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Enzymatic synthesis of oligonucleotides of defined sequence: Synthesis of a segment of yeast *iso-1-cytochrome c* gene

(oligonucleotide synthesis/RPC-5 chromatography/DEAE-cellulose-urea chromatography)

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ABSTRACT **The deoxyribooligonucleotide, d(pT-T-A-G-C-A-G-A-A-C-C-G-G), constituting a segment of yeast *iso-1-cytochrome c* gene, has been synthesized by a combination of chemical and primarily enzymatic methods. The starting primer, d(pT-T-A-G), was chemically synthesized by the phosphodiester method and was extended stepwise, by reactions catalyzed by polynucleotide phosphorylase.**

We have previously reported the synthesis of some simple deoxyribooligonucleotides of defined sequences (1–4) by using polynucleotide phosphorylase (polyribonucleotide nucleotidyltransferase, polynucleotide:orthophosphate nucleotidyltransferase, EC 2.7.7.8) isolated from *Escherichia coli* B. The present study was undertaken with the objective of a simple enzyme procedure for the synthesis of a more complex deoxyribooligonucleotide of defined sequence. The resultant procedures have allowed the addition of nine nucleotides to a tetranucleotide primer. Consequently, this enzymic method provides a new route to deoxyribooligonucleotides of biological interest as exemplified by d(pT-T-A-G-C-A-G-A-A-C-C-G-G) which corresponds to a segment of the yeast *iso-1-cytochrome c* gene (5).

MATERIALS AND METHODS

Aminex A-5 resin was purchased from Bio-Rad Laboratories and Pellionex WAX from Reeve Angel. RPC-5 was donated by G. M. Tener, T4 polynucleotide kinase was a gift of Hans van de Sande. Polynucleotide phosphorylase (polynucleotide nucleotidyltransferase, polynucleotide:orthophosphate nucleotidyltransferase, EC 2.7.7.8) (10–25 units/mg) was isolated from *E. coli* B by the procedures developed by Hsieh (6) with additional chromatography on DEAE-Sephadex and Sephadex G-200 at the final stages of purification. Details of procedures will be published. One unit of enzyme catalyzes the incorporation of 1 nmol of dAMP into polymer in 1 min at 37°. Other chemicals and enzymes were the same as reported (2, 3).

Enzymatic Synthesis of Deoxyoligonucleotides. For each step of the synthesis, it was necessary to carry out a number of small scale reactions under different conditions in order to select the best conditions for the large scale synthesis. Considerable variability was found between optimum conditions for each step and details are presented under *Results*. In general, the reaction mixture contained 50 mM Tris·HCl at pH 8.5, deoxyribonucleoside diphosphate, primer, NaCl, 10 mM 2-mercaptoethanol, $MnCl_2$, and polynucleotide phosphorylase and was incubated at 37°. Samples were taken at various times and the extent of reaction was monitored on an analytical RPC-5 column. The reaction was either stopped by addition of EDTA (2) or continued if the yield of the product had not yet reached its maximum. The reaction mixture was then separated on a DEAE-cellulose column with a linear gradient of NaCl in 7 M urea and 0.04 M Tris·HCl at pH 7.5 (7).

Nucleoside Compositions by Liquid Chromatography. A jacketed stainless steel column (500 mm × 3.2 mm outer diameter/0.5 mm wall) was packed with Aminex A-5 cation exchange resin. The column was developed at 37° with 0.4 M ammonium formate at pH 4.5 with a flow rate of 14 ml/hr (8). The packed resin was maintained under a pressure of 55–70 atmospheres (1.013×10^5 pascal) with a Milton Roy Instrument Minipump and the eluate was monitored with an Altex analytical UV detector at 254 nm; full scale deflection was set for 0.005 absorbance units. A standard solution, 6 μl, containing 0.6 nmol each of dT, dG, dA, and dC was injected to calibrate the column for calculating nucleoside ratios. Areas of the symmetrical peaks were estimated by determining height × width at half-height. Nucleoside compositions were obtained by comparison of the areas under peaks for the digested samples with those given by the standards.

Analytical RPC-5 and Pellionex WAX Chromatography. The conditions used for RPC-5 were based on those of Egan (9) and Singhal (10). The column was developed with an ammonium acetate gradient (50 ml of 0.5 M ammonium acetate at pH 4.5, to 50 ml of 3 M ammonium acetate at pH 4.5), with a flow rate of 20 ml/hr. Dry Pellionex WAX was packed into a stainless steel column (500 mm) and equilibrated with 0.03 M ammonium acetate at pH 4.5. The column was eluted with a gradient of 0.03 M ammonium acetate at pH 4.5 (50 ml) to 50 ml of 0.1 M ammonium sulfate in 0.03 M ammonium acetate at pH 4.5 (50 ml). The flow rate was 60 ml/hr. Both columns were operated as described for Aminex A-5 except that they were run at room temperature with the UV detector set with full scale deflection at 0.01 absorbance units.

RESULTS AND DISCUSSION

Earlier studies (1–4) had shown that, with appropriate concentrations of $MnCl_2$ and NaCl, polynucleotide phosphorylase will catalyze the limited addition of a deoxyribonucleotide, derived from a deoxyribonucleoside-5′-diphosphate, to a deoxyribooligonucleotide primer. Phosphorolysis of the deoxyribooligonucleotides was not a significant side reaction (3). Clearly, if conditions for efficient extension of a primer by the addition of a single nucleotide could be defined, then extended stepwise synthesis would be feasible. Two technical steps were devised to achieve this. First, high pressure ion-exchange chromatography was used for product analysis in establishing optimum conditions. Second, the strategy of isolating unreacted primer from a reaction product and recycling it in a repeated synthetic step was employed routinely. Taken together these two procedures made possible extended stepwise synthesis. Details of the synthesis of d(pT-T-A-G-C-A-G-A-A-C-C-G-G)

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Table 1. Synthesis of d(pT-T-A-G-C-A-G-A-A-C-C-G-G)

Primer (μmol)		Products (μmol)		No. of cycles	Overall yield*
d(pT-T-A-G)	17.7	d(pT-T-A-G-C)	10.9	1	62
d(pT-T-A-G-C)	10.9	d(pT-T-A-G-C-A)	4.5	3	41
d(pT-T-A-G-C-A)	4.5	d(pT-T-A-G-C-A-G)	2.1	5	46
d(pT-T-A-G-C-A-G)	2.1	d(pT-T-A-G-C-A-G-A)	1.6	3	76
d(pT-T-A-G-C-A-G-A)	1.6	d(pT-T-A-G-C-A-G-A-A)	0.55	6	34
d(pT-T-A-G-C-A-G-A-A)	0.55	d(pT-T-A-G-C-A-G-A-A-C)	0.18	3	33
		d(pT-T-A-G-C-A-G-A-A-C-C)	0.23	3	42
d(pT-T-A-G-C-A-G-A-A-C)	0.18	d(pT-T-A-G-C-A-G-A-A-C-C)	0.11	2	61
d(pT-T-A-G-C-A-G-A-A-C-C)	0.21	d(pT-T-A-G-C-A-G-A-A-C-C-G)	0.11	3	52
		d(pT-T-A-G-C-A-G-A-A-C-C-G-G)	0.022	3	10

* The amounts of oligodeoxynucleotides were determined spectrophotometrically after degradation with snake venom phosphodiesterase. The overall yield, at each step, is expressed as % of primer.

are given in Table 1. Representative enzymatic reactions for each step of addition are shown in Figs. 1–3 and are summarized in Table 2. In most steps unreacted primer was recovered and recycled, often several times.

Synthesis of d(pT-T-A-G-C). The shortest deoxyoligonucleotide primer that can be used in the enzymatic synthesis contains at least three nucleotides (3). Chemical synthesis of such primers can be performed with reasonable yield. The primer, d(pT-T-A-G), was chemically synthesized by the phosphodiester method (11). It was shown to be homogeneous on RPC-5 and Pellionex WAX analytical columns.

Among the four nucleoside diphosphates, deoxycytidine diphosphate is the best substrate for unprimed synthesis (6). This activity can be suppressed by salts, such as NaCl. In the synthesis of d(pT-T-A-G-C), the addition of a pdC residue to d(pT-T-A-G) proceeded well. A yield of 62% was obtained and it was only necessary to perform this synthesis once (Fig. 1A).

Synthesis of d(pT-T-A-G-C-A). Reaction conditions for this synthesis are similar to those for the previous step. The product of a single addition was 39% and there was about 13% of multiple addition products (Fig. 1B). Attempts to reduce multiple addition were not successful, even with 0.6 M NaCl.

Synthesis of d(pT-T-A-G-C-A-G). The yields for a single addition of a pdG residue are usually good and only a small quantity of the di-adduct is found. Here, however, under the usual conditions (0.3 M NaCl, 10 mM $MnCl_2$), only 15% of d(pT-T-A-G-C-A-G) was formed while the di-adduct represented 51%. Other primers, $d(pT)_6$ and $d(pA)_6$, under the same

Table 2. Synthesis of d(pT-T-A-G-C-A-G-A-A-C-C-G-G)

Primer	Nucleotide residue added	Product (nucleoside composition)	Yield* at each step
d(pT-T-A-G)	pdC	d(pT-T-A-G-C) dT/dG/dA/dC(2.1/1.12/1.13/1.0)	62
d(pT-T-A-G-C)	pdA	d(pT-T-A-G-C-A) dT/dG/dA/dC(1.84/1.0/1.94/1.0)	39
d(pT-T-A-G-C-A)	pdG	d(pT-T-A-G-C-A-G) dT/dG/dA/dC(2.07/2.2/2.14/1.0)	22
d(pT-T-A-G-C-A-G)	pdA	d(pT-T-A-G-C-A-G-A) dT/dG/dA/dC(1.95/2.0/2.9/0.95)	63
d(pT-T-A-G-C-A-G-A)	pdA	d(pT-T-A-G-C-A-G-A-A) dT/dG/dA/dC(2.0/2.14/4.14/1.0)	12
d(pT-T-A-G-C-A-G-A-A)	pdC	d(pT-T-A-G-C-A-G-A-A-C) dT/dG/dA/dC(0.92/1.0/2.10/1.0)	21
d(pT-T-A-G-C-A-G-A-A-C)	pdC	d(pT-T-A-G-C-A-G-A-A-C-C) dT/dG/dA/dC(0.95/1.0/2.10/1.5)	29†, 52
d(pT-T-A-G-C-A-G-A-A-C-C)	pdG	d(pT-T-A-G-C-A-G-A-A-C-C-G) dT/dG/dA/dC(2.0/3.2/4.3/2.9)	28
		d(pT-T-A-G-C-A-G-A-A-C-C-G-G) dT/dG/dA/dC(2.0/4.1/4.33/2.93)	5

For nucleoside analysis, the oligodeoxynucleotide was hydrolyzed in a mixture (50 μl) which contained oligonucleotide (0.3 A_{260} units; one A_{260} unit is defined as the amount of nucleotide which when dissolved in 1 ml H_2O has an absorbance at 260 nm of 1.0 in a cell with a 10 mm light path), 5 μg of *E. coli* alkaline phosphatase, 5 μg of snake venom phosphodiesterase, 50 mM ammonium formate (pH 9.0), and 2 mM magnesium acetate. The incubation was at 37° for 3 hr.

* The amounts of oligodeoxynucleotides were determined spectrophotometrically after degradation with snake venom phosphodiesterase. The yield was expressed as % of primer and is the yield for the individual enzymic reaction in contrast with Table 1 in which the yield is the sum of the cycles for each step.

† The value is for d(pT-T-A-G-C-A-G-A-A-C-C) obtained by direct addition of two dpC residues to the primer, d(pT-T-A-G-C-A-G-A-A).

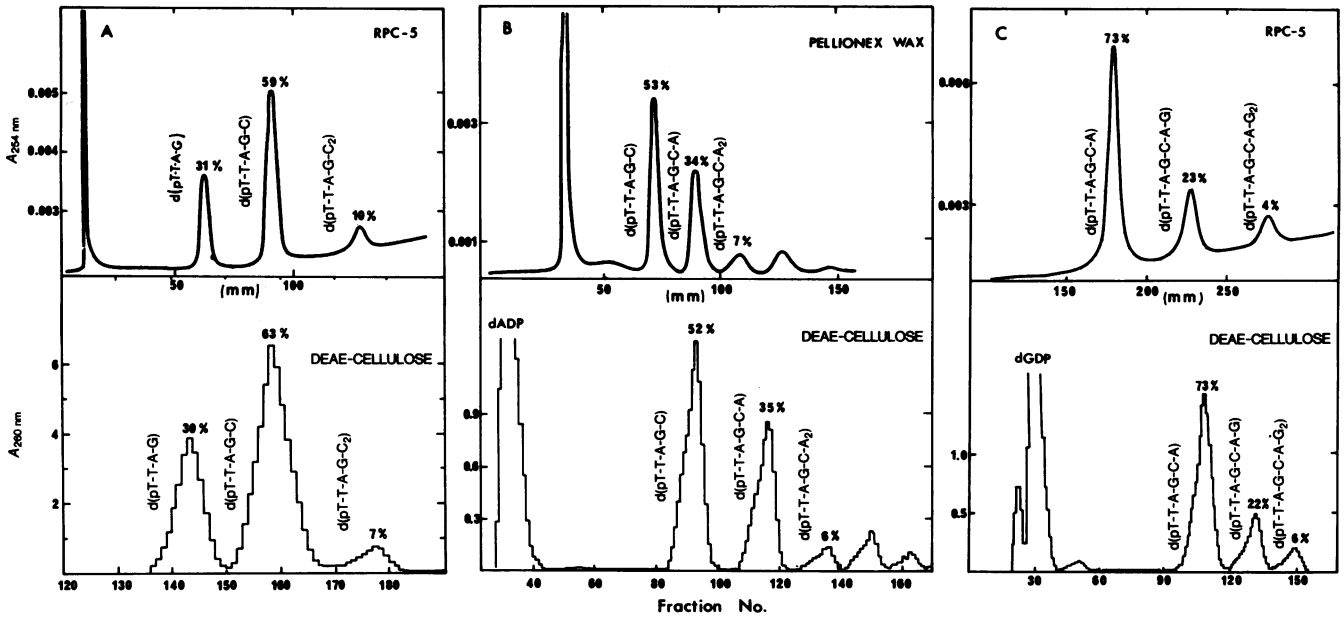

FIG. 1. Synthesis of d(pT-T-A-G-C-A-G). (A) Synthesis of d(pT-T-A-G-C). The reaction mixture (18 ml) containing 17.7 μmol of d(pT-T-A-G), 3 mM dCDP, 20 mM $MnCl_2$, 0.3 M NaCl, and polynucleotide phosphorylase (6 units/ml) was incubated for 4 hr at 37°. The products were fractionated on a DEAE-cellulose column (1.2 × 45 cm) as described in *Materials and Methods*. The gradient was 1000 ml of H_2O to 1000 ml of 0.25 M NaCl. Fractions were collected at 10 ml/15 min. The RPC-5 column was as described in *Materials and Methods*. (B) Synthesis of d(pT-T-A-G-C-A). The reaction mixture (5 ml) containing 2.3 μmol of d(pT-T-A-G-C), 3 mM dADP, 20 mM $MnCl_2$, 0.3 M NaCl, and enzyme (4 units/ml) was incubated for 3 hr. The products were fractionated on a DEAE-cellulose column (1.2 × 45 cm) with a gradient of NaCl (1000 ml each 0.05–0.25 M). Fractions were collected at 8.5 ml/15 min. The column of Pellionex WAX was as described in *Materials and Methods*. (C) Synthesis of d(pT-T-A-G-C-A-G). The reaction mixture (6 ml) containing 2.2 μmol of 6-mer, 2 mM dGDP, 10 mM $MnCl_2$, 0.6 M NaCl, and enzyme (4 units/ml) was incubated for 5 hr. Products were fractionated on a DEAE-cellulose column (1.2 × 40 cm) with a gradient of NaCl (1000 ml each 0.07–0.25 M) at a flow rate of 7.5 ml/10 min. The RPC-5 column was as in *Materials and Methods*. The yields of the products are based on their absorbance.

reaction conditions gave yields of 40% of single addition products and less than 5% of di-addition products. Thus, the tendency to add two residues to d(pT-T-A-G-C-A) seems to be a peculiar property of this oligonucleotide.

In an attempt to improve the yield other concentrations of sodium chloride were used. At 0.4 M, 0.5 M, and 0.6 M NaCl the yields for the single adduct were respectively 24%, 22%, and 22%; for the di-adduct the yields were 20%, 13%, and 6%, respectively. Therefore, 0.6 M NaCl was chosen for this synthesis (Fig. 1C). As the yield was still low, this synthesis was performed five times to give an overall conversion of 40%.

Synthesis of d(pT-T-A-G-C-A-G-A-A). In this step, two pdA residues are added. As it would have been desirable to do this in a single step, many conditions were tested. None gave a high yield of the di-adduct. Conditions favorable to addition of two pdA residues tended to give extended synthesis with addition of up to eight residues. Therefore, the synthesis was carried out as two individual steps. Addition of the first pdA residue proceeded readily with high yield (Fig. 2A). The addition of the second pdA residue was more difficult to control. Under the best conditions found, the yield of the desired product was 12% and there was considerable formation of multiple adducts (Fig. 2B). The overall yield at this step was the lowest in the whole synthesis.

Synthesis of d(pT-T-A-G-C-A-G-A-A-C-C). Here it was possible to find reaction conditions which favored the addition of two pdC residues in high yield. The yields for addition of one and two pdC residues are 21% and 29%, respectively (Fig. 3A). The isolated single addition product was used as primer for addition of a second pdC residue in 0.6 M NaCl (Fig. 3B) and a yield of 52% was obtained. The overall yield of d(pT-T-A-G-C-A-G-A-A-C-C) from d(pT-T-A-G-C-A-G-A-A) was 62%. Stepwise addition can thus still be controlled efficiently with the longer primers, depending on the nucleotide sequence of the primer as well as the deoxyribonucleoside diphosphate used.

Synthesis of d(pT-T-A-G-C-A-G-A-A-C-C-G-G). For the initial study of the addition of pdG residues to the 11-mer (the 11-nucleotide-long oligonucleotide), 0.6 M NaCl and 5 mM $MnCl_2$ were chosen. The yields of the di-, tri-, and tetra-adducts formed were 6.4%, 15%, and 7%, respectively, while the single adduct was only 13%. At 0.3 M NaCl and 4 mM $MnCl_2$, the yield of single adduct was 32% and of the di-, tri-, and tetra-adducts were 5%, 13%, and 15%, respectively. The only reaction condition which produced no multiple adducts was 0.6 M NaCl and 2 mM $MnCl_2$. However, the yield was only 7%. The best results were obtained with 0.3 M NaCl and 1.5 mM $MnCl_2$, which gave yields of single, di-, and tri-adducts of 28%, 6%, and 7%, respectively (Fig. 3C). It is interesting to note that the yield of the desired di-addition product is consistently low under all conditions. Changing the reaction conditions favored only the formation of tri- and tetra-adducts. The higher yields of these adducts compared with the di-adduct suggest a significant difference in the reaction of the 13-mer and 14-mer and illustrate the difficulties to be expected in controlling additions to these longer primers. Addition of a pdG residue to the 12-mer was found to be difficult. Conditions were not found which gave a reasonable yield of the single addition product. Under the best conditions found (0.6 M NaCl and 2 mM $MnCl_2$), only 10% of the single addition product and 2% of the di-adduct were found. Sufficient 13-mer product was obtained by the addition of two pdG residues to the 11-mer for hybridization studies, so that the

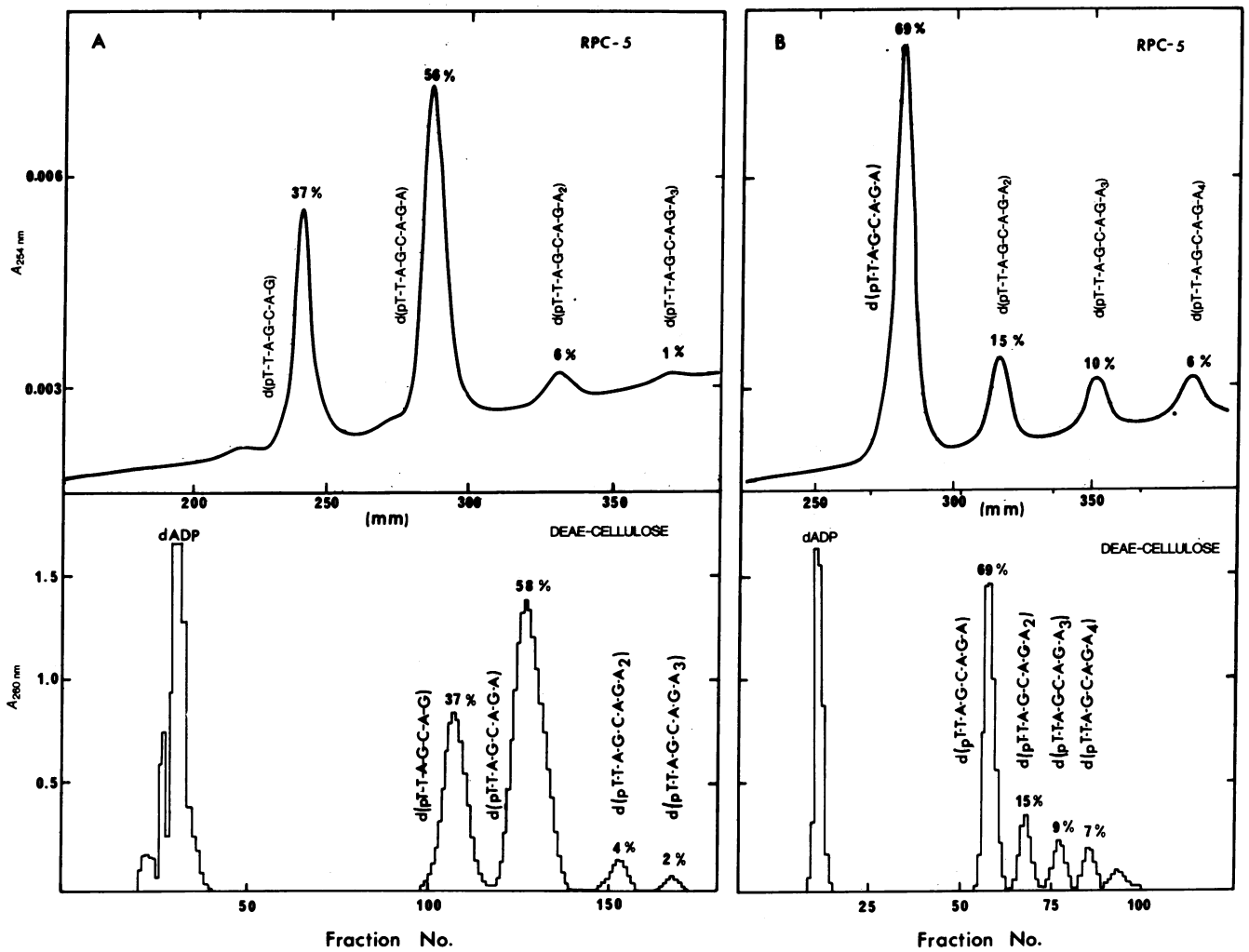

FIG. 2. Synthesis of d(pT-T-A-G-C-A-G-A-A). (A) Synthesis of d(pT-T-A-G-C-A-G-A). The reaction mixture (4.5 ml) contained 1.4 μmol of 7-mer, 1.5 mM dADP, 20 mM $MnCl_2$, 0.5 M NaCl, and enzyme (5 units/ml) and was incubated for 4 hr. The products were separated on a DEAE-cellulose column (1.2 × 37 cm) with a gradient of NaCl (800 ml each 0.1–0.25 M) at a flow rate of 5.5 ml/12 min. The RPC-5 column was as in *Materials and Methods*. (B) Synthesis of d(pT-T-A-G-C-A-G-A-A). The reaction mixture (2.5 ml) contained 0.74 μmol of 8-mer, 0.8 mM dADP, 5 mM $MnCl_2$, 0.6 M NaCl, and enzyme (10 units/ml) and was incubated for 5 hr. The products were fractionated on a DEAE-cellulose column (1 × 32 cm) with a gradient of NaCl (500 ml each 0.13–0.25 M) at a flow rate of 9 ml/12 min. The RPC-5 column was as described in *Materials and Methods*. The yields of the products are based on their absorbance.

addition of the second pdG residue to the 12-mer was not necessary on a preparative scale.

Characterization of Products. At each step of synthesis, the homogeneity of each product was anaylzed on RPC-5 and its nucleoside composition determined by enzymatic hydrolysis and chromatography on Aminex A-5. Nucleoside compositions of the products are given in Table 2. Homogeneity of the 9-mer, 11-mer, and 13-mer was checked by electrophoresis in an acrylamide gel and by homochromatography of the oligonucleotides with A ^{32}P-label at the 5′-terminus. The oligonucleotides were also subjected to partial digestion with snake venom phosphodiesterase, and then by 2-dimensional electrophoresis and homochromatography (12) to determine their nucleotide sequences. Each oligomer gave the expected pattern and appeared to be pure apart from a small amount of d(pT-T-A-G-C-A-G-A-A-C-C-G) in the final product. The details of these analyses will be reported elsewhere.

The concentrations of $MnCl_2$ and deoxyribonucleoside diphosphate used in the earlier stages of the synthesis [up to d(pT-T-A-G-C-A-G)] are higher than in the later steps. The concentration of $MnCl_2$ was decreased about 4-fold after this step. It was found that with longer primers, lower concentrations of $MnCl_2$ reduce the degree of multiple addition. Higher concentrations of NaCl alone do not efficiently prevent successive elongations of the primer. Lowering the concentration of nucleoside diphosphate seems to prevent multiple addition to some extent. Due to the lower concentration of $MnCl_2$ used in the later syntheses, higher concentrations of enzyme were used because the optimal concentration of $MnCl_2$ for the enzymatic reaction is 10 mM (6).

While it is possible to predict what reaction conditions are likely to be best for a particular addition, in some cases the prediction is not reliable. The interaction of polynucleotide phosphorylase with substrates is complex (13). Consequently, the empirical determination of optimum conditions is a necessary feature of the synthetic procedure.

The present results are extremely encouraging but the declining yields as longer deoxyoligonucleotides are used suggest

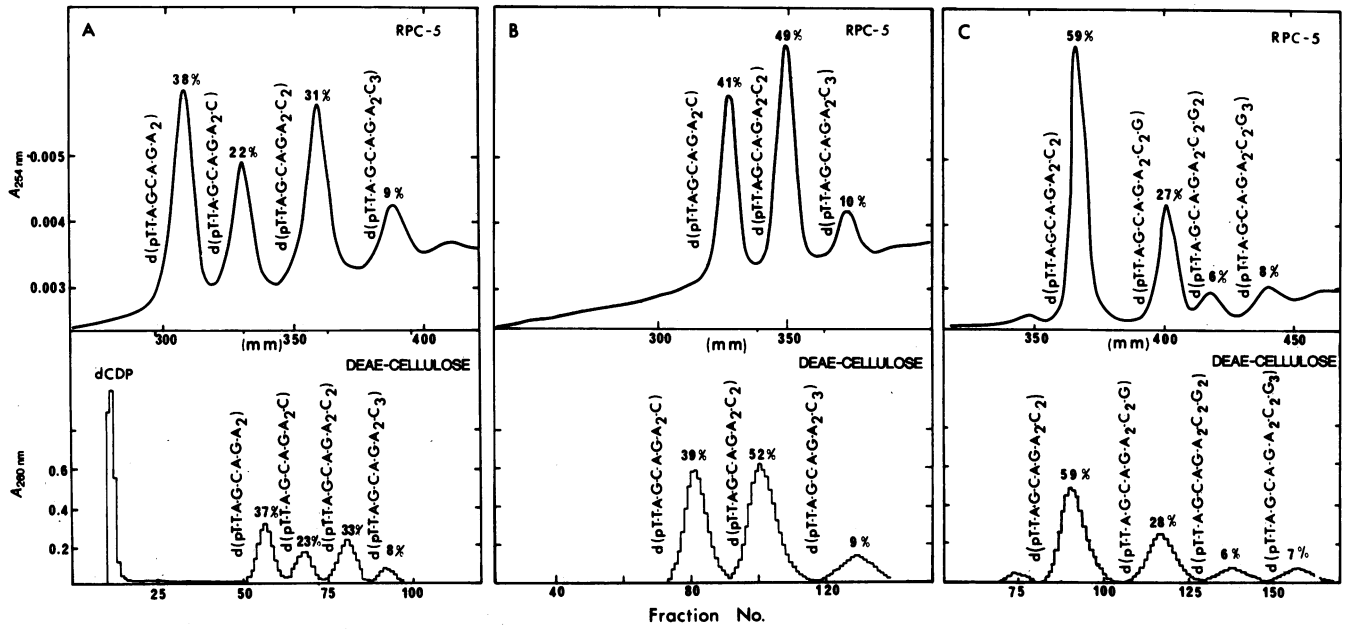

FIG. 3. Synthesis of d(pT-T-A-G-C-A-G-A-A-C-C-G-G). (A) Synthesis of d(pT-T-A-G-C-A-G-A-A-C) and d(pT-T-A-G-C-A-G-A-A-C-C). The reaction mixture (0.6 ml) containing 0.12 μmol of 9-mer, 1.3 mM dCDP, 4 mM $MnCl_2$, 0.3 M NaCl, and enzyme (12 units/ml) was incubated for 4 hr. Products were fractionated on a DEAE-cellulose column (1 × 18 cm) with a gradient of NaCl (300 ml each 0.15–0.25 M) at a flow rate of 2.2 ml/15 min. The gradient for the RPC-5 column was from 50 ml of 0.6 M ammonium acetate (pH 4.5) to 50 ml of 3 M ammonium acetate (pH 4.5). (B) Synthesis of d(pT-T-A-G-C-A-G-A-A-C-C). The reaction mixture (1 ml) containing 0.15 μmol of 10-mer, 1 mM dCDP, 5 mM $MnCl_2$, 0.6 M NaCl, and enzyme (12 units/ml) was incubated for 4 hr. Products were fractionated on a DEAE-cellulose column (1 × 15 cm) as described in (A). Fractions were collected at 1.6 ml/12 min. The RPC-5 column was as described in (A). (C) Synthesis of 13-mer. The reaction mixture (0.9 ml) containing 0.11 μmol of 11-mer, 1 mM dGDP, 1.5 mM $MnCl_2$, 0.3 M NaCl, and enzyme (12 units/ml) was incubated for 7 hr. Products were fractionated on a DEAE-cellulose column (0.6 × 20 cm) and eluted as described in (A). The RPC-5 column was as described in (A). The yields of the products are based on their absorbance.

that further work is required to improve the procedures. The use of the enzyme RNA ligase (14) to join enzymatically synthesized deoxyoligonucleotides is one possible approach.

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