Enzymatic synthesis of deoxyribo-oligonucleotides of defined sequence.
Deoxyribo-oligonucleotide synthesis*

Shirley Gillam, Kimberley Waterman**, Michael Doel*** and Michael Smith

Department of Biochemistry, Faculty of Medicine, University of British Columbia,
Vancouver 8, British Columbia, Canada

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ABSTRACT

An enzyme, which is probably identical with polynucleotide phosphorylase, was prepared from Escherichia coli B. In the presence of Mn^{2+} it catalyzes the addition of one (and to a slight extent more) residue of deoxyribonucleotide residue from the diphosphate to an oligodeoxyribonucleotide primer. The shortest effective primers contained three phosphate residues. Ribodinucleotides were effective as primers and accepted two or three deoxyribonucleotide residues under these conditions. The application of the procedures to the convenient synthesis of certain defined oligodeoxyribonucleotides up to nine residues long is discussed.

INTRODUCTION

Earlier studies have shown that polynucleotide phosphorylase isolated from Escherichia coli B (1,2) or Micrococcus luteus (3) has a limited ability to use deoxyadenosine-5'-diphosphate as a substrate. However, neither of these enzymes has been used in the stepwise synthesis of oligodeoxyribonucleotides. The report by Hsieh (4) of an enzyme activity from E. coli W 3110 pol A1⁻ that polymerizes deoxyribonucleoside-5' diphosphates raised the question of whether the activity could be used in vitro for stepwise synthesis of oligodeoxyribonucleotides. The preceding paper (5) describes some properties of this activity, partially purified from E. coli B. The enzyme appears to be a polynucleotide phosphorylase with its substrate specificity in the presence of Mn^{2+} broadened to include deoxyribonucleoside-5' diphosphates. The enzyme can be used for the primer-dependent synthesis of oligodeoxyribonucleotides under the conditions described in the previous paper. This paper reports the synthesis of a variety of oligodeoxyribonucleotides, illustrating how the extent of the

reaction is dependent on several characteristics of the primer as well as the deoxyribonucleotide being added. The synthesis of high molecular weight deoxyribonucleic acid homopolymers in preparative yields using the enzyme is also described [analogous small scale polymerizations have been described previously (3,4)].

METHODS

Materials - [^{14}C]dGDP, [^3H]dADP and [^3H]dTDP were obtained from Schwarz BioResearch; ribodinucleoside monophosphate from P-L Biochemicals. The enzyme used for nucleotide synthesis was obtained as described in the preceding paper (5).

Deoxyoligonucleotide synthesis - The conditions used for the nucleotide synthesis are described in the preceding paper. The compositions of products were determined by degradation of each oligonucleotide with snake venom phosphodiesterase and then separating the nucleotides by paper chromatography. The reaction mixtures were analyzed on DEAE-cellulose columns (1.2 x 10 cm) in the carbonate form with appropriate ammonium bicarbonate gradients.

Phosphorolysis - The extent of phosphorolysis is expressed as the amount of radioactive nucleotide incorporated into the recovered primer as a percentage ($\mu\text{moles radioactive nucleotide in recovered primer fraction} \times 100 / \mu\text{moles recovered primer}$).

RESULTS

Deoxyribonucleoside diphosphate - Each polymerization step involves two substrates (primer and nucleoside diphosphate) and a metal ion. The formation of complexes between the metal ion, primer, nucleoside diphosphate and enzyme occurs during the reaction. Thus a wide variety of factors is involved in determining the reaction rates of a given system. For the stepwise synthesis of an oligonucleotide with unblocked nucleoside diphosphate, one can not use the reaction conditions that are optimal for the polymerization of nucleoside diphosphate into long polymers. The reaction conditions for the addition of various deoxyribonucleotide residues to the deoxyoligonucleotide primer are given in Table I. It was found that the extent of addition of deoxyribonucleotide to the primer varies, depending upon the reaction conditions and the deoxyribonucleoside diphosphate being added. The effect of NaCl on the addition of p dA residue

Table I

Reaction conditions for different deoxyribonucleoside-5' diphosphates using pd(A₄) as primer

Deoxyribonucleoside-5' diphosphate (mM)		NaCl (M)	Yield	Product (% of primer used)	Recovered primer (% of primer used)	Phosphorolysis (%)
dADP	1.5	0.3	pd(A ₅)	25	35	1.9
dGDP	1.5	0.3	pd(A ₄ G)	32	58	0.5
dCDP	1.5	0.3	pd(A ₄ C)	12	74	- **
	1.5	0.6	pd(A ₄ C)	12	75	- **
	1.5*	0.3	pd(A ₄ C)	18	56	3.7
	1.5*	0.6	pd(A ₄ C)	15	63	2.5
dTDP	1.5	0.05	pd(A ₄ T)	13	75	0.5
	1.5	0.3	pd(A ₄ T)	18	74	0.3
	3.0*	0.3	pd(A ₄ T)	22	64	0.2

The reaction mixture (1 ml) contained the primer, pd(A₄) 0.32 μ moles, 2-mercaptoethanol, 15 μ moles, MnCl₂, 10 μ moles, Tris-HCl pH 8.5, 100 μ moles, and enzyme 1.8 mg.

* The concentration of MnCl₂ was 20 μ moles.

** The extent of phosphorolysis was not determined in these experiments. Determination of phosphorolysis is described under Methods.

to the primer was described in the preceding paper and similar conditions were chosen for the addition of a pdG residue to the primer as this gave a high yield of the monoaddition product. The difference between the addition of pdA and pdG residues to the primer under the same conditions is that the enzyme tends to add more than one pdA residues to the primer while for pdG the major product is the mono-addition product (6).

Among the four deoxyribonucleoside diphosphates, deoxycytidine diphosphate gave the lowest yield of the mono-addition product, though it is the best substrate for the enzyme in the polymerization reaction (4).

For thymidine diphosphate, a higher concentration of substrate favored the addition of a pdT residue to the primer. This could be due to the fact that dTDP is a poor substrate for the enzyme.

Chain length of the primer - Some experiments to examine the efficiency of the addition of different deoxynucleotide residues to various primers are summarized in Table II. Typical separations of the reaction mixtures with two different chain lengths of primer are shown in Fig. 1. The shortest primer accepted by the enzyme was a trinucleotide. The shorter oligonucleotides pd(A₂), pd(T₂), dA-dA and dT-dT were found to be ineffective as primers. Chromatography of products from reactions using high concentrations of a dinucleotide and deoxyribonucleoside diphosphates showed only high molecular weight products resulting from self-initiated synthesis. Higher concentration of enzyme was also found to produce only the un-primed synthesis of the high molecular weight products. Primers ranging from four to nine nucleotides in length were equally efficient acceptors when the same deoxyribonucleotide residue was added. The yield of the mono-addition product seems to depend on the nucleotide residues added and not on the chain length of the primers. Addition of pdA and pdG residues to various primers gave about 30% yield while pdC and pdT gave about 15%.

The effect of chain length on the phosphorolysis of primer can be seen from the comparison of the addition of a pdA or pdG residue to primers of different lengths (Table II). The extent of phosphorolysis seems not to be influenced by the chain length of the primer.

Fig. 1. Fractionation of products of reactions.

A, $pd(T_9)$, 0.2 moles with $[^{14}C]$ -ppdG, 1.5 μ moles (2.27×10^6 cpm/ μ mole).

B, $pd(A_3)$, 0.433 moles with $[^3H]$ -ppdA, 1.5 μ moles (5.37×10^6 cpm/ μ mole).

Reactions (1 ml) were incubated at 37° for 4 hours and analyzed as described in the method. For the reaction of $pd(T_9)$ and ppdG, the column was eluted with a linear gradient of ammonium bicarbonate (gradient from 500 ml 0.2 M NH_4HCO_3 to 500 ml 0.8 M NH_4HCO_3). Fractions were collected at 5 minute intervals at a rate of 1 ml per minute.

Table II

Addition of a single deoxyribonucleotide residue to various primers.

Primer	Product	Yield (% of input primer)	Recovered primer (% of input primer)	Composition of product	Phosphoro- lysis of recovered primer (%)
pd(A ₃)	pd(A ₄)	33	42	pdA/dA 2.81	0.31
pd(T ₃)	pd(T ₃ A)	12	72	pdT/pdA 2.93	0.1
pd(A ₄)	pd(A ₅)	25	35	pdA/dA 3.75	0.7
pd(A ₄)	pd(A ₄ G)	32	58	pdA/pdG 4.04	0.3
pd(A ₄)	pd(A ₄ C)	15	56	pdA/pdC 3.96	0.7
pd(T ₄)	pd(T ₄ G)	30	52	pdT/pdG 3.9	- *
pd(A ₄)	pd(A ₄ T)	18	54	pdA/pdT 4.1	0.1
pd(A ₅)	pd(A ₅ G)	26	51	pdA/pdG 4.77	- *
pd(A ₅)	pd(A ₅ T)	14	66	pdA/pdT 4.93	- *
pd(T ₉)	pd(T ₉ G)	22	50	pdT/pdG 8.7	0.1
pd(A ₇)	pd(A ₇ C)	12	60	-	0.4

Reaction mixtures were prepared as described in Table I using approximately 0.3 μ mole of primer. All were 0.3 M in NaCl and were incubated for a period of four hours at 37°. Composition of products was determined after phosphodiesterase degradation as described in the preceding paper.

* The extent of phosphorolysis was not determined in these experiments. Determination of phosphorolysis is described under Methods.

Effect of a phosphate group on the 5'-end of the primer - It has been proposed that polynucleotide phosphorylase has multiple phosphate binding sites for its substrates (7). Thus, for short primers the addition of a 5'-phosphate group would be expected to favour binding. This was studied for primers of different chain lengths. Results obtained are shown in Tables II and III and Fig. 2. It was found that at the shortest chain lengths which can serve as primers, the presence of a 5'-phosphomonoester group in the primer is important. Thus, the tetranucleoside triphosphate was less effective than the corresponding tetranucleotide. However, as shown in Tables II and III, at longer chain lengths, the state of phosphorylation of the 5'-hydroxy group seems to be unimportant in affecting the priming capacity of an oligonucleotide. The phosphate group on the 5'-end of the primer was found to have no effect on the phosphorolysis of the primer, as there is no significant difference observed between the phosphorylated and dephosphorylated primer (Tables II and III). With such primers negligible formation of di-addition product was observed even when p dA was the residue to be added. This gives the advantage of higher recovery of the unreacted primer.

Effect of the nucleotide on the 3'-end of the primer - Since the yield for the addition of a single deoxyribonucleotide to the 3'-terminus of a deoxyribo-oligonucleotide was found to depend greatly on the nucleotide residue to be added, it was of interest to examine the effect of the residue at the 3'-terminus of the primer on its priming capacity. It was found that this residue can be either a purine or a pyrimidine nucleotide (Table IV). At chain lengths larger than four nucleotides, the base on 3'-end of the acceptors has very little effect on the accepting efficiency. The lower yield obtained with the longer primers was due to the low concentration of primers used in the reaction, and also to the lower recovery obtained from DEAE-cellulose column chromatography. A further cause of low yields will be discussed below.

Use of ribodinucleotides as primers - The shortest oligodeoxynucleotide found to be capable of acting as a primer for the enzyme was a 5'-phosphorylated trinucleotide. However, the ribonucleotides, A-A and A-G were found to accept several deoxy-

Fig. 2. Fractionation of products obtained from dephosphorylated primer.

The reaction mixtures (0.5 ml) contained d(A₄) , 0.305 μ moles, radioactive deoxyribonucleoside-5'-diphosphate 0.75 μ moles; enzyme, 0.8 mg and the other components of the reaction. The specific activities of [^3H]-ppdA and [^{14}C]-ppdG were respectively 5.8×10^6 cpm/ μ mole and 1.54×10^6 cpm/ μ mole. The efficiency was for ^3H , 30% and for ^{14}C , 80%. Fractions were collected at 7 minute intervals at a rate of 0.8 ml per minute.

Table III

Addition of a single deoxyribonucleotide residue to various 5'-dephosphorylated primers.

Primer	Product	Yield (% of primer)	Recovered primer (% of primer used)	Composition of product	phosphoro- lysis of recovered primer
d(A ₄)	d(A ₅)	16	59	pdA/dA (1/3.92)	0.75
d(A ₄)	d(A ₄ G)	15	62	pdA/pdG/dA (3.0/1.1/1.0)	1.3
d(ACT ₅)	d(ACT ₅ G)	30	55	dpT/dpC/dpG/dA (5.24/0.94/0.8/1.0)	- *
d(ACT ₅ G)	d(ACT ₅ GT)	25	77	not analysed	- *

The experimental conditions were as described in Table-1.

* The extent of phosphorolysis was not determined in these experiments. Determination of phosphorolysis is described under Methods.

ribonucleotide residues, as well as ribonucleotide residues, even in the presence of Mn^{+2} . Results obtained with these primers are illustrated in Fig. 3. Table V gives the analysis of the reaction products. It is interesting that the enzyme preferentially added two to three nucleotide residues to the ribonucleotide primers with little mono-addition product being formed under these reaction conditions. It is thus possible to use a ribo-oligonucleotide as a starting primer to accept several deoxyribonucleotide residues at the 3'-end and this can then serve as the primer for the addition of further specific deoxyribonucleotide residues. The ribonucleotide primer can be easily removed after the completion of the synthesis either by alkaline hydrolysis or by digestion with ribonucleases.

The use of A-G as a ribo-oligonucleotide primer to synthesize A-G-d(C-C-G) is illustrated in Figs. 3 and 4 and Table VI. Initially A-G-d(C-C) was synthesized enzymatically from ApG and 3H -dCDP (Fig. 3-c); then it was used as a primer for further addition of a ^{14}C -pdG residue (Fig. 4). Table VI presents the characterization of the products after removal of their ribonucleotide moieties by hydrolysis with alkali or ribonuclease T_1 .

The product of three additions to the ribonucleotide primer can obviously serve as a primer for further addition of deoxyribonucleotide residues.

Synthesis of high molecular weight homopolymers - The enzyme catalyzes the formation of polydeoxyadenylate, polydeoxycytidylate, polydeoxyguanylate and polydeoxythymidylate from corresponding deoxyribonucleoside diphosphates (4). The rates of formation of polydeoxyadenylate and polydeoxycytidylate are about 5 times greater than the rates of formation of polydeoxyguanylate and polydeoxythymidylate (4). We have used this property of the enzyme to synthesize high molecular weight polydeoxyguanylate and polydeoxythymidylate with 13% conversion of the deoxyribonucleoside diphosphate. The conditions for the synthesis of homopolymers were the same as described for the stepwise synthesis of oligonucleotides, except that NaCl was omitted from the reaction mixture. The incubation time was increased to seven hours in order to obtain higher yield. The enzyme was partially digested by trypsin at the end of the reaction and the digested pep-

Table IV

Addition of a single nucleotide to primers with differing 3'-terminus.

Primer	Product	Yield (% of primer used)
r (AG) -d (C ₂)	r (AG) -d (C ₂ G)	20
pd (T ₉)	pd (T ₉ G)	22
pd (A ₅ G)	pd (A ₅ GA)	5
pd (A ₅ T)	pd (A ₅ TA)	10
pd (A ₇)	pd (A ₈)	14
pd (A ₃)	pd (A ₄)	33
pd (T ₃)	pd (T ₃ A)	12

The experimental conditions were as described in the text. The method of product analysis is described in the preceding paper.

Table V

Use of oligoribonucleotides as primers for addition of various nucleotides

Primer	Products Yield (% of input primer)				Primer recovered (% of input primer)
r (A ₂)	r (A ₃) 5	r (A ₄) 15	r (A) 15		13
r (A ₂)	r (A ₂)-da*	r (A ₂)-d (A ₂) 25	r (A ₂)-d (A ₃) 25		15
r (AG)	r (AG)-dC 10	r (AG)-d (C ₂) 22	r (AG)-d (C ₃) 4		30

The experimental conditions were as described in Figure 3.

* r (A₂)-dA were eluted in the same peak as deoxyadenosine-5' diphosphate. The yield was not determined.

Fig. 3. Fractionation of products of reaction with ribodinucleotide monophosphate as primer.

A, Reaction solution (0.5 ml) contained A-A, 0.42 μmoles, [³H]-ppA, 2.5 μmoles (2.5 × 10⁶ cpm/μmole), MnCl₂, 1 μmoles, NaCl, 150 μmoles and enzyme 0.5 mg.

B, Reaction mixture (1 ml) contained A-A, 0.64 μmoles; [³H]-ppdA, 1.5 μmoles (5.8 × 10⁶ cpm/μmole); MnCl₂, 10 μmoles; NaCl, 300 μmoles and enzyme 1.8 mg.

C, Reaction mixture (1 ml) contained A-G 0.9 μmoles, [³H]-ppdC 1.5 μmoles (2.7 × 10⁶ cpm/μmole, MnCl₂, 10 μmoles, NaCl, 300 μmoles and enzyme, 1 mg.

Fractions were collected as described in Fig. 1.

Table VI
Hydrolysis of the synthesized oligonucleotides.

Oligonucleotide	Treatment	Products		
			Rf	μmoles
r(AG)-d(C ₂)	snake venom phosphodiesterase (50 μg/ml) 37°C, 3 hours	A pG pdC	0.14 0.49 0.67	0.049 0.052 0.12
r(AG)-d(C ₂)	0.3 N KOH 18 hours, 37°C	Ap Gp d(C ₂)	0.24 0.16 0.49	0.038 0.043 0.04
r(AG)-d(C ₂ G)	T ribonuclease (100 units/ml), and <i>E. coli</i> alkaline phosphatase (40 μg/ml) 37°C, 3 hours	r(AG) d(C ₂ G)	0.52* 0.38*	0.031 0.03
r(AG)-d(C ₂ G)	None	r(AG)-d(C ₂ G)	0.03*	0.007

The oligonucleotides were synthesized and isolated as described in Figs. 3 and 4. The digests were separated by paper chromatography in ethanol/1 M ammonium acetate (7/3, pH 7.5), except the hydrolysate of r(AG)-d(C₂) with snake venom phosphodiesterase which was separated in saturated (NH₄)₂SO₄/0.6 M sodium acetate/isopropanol (160/36/4). The nucleotides were estimated spectrophotometrically.

* Due to the poor separation of r(AG) and d(C₂G), the chromatogram was over run and the values given are relative to dC. The Rf value dC is 0.76.

Fig. 4. Analysis of the reaction of r(AG)-d(C₂) and ppdG.

The reaction mixture (0.5 ml) contained r(AG)-d(³H-C₂), 0.09 μ moles; [¹⁴C]-ppdG, 0.75 μ moles (9.2×10^6 cpm/ μ mole); MnCl₂, 5 μ moles; NaCl, 150 μ moles and enzyme, 0.6 m μ . The products were analyzed on a DEAE-cellulose column (0.8 x 14 cm) with a linear gradient of ammonium bicarbonate (400 ml H₂O to 400 ml 0.5 M NH₄HCO₃). The efficiency was for ³H, 30% and for ¹⁴C, 60%. Fractions were collected at 7 minute intervals at a rate of 0.3 ml per minute.

Fig. 5. Chromatography of the reaction mixture containing poly dG on a Sephadex G-100 column after trypsin treatment.

The reaction was performed as described in Methods except that NaCl was omitted from the incubation medium. The concentration of dGDP was 2.25 μ moles (9×10^5 cpm/ μ mole) and the incubation was continued for another 2 hours at 30°C before the solution was applied onto a Sephadex G-100 column (1.5 x 50 cm). The column was eluted with 0.02 M Tris-HCl, pH 7.8 and 0.02 M NaCl at a flow rate of 0.3 ml/min. Fraction of 3.0 ml were collected.

tides and unreacted nucleoside diphosphate were separated from the polymers by gel filtration of the reaction mixture on Sephadex G-100 (Fig. 5). Since the enzyme seems to be free of nucleases, it offers a convenient and simple method for obtaining small quantities of these high molecular weight homopolymers.

DISCUSSION

The shortest oligodeoxyribonucleotide primer that can be used contains three nucleotides. Chemical synthesis of such primers may be avoided in a stepwise synthesis since the enzyme will add deoxyribonucleotides to a ribodinucleoside monophosphate. The ribonucleotide portion may subsequently be removed from the oligonucleotide by alkaline hydrolysis or digestion by a ribonuclease (Figures 3-c and 4).

The particular nucleoside at the 3'-end of the primer does not affect the extent of the reaction. However, both the extent and the products of the reaction are affected by the deoxynucleoside 5'-diphosphate present in the reaction mixture. Purine deoxynucleoside diphosphates tend to give approximately 30% conversion and several products, corresponding to the addition of one to three residues (Figures I and II). dTDP give lower conversion producing predominantly mono- with some di-addition product. dCDP gives the lowest conversion of all with formation of almost entirely mono-addition product. If an oligoribonucleotide primer is used, addition is more facile, 2 or 3 residues being added even in the case of the less reactive pyrimidine deoxynucleoside-5' diphosphates.

Two reactions reported here gave particularly low yields (the heptanucleotide synthesis in Table IV). We believe this is not a result of the particular oligonucleotide serving as primer, but rather due to an inhibitor, described below, in the reaction mixture. These two primers were themselves the products of earlier enzymatic reactions (Table II). We have found that sometimes when setting up a reaction mixture incorporating a primer from an earlier enzymatic synthesis instead of a chemically-synthesized primer, a precipitate forms in the reaction mixture upon addition of Mn^{+2} . Formation of this undefined precipitate correlates with low conversion, and must be due to some variable factor in the workup from the earlier enzymatic synthesis. Clearly this pro-

blem must be investigated further.

No synthesis comparable to the chemical syntheses reported by Khorana *et al.* (9) has yet been accomplished using these methods. It should be emphasized that for the synthesis of certain defined oligodeoxyribonucleotides, particularly those having adjacent repeated units, the method is already of practical value. For example, in the heptanucleotide synthesis reported in Table IV the homologous octanucleotides and nonanucleotides were also formed in low yield in the reaction mixture. Their formation represents the conversion of a pentanucleotide, pd(A)₅, to a nonanucleotide in two enzymatic steps (Table II and IV). However the method needs further development before it will allow the synthesis of, for example, a decanucleotide of non-repeating, defined sequence in adequate yields.

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** Present address: Rockefeller University, New York, N.Y. 10021

*** Medical Research Council of Canada Fellow. Present address: G.D. Searle, Research Division, High Wycombe HP12 4HL, England

**** Medical Research Associate of the Medical Research Council of Canada

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