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Purification and Substrate Specificity of Polydeoxyribonucleotide Kinases Isolated From Calf Thymus and Rat Liver

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Abstract Damage to DNA can result in strand breaks with 5′-hydroxyl and 3′-phosphate termini. Before DNA polymerases and ligases can rejoin the broken strands, such termini have to be restored to 5′-phosphate and 3′-hydroxyl groups. Polydeoxynucleotide kinase is an enzyme that may fulfil this function. We have purified the kinases from calf thymus and rat liver to near homogeneity. Based on SDS-polyacrylamide gel electrophoresis and activity gels, the enzymes from both sources are ~60-kDa polypeptides. Both enzymes have an acidic pH optimum (5.5–6.0) for kinase activity, and similar pI values (8.5–8.6), and a specificity for DNA. The calf thymus kinase possesses a 3′-phosphatase activity, as has previously been shown for the rat liver enzyme. The minimum size of oligonucleotide that can be labelled is 7–8 nucleotides in length, but the optimal size appears to be >18 nucleotides. Comparison of phosphorylation of oligo$(dA)_{24}$ and oligo$(dT)_{24}$ with oligonucleotides containing a varied nucleotide sequence indicated that the homopolymers are poorer substrates. Unlike the bacteriophage T4 polynucleotide kinase, the mammalian kinases exhibit no preference for 5′-overhanging termini when acting at DNA termini produced by restriction enzymes. With double-stranded oligonucleotide complexes designed to model single-strand gaps and nicks, the mammalian kinases preferentially phosphorylate the 5′-terminus associated with the gap or nick, in keeping with the idea that the kinases are involved in the repair of DNA single-strand breaks. J. Cell. Biochem. 64:258–272.

Key words: DNA strand break; DNA termini; DNA phosphorylation; polynucleotide kinase; phosphatase; DNA repair

Many cytotoxic agents, including ionizing radiation, generate strand breaks in cellular DNA. Such strand breaks display a variety of chemically distinct termini. In the case of ionizing radiation, the two major end groups observed at the 3′-termini are phosphate and phosphoglycolate [Henner et al., 1983; Bases et al., 1990]. The former are produced under both oxic and anoxic conditions, whereas generation of the latter is dependent on the presence of oxygen or nitroaromatic radiosensitizers [Buchko and Weinfeld, 1993]. The predominant 5′-end groups produced by irradiation of cellular DNA are 5′-phosphate and 5′-hydroxyl groups, with the latter found at 10–14% of strand breaks [Coquerelle et al., 1973; Lennartz et al., 1975].

No matter what form the termini take, the repair of strand breaks requires restoration of the termini to 3′-hydroxyl and 5′-phosphate, before gap filling and strand rejoining can be completed by a DNA polymerase and a DNA ligase, respectively. A 5′-hydroxyl terminus can be converted to a terminus with a phosphate, either by exonuclease-mediated removal of the 5′-terminal nucleoside or, more straightforwardly, by direct phosphorylation catalyzed by a polynucleotide kinase (PNK). At present, it is not known which mechanism is adopted in mammalian cells. Several mammalian enzymes are known to act on the 3′-modifications noted above. In particular, the human AP endonuclease I (HAP1 or APE) and its bovine and murine homologs, BAP and APEX, are capable of removing both 3′-phosphoglycolates and 3′-phosphates [Demple and Harrison, 1994]. However, when 3′-phosphatase activity was purified from rat liver nuclei, the major enzyme activity copu-

Abbreviations: CT, calf thymus; PMSF, phenymethylsulfonylfluoride; PNK, polynucleotide kinase; RL, rat liver; SDS, sodium dodecyl sulfate.

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rified through three or four steps with a polynucleotide kinase [Pheiffer and Zimmerman, 1982; Habraken and Verly, 1988], although it did not remove phosphoglycolate groups [Habraken and Verly, 1988]. Thus, in mammalian cells, PNK has the potential to act at both 3′- and 5′-damaged termini.

The best known PNK is the 136-kDa protein (composed of four identical 34-kDa peptides) from bacteriophage T4 [Midgley and Murray, 1985]. However, this enzyme does not appear to participate in DNA repair in phage-infected *Escherichia coli*, but instead restores tRNA termini damaged by an anticodon nuclease to a ligatable form [Amitsur et al., 1987]. The enzyme is capable of phosphorylating a broad range of substrates, including DNA, RNA, oligonucleotides, and 3′-mononucleotides [Kleppe and Lillehaug, 1979]. It also possesses a 3′-phosphatase activity [Cameron and Uhlenbeck, 1977].

PNKs have been isolated from a variety of eukaryotic sources. Although there is still a degree of confusion, two distinct classes are emerging, one acting on DNA and the other on RNA. The latter proteins appear to be larger than the former and have a higher pH optimum (pH 8 vs. pH 5.5). There have been a number of reports describing the isolation and characterization of PNKs from rat organs, principally liver and testes. The size of the rat liver protein isolated from nuclei has been variously reported as 61 and 80 kDa [Teraoka et al., 1975; Levin and Zimmerman, 1976; Ohmura et al., 1987; Habraken and Verly, 1988], and that of rat testes as 38 kDa [Bosdal and Lillehaug, 1985]. However, all the proteins appear to phosphorylate DNA specifically, labelling RNA either poorly or not at all, and have a pH optimum of ~5.5. PNK has also been purified from calf thymus. Again, there appear to be some discrepancies between the properties reported for this enzyme. Both Austin et al. [1978] and Tamura et al. [1981] describe a protein that phosphorylates DNA but not RNA (or only poorly so), with a pH optimum of 5.5. However, the enzyme in the former report has a molecular weight of 70 kDa, whereas the enzyme in the latter report was estimated to be 56 kDa. In neither case was an accompanying 3′-phosphatase activity determined. More recently, Prinos et al. [1995] purified a PNK from calf thymus with a Stokes radius of 4.5 nm (leading to an estimated molecular mass of 72 kDa), which unlike the enzymes isolated earlier has a pH optimum of 7.5. This enzyme preparation displayed no detectable 3′-phosphatase.

This communication provides a more detailed characterization of calf thymus PNK, about which less is known than the rat liver enzyme. However, the latter was also purified in order to directly compare some of their properties, and thereby resolve some of the inconsistencies noted above. Emphasis was placed on the substrate specificity of the enzymes, including substrates that model DNA damage intermediates.

MATERIALS AND METHODS

Materials

T4 polynucleotide kinase, shrimp alkaline phosphatase, and [γ-^{32}P]ATP (3,000 Ci/mmol) were purchased from Amersham Corporation (Arlington Heights, IL). The restriction enzymes Ahd I, AlwN I, Bpm I, EcoO109 I, Nar I, and Nde I were obtained from New England Biolabs (Beverly, MA); BamH I and Hind III from Gibco-BRL (Grand Island, NY); and Sma I and Kpn I from Pharmacia (Baie D'Urfe, PQ). Micrococcal nuclease was supplied by Worthington. Calf thymus DNA and poly(A) were purchased from Sigma (St. Louis, MO), and poly(dA), ATP, and the oligo(dT)$_{8\text{-}32}$ size markers from Pharmacia. The synthetic oligonucleotides were prepared by the DNA synthesis service of the Microbiology Department of the University of Alberta, and generously provided to us by Dr. Linda Reha-Krantz at the University of Alberta. Protein size markers, including prestained markers, were obtained from Bio-Rad (Hercules, CA), New England BioLabs, and Promega (Madison, WI). Silver nitrate was purchased from BDH (Toronto, ON), and Coomassie brilliant blue R-250 from Sigma. The protease inhibitors, phenylmethylsulfonylfluoride (PMSF), aprotinin, leupeptin, and pepstatin A, were supplied by Sigma. All other chemicals used were reagent grade.

PNK Substrate Preparation

DNA with 5′-hydroxyl termini was prepared according to the procedure of Richardson [1965]: 8 mg calf thymus DNA was dissolved in 10 mM Tris-HCl, pH 8.0, and 1 mM $CaCl_2$, and then digested with 75 U of micrococcal nuclease for 5 min at 37°C in a total volume of 5.3 ml. The reaction was stopped by addition of 50 µl of 500

mM EDTA, and the DNA was subsequently dialyzed with 1.0 M KCl for 12 h, and 0.02 M KCl for 24 h.

Protein Gel Electrophoresis, Staining, and Protein Assay

SDS-polyacrylamide gel electrophoresis was performed on 12% gels using a Bio-Rad Mini-Protean II electrophoresis apparatus [Laemmli, 1970]. Proteins on gels were visualized by Coomassie blue R-250 or silver nitrate staining [Wray et al., 1981]. Protein assays were carried out with a Bio-Rad protein assay kit.

Activity Gels

DNA-kinase activity was detected directly on SDS/polyacrylamide gels following the technique described by Ohmura et al. [1987], with slight modifications: 150 µg of PNK substrate was incorporated in a 12% SDS-polyacrylamide gel, with a 4% stacking gel. The samples were denatured in SDS sample buffer (60 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 1 mM 2-mercaptoethanol, and 0.0025% bromophenol blue) for 10 min at 37°C, loaded on the gel, and run at 80 V for 3 h. After electrophoresis, the proteins were renatured in situ by washing three times, for 30 min each, with 300 ml of 50 mM Tris-HCl, pH 7.5, and 50 µg/ml gelatin at room temperature, with mild agitation, followed by overnight storage of the gel in 50 mM Tris-HCl, pH 7.5, 0.5 mM dithiothreitol at 4°C. The gel was washed with 50 mM sodium succinate, pH 5.5, and 0.5 mM dithiothreitol for 1 h, before being incubated overnight at 37°C in a sealed bag containing 10 ml of the following reaction mixture: 80 mM sodium succinate, pH 5.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 10 µl of [γ-^{32}P]ATP (3,000 Ci/mmol, 10 µCi/µl). The gel was then washed four times for 15 min and then three times for 10–15 h each with 10% trichloroacetic acid and 1% sodium pyrophosphate, with mild agitation at 4°C. Autoradiography of the gel was carried out at −70°C over 1–3 days.

Standard 5′-OH Phosphorylation Assay

The reaction mixture (60 µl), containing 80 mM succinic acid, pH 5.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 8 µg bovine serum albumin, 0.225 µCi [γ-^{32}P]ATP (3,000 Ci/mmol), 10 µM unlabelled ATP, 45 µg PNK substrate, and enzyme, was incubated for 30 min at 37°C. The reaction was stopped by placing the tubes on ice, and the DNA precipitated by addition of 250 µl of 20% trichloroacetic acid and 200 µl of 250 µM sodium pyrophosphate containing 100 µg of bovine serum albumin. The tubes were centrifuged at 10,000 × *g* for 10 min, and after removing the supernatant, the pellets were dissolved in 100 µl of 0.1 M sodium hydroxide and the DNA reprecipitated by addition of 500 µl of 10% trichloroacetic acid. The wash was repeated once more, and the final pellets were directly used for radioactivity measurements. One unit of enzyme is the amount required to incorporate 1 nmol of phosphate from ATP into micrococcal nuclease-treated DNA in 30 min at 37°C under standard assay conditions [Richardson, 1971].

Unless otherwise stated, the buffer for T4 polynucleotide kinase was 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, and 10 mM 2-mercaptoethanol.

Purification of PNK From Calf Thymus

All steps were performed at 4°C. One kilogram of fetal calf thymus, obtained frozen from AnTech (Tyler, TX), was minced for 3 min at the highest speed in an Osterizer blender, in 3 volumes of 10 mM potassium phosphate (pH 7.5), 4 mM 2-mercaptoethanol, 0.2 M KCl, 0.1 mM EDTA, and a cocktail of protease inhibitors (1 mM PMSF, 0.7 µg/ml aprotinin, 0.5 µg/ml leupeptin, and 0.7 µg/ml pepstatin A). Insoluble material in the homogenate was removed, first by centrifugation at 13,000*g* for 35 min, and then by filtration of the supernatant through four layers of cheesecloth. The pH of the filtrate was adjusted to 5.0 with dropwise addition of acetic acid and gentle stirring, held at 4°C for 5 min, and then centrifuged at 13,000*g* for 5 min. The pH of the supernatant was restored to 7.5 by ammonium hydroxide, and solid ammonium sulfate was added to bring the solution to 30% saturation. After stirring the mixture for 20 min, the precipitate was removed by centrifugation at 10,000*g* for 10 min. The supernatant was further treated with solid ammonium sulfate to bring it up to 55% saturation, and kept on ice for 24 h before centrifugation at 10,000*g* for 10 min. The pellet was dissolved in buffer A (10 mM potassium phosphate, pH 6.8, 0.2 M KCl and 4 mM 2-mercaptoethanol), and dialyzed overnight in the same buffer.

The dialysate was applied on a 325-ml SP Sepharose Fast Flow cation exchange column (Pharmacia). The column was washed with 1,000 ml buffer A at a flow rate of 6 ml/min, and then the enzyme was eluted with a 900-ml linear gradient of buffer A containing 0.2–0.8 M KCl. The enzyme eluted at 0.4 M KCl. The active fractions were pooled and concentrated by addition of solid ammonium sulfate to 65% saturation and centrifugation at 10,000*g* for 10 min. The pellet was resuspended in a minimum volume of buffer A (set this time to pH 8.0), and dialyzed overnight against the same buffer. The solution was loaded onto a 15-ml Blue Sepharose CL-6B column (Pharmacia), washed with buffer A (pH 8.0) at a flow rate of 1 ml/min, and the enzyme was eluted with a 60-ml linear gradient of 0.2–1.0 M KCl in buffer A. The active fractions eluted at about 0.6 M KCl. The activity was concentrated by addition of solid ammonium sulfate as described above. The precipitate was dissolved in a minimal volume (~2 ml) of buffer A (set to pH 7.5), and was dialyzed against the same buffer overnight. It was loaded on a HiLoad 16/60 Superdex 200 gel filtration column (Pharmacia), and the proteins were eluted with buffer A (pH 7.5) at a flow rate of 1.0 ml/min. The active fractions were pooled (~12 ml), applied to a 4-ml hydroxyapatite column (Bio-Gel HT, Bio-Rad), washed with 30 ml buffer A (pH 7.5), and eluted using an 80-ml linear gradient of 0.2–0.4 M KCl in buffer A, followed by a 20-ml isocratic step of 0.4 M KCl in buffer A (flow rate of 0.5 ml/min). The active fractions eluted as a single peak close to the boundary between the gradient and isocratic step. The active fractions were pooled and dialyzed for 4 h with several changes of buffer (5 mM potassium phosphate, pH 7.5), and then concentrated fivefold with a Centricon 10 microconcentrator. Glycerol was added to a final concentration of 20%, and the protein was stored at −80°C. The results of a typical purification are summarized in Table I.

Purification of PNK From Rat Liver

The same protocol was applied to the purification of the rat liver protein, but with the following minor modifications: a) Solutions containing rat liver PNK, to which were added ammonium sulfate, were not held at 4°C for prolonged periods and were more extensively dialyzed, because the rat liver protein is more susceptible to inactivation by ammonium sulfate than the calf thymus protein. For the same reason, after the Blue Sepharose step, the rat liver protein was concentrated using Centricon 10 microconcentrators (Amicon). b) As a result of starting with 330 g of tissue instead of 1 kg, the sizes of the SP Sepharose Fast Flow, Blue Sepharose CL-6B, and hydroxyapatite columns were reduced to 80 ml, 6 ml, and 3 ml, respectively. c) For the SP Sepharose and Blue Sepharose, the elution gradient volumes were 250 ml and 30 ml, respectively. The rat liver kinase activity eluted as a single peak from the SP Sepharose column at ~0.3 M KCl (compared to 0.4 M KCl for the calf thymus kinase), and in a single peak from the Blue Sepharose and Sephadex 200 columns (at 100 to 110 kDa), but in two peaks from the hydroxyapatite column (as shown in Fig. 4). The results of a typical purification are summarized in Table II.

Preparation of Nuclei

Rat liver and calf thymus nuclei were prepared by the method described by Teraoka et al. [1975]. Briefly, the tissues were homogenized in 4 volumes of cold 0.25 M sucrose containing 3.3 mM $MgCl_2$, 1 mM PMSF, 0.7 μg/ml aprotinin, 0.5 μg/ml leupeptin, and 0.7 μg/ml pepstatin A, and the homogenate was passed through four layers of gauze and centrifuged at 1,000*g* for 10

TABLE I. Purification of Polynucleotide Kinase from Calf Thymus*

Step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
1. Crude extract	120720	640	0.0053	100	1
2. SP Sepharose	317	53	0.167	8.3	32
3. Blue Sepharose	9.3	35	3.7	5.5	698
4. Sephadex 200	3.2	14.4	4.5	2.3	849
5. Hydroxyapatite	0.15	5.3	35.6	0.8	6717

*The purification protocol is fully described under Materials and Methods.

TABLE II. Purification of Polynucleotide Kinase from Rat Liver*

Step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
1. Crude extract	48610	373	0.008	100	1
2. SP Sepharose	22.3	190	8.5	51	1110
3. Blue Sepharose	6.7	91	13.6	24	1770
4. Sephadex 200	2.9	54.4	18.8	15	2445
5. Hydroxyapatite					
First Peak	0.06	5	83.3	1.3	10860
Second Peak	0.10	4.7	47.0	1.3	6125

*The purification protocol is fully described under Materials and Methods.

min. The pellet was suspended in 9 volumes of 2.2 M sucrose containing 3.3 mM $MgCl_2$, 1 mM PMSF, 0.7 µg/ml aprotinin, 0.5 µg/ml leupeptin, and 0.7 µg/ml pepstatin A, and centrifuged at 100,000*g* for 60 min. The nuclei were washed twice with 0.25 M sucrose containing 3.3 mM $MgCl_2$.

Isoelectric Point Determination

Two-dimensional polyacrylamide gel electrophoresis was carried out on a Mini-PROTEAN I 2-D System (Bio-Rad). The Bio-Rad protocol, based on the method described by O'Farrell [1975], and the Bio-Rad 2-D markers were used to determine the isoelectric point of the calf thymus PNK.

3′-Phosphatase Assay

We made use of the assay described by Habraken and Verly [1983]. ^{32}P-labelled DNA ($\sim 10^9$ dpm/µg) was kindly provided to us by Dr. Malcolm Paterson of the Cross Cancer Institute. It was prepared by random primer extension using the Klenow fragment of DNA polymerase and [α-^{32}P]dCTP. The substrate for the phosphatase was generated by incubation of 5 ng of the labelled DNA with 10 U of micrococcal nuclease in 10 mM Tris-HCl, pH 8.0, and 1 mM $CaCl_2$, for 5 min at 37°C in a total volume of 200 µl. The reaction was stopped by heating at 100°C for 10 min. For the phosphatase assay, the reaction mixture (50 µl) contained 10 µl of the substrate, 8 µg bovine serum albumin, 20 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and one of three different buffers (50 mM Tris-acetate, 80 mM sodium succinate, or 50 mM Tris-HCl, with their pH adjusted by addition of acetic acid, NaOH, or HCl, respectively, to cover a pH range between 3.5 to 9.0), and 10 µl of enzyme solution. After 10 min at 37°C, the reaction was stopped on ice and by adding 250 µl of 10% trichloroacetic acid, 100 µl of a 250 µM sodium pyrophosphate solution containing 100 µg of bovine serum albumin, and 200 µl of 20% activated charcoal (Norit) in water. The tubes were centrifuged 20 min later, and the supernatants were used to measure the radioactivity of the inorganic phosphate released by the enzyme.

RESULTS

Kinase Activity in Crude Cell Extracts

Prior to enzyme isolation, the sizes of polypeptides displaying DNA-kinase activity (at pH 5.5) in calf thymus and rat liver were ascertained by activity gel analysis [Ohmura et al., 1987]. In this technique, proteins are separated on a standard denaturing SDS/polyacrylamide gel, except that the gel contains micrococcal nuclease-digested DNA as a substrate for the kinase. After electrophoresis, the gel is incubated first in a protein renaturation buffer and then in reaction buffer together with [γ-^{32}P]ATP, thereby allowing the kinase to radioactively phosphorylate the DNA in the immediate vicinity of the enzyme. Protein extracts were prepared from whole cells and cell nuclei. Protease inhibitors were included in all preparations. Figure 1 shows the autoradiograms of the activity gels, as well as the controls in which no DNA substrate was included in the gel matrix. The gels indicate that the kinase activities in the whole cell extracts, both calf thymus and rat liver, are associated with 55–60-kDa polypeptides. In the nuclear extracts, there is an additional, and in the case of the calf thymus extract a predominant, 40-kDa DNA-kinase. Another rat liver nuclear extract prepared with PMSF as the sole protease inhibitor also displayed a predominant 40-kDa kinase (shown in lane 4 of Fig. 2). A low level of activity was seen

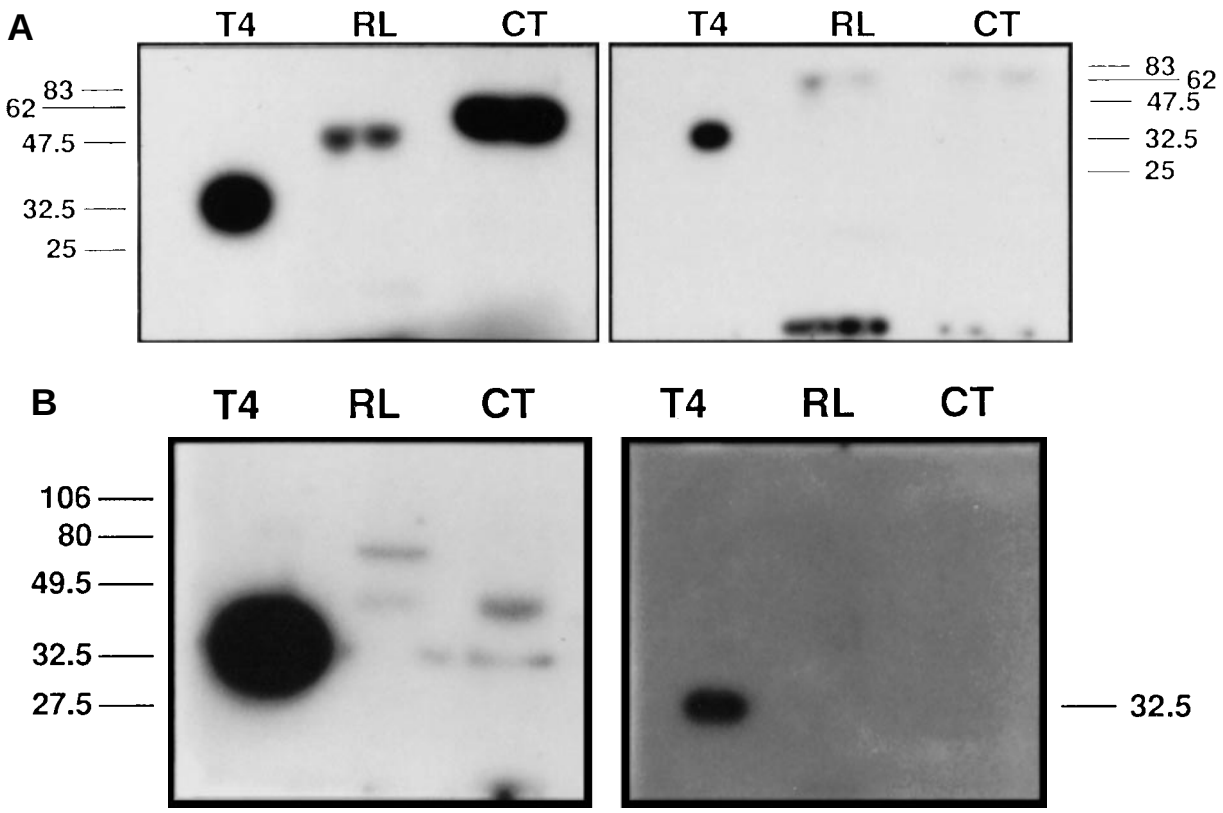

Fig. 1. Activity gels revealing DNA-kinase activity in cell extracts. **Left panel A,B:** The gel contained micrococcal nuclease-digested DNA. **Right panel A,B:** The gel is the control lacking DNA. The migration of protein size markers are indicated in kDa on the sides of the gels. The activity gel methodology is explained in Materials and Methods. A: Kinase activity in whole-cell extracts from rat liver (RL) and calf thymus (CT). Phage T4 kinase (5 units) served as a positive control (T4). B: Kinase activity in nuclear extracts. (See also Fig. 2, lane 4.)

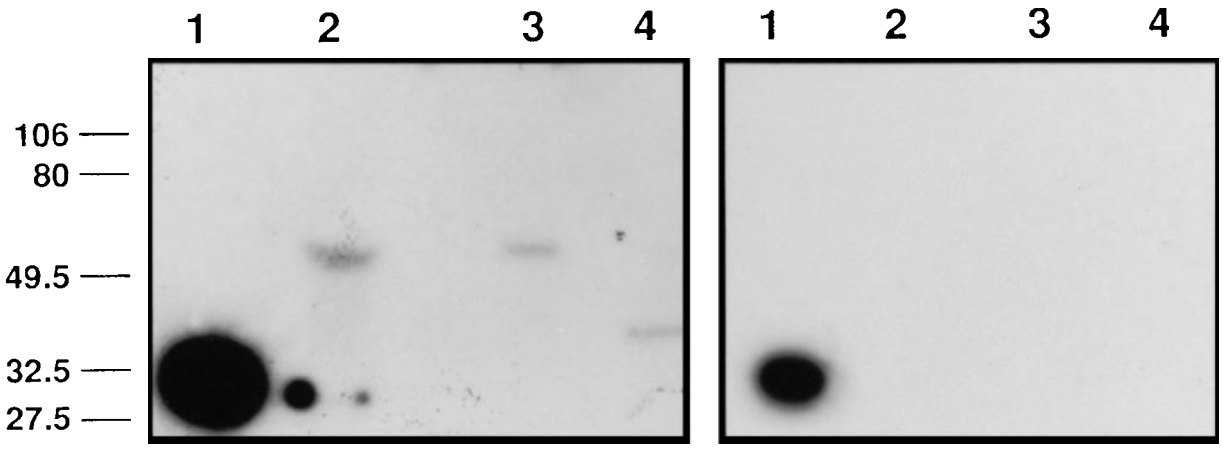

Fig. 2. Activity gel of partially purified calf thymus extract. **Left panel:** The gel contained micrococcal nuclease-digested DNA. **Right panel:** The gel is the control lacking DNA. The migration of protein size markers are indicated in kDa on the sides of the gels. *Lanes 2 and 3:* Samples of calf thymus extract after chromatography on Blue Sepharose (Table I, step 3) and hydroxyapatite (Table I, step 5), respectively. *Lane 1:* 5 units of the phage T4 kinase. *Lane 4:* The kinase activity in a nuclear extract of rat liver isolated with PMSF as the sole protease inhibitor.

in the control gels, especially in the lanes containing T4 PNK. This is most likely due to the formation of enzyme-ATP intermediates, because it is known that the enzymes can bind ATP in the absence of DNA [Kleppe and Lillehaug, 1979].

Purification of Calf Thymus and Rat Liver PNK

The purification protocol was adapted from that used by Tamura et al. [1981] to purify calf thymus PNK. The enzymes were purified from a 30–55% ammonium sulphate cut of the whole-cell homogenate. Results of typical purifications are summarized in Tables I and II. Crude extract refers to the filtrate recovered after passage of the centrifuged tissue homogenate through cheese cloth. In the case of rat liver, this preparation probably contained inhibitors of the kinase, which would partly explain the seemingly high purification obtained by the first chromatographic step.

At each chromatographic step of the purification of the calf thymus enzyme, the DNA-kinase was found in a single peak. The elution volume of the protein on the Superdex 200 gel filtration column indicated a molecular weight of ~125 kDa (data not shown). Figure 2 shows an activity gel of calf thymus protein from the active fractions recovered after the Blue Sepharose (lane 2) and hydroxyapatite (lane 3) steps respectively. The activity was retained in a single ~60-kDa polypeptide. In the case of the rat liver PNK, activity was recovered in single peaks until the final step. As shown in the upper panel of Figure 3, two peaks of activity eluted from the hydroxyapatite column. When these were analyzed on SDS/polyacrylamide gels and silver stained (Fig. 3, lower panel), two polypeptides of 60 and 42 kDa were observed in the fractions from the first peak of activity, and a single polypeptide of 60 kDa in the fractions from the second peak. A direct comparison of

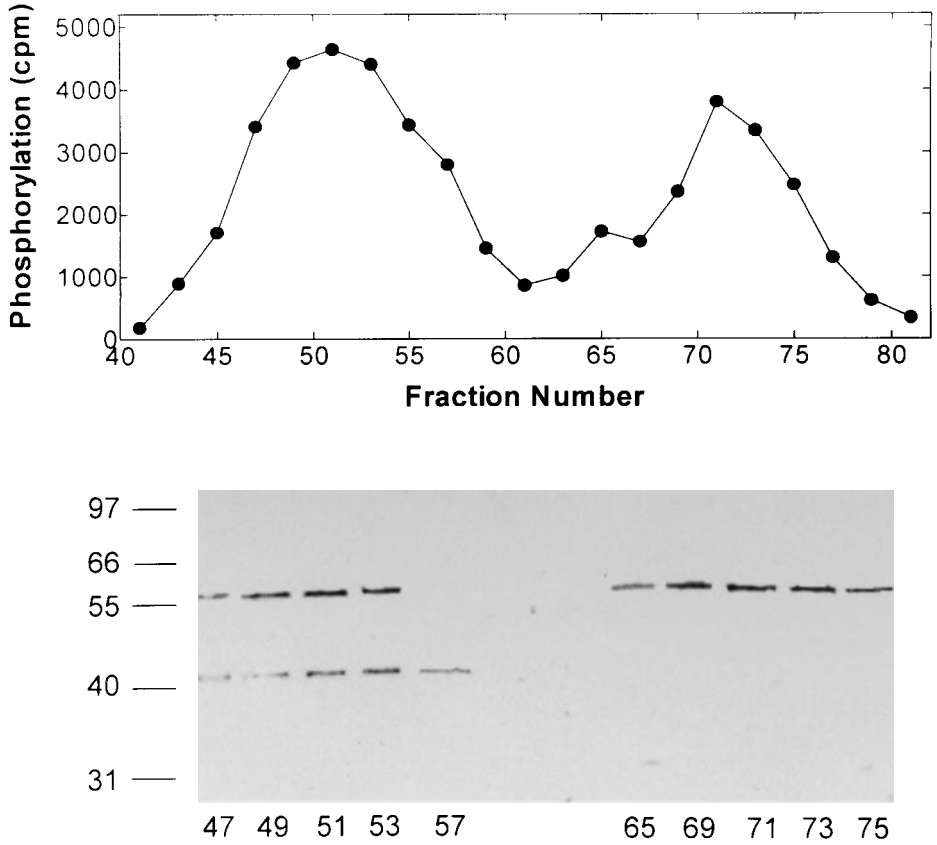

Fig. 3. Elution of rat liver polynucleotide kinase from hydroxyapatite. **Top:** PNK was eluted from a 3-ml hydroxyapatite column in eighty 1-ml fractions using a 0.2–0.4 M KCl gradient (see Materials and Methods for full details). No activity was observed in fractions 1–40. **Bottom:** Silver-stained protein gel of active fractions. The migration of protein size markers are indicated in kDa on the left side of the gel.

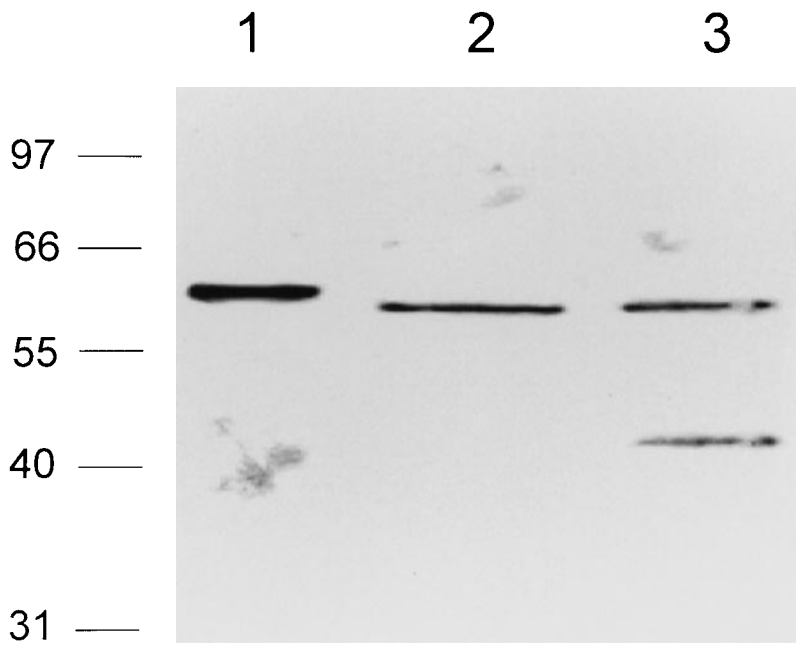

Fig. 4. Silver-stained SDS/polyacrylamide gel of purified calf thymus PNK *(lane 1)* and rat liver PNK *(lane 2,* second hydroxyapatite peak *[lane 2]* and first hydroxyapatite peak *[lane3]).* Protein sizes in kDa are shown on the **left.**

the calf thymus and rat liver proteins (Fig. 4) indicated that the former is approximately 1–2 kDa larger than the latter.

Isoelectric Point

The isoelectric point was determined by two-dimensional gel electrophoresis to be 8.5. This is in close agreement with the value of 8.6 established for the rat liver PNK [Habraken and Verly, 1988].

Optimum pH of the 5′-Kinase and 3′-Phosphatase of Calf Thymus PNK

The variation in kinase activity as a function of pH at 37°C is shown in Figure 5a. In agreement with Tamura et al. [1981], the enzyme showed reasonable activity from pH 5.5 to pH 7.0 with an optimum pH at 6.0. Austin et al. [1978] established a pH optimum of 5.5. The pH optimum for DNA phosphorylation by the rat liver kinase is 5.5 [Levin and Zimmerman, 1976].

Using micrococcal nuclease-digested ^{32}P-labelled DNA as a substrate, we observed that calf thymus PNK, like its rat liver homolog, possesses a 3′-phosphatase activity (Fig. 5b). Furthermore, the pH dependence of this activity displayed a similar profile to that of the rat liver PNK with two distinct optima, at pH 5.0 and 7.0. By comparison, the optima for rat liver PNK are at pH 6.0 and pH 7.5 [Habraken and Verly, 1988].

Phosphorylation of Poly(dA) and Poly(A)

The enzyme purified by Tamura et al. [1981] was unable to phosphorylate RNA. To test the specificity of the present calf thymus PNK preparation, we compared the action of the calf thymus and T4 enzymes toward micrococcal nuclease-digested DNA, poly(dA), and poly(A). The data, presented in Table III, confirmed the inability of the enzyme to phosphorylate poly(A), but also suggested that homopolymeric DNA may be a poorer substrate than the micrococcal nuclease-digested DNA.

Minimum Substrate Size and Sequence Dependence

To assess the size distribution of oligonucleotides phosphorylated by calf thymus PNK, micrococcal nuclease-digested DNA that had been radioactively labelled by the enzyme was analyzed on an 18% polyacrylamide/7 M urea gel (Fig. 6). Unlike the DNA labelled by T4 PNK (lane 2), the DNA labelled by calf thymus (lane 4) and rat liver (lane 6) PNKs display a marked minimum at the octanucleotide size. In addi-

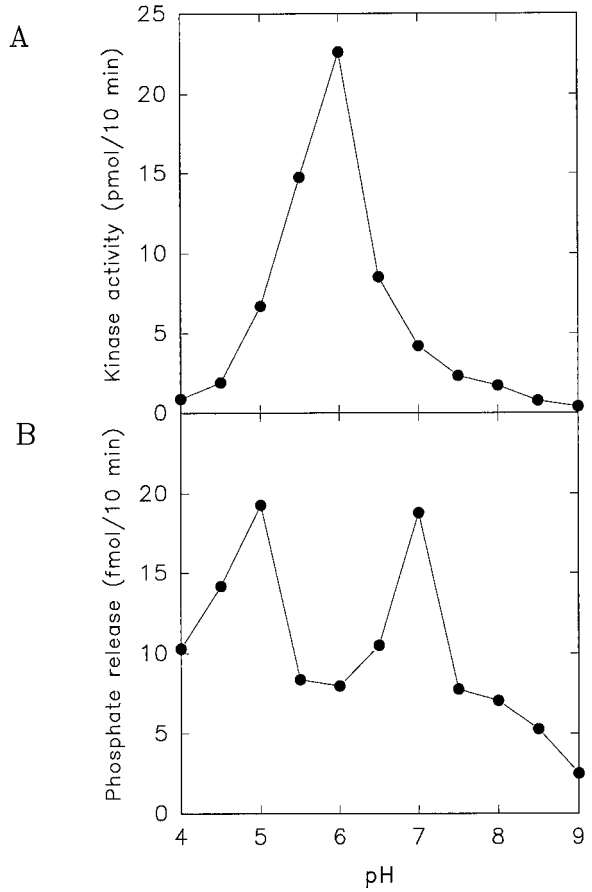

Fig. 5. Dependence of **A)** 5′-kinase and **B)** 3′-phosphatase activities of calf thymus PNK on pH. The phosphorylation and phosphatase assays are described under Materials and Methods. The buffers used for each pH point were: Tris-acetate - pH 4.0–4.5; sodium succinate - pH 5.0–6.0; Tris-HCl - pH 6.5–9.0.

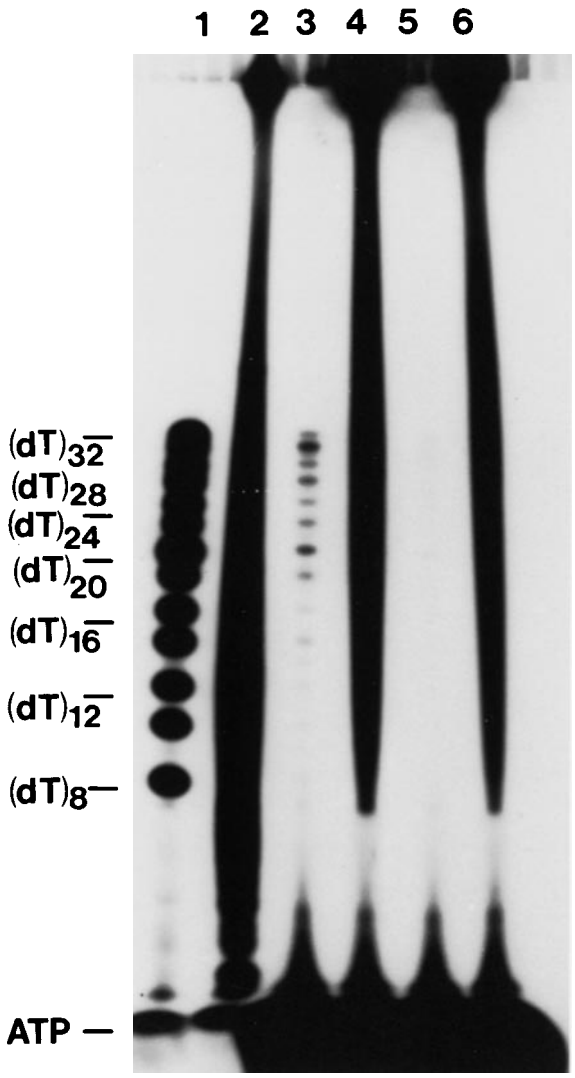

Fig. 6. PNK-catalyzed phosphorylation of micrococcal nuclease-digested DNA and oligo(dT)$_{8\text{-}32}$ size markers. Oligo(dT) size markers (2.7 pmol of total 5′-termini) and micrococcal nuclease-digested DNA (3 µg) were incubated with phage T4 PNK *(lanes 1, 2),* calf thymus PNK *(lanes 3, 4),* or rat liver PNK *(lanes 5, 6)* and 1.5 pmol [γ-^{32}P]ATP (3000 Ci/mmol) in 10 µl of buffer (pH 7.6 for T4 PNK, pH 5.5 for the mammalian PNKs) for 15 min at 37°C. The samples were then analyzed by electrophoresis through an 18% polyacrylamide/7 M urea gel and autoradiography.

TABLE III. Activity of Kinases on DNA and RNA Substrates*

Substrate	T4 kinase activity pmol/10 min	Calf thymus kinase activity pmol/10 min
MN-digested DNA	11.3	10.0
MN-digested DNA (heat denatured)		9.2
Poly(dA)	7.7	1.9
Poly(A)	18.2	0.32
Poly(A) (no enzyme)	0.35	0.35

*The standard assay conditions were used as described under Materials and Methods, except that the precipitated DNA and RNA was redissolved in water rather than NaOH, and the T4 kinase reactions were carried out in the pH 5.5 buffer. Substrate concentrations were chosen so as to give reasonably comparable numbers of termini per µg of substrate. MN-DNA refers to micrococcal nuclease-digested calf thymus DNA.

tion, the intensity of the radioactivity indicates that, of the oligonucleotides able to migrate into the gel, the mammalian kinases preferentially label those in the size range of 20–30 nucleotides in length, whereas the most intense signal produced by the phage PNK centered around oligomers 12–14 nucleotides in length. When the ability of the enzymes to phosphorylate the oligo(dT) size markers was compared, it was

apparent that any appreciable labelling was restricted to oligomers larger than oligo(dT)$_{18}$.

The poor phosphorylation by the mammalian kinases of the oligo(dT) substrates in comparison to randomly sequenced oligonucleotides, represented by the micrococcal nuclease digest of calf thymus DNA, was more rigorously investigated by comparison of the rate of reaction of the calf thymus kinase with the four 24-mers shown in Table IV. The results indicate a 4–5-fold lower rate of phosphorylation of the oligo(dA)$_{24}$ and the mixed-sequence oligonucleotide with a 5′-deoxyadenosine [oligo(dAN$_{23}$)], and a 25-fold difference between oligo(dT)$_{24}$ and the mixed-sequence oligonucleotide with a 5′-thymidine [oligo(dTN$_{23}$)]. From Lineweaver-Burk analysis of the kinetics of phosphorylation of the latter two oligonucleotides (Fig. 7), the apparent K_M values of the enzyme for the two substrates were found to be very similar—1.4 μM for oligo(dT)$_{24}$ vs. 1.7 μM for oligo(dTN$_{23}$)—but the apparent V_{max} values were significantly different—0.16 pmol/min for oligo(dT)$_{24}$ vs. 5.26 pmol/min for oligo(dTN$_{23}$).

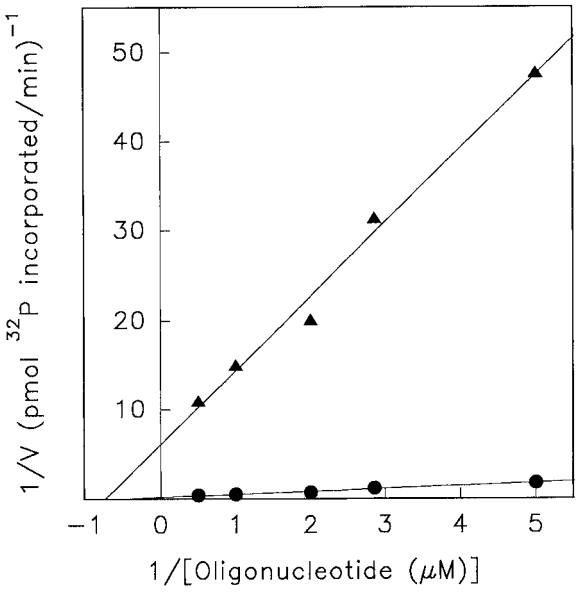

Fig. 7. Kinetics of PNK-catalyzed phosphorylation of oligonucleotides. Between 2 and 20 pmol of oligo(dT)$_{24}$ *(filled triangles)* or oligo(dTN$_{23}$) *(filled circles)* was incubated at 37°C for 4 min with 200 pmol unlabelled ATP, 5 μCi [γ-^{32}P]ATP (3,000 Ci/mmol), and ~0.1–0.2 units of kinase, in 10 μl buffer containing 80 mM succinic acid, pH 5.5, 10 mM $MgCl_2$, and 1 mM dithiothreitol. Samples were analyzed by polyacrylamide gel electrophoresis and autoradiography and then excised from the gel and the radioactivity determined.

TABLE IV. Activity of Calf Thymus Polynucleotide Kinases on Oligonucleotide Substrates*

Oligonucleotide	Kinase activity pmol/min
Oligo(dA)$_{24}$	0.38 ± 0.15
5′-AGCGCCCACCACCACTAGC-TGGCC-3′	1.68 ± 0.32
Oligo(dT)$_{24}$	0.20 ± 0.064
5′-TGCGCCCACCACCACTAGC-TGGCC-3′	4.96 ± 0.79

*80 pmol of each substrate was incubated with 400 pmol of unlabelled ATP, 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol), and ~0.1–0.2 units of kinase, in 20 μl buffer containing 80 mM succinic acid, pH 5.5, 10 mM $MgCl_2$, and 1 mM dithiothreitol at 37°C. Samples were analyzed by polyacrylamide gel electrophoresis and then excised from the gel and counted.

Phosphorylation of DNA Termini Generated by Restriction Enzymes

The mammalian PNKs were compared to the phage PNK in their relative abilities to phosphorylate overhanging and recessed DNA termini, produced by digestion of pUC18 plasmid with a series of different restriction enzymes and exhaustive incubation with alkaline phosphatase. The radioactively phosphorylated DNA molecules were analyzed by agarose gel electrophoresis and autoradiography. The autoradiograms of the gels (Fig. 8) reveal a clear difference in the activities of mammalian PNKs in comparison to the phage enzyme. The patterns of phosphorylation by the two mammalian PNKs were very similar and, unlike T4 PNK, which displays a marked preference for 5′-overhanging termini, showed no discernible selectivity for either overhanging or recessed termini. For example, both the rat liver and calf thymus PNKs labelled the recessed termini produced by AlwN I cleavage to a similar extent as the overhanging termini generated by Nar I. Analysis of the DNA sequence at the restriction recognition sites failed to reveal any sequence preference by the kinases.

Phosphorylation of Nicked and Gapped DNA

Models of potential double-stranded DNA intermediates, upon which the kinase may act in the cell, were prepared from oligonucleotides as detailed in Table V. These constructs modelled a large gap of ~20 bases (A), a short gap of 1 base (B), and a nick with dephosphorylated 3′-

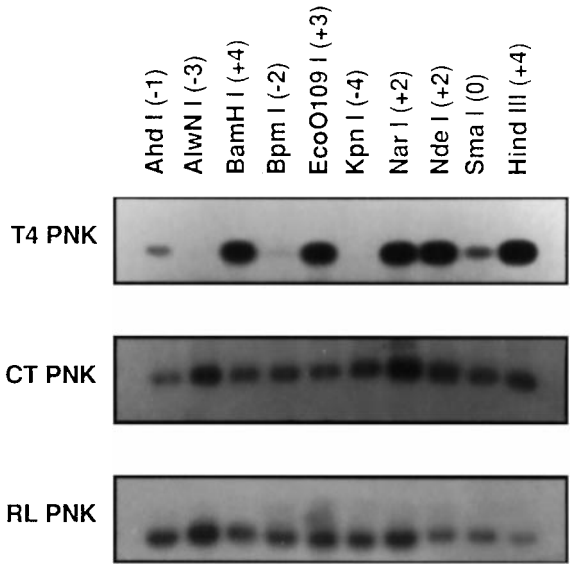

Fig. 8. Phosphorylation of linearized plasmid DNA generated by restriction enzymes. pUC18 plasmid DNA was digested with the restriction enzymes noted in the figure according to the suppliers instruction and then incubated exhaustively with shrimp alkaline phosphatase to remove terminal 5′-phosphate groups. Alkaline phosphatase activity was removed by heating at 65°C for 15 min, followed by phenol/chloroform extraction. For each PNK reaction, 0.4 μg of plasmid DNA (~0.5 pmol 5′-termini) was incubated with 1.5 pmol [γ-^{32}P]ATP (3000 Ci/mmol) and PNK from phage T4, calf thymus (CT), or rat liver (RL) in 10 μl of buffer (pH 7.6 for T4 PNK, pH 5.5 for the mammalian PNKs) for 5 min at 37°C. The products were analyzed by electrophoresis through a 1% agarose gel and autoradiography. The numbers in parentheses refer to the number of overhanging bases at the termini, with positive numbers indicating a 5′-overhang and negative numbers a 3′-overhang.

and 5′-termini (C). To ensure that the 24-mer was quantitatively annealed within each construct, a slight excess of the other oligonucleotides was added to the annealing solutions. Furthermore, in constructs B and C, the other short oligonucleotides (the 20-mer and 21-mer) were in slight excess of the 45-mer to ensure the quantitative formation of the triple-oligonucleotide products. It is important to note that the 5′-termini of all the oligonucleotides had 5′-hydroxyl groups and were therefore available for phosphorylation. After incubation with calf thymus PNK, the products were analyzed on a nondenaturing gel (Fig. 9a). The slower mobility of the radiolabelled products in the annealed mixtures, in comparison to the radiolabelled single oligomers, indicated that the radiolabel was almost entirely associated with the annealed oligonucleotides in A, B, and C. When the same reaction mixtures were subsequently analyzed on a denaturing gel (Fig. 9b) to determine which oligonucleotides within the complexes were labelled, the 24-mer produced a considerably stronger signal than any of the other oligonucleotides. Rat liver PNK gave an identical result (data not shown).

DISCUSSION

This communication describes the purification of polynucleotide kinase from calf thymus and extends the characterization of its properties, especially in regard to its substrate specificity. The observation of an acidic pH optimum of phosphorylation, and the specificity of the enzyme toward DNA as opposed to RNA, confirmed that the calf thymus enzyme purified here was the same as that described by Austin et al. [1978] and Tamura et al. [1981], and not the kinase recently discovered by Prinos et al. [1995]. Other properties of the calf thymus PNK that we observed indicate the great similarity of this enzyme to the rat liver PNKs isolated in several laboratories, including ours. These include a) the similar pI values, b) the identical minimum size of an oligonucleotide that can be phosphorylated, and c) the observation that the calf thymus kinase also has a 3′-phosphatase activity. The unusual bimodal pH dependence of this activity (Fig. 5b) was also very similar to that seen with the phosphatase activity of rat

TABLE V. Composition of Oligonucleotides and Model Substrates*

Oligonucleotide	Sequence
20-mer	5′-ATTACGAATGCCCACACCGC-3′
21-mer	5′-ATTACGAATGCCCACACCGCC-3′
24-mer	5′-GGCGCCCACCACCACTAGCTGGCC-3′
45-mer	5′-GGCCAGCTAGTGGTGGTGGGCGCCGGCGGTGTGGGCATTCGTAAT-3′

Complex	Oligonucleotides
A	45-mer + 24-mer
B	45-mer + 24-mer + 20-mer
C	45-mer + 24-mer + 21-mer

*The complexes were annealed by heating the oligonucleotides in buffer (60 μl total volume) containing 20 mM Tris-HCl, pH 6.8, 2 mM EDTA, 1 mM DTT, 20 mM NaCl and 10 mM $MgCl_2$ at 70°C for 5 min and allowing them to cool to room temperature over 2 h. For each complex the annealing solutions contained the following quantities of oligonucleotide: complex A − 5 pmol 45-mer + 4 pmol 24-mer; complex B − 5 pmol 45-mer + 4 pmol 24-mer + 6 pmol 20-mer; complex C − 5 pmol 45-mer + 4 pmol 24-mer + 6 pmol 21-mer.

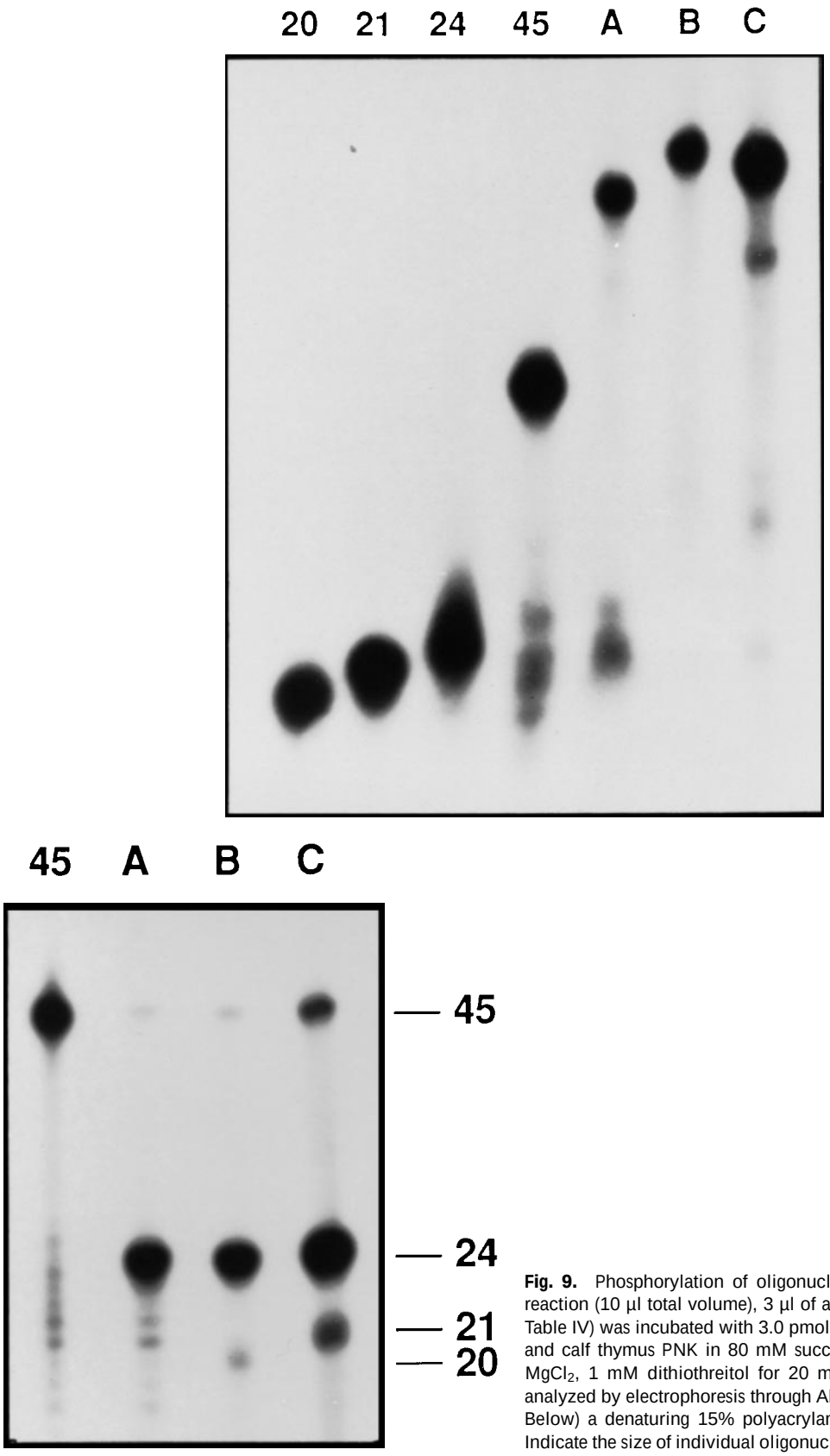

Fig. 9. Phosphorylation of oligonucleotide complexes. In each reaction (10 µl total volume), 3 µl of annealed complex A–C (see Table IV) was incubated with 3.0 pmol [γ-^{32}P]ATP (3000 Ci/mmol) and calf thymus PNK in 80 mM succinic acid (pH 5.5), 10 mM $MgCl_2$, 1 mM dithiothreitol for 20 min at 37°C. Products were analyzed by electrophoresis through Above) a non-denaturing and Below) a denaturing 15% polyacrylamide gel. *Numbered lanes:* Indicate the size of individual oligonucleotides used as markers.

liver PNK. We think it unlikely that this is the result of an artifact associated with the buffers, because the maxima and minima in the curve did not correspond with the changes in the buffers. A mitochondrial endonuclease isolated from bovine heart has also been shown to have a bimodal pH dependence [Cummings et al., 1987].

On the basis of SDS/polyacrylamide gel electrophoresis, the polypeptides of both the calf thymus and rat liver kinase, when purified from whole-cell homogenates, were determined to have molecular weights of close to 60 kDa (Fig. 4). This concurred with our activity gel results (Figs. 1, 2), with the activity gels of PNK activity in various rat organs reported by Ohmura et al. [1987], and is in reasonable agreement with the previous estimate of 70 kDa for the calf thymus enzyme based on a Stokes radius of 4.3 nm [Austin et al., 1978]. On the other hand, the major kinase activity retrieved from calf thymus and rat liver nuclei was associated with a 40-kDa polypeptide (Figs. 1, 2). This is in accord with the size of the polypeptide purified from rat liver by Habraken and Verly [1988], and from rat testes by Bosdal and Lillehaug [1985]. In our view, the most reasonable explanation for these observations is that, under normal circumstances, PNK is a ~60-kDa polypeptide, but that in the course of isolating nuclei (and to a minor extent when purifying PNK from whole-cell homogenate), the protein is subject to specific proteolysis to a 40-kDa polypeptide, even in the presence of protease inhibitors. If in the cell the kinase existed in relatively large quantities in both a 60- and a 40-kDa form, one would anticipate a significant signal at 40 kDa, as well as at 60 kDa, in the activity gels of the whole-cell extracts. No such signal is evident in Figure 1a. However, to fully resolve this issue, antibodies to the proteins are being generated in order to determine the relationship between the various forms of PNK.

Earlier molecular size determination of the rat liver PNK by gel filtration led to the conclusion that the enzyme is composed of a dimer of the polypeptide [Habraken and Verly, 1988]. Our observations tend to support this conclusion. First, the calf thymus and rat liver PNKs eluted from the Superdex 200 column as >100-kDa proteins. Second, the rat liver PNK eluted from hydroxyapatite in two major peaks (Fig. 3). The silver-stained denaturing gel suggested that the first peak was protein composed of a heterodimer between a 60-kDa and a 40-kDa polypeptide, whereas the second peak was the homodimer of the 60-kDa polypeptide.

Examination of the micrococcal nuclease-digested DNA labelled by the calf thymus and rat liver PNKs (Fig. 6) demonstrated that the minimum length for an oligonucleotide substrate for PNK from both sources is 7–8 nucleotides. Levin and Zimmerman [1976], using DEAE-cellulose chromatography, had previously established a minimum substrate size of 10–12 nucleotides for phosphorylation by the rat liver kinase. The comparatively poor labelling by the mammalian PNKs of the oligo(dT) size markers shown in the same figure was confirmed by the kinetic analysis of phosphorylation of defined oligonucleotides (Table IV, Fig. 7). It remains to be determined whether the heterooligomers are better substrates because of an enzyme interaction with a specific sequence within the substrate, which is clearly missing in the homopolymer, or because in comparison to heteropolymers, homopolymers (particularly poly[dT]) tend to lack any form of secondary structure at 37°C [Bloomfield et al., 1974].

Although the biochemical activities of mammalian polydeoxynucleotide kinase are well known, a cellular role for the enzyme has yet to be defined. Pohjanpelto and Hölttä [1996] have recently provided evidence that a PNK may be necessary for phosphorylation of Okazaki fragments during DNA replication. In most other studies, including this one, PNK has been regarded as a potential DNA repair protein involved in strand rejoining. The oligonucleotide complexes A and B were intended to mimic gaps in DNA that may occur in nucleotide excision repair, where the gap is usually of the order of 20–30 nucleotides, and in base excision repair, where a single nucleotide may be replaced [Friedberg et al., 1995]. In the latter repair pathway, a phosphate at the 5′-terminus of a strand gap is not only required for ligation, but also for gap filling DNA synthesis by DNA polymerase β [Singhal and Wilson, 1993]. The 8-kDa tryptic fragment of the polymerase binds to the 5′-phosphate at a gap [Prasad et al., 1994]. Nicked DNA, represented by complex C, may arise as a repair intermediate, but may also be the result of DNA cleavage by an endonuclease.

Several endonucleases nick DNA to generate termini with 3′-phosphates and 5′-hydroxyl groups. If these incised strands are subject to repair, one could anticipate an intermediate such as that modelled in complex C. One nuclease that cleaves DNA in this manner is DNase II. Because this enzyme has recently been implicated in DNA degradation during apoptosis [Barry and Eastman, 1993] and lens cell differentiation [Torriglia et al., 1995], it may suggest a role for PNK in responding to DNA cleavage by DNase II and, therefore, regulating apoptosis.

In previous investigations of the ability of PNK to phosphorylate damaged DNA, the usual substrate has been micrococcal nuclease-nicked DNA. However, this is a relatively ill-defined substrate, because such treatment can generate gaps as well as nicks [Habraken and Verly, 1983]. Habraken and Verly [1986] generated a more defined poly(dA)/oligo(dT) substrate to test the potential direct transfer by PNK of a phosphate from a 3′ to a 5′-terminus. But even this substrate has the potential for gap formation by slippage of the oligo(dT) on the complementary strand. For this reason, we employed oligonucleotides that, when annealed, would produce the clearly defined model substrates described above. An added advantage of this approach was that we were able to compare phosphorylation of all the 5′-termini in the complexes. Thus, it appears that the internal 5′-hydroxyl terminus is markedly preferred to the more sterically accessible overhanging single-stranded terminus of the 45-mer in complex A, or the blunt end double-stranded termini of the 20-mer, 21-mer, and 45-mer in complexes B and C (Fig. 9). It remains to be seen if this has a bearing on the cellular function of polydeoxynucleotide kinase, but it might, for example, indicate that the enzyme acts on termini of single-strand breaks rather than double-strand breaks.

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