Phosphorylation of N-Protected Deoxyoligonucleotides by T_4 Polynucleotide Kinase†

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ABSTRACT: The substrate recognition of T_4-induced polynucleotide kinase has been investigated. It was found that the presence of base protection groups on the 5′-nucleoside moiety of an oligonucleotide did not interfere with the action of the enzyme. The deoxyoligonucleotides d-TpC^{An}pG^{iB}pA^{Bz}pA^{Bz}, d-C^{An}pTpA^{Bz}pC^{An}, d-A^{Bz}pG^{iB}pA^{Bz}pG^{iB}, and d-G^{Bz}pC^{An}pT could be quantitatively phosphorylated at their 5′-hydroxyl terminus. No difference in the rate of phosphorylation was observed between an N-protected and unprotected deoxyoligonucleotide. The use of ^{32}P-labeled protected nucleotide blocks during the chemical synthesis of a polynucleotide provides a simple way to follow the rate of internucleotide bond formation and facilitates the isolation and characterization of the reaction products.

Bacteriophage T_4 induced polynucleotide kinase catalyzes the transfer of the γ-phosphate from a nucleoside 5′-triphosphate to the 5′-hydroxyl terminus of polynucleotides, oligonucleotides, and 3′-mononucleotides (Richardson, 1965). The enzyme has been extensively used for 5′-end group analysis of several bacteriophage DNAs (Weiss and Richardson, 1967; Wu and Kaiser, 1967; Jacquemin-Sablon and Richardson, 1970), for labeling of pyrimidine tracts of DNA (Székely and Sanger, 1969; Southern, 1970), and for the preparation of labeled substrates for polynucleotide ligase (Weiss *et al.*, 1968b; Gefter *et al.*, 1967; Olivera and Lehman, 1967), and 5′-phosphomonoesterases (Becker and Hurwitz, 1967; Weiss *et al.*, 1968a). In this paper we report our findings on the phosphorylation of amino-protected deoxyoligonucleotides by T_4 polynucleotide kinase.

The chemical synthesis of polynucleotides of defined sequence has been greatly aided by the use of preformed deoxyoligonucleotides bearing phosphomonoester groups, which are condensed with oligonucleotidic components carrying hydroxyl end groups (Kössel *et al.*, 1967). This approach has been successfully used in the chemical synthesis of the deoxyoligonucleotide segments corresponding to the structural gene of a major yeast alanine tRNA (Agarwal *et al.*, 1970; Khorana *et al.*, 1972). As the length of the polynucleotide to be synthesized increases, the characterization of the polynucleotides becomes more and more difficult. A tritium-labeled tetranucleotide block was used in the final step of the chemical synthesis of an icosanucleotide to facilitate the isolation and characterization of the reaction products (Weber and Khorana, 1972). The tritium-labeled tetranucleotide block was prepared from the labeled mononucleotide and involved several steps with extensive losses. Oligonucleotide blocks have previously been labeled at the 5′-hydroxyl terminus by the use of ^{32}P-labeled 2-cyanoethyl phosphate and dicyclohexylcarbodiimide (Tener, 1961; Pfitzner and Moffatt, 1964).

The present finding that T_4 polynucleotide kinase will quantitatively phosphorylate N-protected deoxyoligonucleotides provides a facile method to label any deoxyoligonucleotide block prior to its use in a chemical condensation.

Materials and Methods

Chemicals. Pyridine was treated with chlorosulfonic acid (1.5%, v/v) before distillation, redistilled from potassium hydroxide, and stored over molecular sieves (4A). 2,4,6-Triisopropylbenzenesulfonyl chloride was obtained from Aldrich Chemical Co., Inc., and was recrystallized from anhydrous *n*-pentane before use. Inorganic [^{32}P]- or [^{33}P]phosphate was obtained from New England Nuclear Co. γ-^{32}P- or γ-^{33}P-labeled ATP was prepared by the method of Glynn and Chappell (1964).

Enzymes. Hexokinase from yeast was purchased from Sigma Chemical Co. and used as such. Bacterial alkaline phosphatase, snake venom phosphodiesterase, and spleen phosphodiesterase were obtained from Worthington Biochemical Corp. The bacterial alkaline phosphatase was freed of contaminating DNase by passage through a DEAE-cellulose column as described by Weiss *et al.* (1968b). Polynucleotide kinase was isolated from T_4 amN82 phage infected *Escherichia coli* B62 by a modification (Panet *et al.*, 1973) of the method of Richardson (1965). The enzyme had a specific activity of 65,000 units/mg of protein and was free of endonuclease as assayed by alkali zone sedimentation of T_7 DNA according to Weiss *et al.* (1968a).

Chromatography. Thin-layer chromatography (tlc) was carried out on precoated silica gel plates containing fluorescence indicator (Eastman 6020). Whatman No. 1 and DEAE-cellulose ion exchange (Whatman DE-81) papers were used. The following systems were used for tlc and paper chromatography: solvent A, ethanol–1 M ammonium acetate (pH 7.5) (70:30, v/v); solvent B, 1-propanol–concentrated ammonium hydroxide–water (55:10:35, v/v); solvent C, 0.1 M sodium phosphate (pH 6.8)–ammonium sulfate–1-propanol (100 ml:60 g:2 ml); solvent D, 0.35 M ammonium formate; and solvent E, 0.30 M ammonium formate.

DEAE-cellulose (Whatman DE-23) was used for anion exchange column chromatography with appropriate gradients

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in triethylammonium bicarbonate. Gel filtration was carried out using Sephadex G-25 SF or G-50 SF (Pharmacia) with 0.1 M triethylammonium bicarbonate as the elutriator.

Oligonucleotides.[1] The chemical synthesis of the oligonucleotides d-MMTrG^{Bz}pC^{An}pT, d-MMTrG^{iB}pC^{An}pTpC^{An}-pC^{An}pC^{An}pT, and d-pA^{Bz}pG^{iB}pA^{Bz}pG^{iB} has been reported previously (Büchi and Khorana, 1972). The tetranucleotide block d-pC^{An}pTpA^{Bz}pC^{An} was synthesized by Dr. H. Weber; the dinucleotide d-pC^{An}pT and the pentanucleotide d-MMTrTpC^{An}pG^{iB}pA^{Bz}pA^{Bz} were intermediates in the synthesis of an undecanucleotide corresponding to a sequence of the *E. coli* tyrosine suppressor tRNA gene (J. H. van de Sande, unpublished results).

The above oligonucleotides were converted into 5′-hydroxyl-containing compounds by two different processes. (a) Aliquots (~60 OD_{260}) of the 5′-phosphate-containing oligonucleotides, d-pC^{An}pTpA^{Bz}pC^{An}, d-pA^{Bz}pG^{iB}pA^{Bz}pG^{iB}, and d-pC^{An}pT, were dissolved in 0.5 ml of 0.02 M ammonium bicarbonate (pH 8.0) and treated at 37° with 25 μg of bacterial alkaline phosphatase. The dephosphorylation was followed by removing 5-μl aliquots at 15-min intervals and analyzing them by tlc in solvent A. After the reactions were finished (60–90 min), the reaction mixtures were concentrated to 50 μl and chromatographed on Whatman No. 1 in solvent A. The dephosphorylated oligonucleotide spots were cut out and eluted with 0.01 M ammonium hydroxide containing 10% ethanol. Half of each eluate was directly applied to a Sephadex G-25 SF column (1.2 × 95 cm) for further purification; the other half was first treated with concentrated ammonium hydroxide at 50° for 3 hr to remove the N-blocking groups, before passage through Sephadex G-25. The chromatographically pure oligonucleotides d-ApGpApG, d-A^{Bz}pG^{iB}pA^{Bz}pG^{iB}, d-CpTpApC, d-C^{An}pTpA^{Bz}pC^{An}, and d-C^{An}pT were obtained in this manner.

(b) Aliquots (~50 OD_{260}) of the monomethoxytrityl-containing oligonucleotides, d-MMTrG^{Bz}pC^{An}pT and d-MMTrTpC^{An}pG^{iB}pA^{Bz}pA^{Bz}, were dissolved in 1 ml of a mixture of acetic acid–pyridine–water (14:1:3, v/v) and allowed to react at room temperature for 18 hr. Not all of the monomethoxytrityl group is removed under these conditions, but little depurination occurs (J. H. van de Sande, unpublished results). The solutions are concentrated and chromatographed on Whatman No. 1 paper in solvent A. The detritylated oligonucleotide spots are cut out and eluted with 0.01 M ammonium hydroxide containing 10% ethanol. Half of each eluate was directly passed through Sephadex G-25; the other half was first treated with concentrated ammonia to remove the N-blocking groups. Prior to gel filtration, the trinucleotide d-G^{Bz}pCpT was treated with *n*-butylamine in methanol (1:1, v/v) to remove the *N*-benzoyl group, which is not removed by concentrated ammonia.

The chromatographically pure oligonucleotides d-G^{Bz}pC^{An}pT, d-GpCpT, d-TpCpGpApA, and d-TpC^{An}pG^{iB}pA^{Bz}pA^{Bz} were obtained in this manner.

The spectral ratios and chromatographic mobilities of all the protected and unprotected oligonucleotides are shown in Table I. Oligonucleotides terminated in either a 5′-phosphate or 5′-hydroxyl group gave identical spectral ratios. The calculated spectral ratio of an oligonucleotide was obtained by summation of the extinction coefficients of the constituent mononucleotides.

[1] For the system of abbreviations used see Schaller and Khorana (1963a). The additional abbreviation used in this paper and not defined in the text is: iB, isobutyryl group on the guanine ring.

TABLE I: Properties of the Protected and Unprotected Compounds.

Deoxyoligonucleotide	OD_{260}/OD_{280}[b] Obsd	Calcd	R_F[a]
C^{An}pT	0.86	0.89	1.54
pC^{An}pT			1.39
GpCpT	1.16	1.23	0.87
pGpCpT			0.48
G^{Bz}pC^{An}pT	1.06		1.38
pG^{Bz}pC^{An}pT			1.09
ApGpApG	2.60	2.65	0.60
pApGpApG			0.13
A^{Bz}pG^{iB}pA^{Bz}pG^{iB}	0.97	0.92	1.56
pA^{Bz}pG^{iB}pA^{Bz}pG^{iB}			1.33
CpTpApC	1.59	1.67	0.64
pCpTpApC			0.13
C^{An}pTpA^{Bz}pC^{An}	0.81	0.74	1.55
pC^{An}pTpA^{Bz}pC^{An}			1.42
TpCpGpApA	1.92	2.23	0.64
pTpCpGpApA			0.17
TpC^{An}pG^{iB}pA^{Bz}pA^{Bz}	0.88	0.83	1.12
pTpC^{An}pG^{iB}pA^{Bz}pA^{Bz}			0.83

[a] R_F values were determined in solvent B and were recorded relative to d-pT. [b] Absorbance ratios were recorded in 0.02 M Tris-HCl (pH 7.8).

Kinase Reaction. The incubation mixture for the kinase reactions was a slight modification of the one described by Richardson (1965). It contained 0.066 M Tris-HCl (pH 7.6), 0.008 M $MgCl_2$, and 0.01 M 2-mercaptoethanol. The oligonucleotide concentrations, [γ-^{32}P]- or [γ-^{33}P]ATP concentrations, and the units of polynucleotide kinase are described in the legends to the figures. The reactions were carried out as described previously (van de Sande *et al.*, 1972). The kinetics of phosphorylation were followed by taking out 0.1–0.5-μl aliquots and applying them onto DE-81 strips (57 cm) previously spotted with 50 μl of a solution of 1 mM ATP containing 0.05 M EDTA. The strips were subjected to chromatography in solvents D or E. The dried strips were scanned in a Packard 7200 radiochromatogram scanner and the radioactive spots cut out and counted in a liquid scintillation counter. After the reaction is over the excess labeled ATP is destroyed by incubating the reaction mixture for 15 min with a 100-fold excess of both ATP and glucose and 5–10 units of yeast hexokinase (Hänggi *et al.*, 1970). The reaction products are separated by either gel filtration on G-25 or anion exchange chromatography through DEAE-cellulose. Aliquots of fractions were counted in restricted channels for double label experiments and counts were corrected for crossover of ^{32}P into ^{33}P (5–7%). Details of the separation procedures are given in the legends to the figures.

Degradation to 5′- and 3′-Mononucleotides. The procedure for the degradation to 5′-mononucleotides was as reported by Wells *et al.* (1967). Degradation to 3′-mononucleotides was carried out as described by Kleppe *et al.* (1971).

Preparation of d-MMTrG^{iB}pC^{An}pTpC^{An}pC^{An}pC^{An}pT^{32}p-C^{An}pT. A solution of d-MMTrG^{iB}pC^{An}pTpC^{An}pC^{An}-pC^{An}pT, 510 OD_{280} (5.3 μmol), and d-^{32}pC^{An}pToAc, 1200 OD_{280} (49.7 μmol), in pyridine was rendered anhydrous by repeated evaporation with pyridine. 2,4,6-Triisopropylben-

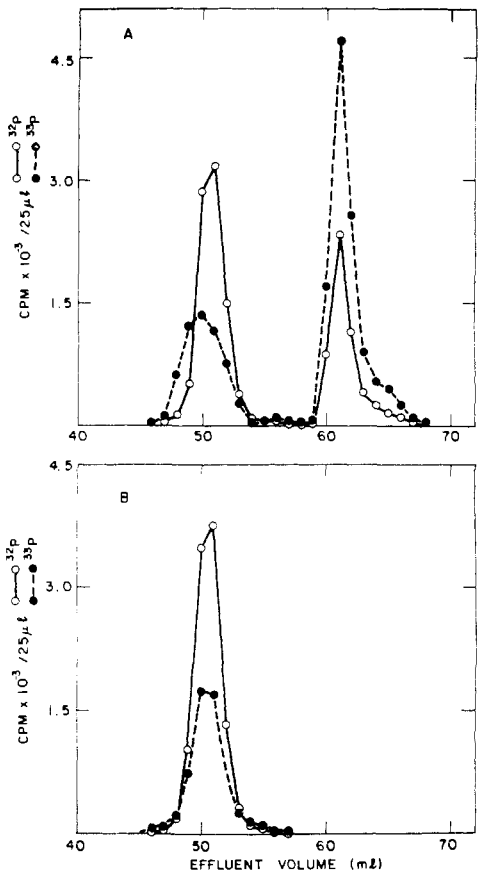

FIGURE 1: Gel filtration of phosphorylated d-TpC^{An}pG^{iB}pA^{Bz}pA^{Bz} and d-TpCpGpApA before (A) and after (B) ammonia treatment. The kinase reaction mixture is described under Materials and Methods. One incubation mixture (75 μl) contained 600 pmol of d-TpC^{An}pG^{iB}pA^{Bz}pA^{Bz} and 1800 pmol of [γ-^{33}P]ATP; the other reaction mixture (75 μl) contained 600 pmol of d-TpCpGpApA and 1200 pmol of [γ-^{32}P]ATP. The reactions were started by adding 2.4 units of polynucleotide kinase and terminated after 40 min by adding EDTA to 0.03 M. The two solutions were mixed, boiled for 3 min, and applied to a column (1.1 × 90 cm) of Sephadex G-50 SF eluted with 0.1 M triethylammonium bicarbonate at 4°. The fractions were counted for ^{32}P and ^{33}P and the elution pattern is shown in part A. The first eluted peak for both ^{32}P and ^{33}P is the phosphorylated oligonucleotide; the second peak for each isotope is the excess ATP. The effluent volume from 47 to 54 ml was concentrated and treated with concentrated ammonia at 50° for 3 hr. After removal of the ammonia the residue was dissolved in 200 μl of water and applied to the column of Sephadex G-50 SF used above. The elution profile is shown in part B.

zenesulfonyl chloride, 57 mg (190 μmol), was added and the reaction was allowed to proceed for 5 hr at room temperature in 1 ml of pyridine. Aliquots, 10 μl, were taken out at 0, 1, 2, and 5 hr and added to 10 μl of water, left at room temperature for 24 hr, and analyzed by paper chromatography in solvent D. The reaction was stopped by adding 0.6 ml of 1 M diisopropylethylamine in pyridine, followed by 1.5 ml of water, and the aqueous pyridine solution was kept for 24 hr at room temperature. The reaction solution was treated with 2 N sodium hydroxide (3.5 ml) at 0° for 10 min, neutralized with pyridinium Dowex, and applied to DEAE-cellulose for chromatography. Details of the chromatographic separation are given in the legend to Figure 7. The nonanucleotide, peak IV, was found to be homogeneous on analysis by paper chromatography in solvent B after removal of the N-protecting groups. The observed ratio E280/E300 was 1.09, the calculated ratio being 1.03. The yield of isolated nonanucleotide was 276 OD_{280} (44%).

Results and Discussion

Phosphorylation of d-TpC^{An}pG^{iB}pA^{Bz}pA^{Bz}. T_4-induced polynucleotide kinase catalyzes the transfer of orthophosphate from ATP to the 5′-hydroxyl termini of polynucleotides, as well as nucleoside 3′-phosphates (Richardson, 1965; Novogrodsky and Hurwitz, 1966; Novogrodsky *et al.*, 1966). A possible interpretation of this substrate specificity is that the enzyme recognizes the nucleoside 3′-phosphate moiety at the 5′-hydroxyl end regardless of whether or not it is part of a polynucleotide chain.

During the chemical synthesis of oligonucleotides no protection of the thymine base is required. A protected oligonucleotide with a thymidine at the 5′-hydroxyl terminus should act as a substrate for the kinase if the enzyme recognizes only the 5′-terminal nucleoside 3′-phosphate group of a polynucleotide. The pentanucleotide d-TpC^{An}pG^{iB}pA^{Bz}pA^{Bz} was subjected to the usual kinase reaction conditions and a transfer of ^{32}P-labeled orthophosphate from [γ-^{32}P]ATP to the pentanucleotide was observed as assayed by DE-81 paper chromatography in solvent D. No difference in the rate of phosphorylation between the protected pentanucleotide, d-TpC^{An}pG^{iB}pA^{Bz}pA^{Bz}, and the unprotected pentanucleotide, d-TpCpGpApA, could be observed. This would be in agreement with the hypothesis that in both compounds only the 5′-hydroxyl thymidine 3′-phosphate is recognized by the polynucleotide kinase and the presence of protection groups in the oligonucleotide beyond that does not inhibit the action of the enzyme. The experiment described in Figure 1 shows conclusively that phosphorylation of the protected pentanucleotide takes place in the desired manner. The protected and unprotected pentanucleotides were separately phosphorylated, d-TpC^{An}pG^{iB}pA^{Bz}pA^{Bz} with [γ-^{33}P]ATP and d-TpCpGpApA with [γ-^{32}P]ATP. After the reactions reached a plateau in the extent of phosphate transfer, the reaction solutions were mixed and the mixture analyzed by gel filtration on Sephadex G-50 (Figure 1A).

The difference in molecular weight (~20%) between the two oligonucleotides is enough to elute the protected pentanucleotide, d-^{33}pTpC^{An}pG^{iB}pA^{Bz}pA^{Bz}, just ahead of the unprotected pentanucleotide, d-^{32}pTpCpGpApA. As expected, the unreacted [γ-^{32}P]- and [γ-^{33}P]ATP elute at the same volume. The fractions containing the ^{33}P- and ^{32}P-labeled pentanucleotides were pooled, concentrated, and treated with ammonia to remove the N-protecting groups from d-^{33}p-TpC^{An}pG^{iB}pA^{Bz}pA^{Bz} to yield a mixture of d-^{32}p- and d-^{33}pTpCpGpApA. Gel filtration of the ammonia treated mixture (Figure 1B) shows coinciding peaks for the radioactivity in ^{32}P and ^{33}P. Analysis of this peak by digestion to 5′-mononucleotides showed that d-pT contained essentially all of the radioactivity in both ^{32}P (7127 cpm out of 7298 cpm) and ^{33}P (6240 cpm out of 6554 cpm). The experiments described above have shown that the presence of N-protecting groups in an oligonucleotide, beyond the substrate nucleoside 3′-phosphate moiety, does not interfere with action of the polynucleotide kinase.

Phosphorylation of d-C^{An}pTpA^{Bz}pC^{An}, d-A^{Bz}pG^{iB}pA^{Bz}pG^{iB}, and d-G^{Bz}pC^{An}pT. The effect of a blocking group on the 5′-hydroxyl terminal nucleoside of an oligonucleotide during a kinase reaction was investigated next. The protected tetranucleotide d-C^{An}pTpA^{Bz}pC^{An} was subjected to the usual kinase reaction conditions and a transfer of ^{32}P-labeled orthophosphate from [γ-^{32}P]ATP to d-C^{An}pTpA^{Bz}pC^{An} was observed as assayed by anion exchange paper chromatography in solvent E. A comparison was made between the

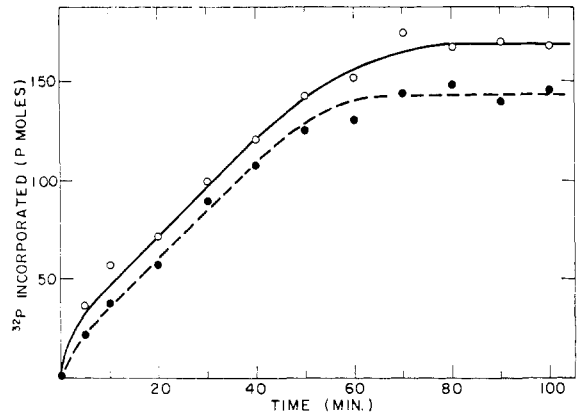

FIGURE 2: Phosphorylation of d-CpTpApC and d-C^{An}pTA^{Bz}pC^{An}. The reaction mixture for phosphorylation is described under Materials and Methods. The concentrations of oligonucleotide and [γ-^{32}P]ATP were 3 and 15 nmol/ml, respectively, in a reaction volume of 0.05 ml. The reactions were started by the addition of 0.2 unit of polynucleotide kinase and the rate of phosphorylation of the oligonucleotides was followed in the usual manner. A second aliquot of enzyme was added after 60 min: (○) d-^{32}pCpTpApC; (●) d-^{32}pC^{An}pTpA^{Bz}pC^{An}.

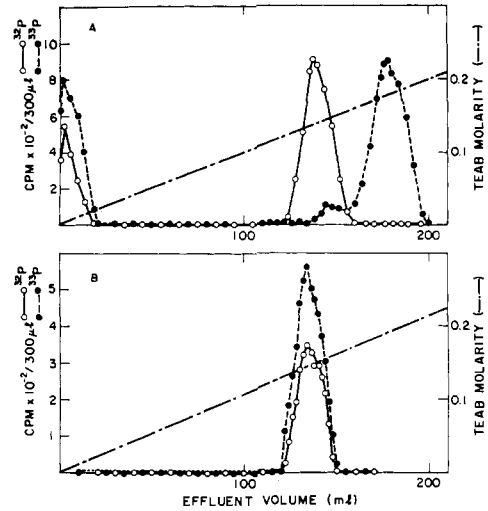

FIGURE 3: Anion exchange chromatography of d-^{33}pC^{An}pTpA^{Bz}pC^{An} and d-^{32}pCpTpApC bdfore (A) and after (B) ammonia treatment. The kinase incubation mixture is described under Materials and Methods. Each incubation mixture (100 μl) contained 700 pmol of either d-CpTpApC or d-C^{An}pTpA^{Bz}pC^{An} and 1200 pmol of [γ-^{32}P] or [γ-^{33}P]ATP, respectively. The reactions were started by adding 6 units of polynucleotide kinase and terminated after 40 min by adding EDTA to 0.03 M. The excess ATP was destroyed with hexokinase as described under Materials and Methods and the two reaction solutions were mixed and applied to a DEAE-cellulose column (1 × 4 cm) preequilibrated with 0.01 M triethylammonium bicarbonate at 4°. A gradient in triethylammonium bicarbonate from 0.01 to 0.2 M, total volume 200 ml, was passed through the column and the elution profile is shown in A. Glucose 6-[^{32}P]- and 6-[^{33}P]phosphate coelute at low salt followed by d-^{32}pCpTpApC and finally d-^{33}pC^{An}pTpA^{Bz}pC^{An}. The peaks of radioactivity corresponding to d-^{32}pCpTpApC and d-^{33}pC^{An}pTpA^{Bz}pC^{An} are pooled together, concentrated, and treated with concentrated ammonia at 50° for 3 hr. After removal of the ammonia the residue was dissolved in 1 ml of 0 01 M triethylammonium bicarbonate and applied to the same DEAE-cellulose column which had been preequilibrated with 0.01 M triethylammonium bicarbonate. A linear gradient (200 ml) from 0.01 to 0.2 M triethylammonium bicarbonate was passed through the column and the elution profile is shown in B.

rate of phosphorylation of the protected tetranucleotide, d-C^{An}pTpA^{Bz}pC^{An}, and the unprotected analog, d-CpTpApC. Equimolar amounts, based on the calculated molar extinction coefficients, of the two oligonucleotides were phosphorylated with [γ-^{32}P]ATP and polynucleotide kinase and the kinetics of phosphate transfer are shown in Figure 2. Little difference was observed in the rate of phosphorylation between the protected and unprotected tetranucleotides. The 10% higher level of phosphate incorporation with the unprotected tetranucleotide can be explained by the fact that no allowance was made in the calculated molar extinction coefficient for any hypochromicity of the unprotected tetranucleotide. The incorporation of radioactive orthophosphate into d-C^{An}pTpA^{Bz}pC^{An} was also shown in the following experiment. The two oligonucleotides, d-C^{An}pTpA^{Bz}pC^{An} and d-CpTpApC, were individually phosphorylated with ^{33}P- and ^{32}P-labeled ATP, respectively, and the excess labeled ATP in each reaction was converted to ADP and glucose 6-phosphate by incubation with hexokinase. The two reaction mixtures were pooled and the resulting solution was analyzed by DEAE-cellulose chromatography as shown in Figure 3A. Glucose 6-[^{32}P,^{33}P]phosphate was eluted first, followed by clearly separated peaks for d-^{32}pCpTpApC and d-^{33}pC^{An}pTpA^{Bz}pC^{An}. The protected tetranucleotide was retarded on the column due to the hydrophobic interactions between the aromatic protection groups and the cellulose matrix. The two tetranucleotide peaks were combined, the blocking groups on d-^{33}pC^{An}pTpA^{Bz}pC^{An} removed by ammonia treatment, and the resulting d-^{32}p,^{33}pCpTpApC rechromatographed on DEAE-cellulose as shown in Figure 3B. The double labeled tetranucleotide was degraded to 5′-mononucleotides with snake venom phosphodiesterase and d-pC was found to contain essentially all the radioactivity in both ^{32}P (1927 cpm out of 2109 cpm) and ^{33}P (933 cpm out of 1041 cpm). Evidently, the anisoyl group on the 5′-terminal nucleoside presents no obstruction for the polynucleotide kinase to phosphorylate the 5′-hydroxyl group.

Similarly, it was found that oligonucleotides containing other protected bases at the 5′ terminus could be phosphorylated by polynucleotide kinase. The rates of phosphorylation of the protected trinucleotide, d-G^{Bz}pC^{An}pT, and the protected tetranucleotide, d-A^{Bz}pG^{iB}pA^{Bz}pG^{iB}, were only slightly lower than those of their respective unprotected compounds. The chromatographic separation of d-^{32}pApGpApG and d-^{33}pA^{Bz}pG^{iB}pA^{Bz}pG^{iB} on DEAE-cellulose is shown in Figure 4A. Again, the ^{32}P- and ^{33}P-labeled glucose 6-phosphates elute first, followed by peaks corresponding to d-^{32}pApGpApG and d-^{33}pA^{Bz}pG^{iB}pA^{Bz}pG^{iB}. After removal of the N-protecting groups on d-^{33}pA^{Bz}pG^{iB}pA^{Bz}pG^{iB}, superimposable peaks were obtained for the two labels, showing that the mixture consisted of d-^{32}p,^{33}pApGpApG (Figure 4B). Again, digestion to 5′-mononucleotides showed essentially all the radioactivity in ^{32}P (3431 cpm out of 3615 cpm) and ^{33}P (1396 cpm out of 1527 cpm) migrated with a d-pA marker in solvent system C.

A similar experiment was carried out with the trinucleotide d-G^{Bz}pC^{An}pT, which showed unambiguously that phosphorylation of this trinucleotide had taken place.

Seemingly, there is no difference between the phosphorylation of an unprotected oligonucleotide and the phosphorylation of oligonucleotides containing large protection groups. The bulky aromatic groups are expected to change the steric as well as the electronic environment of the base moiety, but they do not affect the rate of phosphate transfer from ATP to the deoxyribose part of the protected nucleoside at the 5′-hydroxyl terminus. It is possible that the base at the 5′-hy-

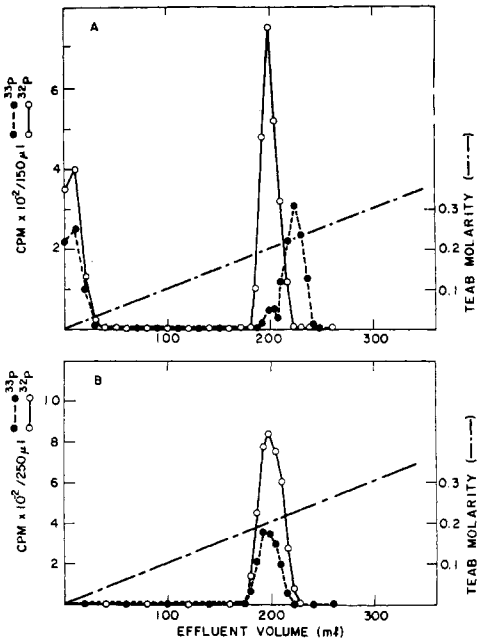

FIGURE 4: Anion exchange chromatography of d-^{33}pA^{Bz}pG^{iB}-pA^{Bz}pG^{iB} and d-^{32}pApGpApG before (A) and after (B) ammonia treatment. The kinase incubation mixture is described under Materials and Methods. Each incubation mixture (75 µl) contained 600 pmol of either d-ApGpApG or d-A^{Bz}pG^{iB}pA^{Bz}pG^{iB} and 1050 pmol of [γ-^{32}P]- or [γ-^{33}P]ATP, respectively. The reactions were started by adding 6 units of polynucleotide kinase and terminated after 40 min by adding EDTA to 0.03 M. The excess ATP was destroyed with hexokinase as described under Materials and Methods and the two reaction solutions were mixed and applied to a DEAE-cellulose column (1 × 6 cm) preequilibrated with 0.01 M triethylammonium bicarbonate at 4°. A gradient in triethylammonium bicarbonate from 0.01 to 0.3 M, total volume 300 ml, was passed through the column and the elution profile is shown in A. The elution of glucose 6-[^{32}P,^{33}P]phosphate is followed by peaks corresponding to d-^{32}pApGpApG and d-^{33}pA^{Bz}pG^{iB}pA^{Bz}pG^{iB}. The latter two peaks are combined, concentrated, and treated with concentrated ammonia at 50° for 3 hr. After ammonia removal, the residue was dissolved in 1 ml of 0.01 M triethylammonium bicarbonate and analyzed by chromatography through DEAE-cellulose under the same conditions as described above. The elution profile is shown in B.

droxyl terminus does not play a part in the substrate recognition by the T_4 polynucleotide kinase. However, polynucleotides containing a thymine dimer at their 5′ end cannot be phosphorylated by polynucleotide kinase (Kushner *et al.*, 1971).[2] The presence of a thymine dimer could impose a rotational restriction around the first internucleotide bond of the polynucleotide, which could result in the observed lack of phosphorylation. Studies are in progress to determine whether the terminal nucleoside 3′-phosphate or only the terminal ribose or deoxyribose 3′-phosphate moiety is involved in the recognition by the enzyme.

Synthesis of d-MMTrG^{iB}pC^{An}pTpC^{An}pC^{An}pC^{An}^{32}pC^{An}pT. The potential use of labeled oligonucleotide blocks was investigated with the synthesis of a nonanucleotide by condensation of a heptanucleotide with a dinucleotide block containing an external ^{32}P label. The presence of the radioactive label in the dinucleotide block could be used to study the rate of internucleotide bond formation in chemical synthesis. The use of a labeled oligonucleotide block in a chemical reaction will also facilitate the characterization of the reaction prod-

[2] The authors wish to thank the referee for bringing this observation to their attention.

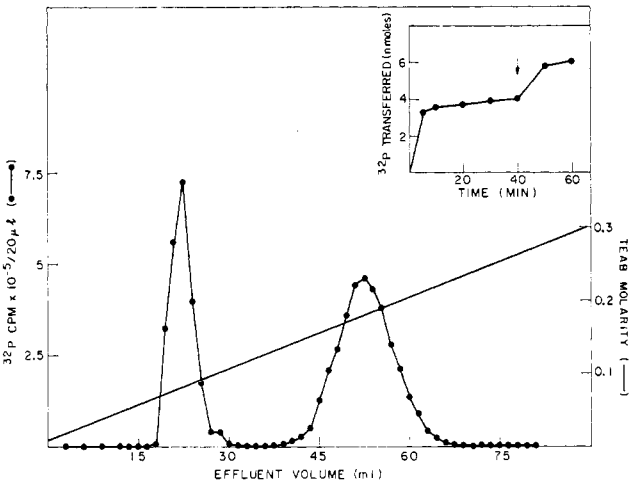

FIGURE 5: Preparation of d-^{32}pC^{An}pToAc. The kinase incubation mixture (500 µl) contained 4 nmol of d-C^{An}pT and 10 nmol of [γ-^{32}P]ATP. The reaction was started by adding 17 units of polynucleotide kinase and the orthophosphate transfer was followed as described under Materials and Methods, and shown in the inset of the figure. After 40 min a second aliquot of d-C^{An}pT (2 nmol) and polynucleotide kinase (17 units) was added. The excess of [γ-^{32}P]ATP was decomposed with hexokinase and the reaction mixture was diluted tenfold with water and applied to a column (1 × 6 cm) of DEAE-cellulose preequilibrated at 4° with 0.005 M triethylammonium bicarbonate. A linear gradient (100 ml) in triethylammonium bicarbonate from 0.01 to 0.3 M was passed through the column at a flow rate of 15 ml/hr. The second peak was pooled, diluted with 50 µmol of d-pC^{An}pT, and then acetylated with acetic anhydride as described previously (Büchi and Khorana, 1972). The dinucleotide block, d-^{32}pC^{An}pToAc, was isolated in 98% yield and had a specific activity of 4270 cpm/nmol.

ucts of the condensation. The dinucleotide block, d-^{32}p-C^{An}pT, was prepared by phosphorylation of 6 nmol of d-C^{An}pT with [γ-^{32}P]ATP of high specific activity (20 Ci/mmol) and then diluted ~8000-fold with unlabeled d-pC^{An}pT. The kinetics of phosphorylation of d-C^{An}pT is shown in the inset of Figure 5. The isolation of d-^{32}pC^{An}pT by DEAE-cellulose chromatography after decomposition of the excess [γ-^{32}P]ATP is also shown in Figure 5. The second peak consisted of the dinucleoside diphosphate which was diluted with 50 µmol of d-pC^{An}pT. The ^{32}P-labeled d-pC^{An}pT was subsequently treated with acetic anhydride to acetylate the 3′-hydroxyl group and the product d-^{32}pC^{An}pToAc was analyzed by paper chromatography in solvent A. The ultraviolet (uv) absorbing spot of d-^{32}pC^{An}pToAc contained essentially all of the radioactivity (273,646 cpm out of 283,046 cpm). The phosphorylation, isolation, and acetylation of this oligonucleotide were carried out within 2 days, which makes it feasible to use this procedure for chemical synthesis.

The dinucleotide block was condensed with the heptanucleotide d-MMTrG^{iB}pC^{An}pTpC^{An}pC^{An}pC^{An}pT as described under Materials and Methods. Samples were withdrawn from the condensation mixture, quenched with water, and analyzed by anion exchange paper chromatography in solvent D. The product, nonanucleotide, is not expected to move in this system and the radiochromatographic scans of the different time points are shown in Figure 6. At zero time there is only radioactivity in d-^{32}pC^{An}pToAc and as the reaction progresses we can see the appearance of a product peak which stays at the origin and a peak which moves behind the dinucleotide block. The latter peak was characterized as the pyrophosphate of the dinucleotide block, which is a well-established intermediate during chemical synthesis (Khorana

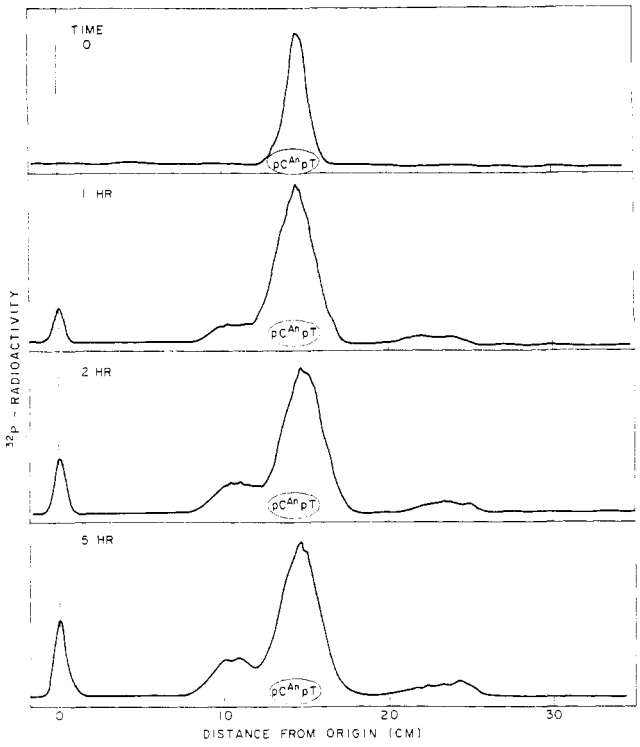

FIGURE 6: Radiochromatographic scans of time points during the chemical synthesis of d-MMTrG^{iB}pC^{An}pTpC^{An}pC^{An}pC^{An}pT^{32}p-C^{An}pT. Aliquots from the reaction mixture after 0, 1, 2, and 5 hr were quenched and chromatographed in solvent D.

and Vizsolyi, 1961). A small amount of radioactivity which moved behind the solvent front appeared during the reaction and is possibly due to inorganic phosphate formed as a breakdown product from the activated dinucleoside diphosphate block.

From the excess of d-^{32}pC^{An}pT used over the amount of heptanucleotide, it can be calculated that the yields of nonanucleotide at 1, 2, and 5 hr were 42, 63, and 73%, respectively. The reaction products were separated by anion exchange chromatography as is shown in Figure 7. The identification of the peaks is given in Table II. The specific activities of the starting block, d-^{32}pC^{An}pT and its pyrophosphate, and the product nonanucleotide were very similar as would be expected. A nearest neighbor analysis on the completely deprotected nonanucleotide showed transfer to d-Tp only (4324 cpm out of 4491 cpm). The starting material, peak III, showed the presence of a trace of radioactivity due to some trailing of the pyrophosphate of d-^{32}pC^{An}pT. The 1 M eluate (peak V) contained a higher level of radioactivity per OD_{280} as did the nonanucleotide product. The specific activity agrees with 2 equiv of d-^{32}pC^{An}pT being attached to the heptanucleotide. The second equivalent of d-^{32}pC^{An}pT can be either attached at the 3′-hydroxyl end of the nonanucleotide because of incomplete protection of the dinucleotide block or by phosphorylation of the thymine ring as has been observed previously (Schaller and Khorana, 1963b). Analysis of peak V by paper chromatography after removal of the protection groups showed radioactivity traveling with the nonanucleotide and the dinucleotide block in a 1:1 ratio. This indicates that phosphorylation of the thymine ring by the dinucleotide block had taken place. The resulting phosphoramidate bond is cleaved on ammonia treatment.

It has been shown that T_4-induced polynucleotide kinase will recognize deoxyoligonucleotides containing protected nucleosides at the 5′-hydroxyl terminus. This method makes it possible to introduce a radioactive label in a protected deoxyoligonucleotide to facilitate characterization during polynucleotide synthesis.

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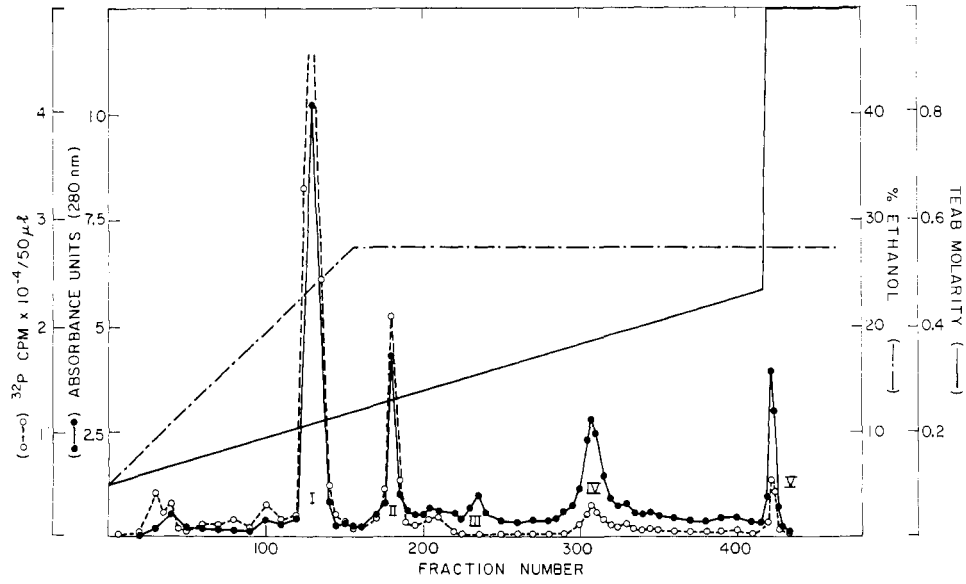

FIGURE 7: Separation of the reaction products in the preparation of d-MMTrG^{iB}pC^{An}pTpC^{An}pC^{An}pC^{An}pT^{32}pC^{An}pT. The aqueous pyridine reaction mixture was applied to a DEAE-cellulose column (1.5 × 75 cm) preequilibrated with 0.05 M triethylammonium bicarbonate at 4°. The first gradient was made up 0.1 M triethylammonium bicarbonate in 10% ethanol (350 ml) in the mixing vessel and 0.25 M triethylammonium bicarbonate in 40% ethanol (350 ml) in the reservoir. The final gradient was made up to 0.25 M triethylammonium bicarbonate in 40% ethanol (750 ml) in the mixing vessel and 0.50 M triethylammonium bicarbonate in 40% ethanol (750 ml) in the reservoir. The column was next washed with 1 M bicarbonate in 40% ethanol (250 ml). The identification of the peaks is given in Table II.

TABLE II: Identification of Products Formed in the Synthesis of d-MMTrG^{iB}pC^{An}pTpC^{An}pC^{An}pC^{An}pT^{32}pC^{An}pT.

Peak	Compound	Radioact. (cpm $\times$ 10^{-2}/OD_{280})	Sp Act. (cpm/nmol)
I	d-^{32}pC^{An}pT	1204	2902
II	d-O(^{32}pC^{An}pT)$_2$	1130	2738
III	d-MMTrG^{iB}pC^{An}pTpC^{An}pC^{An}pC^{An}pT	14	133
IV	d-MMTrG^{iB}pC^{An}pTpC^{An}pC^{An}pC^{An}pT^{32}pC^{An}pT	233	2778
V	d-(MMTrG^{iB}pC^{An}pTpC^{An}pC^{An}pC^{An}pT)(^{32}pC^{An}pT)$_2$	395	5667

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