon UGA would be achieved by substitution of the cytidylic acid residue with a thymidylic acid residue in the sixth position of the complimentary undecadeoxynucleotide. Therefore one of the four oligomers synthesized should be

(1) The system of abbreviations is principally as has been suggested by the IUPAC–IUB commission published in *Eur. J. Biochem.*, **15**, 203 (1970). In this paper the prefix d (for deoxy) in all cases refers to the entire nucleoside residues of the oligonucleotide chains described; for clarity therefore brackets and hyphens usually following the prefix d are always omitted; thus brackets are only used to mark hydroxyl protecting groups. DCC, MS, and TPS refer to *N,N'*-dicyclohexylcarbodiimide, mesitylenesulfonyl chloride and 2,4,6-triisopropylbenzenesulfonyl chloride, respectively. One A_{260} unit is defined as the amount of nucleotide giving an absorbance of 1 at 260 nm when dissolved in 1 ml of solvent and measured in a 1-cm light path quartz cell. Different wavelengths are indicated by the respective subscripts.

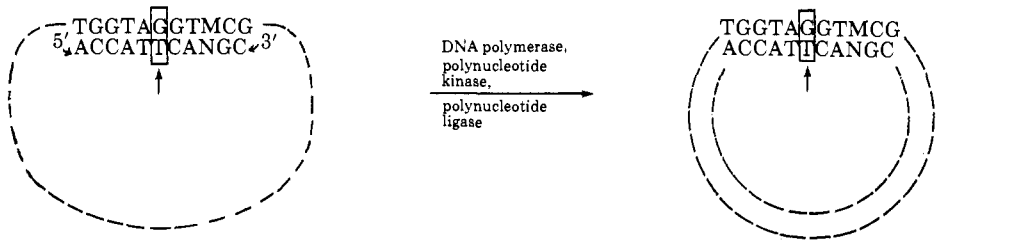
(2) F. Asbeck, K. Beyreuther, H. Köhler, G. v. Wettstein, and G. Braunitzer, *Hoppe-Seyler's Z. Physiol. Chem.*, **350**, 1047 (1969).

(3) (a) M. Nirenberg, T. Caskey, R. Marshall, R. Brimacombe, D. Kellog, B. Doctor, D. Hatfield, J. Levin, F. Rottman, S. Pestka, M. Wilcox, and F. Anderson, *Cold Spring Harbor Symp. Quant. Biol.*, **31**, 11 (1966); (b) H. G. Khorana, H. Büchi, H. Ghosh, N. Gupta, T. M. Jacob, H. Kössel, R. Morgan, S. A. Narang, E. Ohtsuka, and R. D. Wells, *ibid.*, **31**, 39 (1966).

Chart I[6]

H_2N	-------	Ala	Trp	Met	Val ---	COOH	coat protein
5′	-------	GCM	UGG	AUG	GU ---	3′	mRNA
3′	-------	CGN	ACC	TAC	CA ---	5′	DNA, minus strand
		CGN	ACT	TAC	CA		mutated DNA fragments synthesized
Point of mutation →							
5′	-------	GCM	UGA	UG	AUG ---	3′	mutated mRNA
H_2N	-------	Ala	Term.				coat protein fragment

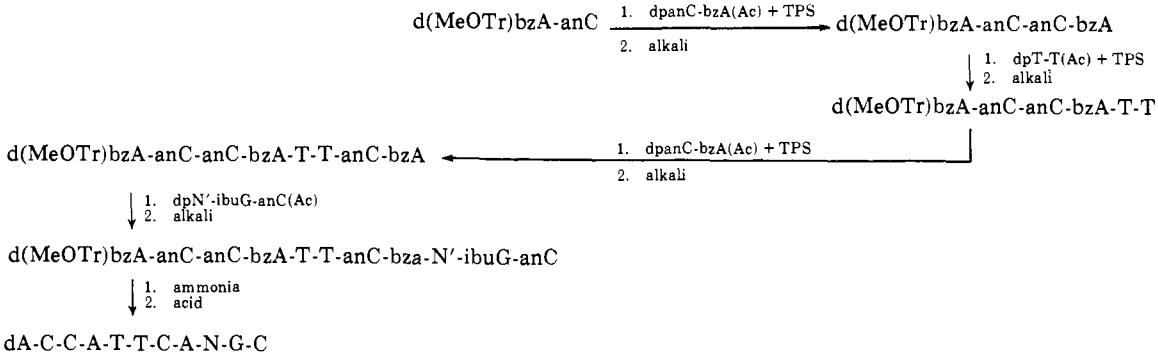
Chart II[6]



Heteroduplex resulting from hybridization of wild type circular fd DNA plus strand with the mutated synthetic minus strand DNA fragment; the nonclassical base pair arising from the mutation in the primer oligonucleotide is indicated by an arrow

Heteroduplex between wild type circular fd DNA plus strand and mutated circular fd DNA minus strand

Chart III.[9] Synthesis of the Undecanucleotides dA-C-C-A-T-T-C-A-N-G-C



able to form a partial heteroduplex structure with the phage DNA plus strand according to Chart II; such a structure could then be complemented to the fully double stranded heteroduplex by the combined action of DNA polymerase, polynucleotide kinase, and polynucleotide ligase according to the general methodology developed by Goulian, *et al.*[4] Subsequent strand separation would yield (besides the wild type plus strand DNA) a mutated minus strand DNA, and mRNA transcribed from the mutated DNA would code in suppressor free systems for a coat protein fragment only, whereas in opal suppressor containing systems production of full length wild type protein would be expected.

The approach, outlined here for the specific induction of a terminator codon, should be generally applicable for stepwise conversions of any known sequences derived from circular single-stranded DNA species; hence substitution of one amino acid by another as well as conversion of base triplets coding for the same amino acid into each other should be possible, as long as the mismatched base pairs do not interfere with the formation of the respective heteroduplex structures.

Preliminary experiments[5] indicate that the oligonucleotides described here serve as specific primers for DNA polymerase when circular plus strand DNA from fd phage is used as template.

Synthesis of the Undecanucleotides dA-C-C-A-T-T-C-A-N-G-C[6] **(N = A, C, G, and T).** The general methodology of connecting preformed oligonucleotide blocks by condensation reactions in the presence of aromatic sulfonyl chlorides[7,8] was applied for the synthesis of the four undecanucleotides. As indicated in Chart III, the octanucleotide sequence dA-C-C-A-T-T-C-A common to all four undecanucleotides was first synthesized in the protected form by using two kinds of protected dinucleotide blocks for the stepwise addition onto the 3′ end of a protected dinucleoside–phosphate. In four individual reactions the protected octanucleotide was then condensed with the four protected trinucleotides dpN′-ibuG-anC(Ac)[9] to yield the four undecanucleotides in the protected form, which were then converted to the free form by

(4) M. Goulian, A. Kornberg, and R. L. Sinsheimer, *Proc. Nat. Acad. Sci. U. S.*, **58**, 2321 (1967); M. Goulian, *ibid.*, **61**, 284 (1968).

(5) S. Kühn, H. Schott, and H. Kössel, unpublished results.

(6) M and N refer to the four possible complementary standard nucleotides corresponding to the degenerate position of the alanine codon.

(7) H. Kössel, M. W. Moon, and H. G. Khorana, *J. Amer. Chem. Soc.*, **89**, 2148 (1967); J. Hachmann and H. G. Khorana, *ibid.*, **91**, 2749 (1969).

(8) (a) H. Kössel, H. Büchi, and H. G. Khorana, *ibid.*, **89**, 2185 (1967); (b) E. Ohtsuka and H. G. Khorana, *ibid.*, **89**, 2195 (1967).

(9) N refers to the four deoxynucleosides A, C, G, and T, respectively. N′ designates deoxythymidine and the three *N*-acyl-deoxynucleosides, bzA, anC, and ibuG, respectively.

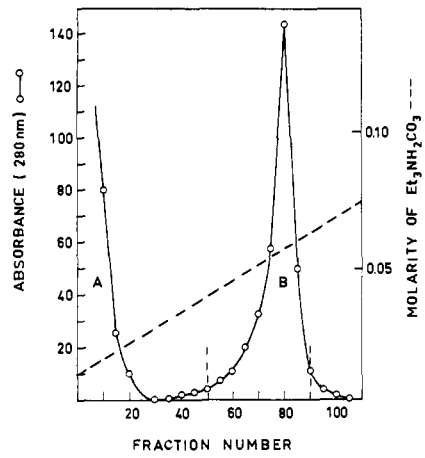


Figure 1. Chromatographic separation of the reaction products resulting from condensation of d(MeOTr)bzA with dpanC(Ac) on a DEAE-cellulose column in the presence of 90% methanol. Peak B contained the desired d(MeOTr)bzA-anC. For explanation, see text.

ammonia and mild acid treatment. The conditions and yields of the individual condensation steps (summarized in Table VI; see Experimental Section) essentially confirm the observations obtained previously[7,8] as concerns reaction times and amounts of condensing agent necessary for optimum yields. In addition, however, satisfactory yields in the syntheses of tetra-, hexa-, and octanucleotides were also obtained, when comparatively low excesses of dinucleotide blocks were reacted with the respective 3′-hydroxyl-bearing components. On the other hand, yields of the final reactions leading from the octanucleotide to the four undecanucleotides were low, which seems to be due (at least partly) to the low excesses and to impurities of the trinucleotide blocks applied. From the practical point of view it is noteworthy that the synthetic steps up to the octanucleotide were carried out with good yields on roughly 10- to 20-fold larger scale as compared with earlier work.[8,10]

Column Chromatographic Separation of Intermediates. Purification of the reaction products from starting material and other side products by preparative column chromatography on DEAE-cellulose becomes a problem if the compounds to be separated carry identical or similar net charges. Additional complications arise from the lipophilic interaction of protected oligonucleotides with the cellulose matrix.[8] As this interaction is favored in the presence of low alcohol content in the eluent, a retarding effect in such solvent is observed especially when tritylated compounds are chromatographed. This effect has often allowed satisfactory separation of trityl containing compounds from trityl free blocks even if both the compounds contained equal net charge (ref 8a and 11); on this basis, for instance, separation of tritylated protected tetranucleoside triphosphates from protected dinucleotide blocks has been possible[8a] although considerable trailing of the more lipophilic tetranucleotides into larger elution volumes had sometimes to be experienced. As, however, observed in the present study, trityl containing oligonucleotides as d(MeOTr)bzA-anC, d(MeOTr)bzA-anC-anC-bzA, and d(MeOTr)bzA-anC-anC-bzA-T-T, the lipophilic character of which is further increased by the presence of a comparatively large number of *N*-benzoyl and *N*-anisoyl groups, may not (or only with extreme trailing) be eluted from DEAE-cellulose unless 90% methanol (Figure 1) or at least 40–50% ethanol (Figures 2 and 3) are added to the elution buffers. Under these alcohol concentrations, on the other hand, the retarding effect of the trityl group is reduced to a minimum so that the two compounds d(MeOTr)bzA-anC-bzA and dpanC-bzA no longer could be separated by one column step (not shown). While problems of this kind, which are especially severe in the separation of protected oligonucleotides of lower chain lengths, can be solved by application of several successive column steps and/or by use of the recently developed trityl-cellulose,[12] a more simple solution is offered by the following new technique: the product mixtures resulting from the respective condensation reactions are first applied on DEAE-cellulose columns in the presence of 20% methanol and the trityl-free products (essentially the dinucleotide blocks given in excess, the corresponding symmetric pyrophosphates, and the hydrolysis products of TPS) are eluted first by linear buffer gradients containing 20% of the more hydrophilic methanol. After reduction of the buffer concentration to 0.04 *M*, a switch from 20% methanol to 50% of the more lipophilic ethanol is made and elution of the tritylated products is then achieved by a second buffer gradient in 50% ethanol. Thus, the difficult separation of the highly lipophilic d(MeOTr)bzA-anC-anC-bzA from dpanC-bzA (same net charge) and from the corresponding symmetric pyrophosphate could be performed in one column step (Figure 2); due to the absence of trailing at 50% ethanol, the two trityl-containing compounds d(MeOTr)bzA-anC and d(MeOTr)bzA-anC-anC-bzA could also be separated from each other with only negligible overlap of the two fractions. This simple procedure is also applicable to the purification of protected oligonucleotides of higher chain lengths as demonstrated in Figures 3 and 4. A protected hexanucleotide and a protected octanucleotide are sufficiently separated from the respective starting blocks and from other side products. Again the trityl-free dinucleotides, dpT-T and dpanC-bzA, and the corresponding symmetric pyrophosphates appear in the 20% methanol containing portion, whereas the tritylated tetra-, hexa-, and octanucleotides are eluted in the 50% ethanol containing buffers.

Besides the fact that the desired trityl compounds could be isolated by one column step in high purity, it is from the practical point of view also interesting that the recovered dinucleotide blocks were sufficiently pure for immediate reutilization. Thus the dinucleotide dpanC-bzA recovered from peak A of Figure 2 after acetylation could be reutilized in the condensation step leading from the protected hexa- to the protected octanucleotide (Figure 4) without making the reaction sluggish. Similarly, the dpT-T block when recovered from peak A of Figure 3 and used in repetitions of the same reaction gave satisfactory yields (see Table VI).

(10) A. Kumar and H. G. Khorana, *J. Amer. Chem. Soc.*, **91**, 2743 (1969).

(11) H. Büchi and H. G. Khorana, *J. Mol. Biol.*, in press.

(12) K. L. Agarwal, A. Yamazaki, P. J. Cashion, and H. G. Khorana, *Angew. Chem., Int. Ed. Engl.*, **11**, 451 (1972).

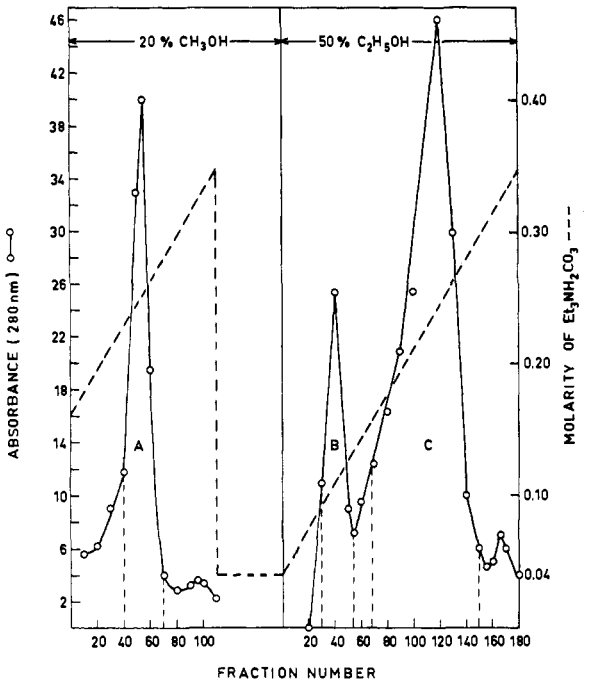


Figure 2. Chromatographic separation of the reaction products resulting from condensation of d(MeOTr)bzA-anC with dpanC-bzA(Ac) on a DEAE-cellulose column. Peak C contained the desired d(MeOTr)bzA-anC-anC-bzA. For explanation, see text.

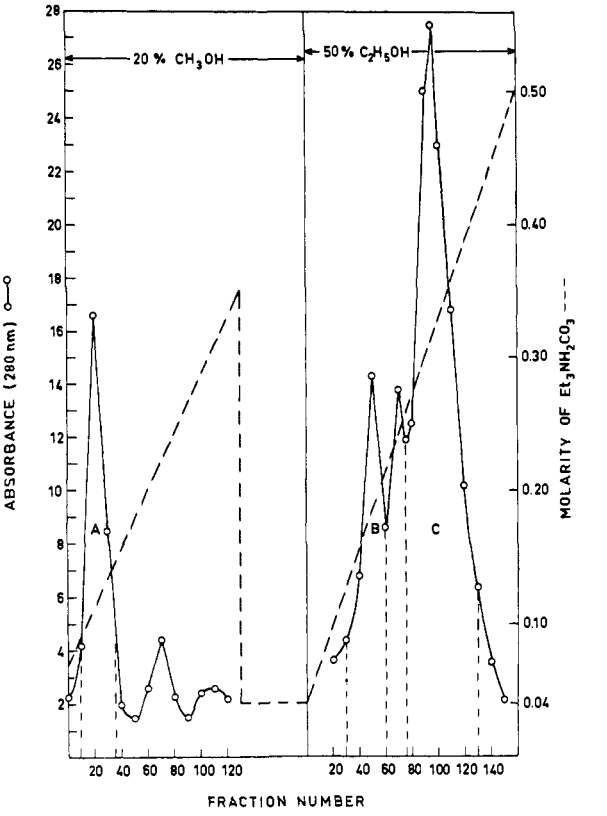


Figure 3. Chromatographic separation of the reaction products resulting from condensation of d(MeOTr)bzA-anC-anC-bzA with dpT-T(Ac) on a DEAE-cellulose column. Peak C contained the desired d(MeOTr)bzA-anC-anC-bzA-T-T. For explanation, see text.

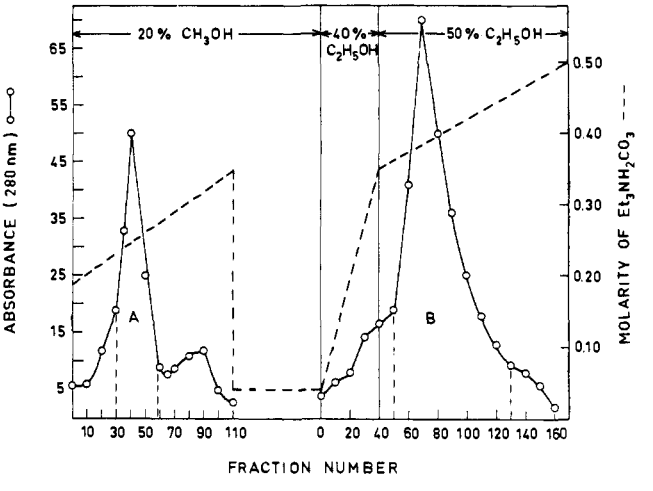


Figure 4. Chromatographic separation of the reaction products resulting from condensation of d(MeOTr)bzA-anC-anC-bzA-T-T with dpanC-bzA(Ac) on a DEAE-cellulose column. Peak B contained the desired d(MeOTr)bzA-anC-anC-bzA-T-T-anC-bzA. For explanation, see text.

Experimental Section

The protected nucleoside d(MeOTr)bzA[13] and the protected mononucleotides dpanC[14] and dpbzA[15] were prepared according to published procedures. dpibuG and dpibuG(IBu) were prepared essentially as described for the acetylated derivatives of guanylic acid.[16,8a] Dry pyridinium dpG (20 mmol) suspended in 200 ml of anhydrous pyridine was reacted with 100 ml of isobutyric anhydride for 3–7 days at room temperature until unreacted dpG was completely solubilized. Methanol (100 ml) was then added under cooling. After the mixture stood at room temperature for 5 hr, the total mixture was concentrated *in vacuo* to ~50 ml of dry pyridine and precipitated into ~1000 ml of dry ether as usual. Thorough washing of the precipitate with dry ether was followed by a second precipitation under identical conditions.

General Method of Condensation and Isolation. Condensations to form internucleotide bonds were carried out according to the following general procedure. Both the nucleotidic reaction components (the compound carrying the free 5′-phosphate group generally being in excess) were dissolved in dry pyridine, and the mixtures were rendered anhydrous by repeated evaporation *in vacuo* in the presence of an excess of dry pyridine. During the last evaporation the minimal amount of pyridine (=reaction volume) necessary for complete solubilization of the reaction components was allowed to remain, whereupon the condensing agents (DCC, MsCl, or TPS) were added to the reaction vessels inside a drybox. When DCC was applied as activating agent small amounts of H-Dowex 50X8 ion exchange resin were also added. The closed reaction vessels were then shaken at room temperature for 3–4 hr (MsCl), 3–10 hr (TPS), or 72–96 hr (DCC). The reactions were terminated by cooling the reaction flasks in an ice bath and adding volumes of 30% aqueous pyridine equal to the total volume of the reaction mixtures. The solutions were then kept at 4° for 2–12 hr. Removal of 3′-*O*-acetyl and 5′-cyanoethyl groups was achieved by treatment with 1 *N* NaOH at 0° for 5–10 min followed by neutralization with excess H⁺-Dowex 50X8 resin; after removal of the resin by filtration the combined filtrates and washings were adjusted to 0.04–0.1 *M* $Et_3NH_2CO_3$ and 20% methanol and then applied onto appropriately sized DEAE-cellulose columns equilibrated with 0.04 *M* $Et_3NH_2CO_3$ in 20% methanol. In all cases column chromatography was carried out at 4°. The columns were first washed with water containing 20% methanol, until the eluates were free from pyridine. Gradients of $Et_3NH_2CO_3$ (pH 7.5–7.9) were then applied, and 15–20-ml fractions were collected at flow rates in the range of 2–6 ml/min. Eluates were monitored by automatic uv recorders and/or by measurement of the absorbancy of individual fractions in a Zeiss spectrophotometer. The fractions containing the desired products or unutilized starting material were combined

(13) H. Schaller, G. Weimann, B. Lerch, and H. G. Khorana, *J. Amer. Chem. Soc.*, **85**, 3821 (1963).
(14) H. G. Khorana, A. F. Turner, and J. P. Vizsolyi, *ibid.*, **83**, 686 (1961).
(15) R. K. Ralph and H. G. Khorana, *ibid.*, **83**, 2926 (1961).
(16) R. K. Ralph, W. J. Connors, H. Schaller, and H. G. Khorana, *ibid.*, **85**, 1983 (1963).

Table I. Calculated and Observed Ultraviolet Characteristics of Protected Oligonucleotides[a]

Compd	λ_{max}, nm	λ_{min}, nm	Absorbance ratio 260/280 Calcd	Absorbance ratio 260/280 Obsd	Absorbance ratio 280/300 Calcd	Absorbance ratio 280/300 Obsd
dpT-ibuG	262	227	1.43	1.45	3.7	3.2
dpT-anC	302	296	0.84	0.95	1.09	1.11
	273	235				
dpanC-bzA	283	238	0.66	0.64	1.28	1.39
dpanC-ibuG	284	270	1.01	0.98	1.09	1.15
	262	233				
dpbzA-ibuG	277	267	0.92	0.99	2.83	2.60
	258	230				
dpibuG-ibuG	257	228	1.45	1.49	2.50	2.10
dpbzA-ibuG-anC	280	267	0.84	0.87	1.45	1.47
	262	238				
dpT-ibuG-anC	262	234	1.08	1.14	1.31	1.21
dpibuG-ibuG-anC	258	234	1.14	1.17	1.29	1.17
dpanC-ibuG-anC	263	237	0.90	0.98	0.95	1.13
d(MeOTr)bzA-anC	283	255	0.66	0.66	1.28	1.40
d(MeOTr)bzA-anC-anC-bzA	283	255	0.66	0.74	1.28	1.22
d(MeOTr)bzA-anC-anC-bzA-T-T	279	243	0.76	0.79	1.49	1.43
d(MeOTr)bzA-anC-anC-bzA-T-T-anC-bzA	279	240	0.72	0.73	1.22	1.20

[a] Measurements taken in 0.04 *M* $Et_3NH_2CO_3$ in 20% methanol (pH 7.2–7.6).

Table II. Calculated and Observed Ultraviolet Characteristics of Unprotected Oligonucleotides[a]

Compd	λ_{max}, nm	λ_{min}, nm	Absorbance ratio 250/260 Calcd	Absorbance ratio 250/260 Obsd	Absorbance ratio 260/280 Calcd	Absorbance ratio 260/280 Obsd
dpT-T	266	234	0.65	0.66	1.38	1.49
dpT-C	267	236	0.73	0.74	1.18	1.22
dpT-G	256	228	0.91	0.96	1.42	1.44
dpC-A	262	228	0.81	0.84	2.39	2.39
dpC-G	255	225	1.00	1.00	1.27	1.25
dpA-G	257	233	0.93	0.95	2.66	2.33
dpG-G	253	223	1.11	1.12	1.46	1.56
dpA-G-C	257	227	0.94	0.93	2.04	2.18
dpT-G-C	258	230	0.89	0.94	1.28	1.36
dpG-G-C	253	229	1.04	1.05	1.31	1.26
dpC-G-C	257	229	0.95	0.99	1.16	1.25
d(MeOTr)A-C	261	227	0.81	0.86	2.39	2.34
dA-C	260	228	0.81	0.82	2.39	2.4
d(MeOTr)A-C-C-A	261	228	0.81	0.83	2.39	2.18
dA-C-C-A	262	228	0.81	0.82	2.39	2.38
d(MeOTr)A-C-C-A-T-T	262	228	0.76	0.74	1.99	1.95
dA-C-C-A-T-T	261	230	0.76	0.78	1.99	1.96
d(MeOTr)A-C-C-A-T-T-C-A	262	232	0.77	0.80	2.07	1.93
dA-C-C-A-T-T-C-A	260	230	0.77	0.81	2.07	2.14
dA-C-C-A-T-T-C-A-T-G-C	258	230	0.79	0.81	1.79	1.70
dA-C-C-A-T-T-C-A-A-G-C	265	230	0.81	0.82	2.04	2.04
dA-C-C-A-T-T-C-A-C-G-C	263	230	0.82	0.84	1.60	1.80
dA-C-C-A-T-T-C-A-G-G-C	259	230	0.85	0.87	1.81	1.82

[a] Measurements taken in 0.1 *M* Tris buffer (pH 7.0).

and concentrated *in vacuo* in the presence of pyridine in order to remove water and $Et_3NH_2CO_3$ buffer. The residual material in the case of protected di- and trinucleotides was dissolved in small volumes of dry pyridine (~1 mmol/5 ml) and precipitated by dropwise addition into 20–40-fold excesses of dry ether under stirring. Protected nucleotides larger than trinucleotides were frequently stored in dry pyridine at −20° for several weeks, whereby no significant decomposition was experienced. The yields of condensation reactions were calculated neglecting the hypochromicity of the products in solution. The molar extinction values employed in neutral solution at 260, 280, and 300 nm, respectively, were as follows:[10] T (8800, 6400, 200); dpanC (12,600, 17,700, 22,300); dpbzA (10,800, 18,300, 5900); dibuG (16,700, 11,500, 4000). Uv characteristics of protected and unprotected di- and trinucleotides are listed in Tables I and II. Removal of protecting groups was achieved by standard procedures.[8]

Identification of Oligonucleotides. For identification and characterization of the compounds from various fractions, paper chromatography was performed at room temperature by the descending technique using Schleicher & Schüll papers 2043a and 2043b in the following solvent systems: solvent A, ethanol–1 *M* ammonium acetate (pH 7.5) (7:3, v/v); solvent B, 1-propanol–concentrated ammonia–water (55:10:35, v/v/v); solvent C, isobutyric acid–concentrated ammonia–water (66:1:33, v/v/v). Purity of the nucleotidic compounds was also routinely checked by paper chromatography in the same systems after the N-protecting groups had been removed by ammonia treatment. In the case of trityl containing compounds, paper chromatography was performed after ammonia treatment as well as after ammonia and acid treatment. Usually less than 10% of impurities could be observed in the partially or fully unprotected compounds unless otherwise mentioned. A summary of paper chromatographic characteristics of the various compounds is given in Tables III and IV.

Base compositions of the unprotected oligonucleotides (after removal of 5′-phosphomonoester groups by alkaline phosphatase in the case of trinucleotides) were determined by enzymatic digestion in the presence of venom or spleen phosphodiesterase.[14] The degradation products were separated by paper chromatography in

Table III. Paper Chromatographic Characteristics of Protected Oligonucleotides in Solvent A

Compound	R_f	R_f relative to pT
dpT-ibuG	0.44	1.16
dpT-anC	0.29	
dpanC-bzA	0.40	
dpanC-ibuC	0.53	1.3
dpbzA-ibuG		1.15
dpibuG-ibuG	0.51	1.35
dpT-T(Ac)		1
dpT-anC(Ac)	0.42	
dpanC-bzA-(Ac)	0.53	
dCNEt-pT-ibuG	0.64	1.63
dCNEt-panC-ibuG		1.83
dCNEt-pbzA-ibuG		1.75
dCNEt-pibuG-ibuG	0.75	1.88
dpbzA-ibuG-anC	0.19	0.74
dpT-ibuG-anC	0.26	0.90
dpibuG-ibuG-anC	0.30	1.11
dpanC-ibuG-anC		0.92
dpbzA-ibuG-anC(Ac)	0.24	0.78
dpT-ibuG-anC(Ac)	0.31	0.93
dpibuG-ibuG-anC(Ac)	0.35	1.15
dpanC-ibuG-anC(Ac)		0.96
d(MeOTr)bzA-anC	0.81	
d(MeOTr)bzA-anC-anC-bzA	0.61	0.86[a]
d(MeOTr)bzA-anC-anC-bzA-T-T	0.29 0.83[a]	0.93
d(MeOTr)bzA-anC-anC-bzA-T-T-anC-bzA		0.78

[a] Solvent B.

Table IV. R_f Values of Unprotected Oligonucleotides

Compd	—Solvent B— I	II[b]	—Solvent C— III[c]	II[b]
dpA-G-C	0.25	0.56		
dpT-G-C		0.71		
dpG-G-C	0.13	0.36		
dpC-G-C	0.23	0.66		
dA-G-C		0.32[a]		
dT-G-C	0.22[a]	0.58[a]		
dG-G-C	0.34	0.72		
dC-G-C	0.46			
d(MeOTr)A-C-C-A	0.27[a]	1.01[a]		
	0.75	1.62		
dA-C-C-A	0.40	0.83		
d(MeOTr)A-C-C-A-T-T	0.6	1.32		
dA-C-C-A-T-T		0.58		
d(MeOTr)A-C-C-A-T-T-C-A	0.39	0.97		
dA-C-C-A-T-T-C-A		0.34		1.04
d(MeOTr)A-C-C-A-T-T-C-A-G-G-C	0.27			
dA-C-C-A-T-T-C-A-G-G-C		0.15	0.24	0.38
d(MeOTr)A-C-C-A-T-T-C-A-C-G-C	0.18			
dA-C-C-A-T-T-C-A-C-G-C			0.38	0.65
dA-C-C-A-T-T-C-A-T-G-C			0.27	0.41
dA-C-C-A-T-T-C-A-A-G-C		0.09	0.19	0.48

[a] Solvent A. [b] R_f values relative to pT. [c] R_f values relative to d(A-C-C-A-T-T-C-A).

system B or by high voltage paper electrophoresis in pH 3.5 buffer containing 10.2 g of ammonium acetate and 9.0 ml of glacial acetic acid dissolved in 90 ml of water. Estimation of the compounds from the individual spots was carried out spectrophotometrically after elution from paper with 1% aqueous ammonia. The results of the enzymatic degradation are listed in Table V. The 3'-terminal end groups of unprotected octa- and undecanucleotides were identified as A or C, respectively, by the terminal addition of [α-^{32}P]ribonucleoside triphosphates as described earlier.[17] The sequence of the undecanucleotide dA-C-C-A-T-T-C-A-T-G-C could also be confirmed by partial sequence determination.[18]

β-Cyanoethylation of the 5'-Phosphomonoester Groups. To anhydrous mixtures of the pyridinium salts of mono- or dinucleotides 30–70-fold molar excesses of β-hydracrylonitrile (CNET), pyridine (once to twice the volume of β-hydracrylonitrile), and 10–20-fold molar excesses of DCC (in respect to monophosphate groups) were added. The resulting suspensions were shaken in the dark at room temperature for 12–24 hr. After addition of water (2 volumes) the aqueous suspensions were shaken for an additional 6–12 hr. The mixtures were then filtered and the insoluble residues washed with 50% aqueous pyridine. To the combined filtrates and washings triethylamine was added until the pH reached a value of 9.0, whereupon the reaction mixtures were kept at this pH for 4 hr at room temperature. The products were finally purified by column chromatography on DEAE-cellulose. For the separation of 0.3–1.5 mmol of cyanoethylated dinucleotides, 350–600-ml columns were used. After the columns were washed with 0.01 M $Et_3NH_2CO_3$ in 20% methanol until the eluates were free from pyridine, they were eluted with linear gradients of 0.04–0.35 M $Et_3NH_2CO_3$ in 20% methanol (2–4 l. total volume). Further work-up of the product-containing fractions and conversion into the pyridinium forms was as usual. Storage in dry pyridine at −20° caused no detectable loss of protecting groups even during a period of several weeks.

Preparation of Protected Dinucleotide Blocks. Mixtures of cyanoethylated 3'-OH-bearing mononucleotides (1–3 mmol) and acetylated 5'-phosphate-bearing mononucleotides (2–5 mmol) were allowed to react under condition of the general methods as described. After the standard work-up including alkali treatment, the aqueous pyridine solutions were applied to DEAE-cellulose columns (3–4.5 cm/40–65 cm). Columns of 600 to 800 ml were generally used for the separation of 15–25 mmol of total nucleotidic compounds plus sulfonic acid and chloride ions derived from the activating agents MsCl and TPS. In some cases two separations were necessary for the final purification of the reaction products. After the columns were washed with 0.04 M $Et_3NH_2CO_3$ in 20% methanol until the eluates were free from pyridine, the columns in all cases were eluted using linear gradients of 0.04–0.35 M $Et_3NH_2CO_3$ in 20% methanol (4 l. total volume). Further work-up of the product-containing fractions and conversion into the pyridinium forms was as usual. The individual products were characterized by uv spectrophotometry (Tables I and II), by paper chromatography (Tables III and IV), and, after the removal of the protecting groups, by enzymatic digestion. dpT-T, dpT-anC, and dpanC-bzA were finally precipitated from dry pyridine solutions into large excesses of dry ether, redissolved in dry pyridine, and acetylated as described.[8] The dinucleotides dpbzA-ibuG, dpanC-ibuG, dpibuG-ibuG, and dpT-ibuG were cyanoethylated according to the general method.

Preparation of Protected Trinucleotide Blocks dpN'-ibuG-anC(Ac).[9] Mixtures of the cyanoethylated 3'-OH-bearing dinucleotides (0.15–0.48 mmol) and of dpanC(Ac) (1.0–3.3 mmol) were condensed under the general conditions described above. After the standard work-up including alkali treatment, the aqueous pyridine solutions were subjected to chromatography on DEAE-cellulose columns. Columns of 350 to 600 ml were generally used for the separation of 6–18 mmol of total nucleotidic compounds plus sulfonic acid and chloride ions derived from the activating agent TPS. After the columns were washed with 0.04 M $Et_3NH_2CO_3$ in 20% methanol until the eluates were free from pyridine, the columns were eluted using linear gradients of 0.04–0.35 M $Et_3NH_2CO_3$ buffer in 20% methanol (2.0–4.4 l. total volume). The combined fractions containing the trinucleotides were worked up as usual and the compounds after conversion to the pyridinium salts and dissolution in small volumes of dry pyridine were finally precipitated by dropwise addition into large excesses of dry ether and stored as dry powders in a vacuum desiccator. Due to incomplete separation, the trinucleotides contained up to 20% of the respective unreacted dinucleotides in terms of absorbancy units. 3'-O-Acetylation of the trinucleotides was carried out essentially as described earlier.[8]

Preparation of d(MeOTr)bzA-anC. Dry pyridine (10 ml) was added to a mixture of d(MeOTr)bzA (3 mmol), dpanC(Ac) (2 mmol), and Dowex 50-X8 ion exchange resin (4.5 g). The resulting suspension was rendered anhydrous by repeated evaporation in the

(17) H. Kössel and R. Roychoudhury, *Eur. J. Biochem.*, **22**, 271 (1971).

(18) H. Schott, D. Fischer, and H. Kössel, manuscript in preparation.

Table V. Characterization of Oligonucleotides by Enzymic Degradation

Compd	Enzyme	A_{260} units degraded	Degradation products	Theoretical ratio	Obsd ratio
dA-G-C	Spleen	5.4	Ap, Gp, C	1:1:1	1:0.93:1.1
dT-G-C	Spleen	5.1	Tp, Gp, C	1:1:1	0.9:0.9:1
dG-G-C	Spleen	2.9	Gp, C	2:1	2.02:1
dC-G-C	Spleen	5.7	Cp, Gp, C	1:1:1	1:1:0.98
dA-C-C-A	Venom	5.6	A, pC, pA	1:2:1	1:1.95:0.95
dA-C-C-A	Spleen	2.2	Ap, Cp, A	1:2:1	1.08:2.08:1
dA-C-C-A-T-T	Spleen	8.9	Ap, Cp, Tp, T	2:2:1:1	1.98:2.05:1.03:1
dA-C-C-A-T-T-C-A	Venom	6.8	A, pC, pT, pA	1:3:2:2	1:2.86:2.09:1.92
dA-C-C-A-T-T-C-A	Spleen	6.1	Ap, Cp, Tp, A	2:3:2:1	2.06:3.01:62:0.72
dA-C-C-A-T-T-C-A-T-G-C	Venom	9.1	A, pC, pT, pA, pG	1:4:3:2:1	1:4.3:2.97:2.15:0.86
dA-C-C-A-T-T-C-A-A-G-C	Venom	9.2	A, pC, pT, pA, pG	1:4:2:3:1	1:4.12:1.96:2.81:0.96
dA-C-C-A-T-T-C-A-C-G-C	Venom	9.4	A, pC, pT, pA, pG	1:5:2:2:1	1:4.93:2.01:1.8:1.17
dA-C-C-A-T-T-C-A-G-G-C	Venom	7.6	A, pC, pT, pA, pG	1:4:2:2:2	1:3.89:1.99:1.94:2.16

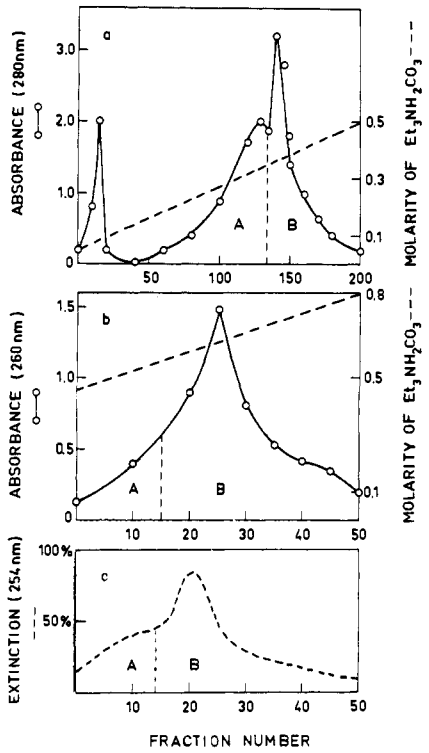


Figure 5. (a) Chromatographic separation of the reaction products resulting from condensation of d(MeOTr)bzA-anC-anC-bzA-T-T-anC-bzA with dpanC-ibuG-anC(Ac) on DEAE-cellulose column a. (b) Elution profile of the ammonia-treated products from section B of part a. (c) Elution profile of the acid-treated oligonucleotide from section B of part b.

presence of excess dry pyridine. After the final evaporation, 4 g of DCC (20 mmol) dissolved in 8 ml of anhydrous pyridine was added to the resulting gum and the solution was then shaken for 4 days at room temperature. After the reaction was stopped with 30% aqueous pyridine, the 3′-*O*-acetyl groups were removed by alkali treatment under standard conditions. After neutralization the solutions were concentrated to 40 ml, diluted with methanol to 400 ml, and subjected to DEAE-cellulose column chromatography (Figure 1) in two 200-ml portions. The column (100 cm × 2 cm) was eluted with a linear gradient using 1 l. of 0.01 *M* $Et_3NH_2CO_3$ in 90% methanol in the mixing vessel and 1 l. of 0.1 *M* $Et_3NH_2CO_3$ in 90% methanol in the reservoir. Fractions of 13 ml were collected every 6 min. Peak B contained the desired product, d-(MeOTr)bzA-anC. After pooling the material as shown by the vertical dotted lines of peak B, evaporation of the solvent and precipitation of the compounds were carried out according to the standard conditions. The yield of the isolated product ranged between 45 and 51%.

Preparation of d(MeOTr)bzA-anC-anC-bzA. An anhydrous mixture of 28,200 A_{300} units of d(MeOTr)bzA-anC (1 mmol) and of 28,200 A_{300} units of dpanC-bzA(Ac) (1 mmol) in dry pyridine (10 ml) was reacted with TPS (4 mmol) for 6 hr at room temperature. After the reaction was stopped and the 3′-*O*-acetyl groups were removed according to the general procedures, the resulting mixture (300 ml) was subjected to DEAE-cellulose column chromatography (Figure 2). The column (50 cm × 4.5 cm) was washed with 0.04 *M* $Et_3NH_2CO_3$ in 20% methanol until the eluates were free from pyridine. Then elution with a linear gradient was started using 2.1 l. of 0.04 *M* $Et_3NH_2CO_3$ in 20% methanol in the mixing vessel and 2.1 l. of 0.35 *M* $Et_3NH_2CO_3$ in 20% methanol in the reservoir. After passing 2.2 l. of the gradient, fractions of 20 ml were collected every 8 min. After elution with the remaining gradient mixtures the column was washed with 800 ml of 0.04 *M* $Et_3NH_2CO_3$ in 20% methanol and then eluted with a second linear gradient using 2 l. of 0.08 *M* $Et_3NH_2CO_3$ in 50% ethanol in the mixing vessel and 2 l. of 0.35 *M* $Et_3NH_2CO_3$ in 50% ethanol in the reservoir. Identification of the three main peaks indicated that peaks A, B, and C contained dpanC-bzA, d(MeOTr)bzA-anC, and the desired tetranucleotide d(MeOTr)bzAanC-anC-bzA, respectively. Material was pooled as shown by the vertical dotted lines.

Preparation of d(MeOTr)bzA-anC-anC-bzA-T-T. An anhydrous mixture of 20,000 A_{300} units of d(MeOTr)bzA-anC-anC-bzA (0.35 mmol) and 21,000 A_{260} units of dpT-T(Ac) (1.2 mmol) in dry pyridine (6 ml) was reacted with TPS (5 mmol) for 7 hr at room temperature. After the reaction was stopped and the 3′-*O*-acetyl groups were removed according to the general procedures, the resulting mixture (140 ml) was subjected to DEAE-cellulose column chromatography (Figure 3). The column (45 cm × 4.5 cm) was washed with 0.04 *M* $Et_3NH_2CO_3$ in 20% methanol until the eluates were free from pyridine. Then elution with a linear gradient was started using 2 l. of 0.04 *M* $Et_3NH_2CO_3$ in 20% methanol in the mixing vessel and 2 l. of 0.35 *M* $Et_3NH_2CO_3$ in 20% methanol in the reservoir. After passing 0.5 l. of the gradient, fractions of ~20 ml were collected every 6 min. After elution with the remaining gradient mixtures, the column was washed with 500 ml of 0.04 *M* $Et_3NH_2CO_3$ in 20% methanol and then eluted with an additional gradient using 1.8 l. of 0.04 *M* $Et_3NH_2CO_3$ in 50% ethanol in the mixing vessel and 1.8 l. of 0.5 *M* $Et_3NH_2CO_3$ in 50% ethanol, in the reservoir. Identification of the three main peaks indicated that peaks A, B, and C contained dpT-T, d(MeOTr)bzA-anC-anC-bzA, and the desired hexanucleotide d(MeOTr)bzA-anC-anC-bzA-T-T, respectively. Material was pooled as shown by vertical dotted lines.

Preparation of d(MeOTr)bzA-anC-anC-bzA-T-T-anC-bzA. An anhydrous mixture of 32,000 A_{300} units of d(MeOTr)bzA-anC-anC-bzA-T-T (0.56 mmol) and of 50,800 A_{300} units of dpanC-bzA (Ac) (1.8 mmol) in dry pyridine (10 ml) was reacted with TPS (8 mmol) for 8 hr at room temperature. After the reaction was stopped and the 3′-*O*-acetyl groups were removed according to the general procedures, the resulting mixture (220 ml) was subjected to DEAE-cellulose column chromatography (Figure 4). The column (50 cm × 4.5 cm) was eluted with a linear gradient using 2.2 l. of 0.04 *M* $Et_3NH_2CO_3$ in 20% methanol in the mixing vessel and 2.2 l. of 0.35 *M* $Et_3NH_2CO_3$ in 20% methanol in the reservoir. After passing 2 l. of the gradient, fractions of ~20 ml were collected every 13 min. After elution with the remaining gradient mixtures, the column was washed with 600 ml of 0.04 *M* $Et_3NH_2CO_3$ in 20% methanol and then eluted with a second gradient using 0.45 l. of 0.04 *M* $Et_3NH_2CO_3$ in 40% ethanol in the mixing vessel and 0.45 l. of 0.35 *M* $Et_3NH_2CO_3$ in 40% ethanol in the reservoir. After elution with the second gradient, the column was eluted with a third linear

Table VI. Summary of Conditions and Yields in the Synthesis of Protected Oligonucleotides

Condensation product	3-OH-bearing component (mmol)	5'-Phosphate-bearing Component (mmol)	Activating agent (mmol)	Pyridine, ml	Time, hr	Yield, %	Absorbancy units isolated in the fully protected form
d(MeOTr)bzA-anC, I	d(MeOTr)bzA	dpanC(Ac)	DCC				
	1 (3.5)	(2)	(20)	8	72	51	
	2 (6.5)	(4)	(40)	20	120	45	
d(MeOTr)bzA-anC-anC-bzA, II	I	dpanC-bzA(Ac)	TPS				
	1 (0.5)	(0.5)	(3)	5	4	35	
	2 (1.0)	(1.0)	(4)	10	6	47	
	3 (1.0)	(1.0)	(5)	10	6	57	32,000[a]
d(MeOTr)bzA-anC-anC-bzA-T-T, III	II	dpT-T(Ac)	TPS				
	1 (0.3)	(1.2)	(6)	5	6	58	
	2 (0.35)	(1.2)	(5)	6	7	65	13,000[a]
	3 (0.48)	(2.0)	(8)	8	7	67	
d(MeOTr)bzA-anC-anC-bzA-T-T-anC-bzA, IV	III (0.56)	dpanC-bzA(Ac) (1.8)	TPS (8)	10	8	70	33,500[a]
d(MeOTr)bzA-anC-anC-bzA-T-T-anC-bzA-T-ibuG-anC	IV (0.020)	dpT-ibuG-anC-(Ac) (0.040)	TPS (0.3)	1.5	10	10[b]	510[c]
d(MeOTr)bzA-anC-anC-bzA-T-T-anC-bzA-bzA-ibuG-anC	IV (0.015)	dpbzA-ibuG-anC(Ac) (0.030)	TPS (0.2)	1.5	10	6[b]	230[c]
d(MeOTr)bzA-anC-anC-bzA-T-T-anC-bzA-anC-ibuG-anC	IV (0.015)	dpanC-ibuG-anC(Ac) (0.020)	TPS (0.2)	1	10	6[b]	230[c]
d(MeOTr)bzA-anC-anC-bzA-T-T-anC-bzA-ibuG-ibuG-anC	IV (0.014)	dpibuG-ibuG-anC(Ac) (0.060)	TPS (0.3)	2	8	10[b]	380[c]

[a] At 300 nm. [b] Corrected for side products as octanucleotides and decanucleotides. [c] At 260 nm; contaminated up to 50% by the octanucleotide and by decanucleotides.

buffer gradient using 1.2 l. of 0.35 *M* $Et_3NH_2CO_3$ in 40% ethanol in the mixing vessel and 1.2 l. of 0.5 *M* $Et_3NH_2CO_3$ in 50% ethanol in the reservoir. Identification of the two main peaks indicated that peaks A and B contained dpanC-bzA and the desired octanucleotide d(MeOTr)bzA-anC-anC-bzA-T-T-anC-bzA, respectively. Material was pooled as shown by the vertical dotted lines.

Preparation of Protected Undecanucleotides. Anhydrous mixtures containing 1200–1700 A_{300} units of the protected octanucleotide d(MeOTr)bzA-anC-anC-bzA-T-T-anC-bzA (0.014–0.020 mmol) and 500–1800 A_{300} units of the respective protected trinucleotides (0.02–0.06 mmol) in dry pyridine (1–2 ml) were reacted with TPS (0.2–0.06 mmol) for 8–10 hr at room temperature (see Table VI). After the reaction was stopped according to the general procedures, the resulting mixtures were subjected to extensive DEAE-cellulose column chromatography and to paper chromatography. As an example, separation of d(MeOTr)bzA-anC-anC-bzA-T-T-anC-bzA-anC-ibuG-anC is shown in Figures 5A, B, and C. The first column (40 cm × 4 cm, Figure 5a) was eluted with a linear buffer gradient using 2 l. of 0.04 *M* $Et_3NH_2CO_3$ in 40% ethanol in the mixing vessel and 2 l. of 0.5 *M* $Et_3NH_2CO_3$ in 50% ethanol in the reservoir. Fractions of ~20 ml were collected every 12 min. Characterization of the two sections indicated that section A contained mostly trityl-negative products, whereas trityl-positive products were predominant in section B. The latter was pooled as shown by the vertical dotted lines and concentrated *in vacuo* to dryness. After removal of the N-protecting groups according to the general procedure, the resulting mixture was again subjected to DEAE-cellulose column chromatography according to Figure 5b. The column (40 cm × 4 cm) was eluted with a linear buffer gradient using 1.5 l. of 0.04 *M* $Et_3NH_2CO_3$ in 20% methanol in the mixing vessel and 1.5 l. of 0.8 *M* $Et_3NH_2CO_3$ in 20% methanol in the reservoir. Trityl-positive products from section B of Figure 5b were pooled and concentrated *in vacuo* to dryness. After removal of the monomethoxytrityl groups according to the general procedure, the resulting mixture was subjected to DEAE-cellulose column chromatography a third time (see Figure 5c). The column (40 cm × 3.5 cm) was eluted with a linear buffer gradient using 1 l. of 0.35 *M* $Et_3NH_2CO_3$ in 20% methanol and 1 l. of 0.9 *M* $Et_3NH_2CO_3$ in 10% methanol in the reservoir. Characterization of sections A and B indicated that A contained mainly octanucleotide whereas the desired undecanucleotide together with contaminating octa- and decanucleotides appeared in fraction B. After passing 1.8 l. of the gradient, fractions of ~16 ml were collected every 10 min. Similar elution profiles were obtained with all four of the undecanucleotides which were finally purified by paper chromatography in solvent systems B and C.

Acknowledgments. We are indebted to the Deutsche Forschungsgemeinschaft for support of the present work by grants and by awarding a research fellowship to one of us (H. S). Valuable technical help is credited to Ms. S. Baars. Helpful criticism of the manuscript by Drs. G. Feix and K. Hilse is gratefully acknowledged.