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Purification of a Polynucleotide Kinase From Calf Thymus, Comparison of Its 3′-Phosphatase Domain With T4 Polynucleotide Kinase, and Investigation of Its Effect on DNA Replication In Vitro

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Abstract Mammalian polynucleotide kinases (PNKs) carry out 5′-phosphorylation of nucleic acids. Although the cellular function(s) of these enzymes remain to be delineated, important suggestions have included a role in DNA repair and, more recently, in DNA replication. Like T4 PNK, some preparations of mammalian PNKs have been reported to have an associated 3′-phosphatase activity. Previously, we have identified in calf thymus glands an apparently novel PNK with a neutral to alkaline pH optimum that lacked 3′-phosphatase activity. In this report, we describe purification of another bovine PNK, SNQI-PNK, with a slightly acidic pH optimum that copurifies with a 3′-phosphatase activity. The enzyme appears to be a monomer of 60 kDa. Mammalian DNA replication reactions were supplemented with T4 PNK or SNQI-PNK, and no significant effect on DNA replication in vitro was observed. Database searches support the earlier mapping of the 3′-phosphatase activity of T4 PNK to the C-terminus and suggest that the 3′-phosphatase domain of T4 PNK is related to the protein superfamily of L-2-haloacid dehalogenases. Exopeptidase digestion experiments were carried out to compare the SNQI-PNK enzyme with T4 PNK and led to the inference that the domain organization of the bovine polypeptide may differ from that of the T4 enzyme. J. Cell. Biochem. 73:188–203, 1999.

Key words: mammalian polynucleotide kinase; T4 polynucleotide kinase; DNA phosphorylation; 3′-phosphatase; L-2 haloacid dehalogenase fold; DNA replication; bovine thymus

Damage to cellular DNA occurs after exposure to a variety of chemical and physical agents. Repair of damaged DNA is a vital task for cells. Some single-strand breaks of DNA from irradiated thymocytes contain 5′-hydroxyl groups [Coquerelle et al., 1973; Lennartz et al., 1975], which are not substrates for DNA ligases. After treatment of cells with ionizing radiation, 3′- phosphate groups and 3′-phosphoglycolate groups, which cannot support DNA repair synthesis, have been observed [reviewed in Friedberg et al., 1995]. Another example of the occurrence of 5′-OH DNA termini is in DNA fragments formed after transient cerebral ischemia [MacManus et al., 1997]. Polynucleotide kinases (PNKs) have been isolated from mammalian sources [reviewed in Zimmerman and Pheiffer, 1981; Tamura et al., 1981; Pheiffer and Zimmerman, 1982; Habraken and Verly, 1983; Habraken and Verly, 1986; Habraken and

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Verly, 1988; Prinos et al., 1995; Karimi-Busheri and Weinfeld, 1997]; PNKs with associated 3′-phosphatase activity [Pheiffer and Zimmerman, 1982; Habraken and Verly, 1983] have been proposed to repair 5′-OH and 3′-P termini to allow subsequent DNA repair synthesis and ligation. Two studies have addressed the function of DNA repair by purified PNKs, using synthetic substrates [Habraken and Verly, 1983; Karimi-Busheri et al., 1998]. The identity of the predominant mammalian DNA-specific 3′-phosphatase in vivo is uncertain [Demple and Harrison, 1994].

Potential physiological roles for mammalian PNKs extend beyond DNA repair. Recently it has been suggested that a mammalian PNK is involved in the phosphorylation of 5′-termini of Okazaki fragments during DNA replication [Pohjanpelto and Hölttä, 1996]. Replication factor C was found to require 5′-P groups in order to bind to the ends of telomeres [Uchiumi et al., 1996]. The *Saccharomyces cerevisiae RLG1* gene, encoding a protein with multiple enzymatic activities including a PNK activity, has been implicated in RNA processing, both in tRNA splicing [reviewed by Phizicky and Greer, 1993] and in splicing of the *Hac1* mRNA during the unfolded protein response [Sidrauski et al., 1996; Sidrauski and Walter, 1997]. The former pathway appears to have been conserved in mammalian cells as a minor contributor to tRNA processing [Phizicky and Greer, 1993].

PNK enzymes were first discovered in *Escherichia coli* infected with bacteriophage T4 and T2 [Richardson, 1965; Novogrodsky and Hurwitz, 1966] and have been found in many eukaryotes [Zimmerman and Pheiffer, 1981, Kornberg and Baker, 1992]. Substantial information is available on the biochemical properties of T4 polynucleotide kinase. The enzyme is a homotetramer of 33 kDa subunits, and important features of the 5′-phosphorylation reaction include a neutral to alkaline pH optimum, use of any nucleotide triphosphate or deoxynucleotide triphosphate as a phosphoryl donor, and broad substrate specificity, including nucleotide 3′-monophosphates [Richardson, 1981]. Additionally, the T4 enzyme has a 3′-phosphatase activity [Cameron and Uhlenbeck, 1977]. The gene encoding T4 PNK, *pseT*, has been cloned [Midgley and Murray, 1985], and the biochemical properties of several *pseT* mutants have been studied [Soltis and Uhlenbeck, 1982a]. On the basis of digestion with trypsin and with exopeptidases, the 5′-phosphorylation activity has been mapped to the N-terminal and the 3′-phosphatase activity to the C-terminal region of the polypeptide [Soltis and Uhlenbeck, 1982b]. There are reports that T4 PNK may play a role in DNA repair and replication [Depew and Cozzarelli, 1974] and in T4 true-late gene expression [Sirotkin et al., 1978]. More recently, a function in reprocessing of host lysine tRNA in conjunction with T4 RNA ligase has been proposed and well documented [Amitsur et al., 1987, 1989].

Among mammals, PNK activity was reported from rat tissues, including liver [reviewed in Zimmerman and Pheiffer, 1981; Pheiffer and Zimmerman, 1982; Habraken and Verly, 1983; Habraken and Verly, 1986, 1988; Karimi-Busheri and Weinfeld, 1997], testis [Bosdal and Lillehaug, 1985], and calf thymus [Austin et al., 1978; Tamura et al., 1981; Prinos et al., 1995; Karimi-Busheri and Weinfeld, 1997]. An RNA kinase from mouse L cells has been reported [Winocov and Button, 1982; Sertic-Pristos et al., 1984]. Shuman and Hurwitz [1979] have isolated a PNK from HeLa cells that phosphorylated 5′-OH polyribonucleotides. In addition, their preparations showed 5′-DNA kinase activity, although it was far less efficient than the 5′-RNA kinase activity. In all these studies, useful properties for distinguishing partially purified PNKs have been pH optimum, substrate specificity, and presence of 3′-phosphatase activity. PNK activity has also been detected in other eukaryotes, signifying a wide distribution and implying an important physiological role or roles [Zimmerman and Pheiffer, 1984].

Attention has been focused chiefly on acid pH optimum PNK activity from rat liver and calf thymus. This type of PNK has been highly purified from rat liver [Habraken and Verly, 1983; Habraken and Verly, 1986, 1988; Karimi-Busheri and Weinfeld, 1997], calf thymus [Tamura et al., 1981], and fetal bovine thymus [Karimi-Busheri and Weinfeld, 1997]. In these studies, substrate specificity has been found to be restricted to DNA of at least four nucleotides, in contrast to the broad substrate specificity of T4 PNK. For rat liver PNK and fetal bovine thymus PNK preparations, an associated 3′-phosphatase activity has been observed [Pheiffer and Zimmerman, 1982; Habraken and

Verly, 1983; Karimi-Busheri and Weinfeld, 1997].

With the ultimate goal of elucidating the physiological function(s) of the mammalian PNKs, we have earlier purified from calf thymus a neutral-alkaline pH optimum enzyme, Polymin P-precipitable polynucleotide kinase (PP-PNK) [Prinos et al., 1995]. In this report, we confirm the presence in the Polymin P supernatant of another three distinct peaks of polynucleotide kinase activity on the basis of their different chromatographic behaviour and describe the purification of one of these peaks of activity, SNQI-PNK, to near-homogeneity. The results of these experiments indicate that the enzyme is a 60-kDa monomer with apparent specificity for DNA substrates and an associated 3′-phosphatase activity, even in the most highly purified fractions. We examined the possible effect of addition of excess PNK on DNA replication in a cell-free mammalian in vitro replication system. Furthermore, we investigated the effect of exopeptidase digestion of SNQI-PNK compared with T4 PNK and infer that the enzymes may differ in the organization of the active sites.

MATERIALS AND METHODS

Materials

Synthetic oligonucleotides were purchased from Bio-Can (Mississauga, Ontario) or Sheldon Biotechnology (McGill University, Montreal, Quebec). T4 polynucleotide kinase (PNK) was purchased from New England BioLabs (Beverly, MA) or Boehringer Mannheim (Laval, Quebec). Nuclease-free bovine serum albumin (BSA) was obtained from New England BioLabs. Carboxypeptidase Y, double-stranded DNA cellulose, phosphocellulose, Polymin P, protease inhibitors (aprotinin, phenylmethylsulfonylfluoride [PMSF], chymostatin, leupeptin, and N-α-p-tosyl-L-lysine chloromethyl ketone [TLCK]), and thymidine 5′-monophosphate were purchased from Sigma Chemical Co. (St. Louis, MO). Ammonium sulfate was from Gibco-BRL (Mississauga, ON). Blue Sepharose CL 6B, Q Sepharose Fast Flow, SP Sepharose Fast Flow, a Superose 12 HR 10/30 column, a MonoS HR 5/5 column, 5′-OH $rA_{12–18}$, 5′-OH $dA_{12–18}$, and 5′-OH $dT_{12–18}$ were obtained from Pharmacia Biotech (Baie D'Urfe, Quebec). Adenosine 5′ triphosphate, dithiothreitol (DTT), herring sperm DNA, imidazole, 3′-phosphatase-free T4 PNK and pyruvate kinase were purchased from Boehringer Mannheim (Laval, Quebec). [γ-^{32}P] ATP (3,000 Ci/mmol), ^{14}C-labeled protein markers and [^{32}P] orthophosphate were purchased from Amersham (Arlington Heights, IL). Polyethyleneimine (PEI) thin-layer chromatography (TLC) plates were from J.T. Baker or Sigma. Strataclean resin was from Stratagene (LaJolla, CA). Calf thymus tissue was obtained from Aliments Danac (LaPlaine, Quebec).

Purification of a PNK Activity from the Polymin P Supernatant

All the purification steps were performed at 4°C. Fresh thymus glands (1 kg) from 6-month-old calves were homogenized within 2 h in Buffer A (50 mM Tris-HCl pH 7.5, 300 mM NaCl, 0.5 mM EDTA, and 0.5 mM DTT) supplemented with protease inhibitors, namely aprotinin (19 µg/ml), PMSF (0.5 mM), chymostatin (5 µg/ml), leupeptin (5 µg/ml), and TLCK (5 µg/ml). The extract was centrifuged and subjected to Polymin P precipitation as described [Prinos et al., 1995]. The NaCl concentration of the polymin P supernatant was lowered to 150 mM by the addition of an equal volume of Buffer A without NaCl (containing 0.5 mM PMSF) and it was batch adsorbed with 1.4 L of pre-equilibrated phosphocellulose and stirred overnight. After washing the phosphocellulose thrice (3 L each time) with Buffer B (50 mM Tris-HCl, pH 7.5, 30 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT and 0.5 mM PMSF), the resin was packed in a large preparative column (4.8 × 120 cm), and the bound proteins were eluted with two column volumes of Buffer B containing 0.6 M NaCl. Ammonium sulfate was added to 75% saturation, and the slurry was centrifuged at 8,000*g* at 4°C for 20 min. The protein precipitate was dialyzed versus four changes of Buffer B before loading onto a pre-equilibrated Q Sepharose Fast flow (Pharmacia) column (60 ml, 2.4 mg protein/ml). The resin was washed with three column volumes of Buffer B and then eluted with a 300-ml linear gradient (0.03–1 M NaCl) in Buffer B. Fractions (6 ml) were collected, and 1-µl aliquots were assayed for DNA kinase activity using a 5′-OH oligo dT_{25} substrate to identify SNQII-PNK and SNQIII-PNK. The wash fraction, containing SNQI-PNK activity was concentrated by ammonium sulfate precipitation. The SNQI-PNK preparation was dialyzed versus four changes of Buffer C (50 mM Tris-HCl, pH 7.5, 30 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF and 10% glycerol)

and loaded onto a pre-equilibrated SP-Sepharose Fast Flow column (66 ml, 2.4 mg/ml) and eluted with a 330-ml linear gradient of 0.03–1 M NaCl in Buffer C. Fractions (6 ml) were collected and assayed as described. The fractions with highest specific activity were pooled and dialysed versus four changes of Buffer D (10 mM K_2HPO_4, pH 8.0, 100 mM KCl, 0.5 mM DTT, 0.5 mM PMSF), loaded onto a pre-equilibrated Blue Sepharose CL 6B column (3 ml , 1.3 mg/ml) and eluted with 15-ml linear gradient (0.1–1 M KCl) in Buffer D. Fractions (0.4 ml) were collected and assayed as described. The fractions with highest specific activity were pooled and dialysed vs four changes of Buffer C, loaded onto a pre-equilibrated double-stranded DNA-cellulose column (3 ml, 0.1 mg/ml) and eluted with 15-ml linear gradient (0.03–1 M NaCl) in Buffer C. Fractions (0.33 ml) were collected and assayed as described. The fractions with highest specific activity were pooled and dialysed versus three changes of Buffer E (Buffer C with 0.15 M NaCl), loaded at a flow rate of 0.25 ml /min onto a pre-equilibrated FPLC Superose 12 (HR 10/30) column connected to a Waters 600/650E HPLC system. Fractions (0.125 ml) were collected and assayed. To calibrate the column, the following standards obtained from Pharmacia were used: aldolase (158 kDa, 4.81 nm), BSA (67 kDa, 3.55 nm), ovalbumin (43 kDa, 3.05 nm) and ribonuclease A (13.7 kDa, 1.64 nm).

In some experiments, the Superose 12 step was performed before the DNA cellulose step (Preparation B), resulting in purified protein containing a 60-kDa polypeptide that correlated well with activity, as well as two smaller polypeptides of about 55 and 40 kDa. Preparation B material was further purified for use in the DNA replication experiments (see below).

Assays for Enzymatic Activity

The oligonucleotide-based assay for 5′-phosphorylation activity was done as reported earlier [Prinos et al., 1995]. In brief, enzyme preparation (1 µl) was added to a 10-µl reaction mixture containing 100 mM Mes, pH 5.5, 10 µM oligo dT_{25}, 0.4 µCi [γ-^{32}P] ATP (3,000 µCi/mmol, Amersham), 20 µM ATP, 1 mg nuclease free BSA, 10 mM $MgCl_2$, and 2 mM DTT. The reaction was incubated for 20 min at 37°C, followed by the addition of EDTA to 25 mM and treatment with Strataclean resin as described by the manufacturer. DNA sequencing stop buffer was added, followed by electrophoresis on 20% acrylamide/7 M urea gel. Enzyme activity (1 U) is defined as 1 nmol of [^{32}P] incorporated in 20 min.

To assay for possible contaminating enzymatic activities, alkaline phosphatase and acid phosphatase were determined using kits purchased from Sigma. Endonuclease activity was assayed by incubating 1 µg of supercoiled plasmid DNA under assay A conditions for 4 h, followed by Strataclean treatment of the DNA and analysis on an 0.6% agarose gel stained with 0.5 µg/ml ethidium bromide.

The 3′-phosphatase assay was carried out as described [Cameron and Uhlenbeck, 1977]. Thymidine 3′ monophosphate (Tp 3′) and 3′-phosphatase-free T4 PNK were used to prepare 5′[^{32}P]pTp. In a reaction volume of 25 µl, 4 µM of 5′[^{32}P]pTp3′ was incubated with 80 mM imidazole-HCl, pH 6.4, 10 mM $MgCl_2$, 2 mM DTT for 20 min at 37°C. Reaction products (0.5 µl) were spotted on a 20-cm × 20 cm polyethyleneimine TLC plates and developed as described [Cameron and Uhlenbeck, 1977; Shuman and Hurwitz, 1979] with slight modification, dried and exposed for autoradiography at −70°C. The amount of radioactivity for 5′[^{32}P]pT and 5′[^{32}P]pTp were determined by liquid scintillation counting. Standards used were thymidine 3′ monophosphate, thymidine 5′ monophosphate, [γ-^{32}P]ATP, and [^{32}P]orthophosphate. One unit of 3′-phosphatase activity is defined as 1 nmol of 3′ phosphate removed in 20 min.

Renaturation Gel

Herring sperm DNA (8 mg) was digested with 3.2 µl (75 U) micrococcal nuclease in 10 mM Tris-HCl pH 8.0, 1 mM $CaCl_2$ in a total volume of 5.3 ml for 5 min at 37°C. The reaction was stopped with 6 µl of 0.5 M EDTA before being dialysed versus 2 L of 1 M KCl at 4°C. After 18–20 h, the dialysis buffer was changed to 0.02 M KCl for another overnight dialysis. This 5′-hydroxyl terminated DNA substrate (1.8 mg) was incorporated into a 10% separating sodium dodecyl sulfate (SDS)-polyacrylamide gel, and a control gel was prepared without the substrate. In both the gels a 4% stacking gel was used. Samples were incubated for 15 min at 37°C with sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS) before electrophoresis at 80 V for 3 h according to Laemmli [1970]. After electrophoresis the renaturation of the gel was done according to Ohmura et al. [1987]

with slight modification. Briefly, the gel was washed three times for 30–45 min with 300 ml renaturing solution (50 mM Tris-HCl, pH 7.5, 50 µg/ml gelatin) at room temperature. It was stored overnight at 4°C in 500 ml of 50 mM Tris-HCl, pH 7.5, 0.5 mM DTT. After washing with 100 ml of kinase buffer (50 mM sodium succinate, pH 5.5, 0.5 mM DTT, 10 mM $MgCl_2$) for 1 h at 4°C, the gel was shaken for approximately 20 h at 37°C in a sealed bag containing 15 ml of kinase buffer and 100 µCi [γ-^{32}P]ATP. The gel was washed for 20 min four times in 500 ml ice-cold 5% TCA and 1% sodium pyrophosphate, and shaken overnight in fresh solution, followed by two 30-min washings with 500 ml of the same solution. In order to detect the ^{14}C radiolabelled protein markers (0.1 µCi, MW 14.3–220 kDa, Amersham), the gel was dried before performing autoradiography. As a positive control, 30 U of T4 PNK (New England BioLabs) was used.

Protein Estimation and Electrophoresis

The protein estimations were done according to Bradford [1976] using Coomassie protein assay reagent (Pierce, Rockford, IL). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done according to Laemmli [1970], and the gels were stained using Coomassie Blue R-250 or silver stained using the Silver Stain Plus kit (Bio-Rad, Mississauga, ON). The protein standards used were the broad range markers from Bio-Rad.

Carboxypeptidase Y Digestion

The reaction mixture (10 µl) contained SNQI-PNK (DNA cellulose pool, 1.6 µg), 200 mM sodium acetate, pH 5.5, 32 ng of Carboxypeptidase Y (HPLC pure, Sigma) and incubated at 25°C for 4 h. Buffer control reactions were also incubated for the same period of time. Immediately after the reaction, 1 µl of this product was taken for 5′-DNA kinase assay or 3′-phosphatase assay. T4 PNK (New England Biolabs, 0.2 µg) digested with 4 ng of Carboxypeptidase Y was used for comparison.

Preparation of DNA Replication Extracts and Addition of Purified SNQI-PNK

HeLa cell extracts were prepared as described previously [Pearson et al., 1991; Matheos et al., 1998]. The plasmid used for the replication assays was p186, a pBR322 derivative containing the *Nde*I-*Rsa*I subfragment of *ors8* (GenBank Accession No. M26221; [Kaufman et al. 1985; Zannis-Hadjopoulos et al., 1985; Rao et al., 1990]), which has been found to replicate autonomously both in vitro and in vivo [Todd et al., 1995]. Preparation B SNQI-PNK was purified on a MonoS HR5/5 column to a specific activity of 16.2 U/mg (Preparation C). In a series of experiments, 1 or 2 U of Preparation C SNQI-PNK was added to the replication extracts. Approximately equivalent units of T4 PNK or varying amounts of BSA equal to the amount of protein in the T4 PNK reaction were used as controls. The PNKs were tested in the in vitro replication system under conditions of preincubation with the p186 template DNA for 20 min on ice before the addition of replication extracts, under conditions of preincubation with the replication extracts for 20 min on ice, or without preincubation. In another series of experiments, SNQI-PNK was purified to a specific activity of 7.3 U/mg by concentration of a DNA cellulose fraction purified using the Preparation B protocol (Preparation D). The purified SNQI-PNK (4.7 U or 47 U) or T4 PNK (4.1 U or 41 U) was added to the replication reactions. BSA was also used as a control. The replication reactions were carried out as previously described [Pearson et al., 1991] with slight modification [Matheos et al., 1998]. Briefly, components were HeLa cytoplasmic and nuclear extracts, 70 µg and 18.5 µg, respectively, 2mM ATP, CTP, GTP, UTP, dATP, and dGTP (100 mM each), 10 µCi [α-^{32}P]-dCTP, 10 µCi [α-^{32}P]-dTTP, 0.2 U pyruvate kinase (Boehringer Mannheim), and 200 ng of p186 DNA; pBR322 DNA was used as a negative contol (data not shown). After the pretreatment (if used), the protein extracts, DNA, nucleotides, pyruvate kinase and a polyethylene glycol solution [Pearson et al., 1991] were combined and incubated at 30°C for 1 h, followed by termination with 30 mM EDTA/1% SDS. Purification of the DNA and *Dpn*I digestion was carried out as described previously [Pearson et al., 1991]. Quantification of the *Dpn*I-digested replication products was done on a PhosphorImager analyzer (Fuji BAS 2000) as previously described [Matheos et al., 1998; Diaz-Perez et al., 1996]. The control p186 replication reaction without addition of PNK or BSA was used to normalize results to 100% replication activity.

RESULTS

Two or More PNKs in Calf Thymus

Our laboratory has earlier reported the purification of a neutral to alkaline pH optimum polynucleotide kinase from the Polymin P-pellet, Polymin P precipitable PNK (PP-PNK). Extensive investigation and partial purification of the PNK activity in the Polymin P pellet indicated that only one activity was present and that it differed from the PNK activity previously described in calf thymus [Prinos et al., 1995]. Furthermore, an isoelectric point of 8.6 had been reported for acid pH optimum PNK from rat liver [Habraken and Verly, 1988], suggesting that the calf thymus enzyme, if similar, would likely be slightly cationic at the pH employed in the Polymin P step. Therefore, we reasoned that the PNK activity reported in the literature would be found in the Polymin P supernatant. Previously, we reported an activity from the Polymin P supernatant that bound only weakly to a Q Sepharose column, designated SNQI-PNK [Prinos et al., 1995]. We sought to determine whether this activity represented the acid pH optimum PNK previously reported by numerous laboratories in rat liver and by several laboratories in calf thymus.

Purification of SNQI-PNK

An assay employing 5′-phosphorylation of synthetic oligo dT_{25} was used to follow the purification of PNKs. The purification scheme for mammalian PNKs (Fig. 1) is novel in that it includes a Polymin P step to precipitate nucleic acids, nucleic acid-binding proteins and acidic proteins [Burgess, 1991]. PNK activity is not detectable in the Polymin P supernatant fraction (data not shown). In order to remove remaining Polymin P, which presumably interfered with the enzymatic assay, the supernatant from the Polymin P step was batch adsorbed to phosphocellulose. After concentration by ammonium sulfate precipitation, the protein was applied to a Q sepharose column. We found DNA kinase activity in the flow through and wash fractions (SNQI-PNK) and DNA kinase activities eluting at two different salt concentrations in the gradient (SNQII-PNK eluted at 0.25 M NaCl, and SNQIII-PNK eluted at 0.5 M NaCl). The occurrence of the three activities was very reproducible, although the relative proportions of SNQII-PNK and SNQIII-PNK were somewhat variable (data not shown).

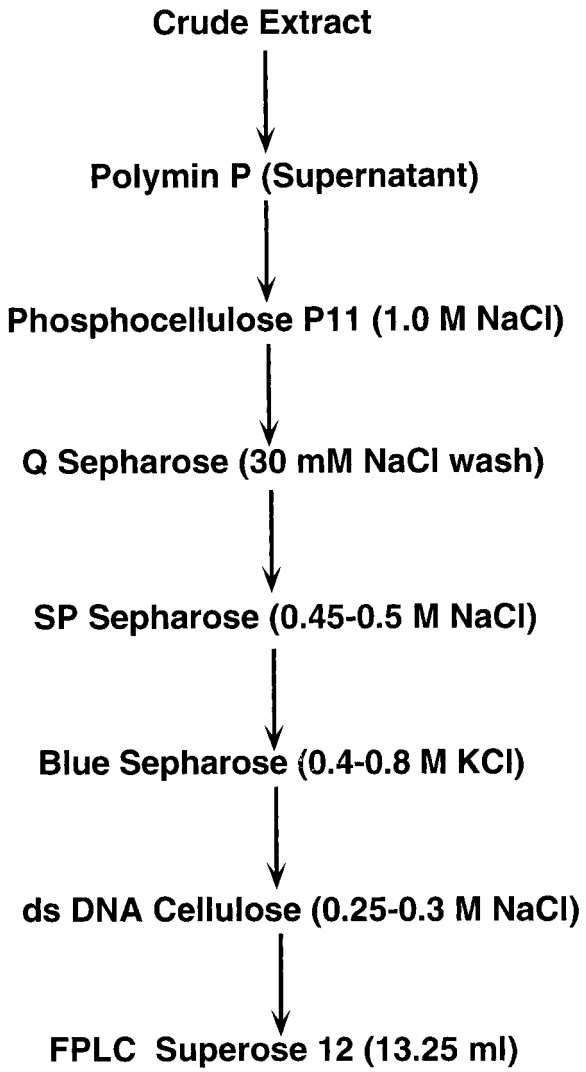

Fig. 1. Purification procedure for SNQI-PNK. The crude extract was subjected to Polymin P precipitation, followed by centrifugation. The supernatant was used to purify SNQI-PNK through six steps. The salt concentration at which SNQI-PNK eluted is shown in parentheses. SNQI-PNK eluted at 13.25 ml on a Superose 12 gel-filtration column.

Further enrichment of SNQI-PNK activity was achieved by sequential purification on SP Sepharose, Blue Sepharose, double-stranded DNA cellulose, and Superose 12 gel filtration columns (see under Materials and Methods) (Fig. 1). After SP Sepharose chromatography, the active fractions were found to be eluted at 0.45–0.5 M NaCl. On the Blue Sepharose column, elution of the activity occurred from 0.4 to 0.8 M KCl. After double-stranded DNA cellulose chromatography, the fractions with the highest DNA kinase activity eluted at 0.25 M NaCl. The pooled fractions were then immedi-

TABLE I. Purification of SNQI-PNK from Calf Thymus as Monitored by 5′-Phosphorylation of Oligo dT_{25}

Step	Protein (mg)	Total activity (U)	Spec act (U/mg)	Yield (%)	Purification (−fold)
Q-Sepharose (flowthrough and wash)	159.8	2.65	0.0166	100	1
SP Sepharose	18.8	17.6	0.935	664	56.3
Blue Sepharose	5.08	1.41	0.277	53.2	16.7
DNA-cellulose	0.276	0.227	0.822	8.57	49.5
Superose 12 (fractions 24–26)	0.0051	0.184	36	6.9	2,200

ately size-fractionated on a Superose 12 column. Table I presents the results of a typical purification of SNQI-PNK using this protocol (Fig. 1). Since we observed multiple activities capable of 5′-phosphorylation of oligo dT_{25} in the early stages of the purification, yield calculations were begun at the Q Sepharose step. At this point in the purification, SNQI-PNK was clearly separated from the other PNKs we have detected, i.e., PP-PNK, SNQII-PNK, and SNQIII-PNK. We were able to purify SNQI-PNK activity about 2,200-fold in this preparation. In tests for contaminating activities, endonuclease, acid phosphatase, and alkaline phosphatase activities were determined, and all were found to be below the limits of detection of the assay (data not shown). A number of purifications were done as described except that the Superose 12 step was performed before the DNA cellulose step (Preparation B). This resulted in a high degree of purification in terms of specific activity except that three major polypeptides (one of which was 60 kDa) were visible upon SDS-PAGE analysis.

Size of SNQI-PNK

The fractions from Superose 12 were subjected to SDS-PAGE analysis (Fig. 2A). As is evident in Figure 2, the occurrence of DNA kinase activity in the Superose 12 fractions (Fig. 2B) correlates with a polypeptide of 60 kDa. Analysis of the fractions from the Superose 12 column showed that the DNA kinase activity had an apparent molecular mass of around 60 kDa compared with globular protein standards, and the Stokes radius of SNQI-PNK was found to be 3.5 nm (data not shown). In fractions 17–22 (the elution expected for 120–60 kDa, according to the calibration) we observed no detectable DNA kinase activity (Fig. 2B, and data not shown); thus, under these conditions, SNQI-PNK elutes as a monomer.

In order to further investigate the active polypeptides in DNA kinase preparations, renaturation gel experiments [Ohmura et al., 1987] were carried out. Figure 3A shows the in situ DNA kinase activity from pooled SP Sepharose-purified SNQI-PNK and a preparation of SNQII-PNK purified using the same protocol. The size of the observed active polypeptide is about 60 kDa in the SNQI-PNK preparation and 40 kDa in the SNQII-PNK preparation. Figure 3B shows the activity from the DNA cellulose pool of another preparation. Again, an active polypeptide of about 60 kDa is evident. There was no detectable activity in a control gel not including substrate (Fig. 3C). In some experiments, a smaller active polypeptide of about 32 kDa that we have ascribed to proteolysis is also present. This smaller polypeptide was observed in experiments performed with samples stored at 0°C (Fig. 3B and data not shown). In the DNA cellulose column step the SNQI preparations were free of any other cellular PNK activity, and we consider it unlikely that this smaller active polypeptide represents another PNK. Taken together, the results of the renaturation gel experiments further support the idea that a 60-kDa polypeptide correlates with enzymatic activity.

Characterization of SNQI-PNK

Characterization experiments used preparations of SNQI-PNK that were purified about 1,750-fold. In order to further delineate the properties of this enzyme and relate it to others described in the literature, the dependence of 5′ kinase activity on pH was monitored. Two buffers, Mes (Fig. 4A) and succinic acid (Fig. 4B) were used for ≤pH 6, and Tris-maleate served as a buffer across the pH range tested. Within the neutral to alkaline pH range, similar re-

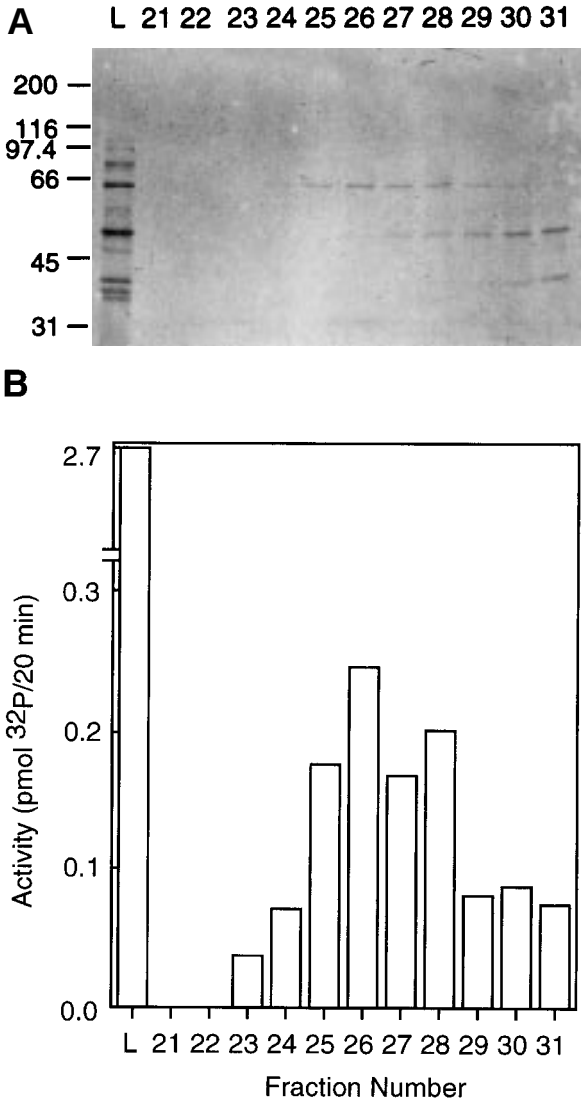

Fig. 2. Analysis of fractions from the Superose 12 step. **A:** SDS-PAGE analysis of Superose 12 fractions Protein (160 µg) from the active DNA-cellulose pool was loaded onto the column and fractions (125 µl) were collected and assayed for the DNA kinase activity. An aliquot (10 µl) of Superose 12 fractions 21–31 was electrophoresed through a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoreses (SDS-PAGE) gel and stained with Coomassie Blue R-250. **B:** DNA kinase activity profile of the Superose 12 fractions An aliquot of 1 µl of each of the fractions was used for the DNA kinase assay. The radioactivity incorporated was measured by excision of the band followed by liquid scintillation counting. There was no detectable activity before fraction 23.

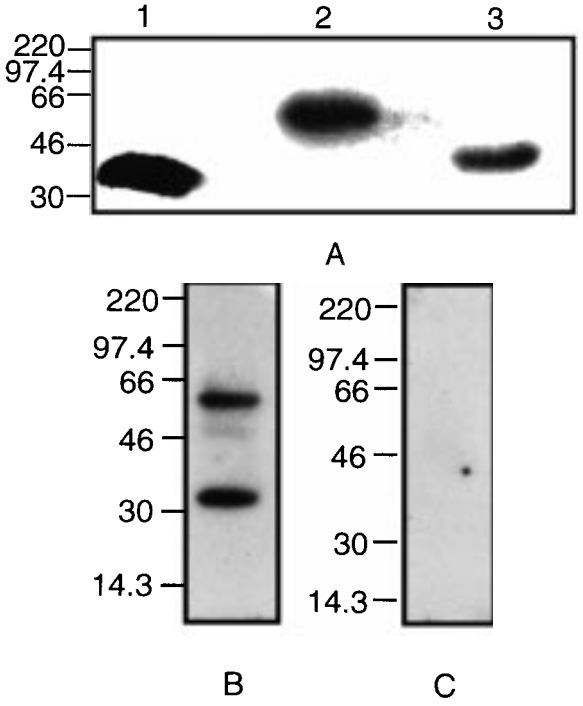

Fig. 3. Identification of polypeptides with 5′ phosphorylation activity by renaturation gel experiments. **A: Lane 1**, T4 polynucleotide kinase; **lane 2**, SP Sepharose-purified SNQI-PNK; **lane 3**, a preparation of SNQII-PNK purified using the same protocol. Positions of ^{14}C size standards are indicated at the left. The autoradiogram was scanned and the signal in lane 1, too intense for size determination in the original autoradiogram, was adjusted using Adobe Photoshop 4.0. **B:** Double-stranded DNA cellulose pool of an another preparation that had been stored for 2 months at 0°C. **C:** Control assay performed in the absence of substrate DNA for the preparation shown in B. Processing of sample, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), renaturation of gels, in situ DNA kinase assay, and autoradiography were done as described under Materials and Methods.

sults were obtained with Tris-HCl buffer (data not shown). A pH optimum of 6.0–6.5 was detected (Fig. 4), classifying SNQI-PNK as an acid-pH optimum DNA kinase. The dependence on oligo dT_{25} was investigated using six concentrations increasing from 0 to 20 µM. The apparent K_m value calculated from Lineweaver-Burk plots was 2.3 ± 1.2 µM (mean ±SD of four experiments, data not shown). The dependence of kinase activity on ATP concentration was studied by varying the ATP concentration from 0 to 100 µM. Lineweaver-Burk plots were constructed, resulting in an estimate of an apparent K_m value of 11.8 ± 6.6 µM for ATP (mean ±SD of four experiments, data not shown). When different constituents of the standard reaction mixture were omitted, presence or absence of BSA and DTT had no effect, absence of oligo dT_{25} resulted in no detectable activity, and absence of $MgCl_2$ left a residual level of incorporation of 13%. Thus DNA and Mg^{2+} are required for enzymatic activity. When the concentration of $MgCl_2$ in the standard assay was varied, the highest activity was found at 10

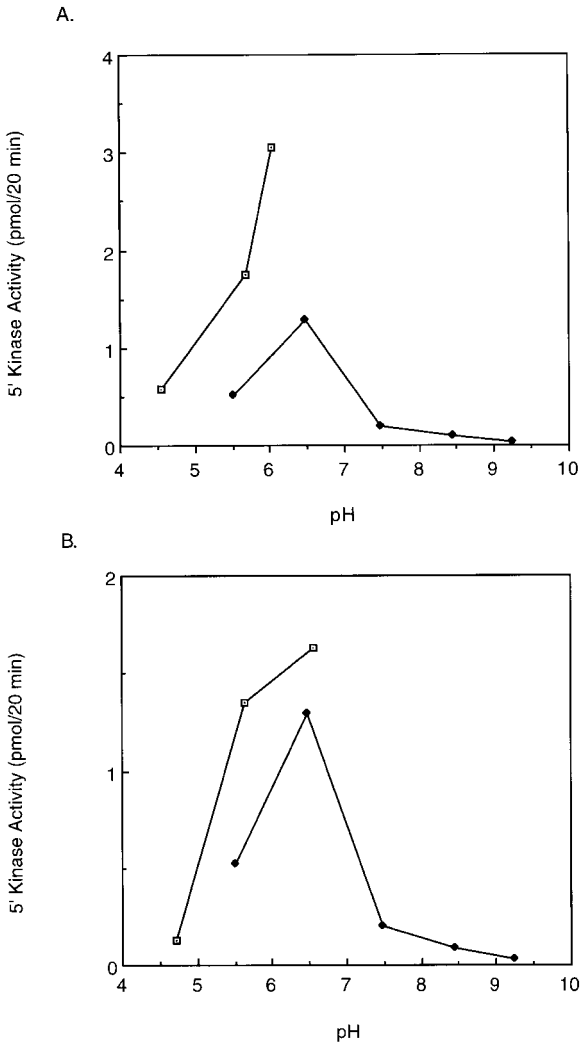

Fig. 4. pH profile of 5′ DNA kinase activity. SNQI-PNK purified 1750-fold was used for these experiments. **A:** pH profile using Mes (open squares, pH 4.6, 5.7, 6.0) and Tris-maleate (closed diamonds, pH 5.5, 6.5, 7.5, 8.4, 9.3). **B:** pH profile using succinic acid (open squares, pH 4.6, 6.0, 6.5) and Tris-maleate (closed diamonds, pH 5.5, 6.5, 7.5, 8.4, and 9.3).

TABLE II. Inhibitors of SNQI-PNK

Compound	Concn for 50% inhibition of activity (mM)
Ammonium sulfate	6 ± 1.6
Sodium sulfate	5 ± 2
Sodium pyrophosphate	8 ± 2
Sodium chloride	150 ± 30
Potassium chloride	68 ± 7

mM. Effects of certain salts on the kinase activity were studied by varying the salt concentrations and detecting the concentration required to inhibit 50% of the DNA kinase activity (IC_{50}). The results are summarized in Table II. Ammonium sulfate, sodium sulfate, and sodium pyrophosphate have similar IC_{50} values of <10 mM. Sodium chloride has an IC_{50} value of about 150 mM.

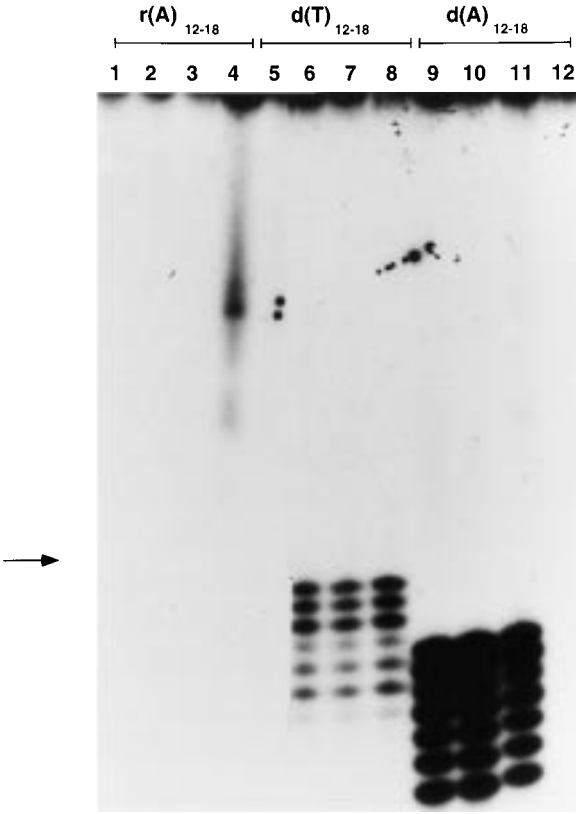

Fig. 5. Substrate specificity of SNQI-PNK. Three homo-oligomer ladders, r($A_{12–18}$), d($T_{12–18}$), and d($A_{12–18}$), were used in the DNA kinase assay. Standard assay conditions (100 pmol 5′ OH termini) were used, and phosphorylated products were analyzed on a 25% polyacrylamide/7 M urea gel. Arrow, migration of oligo rA_{18}. T4 PNK efficiently phosphorylated all these substrates (data not shown). **Lanes 1, 5, 12**, buffer control; **lanes 2–4**, r($A_{12–18}$); **lanes 6–8**, d($T_{12–18}$); **lanes 9–11**, d($A_{12–18}$).

To investigate substrate specificity, SNQI-PNK was incubated with substrates consisting of ladders of 5′-OH r$A_{12–18}$, 5′OH-d$A_{12–18}$, or 5′-OH d$T_{12–18}$. Significant activity was observed only with the latter two substrates (Fig. 5), although T4 PNK 5′-phosphorylated all of these substrates (data not shown). The T4 PNK substrate 5′-OH Tp was not detectably phosphorylated by SNQI-PNK preparations (data not shown). On the other hand, a dephosphorylated *Eco*RI fragment prepared as described by Prinos et al. [1995] was a substrate for SNQI-PNK.

In order to determine whether this enzyme also exhibits a 3′-phosphatase activity, assays were performed using the method of Cameron and Uhlenbeck [1977]. Figure 6 shows the results of a 3′-phosphatase assay. The 3′-phos-

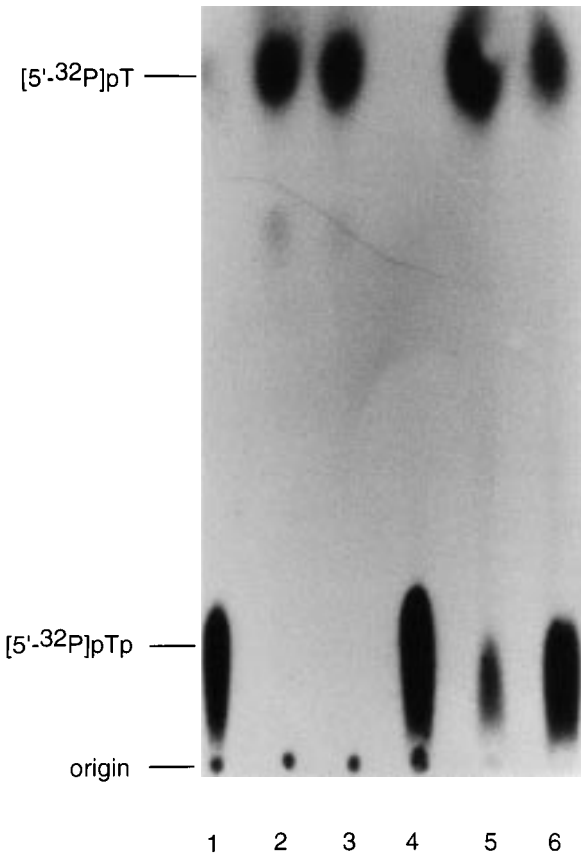

Fig. 6. 3′-phosphatase activity of SNQI-PNK and SNQII-PNK. SNQI-PNK purified 1,750-fold was used for these experiments. Formation of 5′ [^{32}P]pT was analyzed using PEI TLC plates developed in 1.0 M ammonium formate. Units (U) refer to units of 5′ kinase activity. **Lane 1**, 10 U T4 PNK, 3′-phosphatase free (Boehringer Mannheim); **lane 2**, 10 U T4 PNK (Boehringer Mannheim); **lane 3**, 10 U T4 PNK (New England Biolabs); **lane 4**, buffer control; **lane 5**, 0.2 U SNQI-PNK; **lane 6**, 2 µl of an SNQII-PNK preparation. See Table III for determination of 3′-phosphatase activity throughout purification.

phatase activity can be correlated with the 60-kDa polypeptide observed in Figure 2, because significant 3′-phosphatase activity was detectable in fraction 25 (data not shown and Table III). The presence of a 3′-phosphatase activity was reproducible in all steps of the purification. For the same preparation of 5′-kinase activity listed in Table I, Table III shows that 3′-phosphatase activity increased about 1,400-fold. Detection of a 3′-phosphatase activity is in agreement with the results of Karimi-Busheri and Weinfeld [1997], who used a different assay to investigate this question for a DNA kinase purified from fetal calf thymus.

Exopeptidase Digestion Experiments

Soltis and Uhlenbeck [1982b] had assigned the 3′-phosphatase domain to the C-terminus of T4 PNK. It can be very useful to examine structural features conserved among enzymes with a particular function regardless of the specific substrate [Bork and Koonin, 1988]. In order to determine whether any phosphatases would yield homology to T4 polynucleotide kinase, database searches were conducted. Interestingly, when the first 180 aa of the sequence of the *E. coli His7* (*His B*) gene product (the histidinol phosphatase domain) were used to search the NR-AA database with PSI-BLAST search [Altschul et al., 1997], T4 polynucleotide kinase was found to be distantly related, since it was recovered within three iterations with an expected (e) value in the range of 10^{-7}. The reciprocal search using the corresponding region of the T4 PNK sequence also yielded *His7*, within six iterations. The histidinol phosphatase domain of *His7* is a member of the L-2-haloacid dehalogenase protein superfamily [Koonin and Tatusov, 1994], and an alignment with the L-2-haloacid dehalogenase 2 sequence produced by ClustalW[1] [Thompson et al., 1994] is shown in Figure 7. The alignment encompasses amino acids 160–293 of the T4 polypeptide (total length 301), supporting the experimental data that important sequences for the 3′-phosphatase activity of T4 PNK are readily susceptible to carboxypeptidase Y digestion.

To investigate this question for SNQI-PNK, carboxypeptidase Y digestion was carried out for 4 h. T4 PNK was used as a positive control, and 5′ kinase and 3′-phosphatase activities were measured as described in Materials and Methods. In a representative experiment, SNQI-PNK was found to retain 88% of its 5′-kinase activity and 89% of its 3′-phosphatase activity after the carboxypeptidase Y digestion. T4 PNK retained 59% of its 5′-kinase activity and 38% of its 3′-phosphatase activity. We conclude that SNQI-PNK is more resistant than T4 PNK to digestion with this exopeptidase and that again in contrast to the T4 enzyme, there is no apparent difference in the sensitivity of the two activities to exopeptidase.

Effect of Exogenously Added SNQI-PNK on In Vitro DNA Replication

In order to determine whether there was a salient effect of addition of an excess of SNQI-PNK on in vitro DNA replication, experiments were carried out in which SNQI-PNK, T4 PNK, or BSA were added. These experiments were

[1]http//www.ibc.wustl.edu/service/msa/clustal_old.cgi

TABLE III. Co-purification of 3′-Phosphatase Activity With SNQI-PNK[a]

Step	Protein (mg)	Total activity (U)	Spec act (U/mg)	Yield (%)	Purification (−fold)
Q-Sepharose (flow through and wash)	159	6.12	0.0385	100	1
SP-Sepharose	14.8	1130	1.52	462	50
Blue-Sepharose	3.84	81.6	21.2	33.4	14
DNA-cellulose	0.285	12.0	42.2	4.9	28
Superose 12 (fractions 24–26)	0.0051	0.281	55	4.6	1,430

[a]The SNQI-PNK preparation from Table I was assayed for 3′-phosphatase activity as described under Methods and Materials.

```
                        Motif 1

1JUD_Psp    1   MDYIKGIYFD LYGTLFDVHS VVGRCDEYFP GRGREISYLW RQKQLEYTWL
HIS7_Ec     4   ....KYLFID RDGTLISEP. .........P ......SDFQ VDRFDKLAFE
KIPN_BPT4 160   ....KAVIFD VDGTLAKMNG .......RGP .......YD. LEKCDTDVIN
                        *

                                    Motif 2

1JUD_Psp   51   RSLMNRYVNF QQYTEDYLFR TCRHLGLDLD YRTRSTLCDY YLRLYPFSEV
HIS7_Ec    34   PGVIPELLKL QKAGYKLVMI TN........ ...QDG.... ....LGTQSF
KIPN_BPT4 191   PMVVELSKMY ALMGYQIVVV SG........ ...RES.... .....GTKED

1JUD_Psp  101   PDSLRELKRR GLKLYILSNG SPQSIDYVVS HYGLRDGFDH LLSVDPVQVY
HIS7_Ec    65   PQADFDGPHN .LMMQIFTSQ GVQFDEVLIC PH........ .LPADECDCR
KIPN_BPT4 221   PTKYYRMTRK ......WVED IAGVPLVMQC QR........ ....EQGDTR

                        Motif 3

1JUD_Psp  151   KPDNRVYELY EQYLGLDRSY ILFVSSNYWD YTGYRYFGFP TCWINRTGNV
His7_Ec   105   KPKVKLVERY LAEQAMDRAN SYVIGDRATD IQLAENMGIT G......141
KIPN_BPT4 253   KDDVVKEEIF WKHIAPHFDV KLAIDDRTQV VEMWRRIG..  ......290

1JUD_Psp 201    FEEMGQTPDW EVTSLRYVVE LFETAAGKAE KG 232
His7_Ec         ..........  .......... .......... ..
KIPN_BPT4       .......... .......... .......... ..
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Fig. 7. Alignment of the haloacid dehalogenase folds of L-2-haloacid dehalogenase II (*Pseudomonas* sp., 1JUD_Psp), histidinol phosphatase (*Escherichia coli,* HIS7_Ec), and the putative similar region of the T4 PNK polypeptide (KIPN_BPT4). The names used for the proteins are the SWISS-PROT codes. Alignments were initially generated between the *His7 (HisB)* gene product and T4 PNK, using PSI-BLAST [Altschul et al., 1997], as described in the text. Once the region of similarity was found, a three-way alignment was constructed using ClustalW. Motifs 1, 2, and 3 [Aravind et al., 1998] are indicated, and the conserved residues underlined. Asterisk, conserved Asp residue in Motif 1 in the sequence of human phosphomannomutase 1 found to be phosphorylated after incubation with substrate [Collet et al., 1998].

designed to test the hypothesis that SNQI is rate-limiting during in vitro DNA replication. In one experiment, in which the purified PNK was preincubated with the proteins in the replication extract, total DNA replication was 75% of control in the presence of 4.7 U SNQI-PNK, and 92% in the presence of 47 U SNQI-PNK; the corresponding values for 4.1 U and 41 U of the T4 enzyme were 105% and 106%, respectively (data not shown). Overall, no significant effect was observed upon addition of increasing amounts of SNQI-PNK or T4 PNK to the in vitro replication reaction, in four independent experiments. Table IV lists the results of two experiments in which the protein was incubated with the plasmid DNA before the replication reaction. Although a slight decrease in DNA replication at higher levels of SNQI-PNK activity was observed when the protein was preincubated with the DNA, it was not specific for the mammalian enzyme and was also seen when T4 PNK was used. Since addition of BSA also caused a decrease in replication at a level of 230 ng, the observed decrease is most likely nonspecific, possibly due to the high amount of protein present in the in vitro system. A baseline level of DNA kinase activity in the HeLa cell extract used for a DNA replication reaction was determined to be 0.8 U. We conclude that the addition of excess SNQI-PNK neither stimulates nor inhibits DNA replication. However, these data do not rule out a role for PNK activity for DNA replication in vivo, as suggested by Pohjanpelto and Hölttä [1996]; perhaps PNK(s) play a role that could not be detected in vitro. On the other hand, it is conceivable that SNQI-PNK does play a role in vitro, but sufficient amounts are available in the extracts so that it is not rate-limiting.

DISCUSSION

Previous studies [Prinos et al., 1995, Shuman and Hurwitz, 1979] have reported the presence of more than one polynucleotide ki-

TABLE IV. Effect of Addition of Exogenous SNQI-PNK or T4 PNK on DNA Replication In Vitro

SNQI-PNK[a]		T4 PNK[d]		BSA[d]	
U	DNA replication observed[c] (% of control)	U	DNA replication[c] (% of control)	Protein (ng)	DNA replication observed[c] (% of control)
		Experiment 1. SNQI-PNK preparation C[b]			
0	100	0	100	0	100
1	69	1	135	2.5	157
2	112	2	81	5.0	110
		Experiment 2. SNQI-PNK preparation D[b]			
0	100 ± 9.1	0	100 ± 9.1	0	100 ± 9.1
4.7	123 ± 8.5	4.1	90.1 ± 33	23	79.2 ± 40
47	73.5 ± 1.6	41	63.1 ± 11	230	68.9 ± 8.7

[a]SNQI-PNK was purified as described under Materials and Methods, with variations from the standard procedure for preparations C and D.
[b]The specific activities for preparations C and D were 16.2 U/mg and 7.3 U/mg, respectively.
[c]The DNA replication products were purified, treated with *Dpn* I, and replicated DNA was quantitated as described. Data reported for Experiment 1 were the mean of duplicate reactions, and for Experiment 2 the mean and standard deviation of triplicate reactions.
[d]T4 PNK was diluted into buffer containing BSA. The amount of BSA used as a control was equivalent to the amount of BSA in the T4 PNK reaction.

nase in HeLa cells and calf thymus, respectively. Purification and biochemical characterization of a PNK found in the supernatant fraction after Polymin P treatment of extracts prepared from calf thymus glands indicated that this enzyme, SNQI-PNK, has a slightly acidic pH optimum, and is dependent on Mg^{2+} for full activity. In our studies, we found no evidence for detectable 5′-phosphorylation of RNA substrates (Fig. 5, and data not shown), implying a substrate specificity for DNA. We conclude that in contrast to PP-PNK, another bovine PNK studied in our laboratory, SNQI-PNK appears to be closely related to the acidic pH optimum DNA kinases purified from calf thymus [Tamura et al., 1981] and fetal calf thymus [Karimi-Busheri and Weinfeld, 1997]. Furthermore, detection of a 3′-phosphatase activity reinforces the relationship with the fetal calf thymus enzyme and the rat liver DNA kinases studied by many laboratories [reviewed by Zimmerman and Pheiffer, 1981] and more recently purified extensively by Habraken and Verly [1988] and Karimi-Busheri and Weinfeld [1997]. Reconstitution experiments with purified fetal bovine thymus PNK, DNA ligase I, and DNA polymerase β indicated that these enzymes could repair synthetic oligonucleotide substrates containing one base gaps with 3′-P and 5′-OH termini or 3′-OH and 5′-OH termini [Karimi-Busheri et al., 1998].

At the last step of the novel purification reported in this paper, the size of SNQI-PNK is approximately 60 kDa, with a Stokes radius of 3.5 nm, as determined by gel filtration on Superose 12. This finding is in agreement within experimental error with the determination of Tamura et al. [Tamura et al., 1981], who found an apparent molecular mass of 56 kDa and a Stokes radius of 3.3 nm. However, these results for SNQI-PNK from thymus glands of 6-month-old calves are not in accord with an apparent molecular mass of 125 kDa of a PNK from frozen fetal calf thymus upon gel filtration on Superdex 200 [Karimi-Busheri and Weinfeld, 1997]. Possible explanations for this include the different developmental stage of the thymus gland and the measurement of apparent molecular mass by gel filtration before the final stage of purification [Karimi-Busheri and Weinfeld, 1997] as opposed to at the final stage of purification [herein and Tamura et al., 1981]. Habraken and Verly [1988] purified what appears to be a proteolytic fragment of rat liver PNK that was reported to dimerize.

Renaturation gel assay analysis was used to further investigate the nature of the active polypeptide(s). Freshly prepared SP-Sepharose pool SNQI-PNK indicated a single active polypeptide with a size of 56 kDa. A preparation of SNQII-PNK also purified over SP-Sepharose and analyzed concurrently gave a signal of about 40 kDa. It is possible that SNQII-PNK is a proteolytic fragment of SNQI-PNK, but further experimentation will be required to conclu-

sively address this issue. This SNQII-PNK fraction also had an associated 3′-phosphatase activity (Fig. 6). In another experiment, a DNA cellulose pool that had been stored on ice gave a signal at approximately 60 kDa and another signal at about 30 kDa. We judge the smaller signal to be due to proteolysis, because it was found several times with differing intensities when samples of SNQI-PNK or SNQII-PNK were analyzed in the gel renaturation assay. We also note the presence of a similar size band in the autoradiograms of Karimi-Busheri and Weinfeld [1997]. Despite multiple trials, we were unable to obtain a signal on renaturation gels when near-homogeneous fractions of SNQI-PNK were subjected to the procedure. No other laboratory has published renaturation gel data on highly purified preparations of mammalian PNKs. Our results could be attributable to inhibitory effects of components of the electrophoretic gels that are removed by proteins with higher electrophoretic mobility in the impure samples, as noted for a renaturation gel assay for ribonuclease H [Han et al., 1997], or less efficient renaturation in the absence of other polypeptides.

In vitro studies support the hypothesis that mammalian PNKs could function in DNA repair. Another proposed role of mammalian PNKs is in DNA replication [Pohjanpelto and Hölttä, 1996]. As an initial step, we designed experiments to test the hypothesis that SNQI-PNK is rate-limiting during in vitro DNA replication. The results described in the text and in Table IV show that there was no discernable effect of addition of highly purified SNQI-PNK to these in vitro DNA replication reactions. Since it is possible that the enzyme may play a role at the basal levels present in the replication extracts used, future work involving depletion of enzyme activity will be required to address this question more completely. A fraction of Okazaki fragments may require processing by PNKs to allow ligation, as proposed by Pohjanpelto and Hölttä [1996]. We note that in vitro reconstitution experiments using purified enzymes, and isolation of multiprotein replication complexes followed by characterization of the component polypeptides have never indicated a requirement for PNK activity in mammalian DNA replication [reviewed in Waga and Stillman, 1994; Bambara et al., 1997; Zannis-Hadjopoulos and Price, 1998]. However, all in vitro systems are regarded as inefficient compared with cellular DNA replication. Genetic experiments in *S. cerevisiae* revealed that there may be several pathways for Okazaki fragment processing [Bambara et al., 1997]. A true understanding of the possible role of SNQI-PNK in DNA replication in vivo will be achieved only when the gene encoding it is overexpressed or subjected to ablation of expression.

The finding that a 3′-phosphatase activity is exhibited by SNQI-PNK after a high degree of purification is an indication of a close relationship with the PNKs purified from rat liver and fetal calf thymus [Habraken and Verly, 1988; Karimi-Busheri and Weinfeld, 1997]. Having detected this activity, we decided to use it as a tool to compare the possible structural relationship of T4 polynucleotide kinase and SNQI-PNK. Soltis and Uhlenbeck [1982b] had previously found that about 90% of 5′ kinase activity was retained after a 4-h digestion with carboxypeptidase Y in comparison with just less than 19% of 3′-phosphatase activity. Combining these data with information from other experiments, these authors concluded that the 5′-kinase activity probably mapped to the N-terminus of T4 PNK and the 3′-phosphatase activity to the C-terminus. Indeed, these conclusions were substantiated when a Walker consensus ATP binding site was mapped to the N-terminus of the predicted amino acid sequence of the cloned protein [Midgley and Murray, 1985]. However, there have been no subsequent reports describing the 3′-phosphatase domain.

Our results on digestion of T4 PNK with carboxypeptidase Y were qualitatively in accord with those of Soltis and Uhlenbeck [1982b] but quantitatively less striking, with 59% retention of 5′ kinase activity for T4 compared with about 38% of 3′-phosphatase. This may be due to differences in digestion and assay conditions and the preparation of carboxypeptidase Y. However, both the 5′ phosphorylation and 3′-phosphatase activities of highly purified SNQI-PNK displayed a remarkable resilience to carboxypeptidase Y digestion. This may indicate that the amino acid sequence at the C-terminus is not easily digested or that the domain structure of SNQI-PNK differs from T4 PNK, perhaps in that sequences at the extreme C-terminus are not required for 3′-phosphatase activity, in contrast to T4 PNK. It was not possible to separate the two activities of rat liver acid pH optimum PNK by limited digestion with trypsin [Habraken and Verly, 1988], whilst a small domain in the N-terminus of T4 PNK was sensitive to trypsin digestion, with concomitant loss of 5′-

phosphorylation activity [Soltis and Uhlenbeck, 1982b].

Database searches using the PSI-BLAST program [Altschul et al., 1997] to investigate whether known phosphatase domains were related to the sequence of T4 PNK indeed indicated a distant relationship to phosphatases such as histindinol phosphatase of *E. coli (His7)*. To our knowledge, this is the first report of a potential phosphatase domain in the amino acid sequence of T4 PNK. In noting sequence similarity among putative NTP binding sites of T4 PNK, the *RLG1* gene product of *S. cerevisiae*, and mammalian myelin 2′,3′-cyclic phosphohydrolase, Koonin and Gorbalenya [1990] apparently also aligned the COOH termini of these proteins but did not show the alignment and stated that the relevance of the similarity was not clear because the role of specific segments or residues was unknown. Durantel et al. [1998] also noted similarity between T4 PNK and a conceptual translation of the pnk/pnl gene (ORF 86) of the baculovirus *Autographa californica* [Ayres et al., 1994] but did not suggest a specific phosphatase domain. Moreover, the ORF 86 gene product has not been characterized biochemically, although genetic experiments indicated that it is a nonessential, immediate early gene.

The *His7* gene product is known to be a member of the haloacid dehalogenase superfamily of proteins, including several phosphatases, and recently, P-type ATPases [Koonin and Tatusov, 1994; Hisano et al., 1996; Aravind et al., 1998]. When the L-2-haloacid dehalogenase 2 sequence, for which the structure has been determined [Hisano et al., 1996], was aligned with the putative phosphatase domains of His7 from *E. coli* and T4 PNK using ClustalW, conserved residues corresponding to motifs 1, 2, and 3 can be identified (Fig. 7). The importance of this haloacid dehalogenase domain has been shown for L-2-haloacid dehalogenase by site-directed mutagenesis of the conserved residues [Hisano et al., 1996] and in the deletion mutations seen in Menke disease gene and in the base substitution mutations observed in the Wilson disease gene, two other members of the superfamily [Aravind et al., 1998]. When incubated with substrate, the first Asp in the conserved DVDGT motif 1 in human phosphomannomutase 1, another member of the superfamily involved in human disease, has been determined by mass spectrometry to form an acylphosphate [Collet et al., 1998]. It is feasible that T4 PNK has a similar reaction mechanism. The final conserved aspartate residue corresponding to Asp180 of L-2-haloacid dehalogenase is not present in the T4 PNK sequence. It is possible to speculate that T4 PNK may have additional residues inserted between the two conserved Asp positions (Asp300 is the penultimate residue of T4 PNK) or that Glu284 can substitute; the corresponding mutation in L-2-haloacid dehalogenase yielded some residual activity [Hisano et al., 1996]. This domain is very close to the C-terminus of T4 PNK (301 aa total length), possibly explaining why the 3′-phosphatase activity is sensitive to exopeptidase digestion. We have recently obtained a partial cDNA encoding human SNQI-PNK, and the predicted amino acid sequence contains a similar structural domain in which the final conserved residue of motif 3 is located approximately 210 aa from the C-terminus (A. Jilani and D.D. Lasko, unpublished communications). We hypothesize that this haloacid dehalogenase fold domain comprising three motifs is crucial for 3′-phosphatase function in mammalian SNQI-PNK, and in our experiments we found it difficult to inactivate 3′-phosphatase activity with carboxypeptidase Y digestion, consistent with this notion.

In our studies of PNKs in calf thymus glands, we have previously identified an apparently novel activity in the Polymin P pellet fraction. Further study of the Polymin P supernatant fraction has led to the purification and characterization of another enzyme, SNQI-PNK, that has properties shared with the acidic pH optimum DNA kinase purified from the same source using a different approach [Tamura et al., 1981]. DNA replication extracts from HeLa cells were found to contain basal PNK activity, and addition of purified SNQI-PNK neither stimulated nor specifically inhibited DNA replication. At this time, it is not possible to rule out a role for PNKs in DNA replication, although there is only limited evidence in support of this idea. In common with the enzyme purified from fetal bovine thymus glands [Karimi-Busheri and Weinfeld, 1997], SNQI-PNK has a 3′-phosphatase activity. Using database searches, we have identified a potential 3′-phosphatase domain that places T4 PNK in the superfamily of proteins that includes haloacid dehalogenases, a number of phosphatases of different substrate specificity, ATP transporters, and P-type ATPases. This domain maps to the extreme COOH terminus of the T4 PNK polypeptide,

which may explain the sensitivity of the 3′-phosphatase activity to exopeptidase digestion. Such sensitivity is not seen in concurrent experiments using SNQI-PNK, implying a different organization for the mammalian PNK.

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