Phosphorylation of Double-Stranded DNAs by T_4 Polynucleotide Kinase†

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ABSTRACT: The phosphorylation by T_4 polynucleotide kinase of various double-stranded DNAs containing defined 5′-hydroxyl end group structures has been studied. Particular emphasis was placed on finding conditions that allow complete phosphorylation. The DNAs employed were homodeoxyoligonucleotides annealed on the corresponding homopolymers, DNA duplexes corresponding to parts of the genes for alanine yeast tRNA, and a suppressor tyrosine tRNA from *Escherichia coli*. The rate of phosphorylation of DNAs with 5′-hydroxyl groups in gaps was approximately ten times slower than for the corresponding single-stranded DNA. At low concentrations of ATP, 1 μM, incomplete phosphorylation was obtained, whereas with higher concentrations of ATP, 30 μM, complete phosphorylation was achieved. In the case of DNAs with 5′-hydroxyl groups at nicks approximately 30% phosphorylation could be detected using 30 μM ATP. A DNA containing protruding 5′-hydroxyl group ends was phosphorylated to completion using the same conditions as for single-stranded DNA, i.e., a ratio between the concentrations of ATP and 5′-hydroxyl groups of 5:1 and a concentration of ATP of approximately 1 μM. For a number of DNAs containing protruding 3′-hydroxyl group ends and one DNA containing even ends incomplete phosphorylation was found under similar conditions. For all these DNAs a plateau level was observed varying from 20 to 45% of complete phosphorylation. At 20 μM and higher ATP concentrations, the phosphorylation was complete also for these DNAs. With low concentrations of ATP a rapid production of inorganic phosphate was noted for all the latter DNAs. The apparent equilibrium constants for the forward and reverse reaction were determined for a number of different DNAs, and these data revealed that the plateau levels of phosphorylation obtained at low concentrations of ATP for DNAs with protruding 3′-hydroxyl group and even ends is not a true equilibrium resulting from the forward and reverse reaction. It is suggested that the plateau levels are due to formation of inactive enzyme–substrate and enzyme–product complexes. For all double-stranded DNAs tested, except DNAs containing protruding 5′-hydroxyl group ends, addition of KCl to the reaction mixture resulted in a drastic decrease in the rate of phosphorylation, as well as in the maximum level phosphorylated. Spermine, on the other hand, had little influence. Both of these agents have previously been shown to activate T_4 polynucleotide kinase using single-stranded DNAs as substrates (Lillehaug, J. R., and Kleppe, K. (1975), *Biochemistry 14*, 1221). The inhibition of phosphorylation of double-stranded DNAs by salt might be the result of stabilization of the 5′-hydroxyl group regions of these DNAs.

Phage-induced polynucleotide kinases catalyze the transfer of the γ-phosphate residue of ATP to the 5′-hydroxyl group of nucleic acids, oligonucleotides, and 3′-phosphate mononucleotides (Richardson, 1965; Novogrodsky and Hurwitz, 1966). The enzyme from T_4 infected cells has also recently been shown to catalyze the reverse reaction (van de Sande et al., 1973). The latter enzyme has become an indispensable tool in the sequence analysis of DNA and RNA (Weiss and Richardson, 1967; Jay et al., 1974; Szekely and Sanger, 1969; Southern, 1970; Murray, 1973) as well as in the synthesis of certain DNAs such as the genes corresponding to a yeast alanine tRNA (Khorana et al., 1972) and a tyrosine suppressor tRNA from *E. coli* (Caruthers et al., 1973).

We have recently studied the mechanism of action of T_4 polynucleotide kinase in some detail using single-stranded DNAs and 3′-mononucleotides as substrates. The enzyme displayed a marked substrate specificity with regard to the 5′-terminal base residue and, moreover, the mechanism itself was of the ordered sequential type (Lillehaug and Kleppe, 1975a). Salt and polyamines greatly stimulated the reaction at low enzyme concentrations (Lillehaug and Kleppe, 1975b). The overall mechanism in the presence of activators was found to be sequential, but probably of a rapid equilibrium random type.

The present work deals with the phosphorylation of various types of double-stranded DNAs. With regard to the position of the 5′-hydroxyl group to be phosphorylated in a double-stranded DNA one can, in general, distinguish between five different structures, as shown in Figure 1. In structure A and B the 5′-hydroxyl group is situated in a gap and a nick, respectively. In structure C the 5′-hydroxyl group is at the end of the protruding single-stranded DNA. In the case of structure D the ends are even, and in structure E the 3′-hydroxyl group is situated at the protruding single-stranded end. DNAs of all these categories were employed in the present work. Most of the DNAs used corresponded to parts of the genes for yeast alanine tRNA and tyrosine suppressor tRNA from *E. coli*. The phosphorylation of the various DNAs by T_4 polynucleotide kinase was examined with regard to the rate and completeness of phosphorylation, effect of ATP concentration, reversibility and equilibrium constants, specifity of phosphorylation, and influence of salt and polyamines. For comparison the phosphorylation of certain single-stranded DNAs was also included.

Materials

Enzymes. T_4 polynucleotide kinase and ligase were isolated as previously described (Panet et al., 1973). Both enzyme

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‡ Abbreviations used are: HO-DNA, a DNA carrying a free 5′-hydroxyl group; P-DNA, a DNA carrying a 5′-phosphate group; DEAE, diethylaminoethyl; P_i, inorganic phosphate.

preparations were free of nucleases when tested with synthetic DNA fragments, [^{14}C]poly[d(A-T)] and [^{3}H]T_7 DNA. 3-Phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase were products of Boehringer Mannheim. Bacterial alkaline phosphatase, micrococcal nuclease, and spleen phosphodiesterase were from Worthington Biochemical Corp. Pancreatic deoxyribonuclease (DNase I) and venom phosphodiesterase were obtained from Sigma Chemical Co.

DNAs. The synthetic poly- and oligodeoxynucletides $(dA)_n$, $(dC)_n$, $d(pT)_{10}$, and $d(pG)_{10}$ were purchased from P-L Biochemicals, Inc. The oligonucleotides were dephosphorylated as previously reported (Lillehaug and Kleppe, 1975a).

The structure of the DNAs of known sequence used, corresponding to parts of the genes for yeast alanine tRNA and tyrosine suppressor tRNA from *E. coli*, are shown in Figure 2. All the single-stranded segments from which these double-stranded DNAs were made, as well as DNA II and DNA III, were generously donated by Dr. H. G. Khorana, Massachusetts Institute of Technology. The double-stranded DNAs were prepared from the single-stranded segments by the enzymes T_4 polynucleotide kinase and ligase. The preparation of DNA II and DNA III are described by Loewen et al. (1975) and by Caruthers et al. (1975), respectively. The other double-stranded DNAs used in this work were prepared by the authors. DNA I was made as described by Sgaramella et al. (1972). The experimental conditions used for the preparations of DNA IV were essentially the same as those employed for DNA I except that fragments 5, 6, and 7 of the gene for yeast alanine tRNA were used. DNA V and DNA VI were prepared as previously described (van de Sande et al., 1972). The purity of the DNAs was checked by a variety of methods including gel electrophoresis and nearest neighbor analysis.

[γ-^{32}P]ATP. The radioactive [γ-^{32}P]ATP was made essentially as described by Glynn and Chappell (1964). The specific activity was approximately 5 Ci/mmol.

Methods. The standard reaction mixture contained 60 mM Tris-HCl, pH 8.0, 15 mM β-mercaptoethanol, 9 mM $MgCl_2$, 30 μM [γ-^{32}P]ATP, 0.15–0.50 μM DNA (5′-hydroxyl ends), and 12 units/ml of enzyme if not otherwise stated. The time course of phosphorylation was followed by removing aliquots at various time intervals and the aliquots were applied on DEAE paper strips. These were then subsequently developed in 0.3 M ammonium formate, dried, and scanned for radioactivity using a paper-chromatogram scanner. The ^{32}P-labeled oligonucleotides remain at the origin. In this system the inorganic phosphate (P_i) could also be separated from ATP.

Nearest neighbor and 5′-mononucleotide analyses were carried out essentially as previously published (Kleppe et al., 1971).

Gel electrophoresis of the DNAs was performed as described by Raae et al. (1975a).

Results

Phosphorylation of 5′-Hydroxyl Groups in Gaps and Nicks. The substrates used for phosphorylation of gaps and nicks were homodeoxyoligonucleotides, such as $dT(pT)_9$ and $dG(pG)_9$ annealed to the corresponding homopolymers $(dA)_n$ and $(dC)_n$. The properties of such DNAs have recently been examined in detail (Tamblyn and Wells, 1975). When $d(pT)_{10}$ is annealed to $(dA)_n$ in a ratio of 1:1 a double-stranded DNA is formed containing nicks and some gaps. Recent studies with T_4 polynucleotide ligase have shown that approximately 65% of the $d(pT)_{10}$ molecules in the latter DNA can be joined by this enzyme, suggesting that for the remaining molecules gaps on one or both sides occur (Raae et al. 1975b). The time course

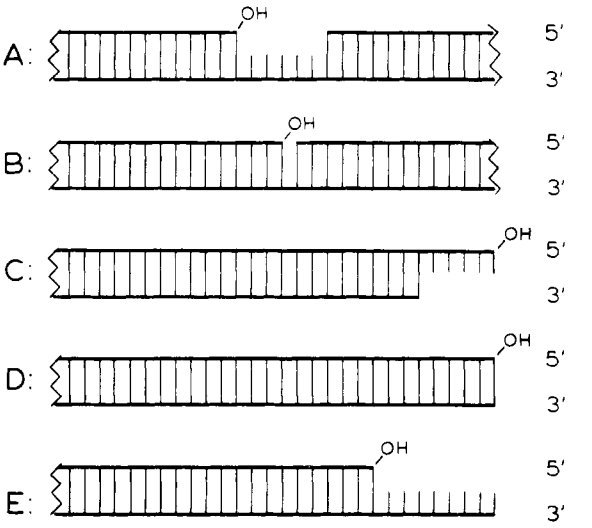


FIGURE 1: Schematic structure of different double-stranded nucleic acids.

of phosphorylation of $dT(pT)_9$ alone and annealed to $(dA)_n$ in various ratios is shown in Figure 3. These experiments were all carried out at 10 °C in order to maintain a stable bihelical structure of the DNA (Cassani and Bollum, 1969), and the concentration of ATP was relatively high, 30 μM. Under these conditions complete phosphorylation of $dT(pT)_9$ alone was achieved in approximately 15 min. When $dT(pT)_9$ was annealed to $(dA)_n$ in a ratio of 1:1 (based on mononucleotide), approximately 60% phosphorylation was obtained after 100 min of incubation. When the same compound was annealed in a ratio of 1:2, complete phosphorylation was achieved in the same period. In the latter case the 5′-hydroxyl group to be phosphorylated should be situated only in gaps. The relative initial rates in the three cases, i.e., no $(dA)_n$, in a ratio of 1:1 and in a ratio of 1:2, were estimated to be approximately 20, 1, and 2, respectively. The presence of salt, 0.125 M KCl, resulted in a decrease in both the initial rate as well as in the final plateau value of phosphorylation. At 3 μM ATP a 10% decrease in the final plateau values was observed for the oligonucleotide in the annealed form but not for $dT(pT)_9$ alone (results not shown).

The question may be raised as to whether it is gaps or nicks which is phosphorylated in the substrate $(dA)_n$–$dT(pT)_9$ annealed in a ratio of 1:1. To resolve this issue a number of experiments with T_4 polynucleotide ligase were carried out. T_4 polynucleotide ligase (80 units/ml) was added to the reaction mixture described in Figure 3; $(dA)_n$ and $dT(pT)_9$ annealed 1:1 both in the presence and absence of salt and after having been exposed for 100 min to T_4 polynucleotide kinase. The joining reactions at 10 °C were then monitored by removing aliquots at 5-min intervals and subjecting these to bacterial alkaline phosphatase at 65 °C (Raae et al., 1975b). At the end of a 20-min period less than 3% of the radioactivity in the phosphorylated samples had become resistant to bacterial alkaline phosphatase in both cases. In the control sample, 5′-[^{32}P]$d(pT)_{10}$ annealed to $(dA)_n$ in a ratio of 1:1, and maximal resistance to bacterial alkaline phosphatase of approximately 65% was reached in 2 min. These results suggest that T_4 polynucleotide kinase predominantly phosphorylates 5′-hydroxyl groups located in gaps on the DNA molecule. Furthermore, hydroxyl groups located in nicks are extremely difficult to phosphorylate under conditions where a double-stranded structure is maintained. By phosphorylation at elevated temperatures, where some denaturation is allowed, the

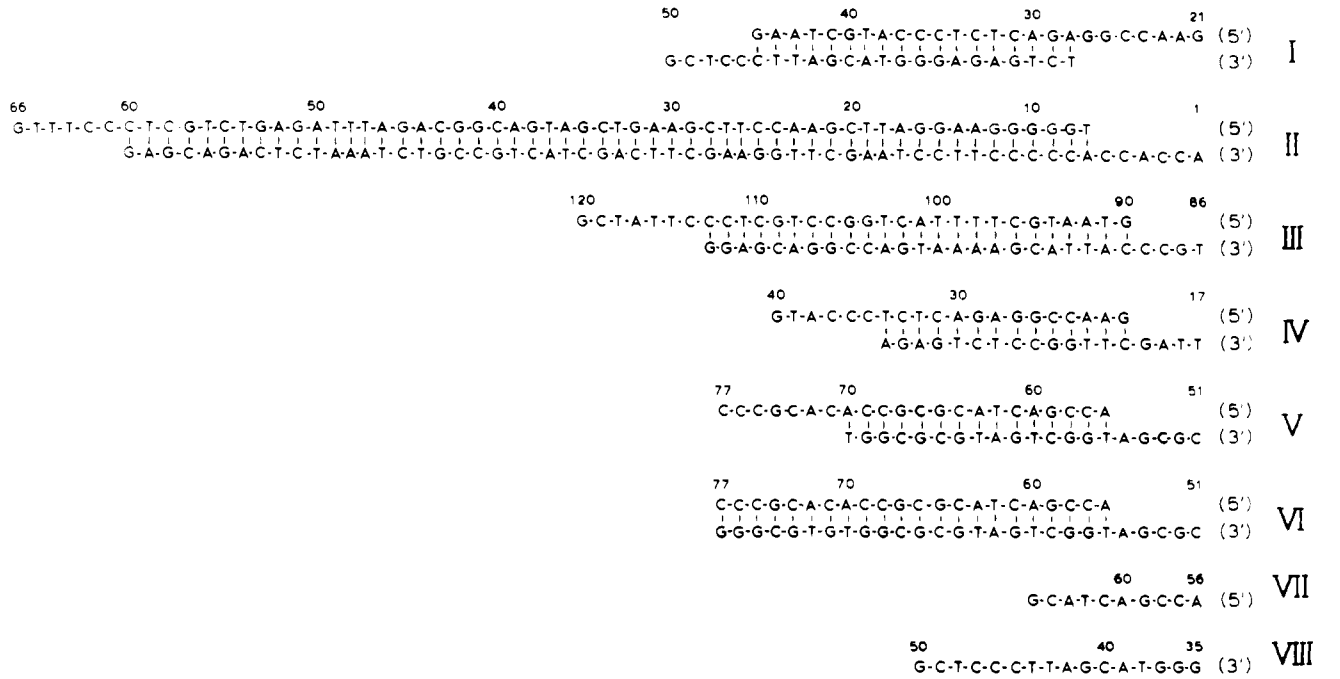


FIGURE 2: Structure of different double- and single-stranded DNAs of known sequence used in this work. DNA II and DNA III are part of the gene for a tyrosine suppressor tRNA from *E. coli* while all the other DNAs listed are part of the gene for alanine tRNA from yeast. The numbers above the different DNAs refer to the position of a given mononucleotide residue in the original tRNA structure starting from the ACC end. DNA VIII belongs to the complementary strand.

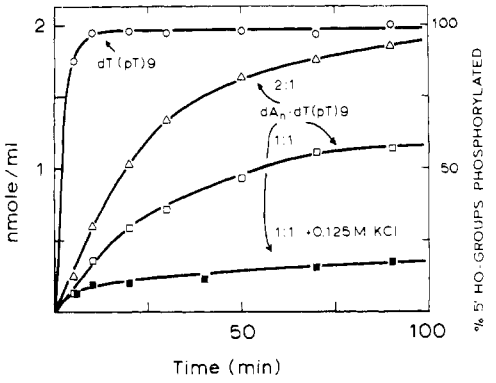


FIGURE 3: Time course of phosphorylation of $dT(pT)_9$ in the presence and absence of the template $(dA)_n$. The assay conditions were as described under Methods except that the temperature was 10 °C. The duplex DNA was made by heating the mixture of $dT(pT)_9$ and $(dA)_n$ to 37 °C for 30 min followed by slow cooling to 0 °C.

degree of phosphorylation increases considerably (Weiss and Richardson, 1968).

Similar experiments to those described above were also carried out with mixture of $dG(pG)_9$ and $(dC)_n$. Under the phosphorylation conditions used, $dG(pG)_9$ alone tends to aggregate (Lipsett, 1964). Taking this into account, the results with the annealed substrates, in general, were the same as for the $dT(pT)_9$-$(dA)_n$ system. Addition of salt, however, did not affect the phosphorylation to the same extent as it did for $dt(pT)_9$-$(dA)_n$ DNA. The latter results are to be expected since the $dG(pG)_9$-$(dC)_n$ DNA is a more stable duplex than $dT(pT)_9$-$(dA)_n$.

Phosphorylation of DNAs with Protruding 5'-Hydroxyl Group Ends. DNA I, Figure 2, corresponds to part of the gene for yeast alanine tRNA and has two protruding 5'-hydroxyl group ends, the protruding ends being five and seven mononucleotide residues in length. The time course of phosphorylation of this DNA at 20 and 37 °C and using a low concentration of ATP, 0.65 μM, which is a fourfold excess over the concentration of 5'-hydroxyl group present, resulted in a very rapid rate of phosphorylation, and complete phosphorylation was achieved after approximately 10 min at both temperatures (data not shown). The rate and extent of phosphorylation were found to be very close to that obtained for the corresponding single-stranded deoxyoligonucleotides under similar conditions. Addition of salt or spermine increased the initial rate of phosphorylation by approximately 10% but did not affect the maximum extent of phosphorylation (results not shown). These results again are similar to those observed for single-stranded DNAs.

Phosphorylation of DNAs with Protruding 3'-Hydroxyl Group and Even Ends. Numerous experiments performed during the synthesis of the genes for yeast alanine tRNA and *E. coli* suppressor tyrosine tRNA have shown that DNAs of type D and E (Figure 1) are difficult to phosphorylate completely using standard phosphorylation conditions, i.e., two- to fivefold excess of [γ-^{32}P]ATP over the concentration of 5'-hydroxyl group ends. The phosphorylation of these types of DNAs was therefore investigated in more detail. DNAs II–V all contain two protruding 3'-hydroxyl group ends, the protruding end being from four to seven mononucleotide residues in length, while DNA VI contains one even end and one end having a protruding 3'-hydroxyl group end. The time course of phosphorylation of DNA II at two different concentrations of ATP, in the presence and absence of KCl or spermine, is shown in Figure 4. With low concentrations of ATP, 1 μM, Figure 4A, only approximately 45% phosphorylation was achieved both in the presence and in the absence of spermine. Addition of more enzyme did not increase the yield of phosphorylated product significantly. Using a concentration of ATP 20-fold higher than in Figure 4A, 20 μM, complete phosphorylation was obtained, and 90% completion was reached in the presence of spermine, (Figure 4B). The presence of 0.125 M KCl in the reaction mixtures produced a dramatic decrease in the maximum level of phosphorylation, in partic-

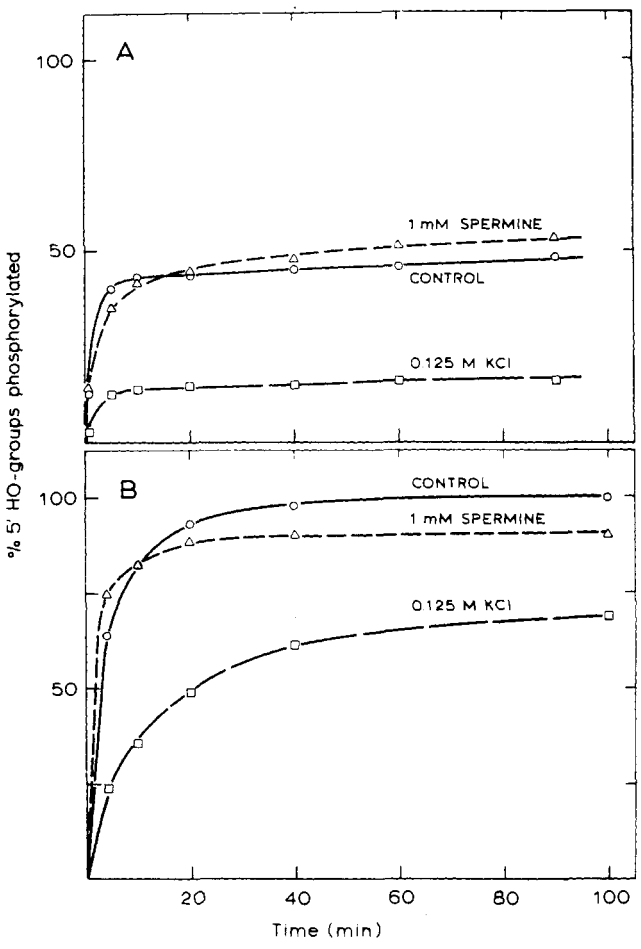


FIGURE 4: Time course of phosphorylation of DNA II at two different concentrations of ATP and in the absence and presence of KCl or spermine. Assay conditions were as described under Methods, except that the concentrations of ATP was A, 1 μM, and B, 20 μM. The concentration of DNA was 0.2 μM (5'-hydroxyl group ends) in both cases.

ular with low concentration of ATP where maximum phosphorylation only reached approximately 15%, while in the presence of high concentrations of ATP, the maximum level of phosphorylation reached was approximately 65%.

Identical experiments to those described above were also carried out with DNA III, IV, V, and VI and in general similar results were obtained. Using low concentrations of ATP the maximum level of phosphorylation reached varied from 20% to 45%. In the presence of KCl 20–50% lower plateau values were obtained. Initial rate studies revealed that the rates of phosphorylation of these double-stranded DNAs were slightly higher in the presence of 1 mM spermine, whereas in the presence of 0.125 M KCl up to 60% decrease in initial rate was noted (results not shown).

Influence of ATP Concentration on Maximum level of phosphorylation. The data given above suggested that for DNA duplexes with protruding 3'-hydroxyl group ends a much higher ATP concentration was needed to achieve complete phosphorylation than for single-stranded DNAs and double-stranded DNAs with protruding 5'-hydroxyl group ends. The effect of ATP concentration on maximum level of phosphorylation was investigated for several DNAs with protruding 3'-hydroxyl group ends. An example with DNA II is shown in Figure 5. It is evident that, under the experimental conditions used, a concentration of at least 20 μM must be used in order to achieve complete phosphorylation of this DNA. Similar data were also obtained for the other DNAs tested.

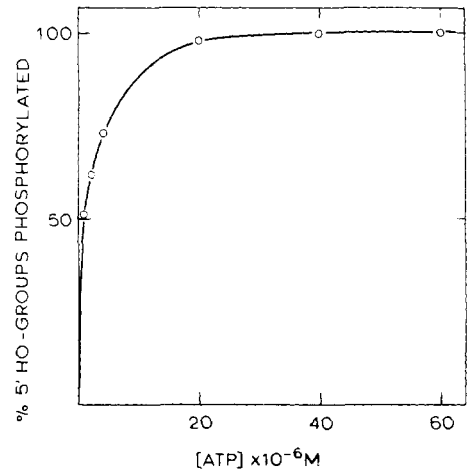


FIGURE 5: Influence of ATP concentration on final plateau levels reached for phosphorylated DNA II. The assays were as described under Methods, except that concentration of ATP varied as shown above. For each experimental point, time course reactions were run and the final plateau value obtained was then used. The concentration of DNA was 0.2 μM (5'-hydroxyl group ends).

Specificity in Phosphorylation of Double-Stranded DNAs. It has previously been shown that T_4 polynucleotide kinase exhibits a marked base specificity with regard to phosphorylation of single-stranded DNAs and 3'-mononucleotides (Lillehaug and Kleppe, 1975a). Such a specificity might also be expressed during phosphorylation of double-stranded DNAs, although in this case it is probable that other structural features play important roles. Specificity in phosphorylation was investigated with DNAs possessing different base residues at the 5'-hydroxyl group ends, namely DNA II, IV, V, and using a low concentration of ATP, 1 μM. Under these conditions plateau levels corresponding to less than 50% completion of phosphorylation were achieved. With these DNAs it is therefore possible that one has uneven phosphorylation at the two ends. In the case of DNA II, samples were removed from the reaction mixture at various times during the reaction; the polynucleotide kinase was then inactivated by heating at 95 °C for 5 min and each sample was submitted to gel filtration to remove ATP and other components of the reaction mixture. The DNA was then degraded to 5'-mononucleotides and these were separated by paper chromatography. The result of this analysis for DNA II is shown in Table I. In the control, as well as in the sample containing 1 mM spermine, the amount of radioactivity found in dpG and dpT was the same at the different time intervals. In the presence of 0.125 M KCl, however, the 5'-thymidine residue was phosphorylated much more rapidly than the 5'-guanosine residue. Thus, after 3 min of reaction the ratio in counts between dpT and dpG was 5.7 and after plateau level had been established this ratio had changed to 1.9.

Similar studies were also carried out with the two other double-stranded DNAs, IV and V. The results obtained at plateau levels are shown in Table II. In these cases approximately equal phosphorylation was achieved at both ends, both in the presence and absence of salt or spermine. Thus, of the DNAs tested, preferential phosphorylation at one end occurred only in the case of DNA II in the presence of salt. The reason for this is not clear, but presumably addition of salt must lead to stabilization of a particular structure at the left end of the duplex molecule which is then less susceptible to phosphorylation.

Production of Inorganic Phosphate, P_i, during the Phos-

TABLE I: 5′-Mononucleotide Analysis of DNA II at Different Times during Phosphorylation.[a]

	Time (min)					
	3		10		90	
	% Distribution of radioactivity					
	dpG	dpT	dpG	dpT	dpG	dpT
Control	50.2	49.8	52.7	47.3	54.0	46.0
+ 0.125 M KCl	14.9	85.1	20.0	80.0	34.8	65.2
+ 1 mM spermine	50.3	49.7	53.7	46.3	54.7	45.3

[a] The experimental conditions were as described under Methods and in the legend to Figure 4. Samples were withdrawn at the time intervals given, the enzyme was inactivated by heating, and the DNA was separated from ATP and other small-molecular-weight compounds present in the reaction mixture by gel filtration on a column of Sephadex G-50 equilibrated with 50 mM triethylammonium bicarbonate. The DNA, which eluted as a sharp peak, was then concentrated to dryness by freeze-drying and subjected to 5′-mononucleotide analysis as described under Methods. The radioactivity present in dpC and dpA was less than 0.1%.

TABLE II: 5′-Mononucleotide Analysis of DNA IV and DNA V.[a]

	% Distribution of counts								
	Control			+ 0.125 M KCl			+ 1 mM spermine		
DNA	dpG	dpT	dpA	dpG	dpT	dpA	dpG	dpT	dpA
DNA IV	46.9	0	53.1	46.7	0	53.3	47.4	0	53.6
DNA V	0	51.7	48.3	0	52.1	47.9	0	50.3	49.7

[a] The conditions of phosphorylation were as described under Methods and in the legend to Table I, the time of incubation being 90 min. Only negligible amounts of radioactivity was found in dpX not present at the 5′-hydroxyl ends of the DNAs tested. A 0 value indicates less than 0.1%.

phorylation of Various DNAs. During the phosphorylation of DNAs with protruding 3′-hydroxyl group ends it was observed that the amount of inorganic phosphate in the reaction mixtures increased markedly. Such a liberation of P_i during phosphorylation and reversal of phosphorylation has earlier been described (van de Sande et al., 1973) and is not due to contamination by a phosphatase enzyme. A systematic survey of the amount of P_i liberated during phosphorylation of various DNAs was therefore undertaken. Figure 6A shows the time course of production of P_i during the phosphorylation of DNA II. The synthesis of P_i does not reach a plateau level such as the phosphorylation reaction. After 90 min approximately 15% of the total radioactivity originally present in ATP was found in P_i. In the case of the single-stranded DNA VIII, Figure 6B, very little was present in P_i after phosphorylation was completed. The same results were obtained with other DNAs of similar structures to those described above.

Determination of Apparent Equilibrium Constants. The fact that incomplete phosphorylation was obtained for DNA duplexes with protruding 3′-hydroxyl group ends at low concentrations of ATP might suggest that some kind of equilibrium had been established. Inactivation of the enzyme can be ruled out as explanation since addition of more enzyme did not increase significantly the percent phosphorylation. The apparent equilibrium constant for the forward reaction:

$$\text{HO-DNA} + \text{ATP} \rightleftharpoons \text{ADP} + \text{P-DNA}$$

$$K_{\text{eq.forw}} = \frac{[\text{ADP}][\text{P-DNA}]}{[\text{ATP}][\text{HO-DNA}]}$$

was estimated for a number of different DNAs, both in the presence and in the absence of 0.125 M KCl, and the results

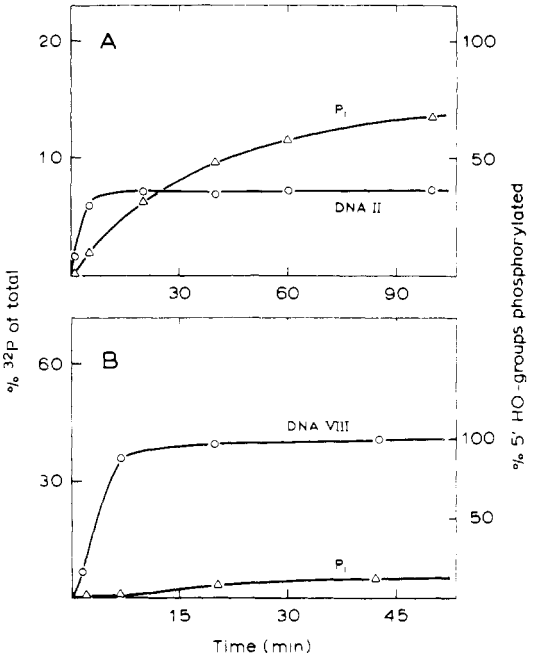


FIGURE 6: Production of P_i during phosphorylation of A, DNA II, and B, DNA VIII. Assays were carried out as described under Methods except that the concentration of ATP was 1 μM in A and 3.3 μM in B. The concentration of DNA was 0.2 μM (5′-hydroxyl group ends) in A and 1.4 μM in B.

are shown in Table III. For comparison the apparent equilibrium constants for the single-stranded DNA VII, DNA VIII, and $dT(pT)_9$ as well as for the double-stranded DNA I are also included. It is evident that DNA duplexes with protruding

TABLE III: Apparent Equilibrium Constants, $K_{eq.forw}$,[a] Determined from Forward Reactions for Various DNAs.[b]

DNA	$K_{eq.forw}$	
	Control	+ 0.125 M KCl
DNA I	33	
DNA II	4.9×10^{-2}	0.9×10^{-2}
DNA III	3.1×10^{-2}	$0.3 \times 10^{-}$
DNA IV	2.2×10^{-2}	1.2×10^{-2}
DNA V	4.4×10^{-2}	1.6×10^{-2}
DNA VI	3.3×10^{-2}	0.6×10^{-2}
DNA VII	50	c
DNA VIII	40	
$dT(pT)_9$	50	

[a] $K_{eq.forw}$ = [P-DNA][ADP]/([HO-DNA][ATP]). [b] The apparent equilibrium constants were determined from plateau levels obtained with a fivefold excess of ATP. The 5′-hydroxyl end concentration used for the double-stranded DNAs was 0.2 μM and the concentration of ATP was 1 μM. The assays was performed as described under Methods. For the single-stranded DNAs and DNA concentration (5′-hydroxyl) was 2 μM and the concentration of ATP 5 μM. [c] Equilibrium constant not determined.

TABLE IV: Apparent Equilibrium Constants, $K_{eq.rev}$,[a] Determined from the Reverse Reaction for Various DNAs.[b]

DNA	$K_{eq.rev}$
DNA I	2.7×10^{-2}
DNA II	4.9×10^{-4}
DNA III	0.36×10^{-4}
DNA IV	13.4×10^{-4}
DNA V	7.1×10^{-4}
DNA VI	1.2×10^{-4}
DNA VII	6.2×10^{-2}
DNA VIII	5.4×10^{-2}
$dT(pT)_9$	20.3×10^{-2}

[a] $K_{eq.rev}$ = [HO-DNA][ATP]/([P-DNA][ADP]). [b] The apparent equilibrium constants were determined from plateau levels. The reaction mix contained 60 mM Tris-HCl, pH 8.0, 9 mM $MgCl_2$, 20 μM ADP, 15 mM β-mercaptoethanol, 0.05 μM P-DNA and 12 units/ml of T_4 polynucleotide kinase. The assays were performed as described under Methods.

TABLE V: Distribution of Radioactivity at 5′-Phosphate Ends at Plateau Levels Using the Reverse Reaction.[a]

DNA	% Distribution of Radioactivity			
	dpA	dpG	dpT	dpC
DNA II	0	62.3	37.7	0
DNA III	2.8	96.5	0.7	0
DNA IV	48.9	51.1	0	0
DNA V	53.5	2.0	44.5	0
DNA VI	56.2	43.8	0	0

[a] The experimental conditions were as described in the legend to Table IV. After plateau levels had been established, the enzyme was inactivated by heating and the DNAs were separated from other components of the reaction mixture by gel filtration as described in the legend to Table I. The 5′-mononucleotide analysis were carried out as described under Methods. 0 indicates values less than 0.1%.

3′-hydroxyl group ends have equilibrium constants which are on the average 1000-fold less than the apparent equilibrium constants for the single-stranded DNAs and DNA I. The presence of salt leads, as expected, to a further decrease in the apparent equilibrium constants for DNAs with internal 5′-hydroxyl groups. It is interesting to note that DNA VI which has one 5′-hydroxyl group situated at an even end and one internal has an apparent equilibrium constant which is approximately the same as for the other DNAs with internal 5′-hydroxyl group ends. The hydrogen bonds between the terminal base residues obviously lead to a structure which is difficult to phosphorylate.

It has recently been shown that T_4 polynucleotide kinase also can catalyze the reverse reaction, i.e., synthesis of ATP in the presence of ADP (ATP) and a phosphorylated oligonucleotide (van de Sande et al., 1973). The question then arises whether the low equilibrium constants observed for DNAs with internal 5′-hydroxyl groups are caused by a rapid reversal of the phosphorylation. To clarify this aspect in more detail a number of dephosphorylation experiments were carried out. In these experiments 5′-^{32}P-labeled oligonucleotides were mixed with ADP and T_4 polynucleotide kinase, and the kinetics of formation of [γ-^{32}P]-ATP measured. The apparent equilibrium constants for the reverse reaction

$$K_{eq.rev} = \frac{[ATP][HO\text{-}DNA]}{[ADP][P\text{-}DNA]}$$

were calculated from the plateau levels reached, and the results for the various DNAs are presented in Table IV. The $K_{eq.rev}$ for the DNAs with internal 5′-hydroxyl group ends are on the average 100-fold lower than from the single-stranded DNAs and DNA I. If the plateau levels observed for the DNAs with internal 5′-hydroxyl groups are due to true equilibrium situation, then one would expect that $1/K_{eq.forw} = K_{eq.rev}$. It is quite clear that this relationship does not hold for the DNAs with internal 5′-hydroxyl groups. For the single-stranded DNAs and DNA I, on the other hand, it would appear that the equilibrium obtained is close to a true equilibrium. Thus, for DNA I $1/K_{eq.forw} = 0.03$. The experimentally determined $K_{eq.rev}$ was estimated to be 0.027. For the DNAs with internal 5′-hydroxyl groups, one therefore has to invoke other explanations for the incompletness of phosphorylations.

The dephosphorylation of the double-stranded DNAs was further studied by analyzing the distribution of ^{32}P labeled in the 5′ ends. Degradation to 5′-mononucleotides was performed and analyzed as described under Methods. The data for five double-stranded DNAs are given in Table V. From these results one may conclude that the readiness of dephosphorylation for internally situated 5′-phosphate ends is in this order: $T > A > G >$.

Discussion

The present work is an extension of our previous studies on the substrate specificity of T_4 polynucleotide kinase and describes the phosphorylation of various types of double-stranded DNAs by this enzyme. Special emphasis has been placed on practical aspects such as completion of phosphorylation since this enzyme is widely used to label nucleic acids. Using a low concentration of ATP, two to fivefold excess over the concentration of 5′-hydroxyl groups to be phosphorylated, only double-stranded DNAs containing protruding 5′-hydroxyl group ends were completely phosphorylated. The action of the enzyme on these types of DNAs resembles that on single-stranded nucleic acids both with regard to kinetic parameters as well as the effect of salt and polyamines. For all the other

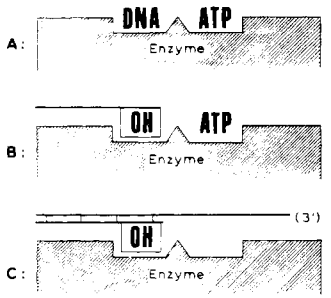
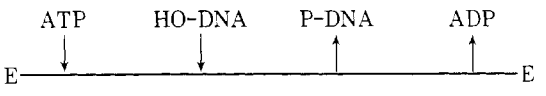


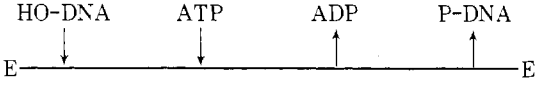
FIGURE 7: Schematic drawing of the active site of T_4 polynucleotide kinase. A, DNA and ATP binding sites; B, binding of single-stranded DNA and ATP; C, binding of double-stranded DNA with protruding 3'-hydroxyl group end.

types of double-stranded DNAs investigated incomplete phosphorylation was observed under the same phosphorylation conditions. Complete phosphorylation was, however, achieved for DNAs with protruding 3'-hydroxyl group ends, even ends, and gaps by increasing the concentration of ATP to at least 20 μM, the ratio of ATP to 5'-hydroxyl groups then being 100:1. In the case of DNAs with 5'-hydroxyl groups located at nicks in the DNA, incomplete phosphorylation was seen even at high concentrations of ATP, which is in agreement with similar observations by Richardson (1971).

The fact that for DNAs with protruding 3'-hydroxyl group ends a plateau level below 100% phosphorylation was established with low concentration of ATP might, at first hand, suggest a true equilibrium position had been reached between phosphorylation and dephosphorylation of the substrate. The studies carried out to determine equilibrium constants for the forward and reverse reaction clearly showed that such an equilibrium had not been established. We suggest as an explanation for this plateau level that the HO-DNA substrate and, in particular, the P-DNA product forms a complex with the enzyme which does not allow ATP to bind to its proper site. The binding sites for the DNA substrate and ATP can be visualized as shown in Figure 7A. In the case of single-stranded DNAs HO-DNA and ATP can bind independently (Figure 7B). With substrates containing 3'-hydroxyl group protruding ends, or 5'-hydroxyl groups located in gaps on the DNA, the protruding end might partially cover up the ATP binding site so that ATP cannot bind correctly. We suggest, however, that ATP is capable of binding to such a complex, but the correct transition complex cannot be formed, and the net result is the formation of P_i and ADP rather than P-DNA and ADP. The plateau levels observed at low concentration of ATP might be the results of formation of complexes of the types E·(P-DNA-ATP) and E·(HO-DNA-ADP) which could be considered to be dead-end complexes. In order for double-stranded DNAs containing protruding 3'-hydroxyl group ends to be phosphorylated completely, we suggest that ATP must bind first and the following reaction sequence must apply:



For single-stranded DNAs we have previously shown (Lillehaug and Kleppe, 1975a) by detailed kinetic evidence that the enzyme reacts according to a sequential ordered mechanism where HO-DNA binds first as shown below:



This is the favored reaction pathway and presumably also applies to double-stranded DNAs with protruding 5'-hydroxyl group ends. However, we also showed that the enzyme may react according to a rapid-equilibrium, random-type mechanism. The fact that one needs much higher ATP concentration to achieve complete phosphorylation for double-stranded DNAs with protruding 3'-hydroxyl ends suggests that the enzyme is forced to use the less favorable mechanism, as indicated above. A mechanism involving cooperative interaction between the various active sites on the enzyme might at least partially also explain the results. No such interactions were, however, detected using single-stranded DNAs (Lillehaug and Kleppe, 1975a).

The influence of salt on the phosphorylation of double-stranded DNAs with protruding 3'-hydroxyl ends and gaps appears to be quite different from the effect on single-stranded nucleic acids, where this compound greatly stimulated the initial velocity of phosphorylation without affecting the maximum level (Lillehaug and Kleppe, 1975b). Both polyamines and salt are known to stabilize bihelical DNA structures, and the marked inhibiting effect of salt in the present case might, therefore, be caused by a stabilization of the 5'-hydroxyl region of the DNA substrate. It is possible that for phosphorylation to occur some degree of single strandedness around the 5' terminus must be present. The results with the DNA with even ends as well as with nicks also support this view. An alternative explanation is that salt drastically changes the affinity of the enzyme for such substrates. In order to prove this, detailed kinetic experiments will have to be carried out. Such studies as well as product inhibition experiments are difficult to perform at the present time due to the limiting amounts of substrates available. Initial structural studies on T_4 polynucleotide kinase have indicated that salt and polyamines both have marked effects on the enzyme conformation.

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Partial Denaturation of Mouse DNA in Preparative CsCl Density Gradients at Alkaline pH†

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ABSTRACT: A new technique—partial denaturation of DNA in equilibrium CsCl density gradients at pH 11.4—is used to determine the distribution of intermediate states in the melting of mouse DNA. When the technique is applied in the preparative ultracentrifuge, the DNA is fractionated according to stability. Neutralization of the partially denatured fractions results in the recovery of most of the DNA in its native form. The individual fractions are more homogeneous than the total DNA: they have decreased density heterogeneity (smaller band widths), neutral CsCl buoyant densities that differ from the average, and more homogeneous melting profiles with melting temperatures that differ from the average.

We have studied intermediate states in the helix-coil transition or "melting" of DNA by partial denaturation in analytical CsCl density gradients at alkaline pH (Wiesehahn et al., 1976). This method gives high resolution because the alkaline titration of DNA is accompanied by a large increase (58 mg/cm^3) in the DNA buoyant density (Baldwin and Shooter, 1963; Vinograd et al., 1963). Of the DNAs we studied, mouse DNA gave a particularly broad distribution of intermediate states during its denaturation. Here we use a preparative version of the same technique to fractionate the mouse genome according to stability. The same partially denatured DNA distributions seen in analytical CsCl gradients are reproduced in the preparative gradients.

After fractionation, the alkaline DNA solutions are neutralized. Any DNA molecules which have not been completely denatured (i.e., strand-separated) will still have their complementary strands in register. These molecules are expected to undergo rapid and complete "type I reversibility" (Geiduschek, 1962) or "rezippering" when the denaturing conditions are removed. To test this expectation, the reversibility of the partial denaturation is investigated by S_1-nuclease resistance, electron microscopy, and denaturation experiments performed on the isolated mouse DNA fractions.

The components of mouse DNA that are isolated by the partial denaturation technique are more homogeneous than the total genome. This increased homogeneity is evident both from neutral CsCl density gradient profiles and from melting profiles of the DNA fractions.

These experiments allow some conclusions to be drawn about the range of molecular weight at which base-compositional heterogeneity occurs in the mouse genome. In addition, the partial denaturation technique is a useful method for fractionating the heterogeneous mouse genome. It gives better resolution of density components than either neutral CsCl or Ag^+-Cs_2SO_4 density gradient centrifugation.

Materials and Methods

DNA Preparation. DNA was extracted from [^{3}H]thymidine-labeled SVT2 mouse tissue culture cells, obtained from Theodore Gurney, Jr. (University of Utah). Purification steps included chloroform-isoamyl alcohol extractions, ethanol precipitations, and Pronase and RNase treatments (Cech et al., 1973). The specific activity of the DNA preparation used in most of the experiments described here was 4.0×10^4 cpm/μg. It contained 10.2% satellite DNA, as determined by analytical CsCl density gradient centrifugation. The DNA had a native molecular weight of 6.2×10^7 and a single-stranded molecular weight of 1.5×10^7. A few experiments were done with Balb/c mouse DNA, isolated according to the procedure of Flamm et al. (1966a). Molecular weights were determined

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