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5′ Phosphorylation of DNA in Mammalian Cells: Identification of a Polymin P-Precipitable Polynucleotide Kinase

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Abstract Proteins that catalyze 5′ phosphorylation of an oligodeoxyribonucleotide substrate can be fractionated by polymin P treatment of whole cell extracts of calf thymus glands. Anion exchange chromatography on Q-Sepharose revealed three separable peaks of activity in the polymin P supernatant fraction, and one peak of activity in the Polymin P pellet fraction. The latter activity, Polymin P-precipitable polynucleotide kinase (PP-PNK), was further purified with a 1,500-fold increase of specific activity compared to the crude Polymin P pellet fraction. Oligonucleotides, a dephosphorylated 2.9-kb *Eco*RI fragment, and poly(A) were phosphorylated by the enzyme preparation, but thymidine 3′ monophosphate was not a substrate. PP-PNK preparations exhibited an apparent K_M of 52 μM for ATP and 8 μM for oligo dT_{25}. The enzyme preparation displayed no detectable 3′ phosphatase or cyclic 2′,3′ phosphohydrolase activities. The sedimentation coefficient of the PP-PNK activity was 3.8S as determined by sucrose density gradient analysis; the Stokes radius was 45 Å, leading to an estimated molecular mass of 72 kDa. The enzyme had a pH optimum in the neutral to alkaline range in several buffer systems and is distinct from the DNA kinase with an acidic pH optimum previously described in calf thymus.

Key words: polynucleotide kinase, DNA kinase, mammalian cells, calf thymus

The mammalian counterparts of bacteriophage T4 DNA metabolic enzymes often have important cellular functions. Examples include the human polymerase cofactor, PCNA (gp45), human SSB (gp32), and a subunit of the human primer recognition factor RF-C (gp44) [Tsurimoto and Stillman, 1990; Chen et al., 1992]. Polynucleotide kinase (PNK), first described in bacteriophages T4 and T2 [Richardson, 1965; Novogrodsky and Hurwitz, 1966; Novogrodsky et al., 1966], catalyzes the transfer of a γ-phosphoryl moiety of a nucleoside triphosphate to a 5′ hydroxyl terminus of a nucleic acid. The biochemical properties of T4 PNK and the genetics of the *pse*T gene encoding this enzyme have been studied extensively [reviewed in Richardson, 1981; Snyder, 1983]; the *pse*T gene has been cloned [Midgley and Murray, 1985]. Physiologically, T4 PNK has been proposed to function in re-processing of host tRNA to adapt to phage codon requirements [Amitsur et al., 1987, 1989] or to play a role in DNA metabolism and expression of late genes in certain *Escherischia coli* genetic backgrounds [Depew and Cozzarelli, 1974; Sirotkin et al., 1978, and Runnels et al., 1982]. Multiple physiological roles are not uncommon for bacteriophage proteins, as evidenced by the catalytic role of T4 RNA ligase in tRNA metabolism and its structural role in tail fiber attachment [Kornberg and Baker, 1992].

Over the past several decades, enzymatic activities sharing some characteristics with T4 polynucleotide kinase have been purified from the following mammalian sources: DNA kinases from rat liver nuclei [Novogrodsky et al., 1966; Teraoka et al., 1975; Levin and Zimmerman, 1975; Habraken and Verly, 1983], rat testis [Bosdal and Lillehaug, 1985], calf thymus [Austin et al., 1978; Tamura et al., 1981], and 5′

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hydroxyl polyribonucleotide kinase from HeLa cells [Shuman and Hurwitz, 1979]. Among reports describing a mammalian DNA kinase activity, there is a general concordance that divalent cations are required for activity, that the K_M for ATP is 2–4 μM, that the pH optimum is 5.5–6.0, and that DNA is phosphorylated to a greater extent than RNA. Because of its activity at single-stranded breaks in DNA, the acid-pH optimum DNA kinase has been postulated to function in DNA repair [reviewed in Zimmerman and Phieffer, 1981]. Indeed, 5′ OH terminated strand breaks have been observed in DNA isolated from thymocytes or mouse thymus glands following exposure to ionizing radiation [Lennartz et al., 1975], although 5′ OH termini were not detected in a study of DNA irradiated in vitro [Henner et al., 1982]. Other potential sources of 5′ OH termini requiring repair can be generated by nucleases [Slor and Lev, 1971] and class III AP endonucleases, one example of which was reported in *Drosophila* embryos [Spiering and Deutsch, 1986]. It is important to note that 5′ phosphoryl transfer is not the only potential mechanism of repair of 5′ OH termini in DNA; for instance, 5′ to 3′ exonuclease activity followed by resynthesis is another feasible pathway. In one study [Shuman and Hurwitz, 1979], nuclear DNA and RNA kinase activities were fractionated by ammonium sulfate precipitation and the RNA kinase activity, which was active on DNA at a much lower efficiency, was further purified. Consequently, it is necessary to consider many potential activities to be more complete in assignment of function to enzymes capable of 5′ phosphorylation of nucleic acids.

A multifunctional RNA ligase involved in tRNA splicing has been described in eukaryotes [reviewed in Phizicky and Greer, 1993]. The pathway in which this enzyme participates is present in human cells [Zillman et al., 1991] despite the fact that it is not the major tRNA splicing pathway in vertebrates [Laski et al., 1983; Filipowicz and Shatkin, 1983]. This ligase was designated tRNA ligase in *S. cerevisiae* [Greer et al., 1983], in which it is essential for viability [Phizicky et al., 1992], and a similar enzyme was also purified from wheat germ [Konarska et al., 1981, 1982; Gegenheimer et al., 1983; Pick and Hurwitz, 1986a,b]. The tRNA ligase genes from *S. cerevisiae* [Phizicky et al., 1986] and *C. albicans* [Baymiller et al., 1994] have been cloned. The *S. cerevisiae* tRNA ligase and the wheat germ RNA ligase contain a polynucleotide kinase activity [Greer et al., 1983; Konarska et al., 1982; Pick and Hurwitz, 1986a]; therefore, any thorough consideration of 5′ phosphorylation of nucleic acids in mammalian cells must include the potential role of RNA ligases. Such analyses are facilitated by the fact that these RNA ligases form an enzyme-adenylate intermediate that can be labeled with α-[^{32}P]-ATP and analyzed on SDS-polyacrylamide gels [Greer et al., 1983; Pick and Hurwitz, 1986b]. Other mammalian enzymes that form adenylated intermediates are DNA ligases I, II, and III [Lindahl and Barnes, 1992] and RNA cyclase [Reinberg et al., 1985]. Small stretches of amino acid homology have been reported [Koonin and Gorbalenya, 1990] in the nucleotide binding domains of T4 polynucleotide kinase, yeast tRNA ligase, and the mammalian myelin 2′,3′-cyclic nucleotide phosphohydrolases R, B, and H. A single report [Sprinkle et al., 1987] indicated that the human myelin 2′,3′-CNPase, a protein considered important in CNS myelination [Sprinkle, 1989; De Angelis et al., 1994], is able to phosphorylate tRNA.

In order to gain a comprehensive overview of the enzymatic activities in calf thymus capable of 5′ phosphorylation of DNA, we used an oligodeoxynucleotide substrate to establish a highly sensitive and specific assay. Whole cell rather than nuclear extracts of calf thymus were prepared in order to avoid possible bias against detection of activities that may leach out of nuclei during purification of this organelle. A similar strategy was successful for the identification and purification of three DNA ligases from mammalian cells, including DNA ligase II, which tightly binds to chromatin [Lasko et al., 1990b; Tomkinson et al., 1992]. We utilized a specific assay for the enzymes capable of transfer of a phosphoryl group to DNA, employing the synthetic oligonucleotide substrate, oligo dT_{25} (assay A). Sample cleanup steps and gel electrophoresis were used to assure that only 5′ phosphorylation of DNA was monitored. The preparation that we have purified from calf thymus in this study has significant activity on an RNA substrate and has properties distinct from those of mammalian polynucleotide kinases previously described in the literature.

MATERIALS AND METHODS

DNA Kinase Enzyme Assays

Assay A. Oligo dT_{25} was purchased from the Sheldon Biotechnology Centre (Montreal, Que-

bec). To follow the purification, reactions in a total volume of 11 μL of 100 mM MES, pH 5.5 contained 10 μM oligo dT_{25}, 0.4 μCi γ-[^{32}P]-ATP (3000 Ci/mmol, Amersham PB10168), 20 μM unlabelled ATP (Boehringer-Mannheim), 1 μg nuclease-free BSA (New England Biolabs), 10 mM $MgCl_2$, and 2 mM DTT. In experiments with T4 PNK (New England Biolabs) as a positive control, 0.1 U (units as defined by Richardson [1965]) was used. In characterization experiments, 100 mM Tris pH 7.5 was sometimes substituted for 100 mM MES, pH 5.5. The reactions were incubated at 37°C for 20 min, then 0.5 μl 0.5M EDTA was added. The samples were treated once with 3 μl of resuspended Strataclean Resin (PDI Biosciences) according to the manufacturer's instructions. DNA sequencing stop buffer (5 μl, [Sambrook et al., 1989]) was added, and the tubes were heated to 90°C for 3 min. The samples were analyzed by 20% polyacrylamide/7 M urea gel electrophoresis in 1× TBE in a MiniProtean II apparatus (BioRad). The gel was exposed to Fuji X-ray film at −70°C with two intensifying screens for 18–90 h. Activity was quantified by liquid scintillation counting of excised bands. The limit of detection of the assay was 0.02 pmol ^{32}P/20 min.

Assay B. Preparations of pUC19 DNA were made using a Magic Maxiprep kit (ProMega). The purified DNA was cleaved with *Eco*RI (Pharmacia), and dephosphorylated with calf intestinal phosphatase (Boehringer-Mannheim, molecular biology grade) following the manufacturer's instructions. Residual protein was removed with Strataclean resin. DNA kinase reactions were carried out as described for assay A except that each assay contained 1.0 pmol of 5′ hydroxyl ends and 1 μCi γ-[^{32}P]-ATP (3,000 Ci/mmol, Amersham PB10168). EDTA was added, and the reactions were processed with Strataclean as described for assay A. The reaction products were precipitated with ethanol [Sambrook et al., 1989], redissolved in 10 μl sterile H_2O, and electrophoresed through an 0.8% agarose gel. The gels were dried and exposed to X-ray film for at least 24 h.

Assays for Other Enzymatic Activities

3′ Phosphatase activity was tested with thymidine 3′,5′-diphosphate prepared by phosphorylation of thymidine 3′ monophosphate with γ-[^{32}P]-ATP [Cameron and Uhlenbeck, 1977]. Phosphorylation of RNA was measured in 50 mM Tris–HCl, pH 7.5, using 5′ OH poly(A) (Pharmacia; polymer size 630–690b, as reported by the manufacturer) as described previously [Shuman and Hurwitz, 1979]; 50 μg of RNA substrate was used in a total reaction volume of 20 μl. Identification of adenylated polypeptides was carried out as described previously [Lasko et al., 1990a]. Endogenous protein kinase activity was assessed by carrying out Assay A with and without DNA substrate, then running the products on a 10% SDS–polyacrylamide gel. Endonuclease activity was assayed by incubating 1 μg of pUC19 supercoiled DNA under assay A conditions for 4 h, Strataclean treatment of the DNA, and analysis on an 0.8% agarose gel stained with 0.5 μg/ml ethidium bromide. Kits for analysis of acid and alkaline phosphatase by spectrophotometric methods were obtained from Sigma. 2′,3′-cyclic nucleotide 3′-phosphodiesterase (EC 3.1.4.37; CNPase) activity was assessed using 2′,3′ cyclic $NADP^+$ as substrate [Sogin, 1976].

Cell Fractionation and Chromatography

Buffer A was 50 mM Tris–HCl, pH 7.5, 30 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA. Thymus glands from calves about 6 months old were obtained from Aliments Danac (LaPlaine, Quebec) and processed within 2 h. The crude extract was prepared with a cocktail of protease inhibitors as described [Tomkinson et al., 1990] except that 0.3 M NaCl was used in the extraction buffer. Preliminary experiments indicated that this salt concentration efficiently extracted DNA kinase activity. The tissue (600 g) was extracted in a total volume of 1.2 L. The extract was centrifuged at 8,000*g* for 20 min at 4°C. The supernatant was filtered through glass wool and diluted to 0.15 M NaCl with the slow addition of buffer A without salt. A 5% stock solution of Polymin P (purchased as a 50% solution from Sigma and prepared as described [Burgess, 1991]) was added to a final concentration of 0.5% (v/v). The mixture was stirred for 30 min at 4°C, and centrifuged at 8,000*g* for 20 min at 4°C. The supernatant was removed and the precipitated material stored at 0°C overnight.

Q-Sepharose Chromatography of the Polymin P Supernatant Fraction

The DNA kinase activity from the supernatant fraction was adjusted to 50 mM NaCl, adsorbed to phosphocellulose, and eluted from phosphocellulose with buffer containing 1 M NaCl. The salt concentration was adjusted to 30 mM NaCl and the protein was applied to a

Q-Sepharose column, and eluted with a 30-mM to 1-M NaCl gradient.

Further Purification of the Polymin P-Precipitable Polynucleotide Kinase

The pellets were washed with 200 ml of buffer A containing no salt, then 200 ml of buffer A containing 0.6 M NaCl. Pilot experiments determined that this was the optimal salt concentration for recovery of DNA kinase activity from the Polymin P pellet. The NaCl concentration was adjusted to 1 M, and the Polymin P eluate was passed over a phosphocellulose P11 (Whatman) column (16 mg protein/ml resin), which was washed with an additional column volume of 1 M NaCl buffer. The following column sizes are for the preparation described in Table 1 (Preparation IV), representative of five preparations. The resulting protein was dialyzed overnight in buffer A, then loaded onto a Q-Sepharose (Pharmacia) column (12 mg protein/ml resin). The column was washed extensively with the starting buffer, and then a 5-column volume gradient of 30 mM to 1 M NaCl in Buffer A was applied. Active fractions eluted at about 0.5 M and were pooled. Potassium phosphate (pH 7.5) was added to a final concentration of 1 mM, and the proteins were applied to a hydroxyapatite (BioRad, type HT; 2 mg protein/ml) column pre-equilibrated in the same buffer. The column was eluted with steps of 2 column volumes each of 1 mM, 50 mM, 150 mM, and 400 mM potassium phosphate, pH 7.5, 0.5 mM DTT. DNA kinase activity eluted in the 150 mM potassium phosphate step. Active fractions (10 mg/ml) were dialyzed against buffer A containing 10 mM potassium phosphate and passed through a 1-ml phosphocellulose column. The flowthrough and 2 ml of wash from the phosphocellulose column (10 mg protein) was applied to a 2.5 ml column of Blue–Sepharose (Pharmacia). A gradient of 5 column volumes was applied ranging from 30 mM to 1 M NaCl. Active fractions eluted at about 600 mM NaCl and were pooled. Other resins tested for PP-PNK binding after pre-equilibration with buffer A included ATP-agarose (attached at the ribose hydroxyls with a 22-atom spacer (Sigma A-4793) and heparin–agarose (Sigma H-6508). Chromatography on MonoQ was performed as follows: protein from the Blue–Sepharose step was dialyzed overnight against buffer A adjusted to pH 7.0. The sample (1.9 ml; 0.25 mg) was loaded onto an FPLC MonoQ (HR5/5, Pharmacia) column connected to a Waters 650E protein purification system with a variable wavelength 440 detector and pre-equilibrated in buffer A, pH 7.0. Absorbance at 280 nm was monitored. The flow rate was 0.5 ml/min. The column was eluted with 5.0 ml buffer A, pH 7.0, followed by a linear 15 ml gradient from 30 mM to 1 M NaCl in the same buffer, and a final 5 ml of buffer A containing 1 M NaCl. Fractions (0.5 ml) were collected. When necessary, fractions were routinely concentrated using Centricon 30, Centriprep 30, or Microcon 30 columns (Amicon).

Protein Characterization

Identity of the phosphorylated product. Two types of experiments established the nature of the phosphorylated oligonucleotide. In the first, T4 PNK-phosphorylated oligo dT_{25} was prepared under standard conditions for assay A at pH 7.5, with the reaction scaled up 10-fold. The phosphorylated oligonucleotide was separated from unincorporated ATP by chromatography on a C_{18} Sep-Pak cartridge [Sambrook et al., 1989]. This 5′ phosphorylated oligonucleotide was then tested in assay A with the Blue–Sepharose fraction. In other experiments, radiolabeled, phosphorylated product (1 μg) was incubated at 37°C for 16 h with snake venom

TABLE I. Purification Table*

Sample	Total protein	Total activity	Specific activity	Yield (%)	Fold purification
1. Crude extract	33.5 g	—	—	—	—
2. Polymin P eluate/phosphocellulose flowthrough	2.6 g	5.7	0.0021	100	—
3. Q-Sepharose	33 mg	5.7	0.175	100	83
4. Hydroxyapatite	11.9 mg	5.2	0.44	91	210
5. Blue–Sepharose	0.6 mg	2.0	3.3	35	1570

*Protein concentration was determined using a commercial Bradford reagent (Pierce). Activity (nmol P/20 min/mg) was determined at pH 5.5. The activity values represent the mean of four determinations differing by less than 20%. When assayed at pH 7.5, the specific activity of the Blue–Sepharose fraction was 40% higher.

phosphodiesterase (Boehringer Mannheim, 15 mU) in 100 mM Tris–HCl, pH 9.0, 10 mM $MgCl_2$ and analyzed on 20% polyacrylamide gels and by TLC using polyethylenimine plates and 1 M LiCl as a buffer system.

Kinetics and inhibition experiments. These experiments were carried out at pH 7.5. In time course experiments, DNA kinase assays were carried out as described for assay A, except that incubation times were varied from 0 min to 240 min. The 20-min incubation time used for substrate dependence and inhibition experiments was in the linear range of the time course. In substrate dependence experiments, the oligo dT_{25} concentration was varied from 0 to 50 μM. In ATP-dependence experiments, the ATP concentration was varied from 0 to 500 μM. The apparent K_M for oligo dT_{25} and ATP were determined by double-reciprocal (Lineweaver-Burk) plots. Curves were fitted using the linear regression function of the CricketGraph (CA Associates) program run on a Macintosh SE30 computer. The K_M was calculated from the X-intercept as determined by the equation for the fitted curve. Three independent experiments were used to determine the K_M; the mean and the standard deviation were calculated. All inhibition experiments were carried out in 100 mM Tris–HCl, pH 7.5. Nine different concentrations of $MgCl_2$ from 0 to 100 mM were assayed. Six concentration points of NaCl or KCl were assayed, from 0 to 500 mM. Increasing amounts of ammonium sulfate, sodium sulfate, or sodium phosphate were added up to a concentration of 100 mM; a total of six points were assayed. In the sodium pyrophosphate inhibition experiments, seven concentration points were assayed from 0 to 20 mM.

Sedimentation Gradient Analysis. Samples of Blue–Sepharose-purified material (0.1 ml; 20 μg) were applied to 4.7 ml of a 5–20% sucrose gradient prepared in buffer A plus 300 mM NaCl. The gradients were centrifuged for 20 h at 39,000 rpm in an SW55 rotor at 4°C. The gradients were fractionated into 0.2-ml fractions. PP-PNK activity was detected using assay A carried out at pH 7.5 after 10-fold concentration of the samples by ultrafiltration using a Microcon 30 unit (Amicon). The sedimentation coefficient ($S_{20,w}$) relative to standards was estimated as previously described [Martin and Ames, 1961]. Migration of PP-PNK was compared to standards purchased from Pharmacia that were run in parallel gradients [catalase (11.3S), aldolase (7.8 S), bovine serum albumin (4.4 S), ovalbumin (3.5 S), and chymotrypsinogen (2.5 S)]. Standards were detected by SDS–PAGE, followed by Coomassie blue staining.

Gel filtration chromatography. Active fractions were centrifuged for 10 min at 4°C prior to loading. Sample (200 μl; 100–400 μg) was loaded onto a Superose 12 Column (Pharmacia) connected to a Waters 650E protein purification system with a variable wavelength 440 detector and pre-equilibrated with buffer A containing 300 mM NaCl. Absorbance at 280 nm was monitored. The flow rate was 0.25 ml/min. Void volume was estimated to be 7.97 ml using Blue Dextran. The column fractions were monitored using assay A. Standards were purchased from Pharmacia and were catalase (230 kDa), aldolase (160 kDa), ovalbumin (45 kDa), and chymotrypsinogen A (24 kDa).

pH optimum. For pH profile experiments, assays were carried out at a number of different pH values in several buffer systems. For pH values below 7, sodium acetate and [N-morpholino]ethanesulfonic acid (MES) buffers were used. Tris–maleate buffer was used from pH 5.2 to 9.5. Tris–HCl buffer was used in the pH range 6.2–10.0. The values for pH are for 100 mM buffer at room temperature, including the amount of 50 mM Tris HCl buffer contained in the reaction due to the addition of the enzyme (1/10th vol).

RESULTS

Fractionation of Whole Cell Extracts of Calf Thymus With Polymin P and Identification of a Polymin-P-Precipitable Polynucleotide Kinase (PP-PNK) Activity

Whole cell extracts of calf thymus were fractionated by the addition of Polymin P (polyethyleneimine) (Fig. 1). The Polymin P supernatant fraction of the calf thymus extract was analyzed for oligonucleotide kinase activity. Activity was detected in the Polymin P supernatant only after phosphocellulose chromatography. The preparation was then applied to a Q-Sepharose column (Fig. 2). Activity eluted in the flow through and wash fractions (SNQI) and early in a 30 mM to 1 M NaCl gradient (fractions 7–8; SNQII). A small amount of activity eluted later in the gradient (fractions 14–16; SNQIII). These data are consistent with the presence of multiple, chromatographically distinct activities in calf thymus capable of phosphorylation of oligo dT_{25}. Fraction SNQI displayed an acidic pH opti-

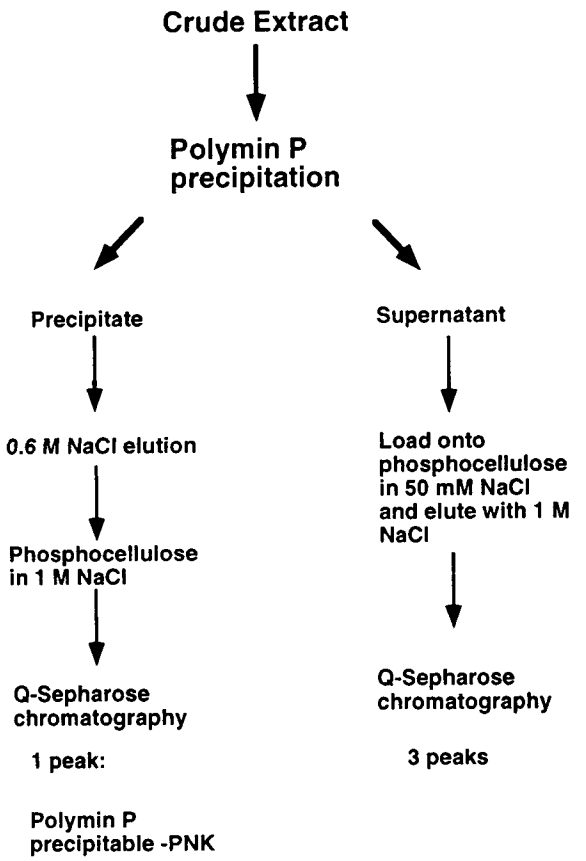

Fig. 1. Fractionation scheme for DNA kinase activity from whole cell extracts of calf thymus. In order to remove nucleic acids, the extract was made 0.5% with respect to Polymin P. Protein extracted from the pellet with 0.6 M NaCl was adjusted to 1.0 M NaCl and applied to a phosphocellulose column preequilibrated in the same buffer. The eluate was adjusted to 30 mM NaCl and applied to a Q-Sepharose column, and eluted with a 30 mM to 1 M NaCl gradient. The DNA kinase activity from the supernatant fraction was adjusted to 50 mM NaCl, adsorbed to phosphocellulose, and eluted from phosphocellulose with buffer containing 1 M NaCl. The salt concentration was adjusted to 30 mM NaCl and the protein was applied to a Q-Sepharose column, and eluted with a 30 mM to 1 M NaCl gradient.

mum in preliminary experiments (C. Slack and D. Lasko, unpublished results). The relative proportion of activities in fractions SNQI, SNQII, and SNQIII was somewhat variable in three different preparations from the Polymin P supernatant; in one experiment, very little SNQIII activity was detected (data not shown). The activity in fraction SNQI was stable for about 2 weeks when stored on ice in buffer A. The SNQII activity was considerably more stable, with activity still detectable after storage for several months on ice.

In pilot experiments, the Polymin P pellet fraction was examined for 5′ phosphorylation activity by elution of the pellet with 0, 0.25, 0.5, and 1.0 M NaCl. Substantial activity eluted in the 0.5 M fraction (data not shown). Subsequently, 0.6 M NaCl was used to elute 5′ phosphorylation activity from the Polymin P pellet in larger scale experiments. After passage through a phosphocellulose column to remove residual Polymin P, the protein preparation was applied to a Q-Sepharose column. The Polymin P-precipitable activity bound to the Q-Sepharose column, eluting as a single peak at about 0.5–0.6 M NaCl in step gradients or linear gradients (Fig. 3). This activity was designated Polymin P-precipitable polynucleotide kinase (PP-PNK) and was further purified as described below.

Although Figure 2 indicates that there is substantial activity in the Polymin P supernatant, the results are not directly comparable to those in Figure 3 because these data are from separate preparations and the samples were processed in a different manner. Proteins in the supernatant were adsorbed to phosphocellulose at low ionic strength and eluted with 1 M NaCl prior to the Q-Sepharose column; the proteins in the pellet fraction were passed over phosphocellulose in 1 M NaCl and then subjected to Q-Sepharose chromatography.

Purification and Chromatographic Properties of the DNA Kinase Activity From the Polymin P Pellet Fraction

PP-PNK activity eluted from the Q-Sepharose column was applied to hydroxyapatite and Blue–Sepharose columns. Elution from hydroxyapatite occurred in the 150 mM potassium phosphate step, with only trace amounts of activity eluting in the 50- or 400-mM fractions. The activity eluted as one peak late in the gradient on Blue–Sepharose chromatography (Fig. 4). The activity did not bind (eluted in the flowthrough and wash) when applied to phosphocellulose and ATP-agarose columns pre-equilibrated with buffer A (data not shown). After the five purification steps, the specific activity increased 1,500-fold above that in the Polymin P pellet (a preparation typical of five purifications is summarized in Table 1). Activity was not detected in the crude extract, consequently the yield was quantified starting from the Polymin P pellet fraction. The increase in specific activity is therefore

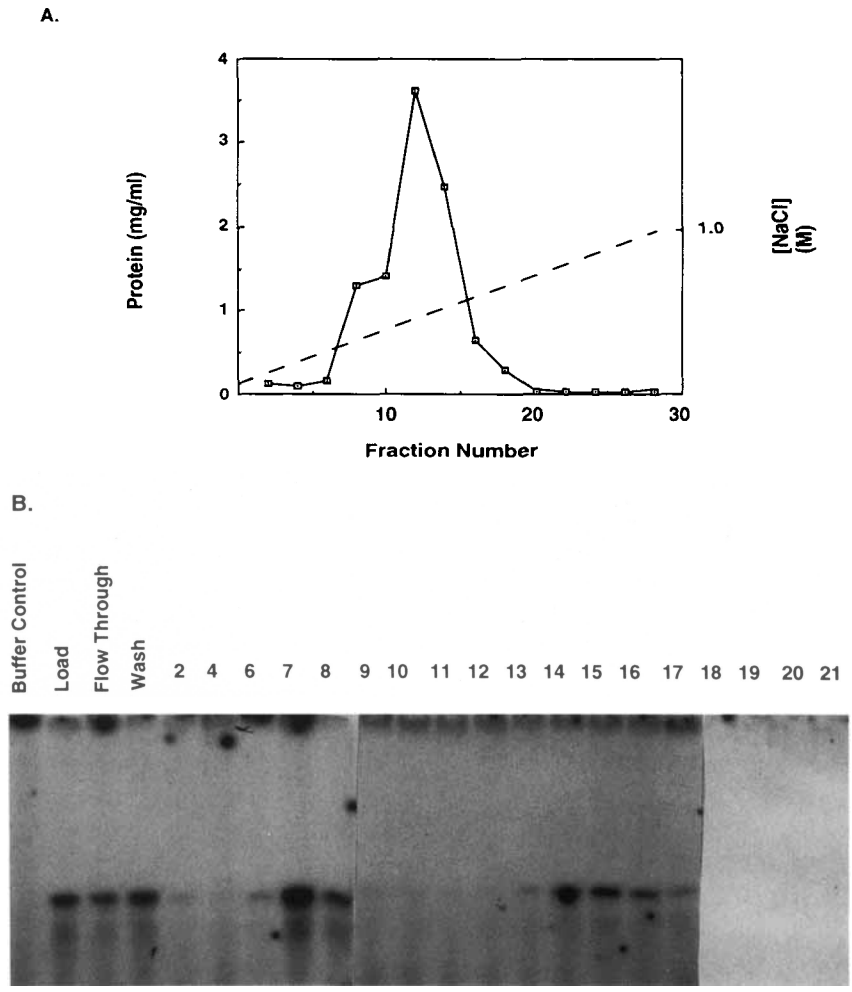

Fig. 2. Q-Sepharose profile, Polymin P supernatant. **A:** Protein profile. Protein from the Polymin P supernatant fraction was batch adsorbed onto phosphocellulose (1L). The phosphocellulose was eluted with buffer A containing 1 M NaCl. A portion of the phosphocellulose eluate (1.4 L) sample was desalted and concentrated to 220 ml using Centriprep 30 units. The NaCl concentration was adjusted to 30 mM, and the sample (363 mg) was loaded onto a 65-ml column of Q-Sepharose pre-equilibrated in buffer A. The column was washed with 2 column volumes (130 ml) of buffer A, then eluted with a 330-ml gradient from 30 mM to 1 M NaCl. Fractions (11 ml) were collected and assayed for protein concentration using a commercial Bradford reagent. The concentrations of protein in the loaded sample was 1.65 mg/ml, in the flowthrough 0.56 mg/ml, and in the wash 0.65 mg/ml. **B:** Assay A profile. An aliquot (1.5 μl) from each fraction was assayed for DNA kinase activity under the standard conditions for assay A. The autoradiograph was exposed for 18 h; a 3-day exposure revealed no further peaks of activity.

likely to be underestimated. The level of activity in Blue–Sepharose purified samples was stable for at least 2 months when stored at 0°C with a protein concentration of at least 100 μg/ml and an NaCl concentration of 0.1–0.5 M. SDS-polyacrylamide gel analysis of the protein preparation (data not shown) revealed that the Blue-Sepharose fraction was heterogeneous, with about 20 polypeptides present upon staining with Coomassie Blue, and there were no obvious candidate polypeptides for the PP-PNK activity.

Further purification (2–3-fold) could be achieved with heparin–agarose, to which PP-PNK bound, Superose 12, or FPLC MonoQ columns, but the activity was significantly less stable after these further steps (data not shown). Therefore, the Blue–Sepharose fraction was utilized for characterization of PP-PNK. Following MonoQ chromatography as described in Materials and Methods, PP-PNK activity was present in fractions 39–41, with the peak in fraction 39. The protein concentration of this further purified sample,

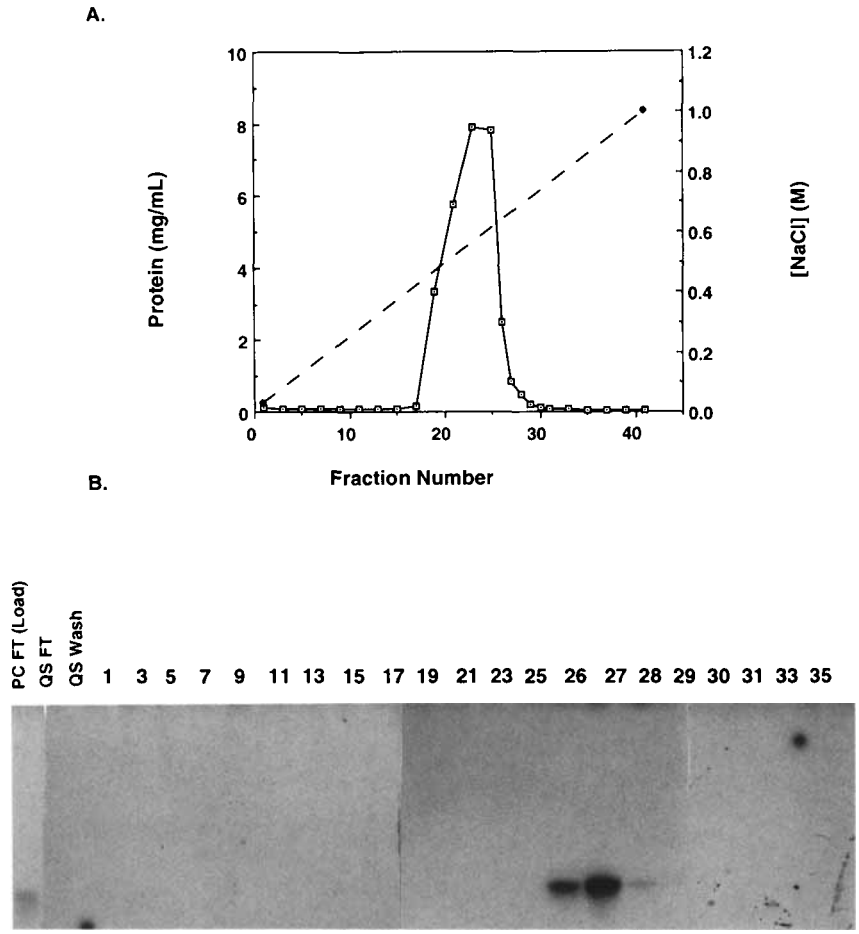

Fig. 3. Q-Sepharose profile, Polymin P pellet. **A:** Protein Profile. Protein (700 mg) was applied to a 130-ml column of Q-Sepharose equilibrated in buffer A. The column was washed with 2 column volumes of buffer A, then eluted with a 600-ml gradient from 30 mM to 1 M NaCl. Fractions (10.8 ml) were collected and assayed for protein concentration using a commercial Bradford reagent. The concentration of protein in the load fraction was 3.6 mg/ml, in the flowthrough fraction 0.09 mg/ml, and in the wash fraction, 0.23 mg/ml. **B:** Assay A profile. An aliquot (1.5 μl) from each fraction was assayed for DNA kinase activity under the standard conditions for assay A. The autoradiograph was exposed for 18 h. A 6-day exposure did not reveal any additional peaks of activity.

which contained about 15 polypeptides from 30 to 200 kDa when analyzed by SDS–PAGE, was 51 ng/μl and the specific activity in assay A was 7 nmol/20 min/mg protein.

Product Analysis

It was important to address the question of whether the 5′ phosphoryl moiety was transferred to the 5′ OH or 3′ OH terminus of the oligodeoxyribonucleotide substrate. The Blue–Sepharose fraction was unable to phosphorylate an oligonucleotide previously 5′ phosphorylated by T4 PNK (Fig. 5), indicating similarity of the reaction products of T4 PNK and PP-PNK. These results are consistent with 5′ phosphorylation of the oligonucleotide substrate. Oligo dT_{25} phosphorylated by the Blue–Sepharose fraction was digested to completion by snake venom phosphodiesterase (data not shown). This digestion pattern indicated that the product of phosphoryl transfer by the PP-PNK preparation was a linear oligonucleotide phosphorylated at the 5′ terminus [Razzell and Khorana, 1959].

pH Profile and Requirements for 5′ Phosphoryl Transfer to dT_{25}

Among the useful parameters for distinguishing an enzymatic activity from published reports is the pH optimum. When the pH was varied (Fig. 6), the PP-PNK preparation displayed activity over a wide pH range, with greatest activity at neutral to alkaline pH (pH 7–9). Neither the hydroxyapatite (data not shown) fraction nor the Blue–Sepharose fraction (Fig.

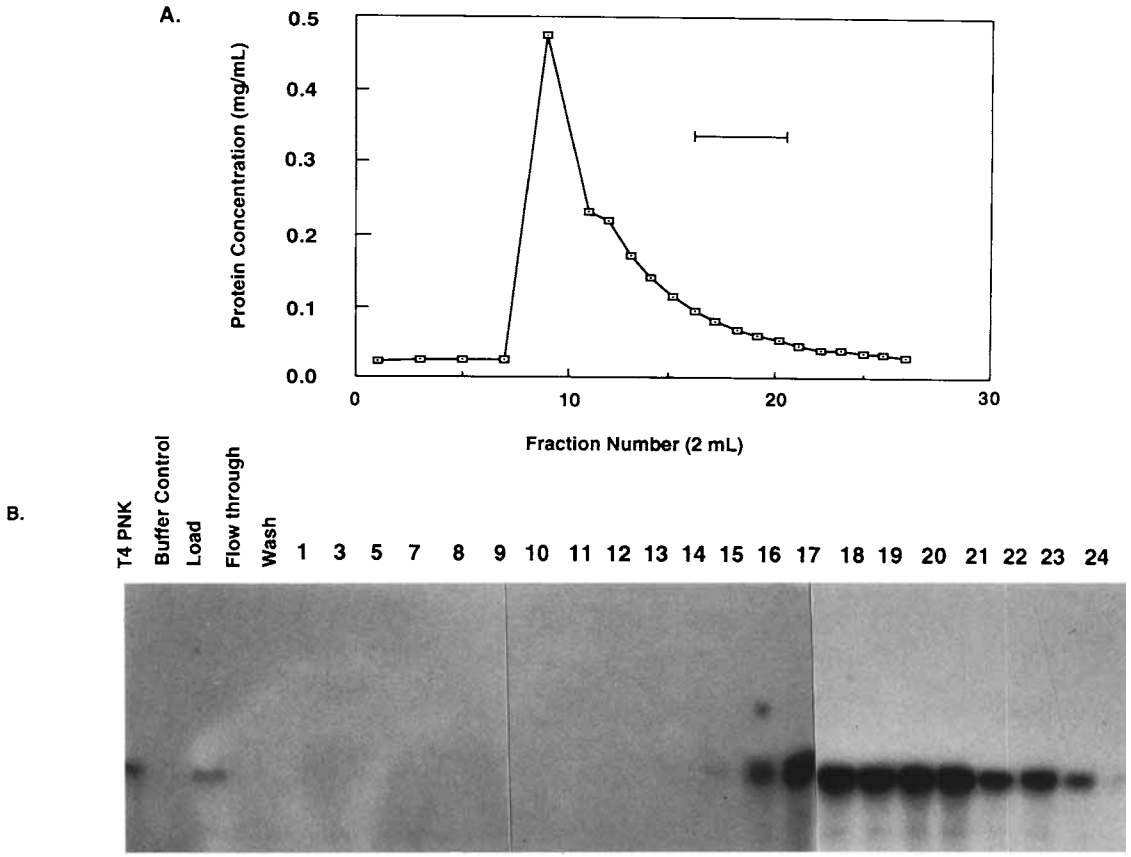

Fig. 4. Blue–Sepharose chromatography of PP-PNK. **A:** Protein profile. In this experiment, a 10-ml Blue–Sepharose column was loaded and washed with 2 column volumes of loading buffer. A 30 mM to 1 M NaCl gradient (50 ml) was applied to elute proteins from the column, and 2 mL fractions were collected. Bar indicates fractions containing highest activity. **B:** Assay A profile. Blue Sepharose activity profile for PP-PNK. An aliquot (1.5 μl) of each fraction was subjected to analysis using assay A at pH 5.5.

6) displayed a peak of activity at acidic pH. Other buffers tested (data not shown) were Tris-maleate (pH 5.2–9.5) and sodium acetate (pH 4.5–6.5); activity levels were similar to those seen for MES and Tris-HCl, and a neutral–alkaline pH optimum was observed. Because of the observed neutral–alkaline pH optimum for phosphorylation of oligo dT_{25}, subsequent experiments were carried out at pH 7.5.

The reaction components required for activity were studied (Table 2), revealing an absolute requirement for ATP and 5′ OH oligo dT_{25}. Omission of DTT resulted in 54% of activity remaining. When the concentration of Mg^{2+} was varied, 1 mM was found to be optimal although activity was also observed in the absence of added Mg^{2+}. This low optimum Mg^{2+} concentration is singular for PP-PNK among reported mammalian polynucleotide kinases. Incubation with increasing concentrations of EDTA resulted in no detectable activity with assay A after the 25 mM point. Treatment with DNase-free RNase A (20 μg) did not affect the amount of product observed in assay A.

Substrate Specificity of the PP-PNK Preparation

In order to compare PP-PNK with previously reported polynucleotide kinases, other substrates were also investigated and included 5′ OH *Eco*RI-cleaved, dephosphorylated pUC19 (Fig. 7), 5′ OH poly(A) (Table 3) and thymidine 3′ monophosphate (not shown). The latter substrate was not efficiently phosphorylated in 50 mM Tris–HCl, pH 7.5, or in 40 mM imidazole-HCl, pH 6.4; no product formed that was detectable when reaction products were analyzed on polyethyleneimine TLC plates. The substrate consisting of a double-stranded DNA molecule with a 5′ OH 4-base protrusion was phosphorylated, although an increased amount of T4 PNK or PP-PNK was required for this reaction (Fig. 7). The enzyme preparation exhibited RNA ki-

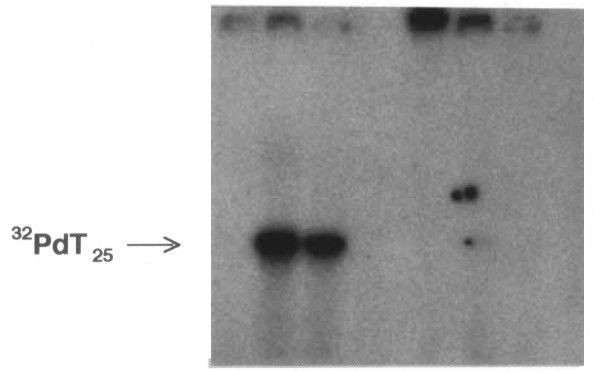

Fig. 5. Similarity of the PP-PNK phosphorylation product to that of T4 PNK. Oligo dT_{25} was phosphorylated using T4-PNK in the presence of unlabelled ATP. A buffer control was carried out in parallel. The phosphorylation products were deproteinized and purified on a Sep-Pak column. The 5′ phosphorylated oligonucleotide was used as a substrate for PP-PNK. Lanes 1–3, 5′ OH oligo dT_{25} control; lanes 4–6, 5′ P oligo dT_{25} phosphorylated with T4 PNK. *Lane 1, buffer control; lane 2, T4-PNK; lane 3,* Blue–Sepharose fraction; lane 4, buffer control; lane 5, T4 PNK; *lane 6,* Blue–Sepharose fraction.

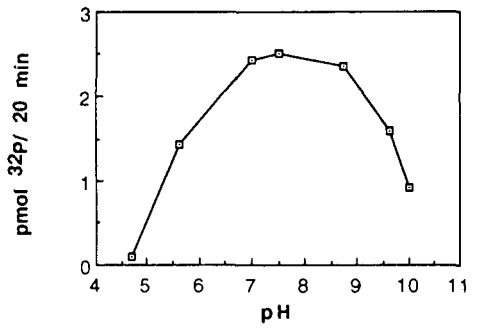

Fig. 6. pH profile of PP-PNK activity. The enzyme activity in the Blue–Sepharose fraction was determined in MES buffer (pH 4.7, 5.5) or Tris-HCl buffer using assay A. The values given for pH are for 100 mM buffer at room temperature, with the volume of 50 mM Tris–HCl pH 7.5 buffer in the added enzyme.

nase activity with 5′ OH poly (A) as a substrate, and the DNA and RNA phosphorylation activities were both present in the peak fraction from an FPLC MonoQ column (Table 3).

Kinetic experiments were carried out at pH 7.5, within the optimal range for the observed DNA kinase activity. When the concentration of ATP was varied, an estimate for apparent K_M of 52 ± 11 μM was calculated (mean ± SD; Fig. 8A). This is higher than the reported values for other mammalian DNA kinases [Zimmerman and Pheiffer, 1981]. An estimate of 8 ± 3 μM (mean ± SD) was obtained for the apparent K_M for oligo dT_{25} (Fig. 8B). This value is not directly

TABLE II. Requirements for 5′ Phosphoryl Transfer to Oligo dT_{25} by PP-PNK*

Reaction	Activity (pmol/20 min) (% of complete reaction)
Complete	100
Omit DTT	54
Omit ATP	N.D.
Omit oligo dT_{25}	N.D.
Omit $MgCl_2$	78
1 mM $MgCl_2$	100
10 mM $MgCl_2$	80
Add EDTA (25 mM)	N.D.
Add DNAse-free RNase A (20 μg)	100

*PP-PNK from the Blue–Sepharose pool was used for these experiments. Assay A was performed as described in Materials and Methods. ND, no activity detected. Reactions were carried out in 1 mM $MgCl_2$ and at pH 7.5, unless otherwise noted. Results are normalized to the standard reaction mixture as 100%.

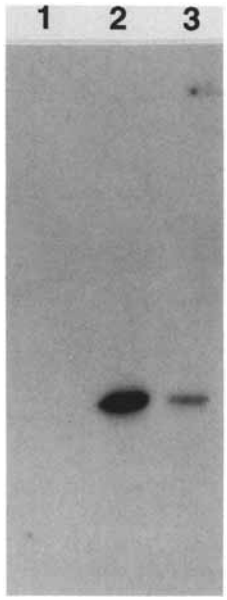

Fig. 7. *Eco*RI cleaved, dephosphorylated pUC19 as a substrate for PP-PNK. DNA kinase assays were done as described in Materials and Methods (assay B). Lane 1, buffer control; lane 2, T4 PNK (10 U); lane 3, 2.8 μg 150 mM HAP fraction.

comparable with previous reports because of the difference in substrate.

The effects of various salts on enzyme activity were determined (Table 4). Sodium pyrophosphate was found to be the most potent inhibitor of enzyme activity under these conditions, consistent with previous studies on DNA kinases from calf thymus [Austin et al., 1978; Tamura et

TABLE III. Phosphorylation of 5′ OH Poly A by the PP-PNK Preparation*

Sample	Net activity (pmol/30 min)–buffer control
5 U T4 PNK	3.6
0.2 μg Blue–Sepharose pool	3.2
0.4 μg Blue–Sepharose pool	6.5
0.1 μg MonoQ fraction 39	2.8

*5′ Phosphorylation of poly A was measured as described in Materials and Methods. These results are representative of three experiment and are the means of duplicates that did not differ by more than 15%. Activity was calculated as net activity after subtraction of the buffer control (0.47 pmol/30 min). The mean of the no RNA controls was 0.44 pmol/30 min.

al., 1981]. In contrast to reports [Zimmerman and Pheiffer, 1981] for the mammalian acid-pH-dependent DNA kinase from rat liver, sulfate anions were not strongly inhibitory. This was reported previously for DNA kinases isolated from calf thymus [Austin et al., 1978; Tamura et al., 1981].

Hydrodynamic Properties of Native PP-PNK

In sedimentation experiments, PP-PNK activity migrated through linear sucrose gradients slightly faster than ovalbumin and slightly slower than bovine serum albumin, with an estimated sedimentation coefficient of 3.8 ± 0.3 S compared to the standards (Fig. 9). The sedimentation behavior of T4 PNK was distinctly different under these conditions, with more than 90% of the activity giving an estimated sedimentation coefficient of 7.0 S (Fig. 9). Blue–Sepharose fraction PP-PNK was applied to a Superose 12 column to estimate Stokes radius as compared to globular protein standards. A K_{av} value of 0.27 (data not shown) was determined in these experiments, performed in buffer containing 0.3 or 0.1 M NaCl. The Stokes radius as determined by this technique was 45 Å. When the sedimentation and Stokes radius data were combined as described by Siegal and Monty [1966], an estimate of 72 kDa for the molecular mass of PP-PNK was obtained.

Assays for 3′ Phosphatase, Cyclic 2′,3′ Phosphodiesterase, and Adenylated Polypeptides

The DNA kinase from rat liver has been reported to exhibit a 3′ phosphatase activity, in common with T4 PNK. Therefore, the 3′ phosphatase activity of the PP-PNK preparation was of interest. Assays for 3′ phosphatase activity revealed no detectable activity in the PP-PNK Blue–Sepharose fraction (<0.02 pmol 3′ phosphate released/18 mU/30 min). Yeast tRNA ligase contains a 2′,3′ cyclic phosphodiesterase

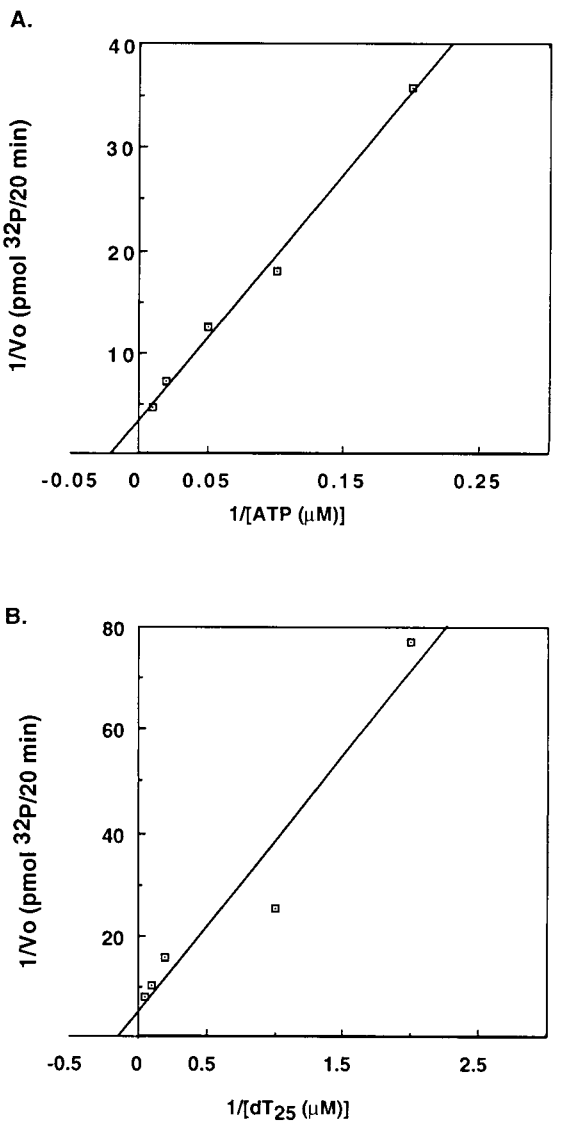

Fig. 8. Determinations of K_M. **A:** Effect of ATP concentration on PP-PNK activity. Assays employing Blue–Sepharose pooled fractions were carried out as described (assay A), except that 50 mM Tris–HCl, pH 7.5 was used and the concentration of ATP was varied from 0 to 100 μM. The K_M for ATP calculated from the double reciprocal plot shown here was 50 μM. The mean of three determinations was 52 ± 11 μM. **B:** Effect of oligo dT_{25} concentration on PP-PNK activity. Assays were carried out as described for assay A, except that the oligo dT_{25} concentration was varied from 0 to 40 μM. The K_M calculated from this plot was 6.8 μM. The mean of three determinations was 8.0 ± 3.7 μM.

activity, as does myelin CNPase, reported to phosphorylate tRNA [Sprinkle et al., 1987], prompting us to investigate this activity in the PP-PNK preparation, since thymocytes have been reported to contain CNPase activity [Sprinkle, 1989]. The Blue–Sepharose fraction did not contain 2′,3′ cyclic phosphodiesterase activity above background levels (E. Gao and P. Braun, personal communication); in immunoblotting experiments with an antiserum directed against bovine myelin CNPase [Braun et al., 1988], no cross-reacting polypeptide was observed (E. Gao and P. Braun, personal communication). A preparation of rat myelin CNPase, isoform 1, expressed as a GST fusion protein in *E. coli*, active in a 2′,3′ cyclic phosphodiesterase assay (E. Gao and P. Braun, personal communication), showed no detectable activity in Assay A for DNA kinase activity carried out at pH 7.5.

To investigate the occurrence of RNA (and DNA) ligases in the PP-PNK preparation, the hydroxyapatite and Blue–Sepharose fractions and a fraction further purified on an FPLC Mono Q column were assessed for polypeptides capable of forming an adenylated intermediate [Lasko et al., 1990a]. Adenylated polypeptides were detected in the hydroxyapatite and Blue–Sepharose fractions (Fig. 10, lanes 3 and 4). The estimated size of these polypeptides compared to standards were 120, 100, and 85 kDa. The MonoQ peak fraction, containing polynucleotide kinase activity with RNA (Table 3) and on oligo dT_{25} as substrates, did not exhibit detectable levels of adenylated polypeptides (Fig. 10, lane 2).

TABLE IV. Inhibition by Inorganic Salts*

Inhibitor	Concn for 50% inhibition in assay A (mM)
Sodium pyrophosphate	2.2
Sodium phosphate	11
Ammonium sulfate	8.5
Sodium sulfate	5.7
Sodium chloride	67
Potassium chloride	71

*Inhibition experiments were carried out as described in Materials and Methods, and the concentrations for 50% inhibition were obtained by interpolating the individual plots.

Other Contaminating Enzymatic Activities in the Blue–Sepharose Purified PP-PNK

Endogenous protein kinase activity in the Blue–Sepharose-purified preparation was assessed by examination of reaction products with and without DNA on an 10% SDS–polyacrylamide gel with the result that no labelled poly-

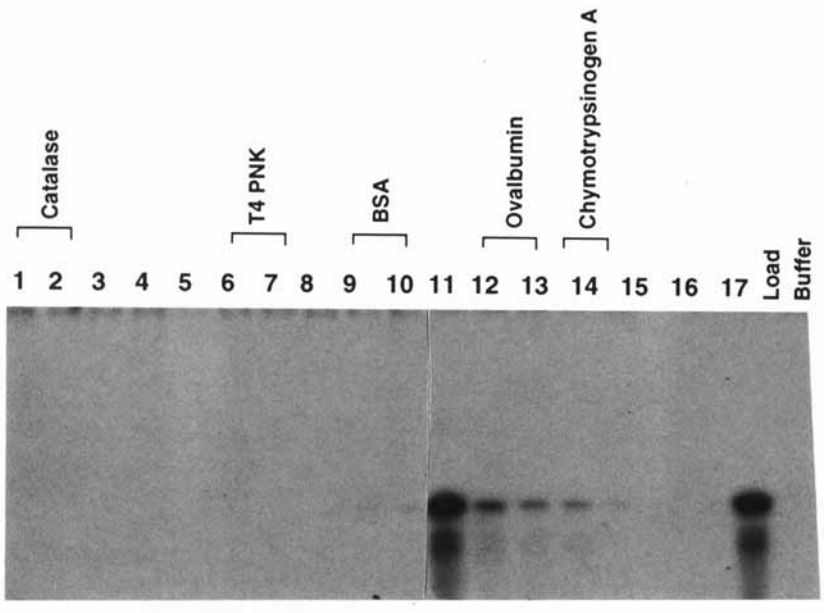

Fig. 9. Sucrose density gradient sedimentation analysis. The sedimentation coefficient of PP-PNK was determined by centrifugation in 5–20% sucrose gradients containing 0.1 M NaCl. By comparison with standards of known sedimentation coefficient, PP-PNK was found to have an S value of 3.8 ± 0.3 (mean ± SD of four determinations).

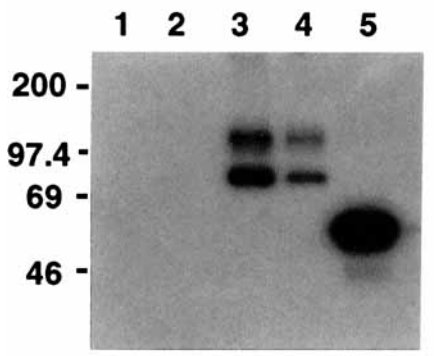

Fig. 10. Adenylated polypeptides present in samples of the PP-PNK preparation. Adenylated polypeptides were ^{32}P labeled as described in Materials and Methods and electrophoresed through a 7.5% SDS-polyacrylamide gel. Lane 1, buffer control; lane 2, 100 ng Mono Q fraction 39; lane 3, 500 ng Blue–Sepharose pool; lane 4, 500 ng μl hydroxyapatite pool; lane 5, T4 DNA ligase-positive control (1 U). There was twice as much activity in assay A in the sample in lane 4 compared to the sample in lane 2.

peptides were detected following a 24-h exposure of the autoradiogram. Assays for endonuclease and alkaline and acid phosphatase activities revealed no significant activity.

DISCUSSION

Whole cell extracts of calf thymus were found to contain several chromatographically distinct activities that transferred a 5′ phosphoryl group to synthetic oligo dT_{25}. The Polymin P supernatant fraction contained three separable activities that respectively eluted in the flowthrough and wash (SNQ I), eluted early in a 30 mM to 1 M NaCl gradient (SNQII) and eluted late in a 30 mM to 1 M NaCl gradient (SNQIII) when subjected to chromatography on Q-Sepharose, a strong anion exchanger. It is not known whether these activities represent chromatographically distinct isoforms of the same enzyme or are related in any other way. The biochemical properties of the SNQI, SNQII, and SNQIII fractions are under investigation.

The Polymin P pellet fraction contained a 5′ phosphorylation activity that could be eluted with 0.6 M NaCl. When subjected to Q-Sepharose chromatography, this fraction revealed one peak of activity that eluted after the protein peak (PP-PNK). PP-PNK was the major activity found in the Polymin P pellet fraction. The possible relationship with SNQIII (Fig. 2) in the Polymin P supernatant fraction, which eluted slightly earlier on Q-Sepharose, is under investigation. The PP-PNK activity was further purified and found to be capable of 5′ phosphorylation of oligo dT_{25} and poly(A). Therefore, this preparation contains polynucleotide kinase activity. However, we have not rigorously demonstrated that the phosphorylation of DNA and RNA that were observed were due to a polynucleotide kinase activity that is contained within one polypeptide, although both substrates were phosphorylated by protein from the peak fraction after further purification on a MonoQ column.

The hydrodynamic properties of the PP-PNK activity were investigated using the Blue–Sepharose fraction. The sedimentation behavior indicated a sedimentation coefficient of about 3.8 S compared to standards and the gel filtration data indicated a Stokes radius of 45 Å. Taken together, an estimate of molecular mass of 72 kDa was obtained using the method of Siegal and Monty [1966]. In both of these techniques, the same globular protein standards were used; however, there is an interesting disparity between the gel filtration results, which correlate with a 120- to 130-kDa protein and the sedimentation analysis, indicating a protein of about 50 kDa. Among the possible explanations are the following: (1) a polypeptide that forms close associations with other polypeptides under some conditions; (2) a monomer polypeptide displaying anomalously slow sedimentation, and (3) a monomer protein with an elongated shape resulting in anomalously early elution from the Superose 12 column (similar to observations for DNA ligase I [Tomkinson et al., 1990]). Another possibility is proteolysis during the sucrose gradient analysis, although we found no evidence for this in our SDS–PAGE of peak fractions from the sucrose gradient, which revealed about 15 polypeptides from 35 to 70 kDa (data not shown).

This search for functional homologues of T4 polynucleotide kinase in calf thymus glands has revealed an enzyme preparation that in common with T4 PNK phosphorylates oligonucleotides, 5′ dephosphorylated double-stranded DNA, and RNA, with a neutral/alkaline pH optimum. In contrast to T4 PNK, the PP-PNK preparations lacked detectable 3′ phosphatase activity and were unable to 5′ phosphorylate thymidine 3′ monophosphate. Thus, the bacteriophage T4 and T2 enzymes remain the sole known polynucleotide kinases able to phosphorylate 3′ mononucleotides [Richardson, 1981], rendering them

useful in postlabeling analysis for damaged DNA [Randerath et al., 1981].

To our knowledge, this is the first report of multiple 5′ phosphorylation factors in calf thymus. However, we anticipated detecting more than one 5′ phosphorylation activity in this tissue, since a number of laboratories have purified a DNA kinase activity from this and other mammalian sources, while a 5′ hydroxylkinase activity was fractionated away from DNA kinase activity in HeLa cells [Shuman and Hurwitz, 1979]. Given the apparent conservation [Zillman et al., 1991] from fungi to mammalian cells of the pathway in which yeast tRNA ligase participates, a mammalian homologue of this RNA ligase with its associated polynucleotide kinase would likely be detected in a study of 5′ phosphoryl transfer to nucleic acids. Consequently, it is important to examine the possible relationship between PP-PNK and each of other enzymes reported in the literature or postulated by us to be present in mammalian cells.

There are three independent criteria that differentiate PP-PNK from the previously reported DNA kinases from mammalian sources: its pH profile, its efficient phosphorylation of an RNA substrate, and its clearly different chromatographic properties. Compared to a highly purified preparation of DNA kinase from calf thymus nuclei [Tamura et al., 1981], PP-PNK had contrasting chromatographic properties: (1) PP-PNK did not bind to phosphocellulose and the DNA kinase bound, (2) PP-PNK bound to hydroxyapatite, in contrast to the DNA kinase, and (3) PP-PNK eluted later in the salt gradient when applied to a Blue–Sepharose column than the DNA kinase. There was no detectable 3′ phosphatase activity in the PP-PNK preparation, an additional distinction, albeit a negative one, from the DNA kinase from rat liver nuclei (the 3′ phosphatase activity of the DNA kinase from calf thymus with an acid pH optimum has not been reported).

Determination of the effect of varying the pH is a useful basis for comparison of an enzyme preparation with published reports. Despite its isolation on the basis of ability to 5′ phosphorylate an oligonucleotide substrate at pH 5.5, PP-PNK was shown to have a neutral to alkaline pH optimum in several buffer systems. The difference in pH profile between PP-PNK and the DNA kinases with an acidic pH optimum is not solely due to the use of an oligonucleotide substrate; Bosdal and Lillehaug [1985] investigated this point and found that the enzyme they purified had an acidic pH optimum for oligonucleotide as well as randomly nicked DNA substrates. Furthermore, we have identified another DNA kinase activity in the Polymin P supernatant, designated SNQI in our study, that (1) binds to phosphocellulose, in contrast to PP-PNK, which does not bind; (2) does not bind to Q-Sepharose, in contrast to PP-PNK, which binds strongly (Fig. 2); and (3) has an acidic pH optimum for 5′ phosphorylation of oligo dT_{25} (C. Slack and D. Lasko, unpublished results). Taken together, our results indicate that PP-PNK does not correlate with the DNA kinases with an acidic pH optimum that have been previously found in mammalian cells.

In a prior investigation [Shuman and Hurwitz, 1979], polynucleotide kinase activities from HeLa nuclei were fractionated by ammonium sulfate precipitation. The bulk of the activity with a 5′ hydroxyl RNA substrate, assayed at pH 8.4, was found in the 0–35% ammonium sulfate fraction and the majority of activity on 5′ hydroxyl DNA, assayed at pH 5.5, was found in the 35–75% fraction. The 5′-hydroxylpolyribonucleotide kinase activity was further purified; two peaks of activity were found after phosphocellulose chromatography and designated PCI and PCII. The PCII peak of the this activity bound to phosphocellulose and thus is distinct from PP-PNK in this respect. Also arguing against a correlation of PP-PNK with PCII RNA kinase is the high K_M for ATP of 500 μM exhibited by the PCII fraction and the sedimentation coefficient of 5.6 S compared to 3.8 S for PP-PNK. PCII lacked detectable 3′ phosphatase activity, as did PP-PNK. About 10% of the HeLa cell RNA kinase activity, the PCI fraction, eluted very early on a phosphocellulose gradient and thus displayed similar behavior on this resin to PP-PNK, which does not bind to phosphocellulose. The PCI fraction was not further described, and kinetic and substrate specificity experiments are not available for comparison. The estimated sedimentation coefficient for PCI (4.4 S) differs somewhat from our estimate for PP-PNK (3.8 S). Because of the lack of information about the PCI fraction, it is difficult to definitively rule out some relationship between it and PP-PNK.

Characterization of RNA ligase from a mammalian source has not been reported; thus, a detailed experimental comparison to PP-PNK is not possible. Yeast tRNA ligase also has poly-

nucleotide kinase and 3′ phosphatase activities which can be isolated in separate domains [Xu et al., 1990]. Thus, in principle, a polypeptide lacking 3′ phosphatase activity could have been derived from proteolysis of a bovine homologue of yeast tRNA ligase. A preparation of wheat germ RNA ligase purified 6,000-fold from the Polymin P supernatant of a crude extract had polynucleotide kinase activity with phosphorylation of DNA to a lesser extent than RNA, but no detectable 3′ phosphatase activity [Pick and Hurwitz, 1986]. Consequently, 3′ phosphatase activity is not necessarily a hallmark of RNA ligase in all species. However, to our knowledge, no fragment of yeast tRNA ligase or wheat germ RNA ligase has been reported containing polynucleotide kinase activity that does not form an enzyme-adenylate intermediate, and our experiments revealed no detectable adenylated polypeptides in MonoQ purified fractions of PP-PNK that are capable of phosphorylation of poly(A). Immunoblotting studies revealed that the DNA ligase I activity in EBV-transformed human cell lines was partially precipitated with Polymin P [Lasko et al., 1990b]. The 120-, 100-, and 85-kDa adenylated polypeptides seen in the hydroxyapatite and Blue–Sepharose fractions are consistent with the presence of small amounts of DNA ligase I, DNA ligase III and a proteolytic fragment of DNA ligase I, respectively [Tomkinson et al., 1990, 1992]; these enzymes would have been purified away from PP-PNK on the MonoQ column. In hamster cell lines, Caldecott et al. [1994] noted a 100-kDa adenylated polypeptide that was ascribed to DNA ligase III, as well as another 100 kDa polypeptide that was found not to be a DNA ligase, but not conclusively identified. We did not observe a polypeptide that could be ascribed to RNA cyclase [Reinberg and Hurwitz, 1985]. We concluded that based on the properties reported thus far for eukaryotic nonmammalian RNA ligase enzymes, a relationship with PP-PNK is unlikely.

The characteristics of PP-PNK are singular with respect to previously described enzymatic activities from calf thymus. The PP-PNK preparation has a combination of pH optimum, chromatographic, and kinetic properties unique for a mammalian polynucleotide kinase. The data reported here suggest that the DNA kinase with an acidic pH optimum remains in the Polymin P supernatant and is efficiently removed from the PP-PNK preparation in the first purification step. Given its broad substrate specificity, the physiological role of PP-PNK may occur in DNA metabolism or in RNA metabolism, or both. PP-PNK does not seem to be related to myelin CNPase. Further investigation aimed at development of molecular probes directed against this enzyme will reveal more about the cellular function. The dissimilarities between PP-PNK and previously reported mammalian DNA kinases and polynucleotide kinases, as well as our preliminary results on fractionation of polynucleotide kinase activities in calf thymus, indicate the presence of multiple factors in mammalian cells capable of 5′ phosphorylation of nucleic acids. Final determination of the character of PP-PNK and its distinction from similar types of enzymes awaits the availability of antibodies and cDNAs for the mammalian polynucleotide kinases.

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