Purification and Characterization of a Mammalian DNA kinase

by

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ABSTRACT

Using a novel purification scheme and a new assay for detection of DNA kinase activity, a Polymin P-precipitable DNA kinase has been identified and characterized from calf thymus extracts The DNA kinase activity was able to phosphorylate RNA as well as single-stranded and double-stranded DNA, therefore it has been termed Polymin P-precipitable polynucleotide kinase (PP-PNK) The enzyme had a neutral to alkaline, broad pH optimum that distinguished it from the previously described mammalian DNA kinases that have an acidic pH optimum The sedimentation coefficient of the enzyme was 3.4-3.8 S, indicating a molecular weight of about 50 kDa. Estimates for the K_M for ATP were 52 μM and for the oligonucleotide substrate 8 μM. The activity was inhibited by pyrophosphate anions and to a lesser extent by sulfate anions. These results differentiate PP-PNK from other mammalian polynucleotide kinases

RÉSUMÉ

Nous avons identifié et caractérisé une nouvelle ADN kinase à partir d' extraits thymiques du veau en utilisant des méthodes de purification et d' essais enzymatiques nouveaux L' ADN kinase, qui est précipitable par Polymine P, demontre une activité qui est capable de phosphorer de l' ARN et de l' ADN a brin simple et double. Cette nouvelle ADN kinase est appelée PP-PNK, pour Polymine précipitable polynucleotide kinase L' activité enzymatique se distingue des autres ADN kinases mammifères de son large pH optimal qui fluctue de neutre à alkalin Le coefficient de sedimentation de l' enzyme est de 3.4 à 3.8 S, indiquant un poids moléculaire de 50 kDa L' activité enzymatique a une K_M pour l'ATP de 52 μM et de 8 μM pour un substrat oligonucleotidique L' enzyme est inhibié par des anions pyrophosphatidiques et, à un moins degrée, sulfateux. Ces résultats démontrent que PP-PNK est une enzyme différent des autres kinases mammifères.

LIST OF ABBREVIATIONS

CNPase, 2', 3'-cyclic nucleotide 3'-phosphohydrolase

DTT, dithiothreitol

dNTPs, deoxynucleotide triphosphates

dT_{25}, oligodeoxythymidine 25

EDTA, ethylenediaminetetraacetate

FPLC, fast protein liquid chromatography

HPLC, high pressure liquid chromatography

kb, kilobase

kDa, kilodalton

LB plot, Lineweaver-Burk plot

LiCl, lithium chloride

MES, [N-Morpholino]ethanesulfonic acid

M. W, molecular weight

Mono Q, monoquaternary amine

mU, milli-units

NTPs, nucleotide triphosphates

PEI, polyethyleneimine

PP-PNK, polymin P-precipitable polynucleotide kinase

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

T4-PNK, bacteriophage T4 polynucleotide kinase

TAE, Tris-acetate EDTA

TBE, Tris-borate EDTA

TEMED, N, N, N', N'-Tetramethylethylene-diamine

TLC, thin layer chromatography

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INTRODUCTION

Reaction catalyzed by DNA kinase.

Mammalian DNA kinase is an enzyme that catalyzes the reversible transfer of a γ-phosphoryl group between a nucleoside triphosphate, usually ATP, and the 5'-OH terminus of a DNA chain (Zimmerman and Pheiffer, 1981). The reaction products are the nucleoside 5'-diphosphate and a 5'-phosphorylated DNA molecule.

$$ATP + 5'\text{-}OH\text{-}DNA \longrightarrow ADP + 5'\text{-}P\text{-}DNA$$

The reaction is routinely assayed in the forward direction by measuring the extent of transfer of the radioactive phosphoryl group from γ-^{32}P ATP to an acid insoluble form in the presence of a DNA acceptor containing 5'-OH termini (Zimmerman and Pheiffer, 1981). This assay should be used cautiously because other enzymes can transfer the terminal phosphoryl group of ATP to acid-precipitable acceptors that may be present in crude extracts (Zimmerman and Pheiffer, 1981). Other, more specific assays can be used employing relatively low molecular weight acceptors that are not acid precipitable, for example synthetic oligonucleotides (see Materials and Methods, assay A). In this case the extent of phosphorylation can be monitored by gel electrophoresis or thin layer chromatography analysis and autoradiography.

Bacteriophage T4 polynucleotide kinase.

DNA kinase activity was first observed in T2 and T4 phage-infected *E coli* (Novogrodsky and Hurwitz, 1966, Richardson, 1965). This 5'-OH polynucleotide kinase activity has a remarkable lack of specificity, both in the phosphate donor and in the recipient. All dNTPs and NTPs can serve as donors of the γ-phosphate group. DNA and RNA can serve equally well as the acceptors; oligonucleotides and nucleoside 3'-monophosphates are substrates as well (Richardson, 1981).

The T4 enzyme has been purified (Novogrodsky and Hurwitz, 1966, Richardson, 1965, Panet et al, 1973, Cameron and Uhlenbeck, 1977) and the gene cloned (Midgley and Murray, 1985). It encodes a 33kd polypeptide which in the functional protein forms a tetramer The T4 early gene pseT, which encodes the polynucleotide kinase, maps closely to the T4 DNA ligase, RNA ligase and T4 thymidilate synthase genes (Depew and Cozzarelli, 1974)
The polynucleotide kinase has an endogenous 3'-phosphatase activity (Cameron and Uhlenbeck, 1977), which is also a cyclic 2',3'-phosphodiesterase The enzyme catalyzes the hydrolysis of 3'-phosphoryl groups of deoxynucleoside 3'-monophosphates, deoxynucleoside 3', 5'-diphosphates, and of 3'-phosphoryl polynucleotides to yield inorganic orthophosphate and a 3'-hydroxyl group. The pH optimum for the kinase reaction is 7.6 in Tris-HCl buffer, and the enzyme requires Mg^{2+} ions for full activity (Richardson, 1981). The T4 polynucleotide kinase (T4-PNK) is strongly

inhibited by pyrophosphate and sulfate (Novogrodsky et al., 1966). In addition, the kinase reaction is reversible (Van de Sande et al., 1973, Chaconas et al., 1975). In the presence of ADP, a 5'-phosphoryl polynucleotide is dephosphorylated by T4-PNK to yield a 5'-hydroxyl terminated polynucleotide and ATP.

Searches for protein homologies have revealed significant homology between the polynucleotide kinase and the N-terminal region of adenylate kinase, an enzyme that catalyzes the phosphorylation of AMP by ATP (Midgley and Murray, 1985). Walker (Walker et al., 1982) has reported the presence of related sequences in enzymes that bind ATP or ADP, like ATP synthase, adenylate kinase, and other kinases, implying the presence of a common adenine nucleotide binding motif in all of these proteins. Another homology search by Koonin and Gorbalenya (Koonin and Gorbalenya, 1990) has revealed related domains between T4-polynucleotide kinase, yeast tRNA ligase (see below), T4 RNA ligase and 2', 3'-cyclic nucleotide 3'-phosphohydrolase (CNPase), a major protein in the mammalian nerve myelin sheath, suggesting a putative nucleotide binding motif in all these proteins.

Together with T4 RNA ligase and an anticodon endonuclease, the T4 polynucleotide kinase has been shown to reprocess host *E. coli* lysine tRNA in vivo (Amitsur et al , 1987). At first, a phage-induced nuclease cleaves specifically the bacterial lysine tRNA 5' to the wobble position, creating a 5'-OH and 2', 3' cyclic phosphate ends. T4 polynucleotide kinase is required to convert them into a 3'-OH and 5'-P pair that can be subsequently joined by RNA

ligase (Amitsur et al., 1987). Such cleaved, but not reprocessed host lysine tRNAs were observed in the *E coli prr* strains, which restrict polynucleotide kinase or RNA ligase phage mutants (Snyder, 1983). This is believed to be one physiological role of the phage enzyme.

In addition, phage mutants in T4-PNK activity have been reported which cannot grow in certain host strains (Sirotkin et al., 1978) Most mutations in the T4 pseT gene inactivate both the 5'-kinase and the 3'-phosphatase activities. A bacteriophage T4 mutant deficient in 3'-phosphatase activity has been isolated (Depew and Cozzarelli, 1974) which could not grow in the T4 sensitive *E coli* strain CTr5x. In these mutants a 50% reduction in the rate of T4 phage DNA synthesis was observed, together with a decrease in the length of the DNA product to half the mature length. These data suggest involvement of T4 polynucleotide kinase in DNA synthesis.

Moreover, *E. coli* mutant strains have been identified which restrict the growth of T4 phages mutant in T4-polynucleotide kinase activity but not of wild type phages (Cooley et al., 1979). These bacterial mutants are called *lit* mutants (*lit* stands for late inhibitor of T4), because the T4 late gene expression is defective in these strains. Late transcription of T4 phage was shown to be replication coupled (Riva et al., 1970), in addition T4-PNK is sometimes required in T4 late gene expression, possibly by helping establish the required intracellular DNA structure (Sirotkin et al , 1978) Furthermore, the defect seems to be associated with a gene product involved in DNA nicks or single stranded gaps (Cooley et al., 1979), suggesting that a similar enzymatic activity might be

present in bacteria.

Yeast tRNA ligase.

In *S cerevisiae*, a multifunctional protein, yeast tRNA ligase (Phizicky et al., 1986), has been reported to contain a polynucleotide kinase activity (Westaway et al., 1988). This enzyme is involved in tRNA splicing in yeast (Greer et al., 1983, Xu et al., 1990). Splicing of pre-tRNAs in yeast proceeds in two separable steps. An endonuclease cleaves specifically at the splice sites generating a 5'-half molecule with a 2', 3'-cyclic phosphate terminus and a 3'-half molecule with a 5'-OH group. Joining of the tRNA half molecules is carried out by the multifunctional yeast tRNA ligase in several steps. First, the cyclic phosphate of the 5'-half molecule is opened leaving a 2'-phosphate; then the 5'-OH group of the 3'-half molecule is phosphorylated, finally, the two tRNA half molecules are ligated together (Peebles et al., 1979).

The yeast tRNA ligase protein is a monomer of 90 kDa (Phizicky et al., 1986). It contains three dinstinct enzymatic activities. a polynucleotide kinase, a cyclic phosphodiesterase and an RNA ligase. These distinct activities have been correlated with different domains of the enzyme: the amino-terminal domain is required for adenylylation, the carboxyl-terminal region is sufficient for cyclic phosphodiesterase activity, and the central region is associated with kinase activity (Apostol et al., 1991). The yeast tRNA ligase gene has been cloned (Westaway et al., 1988) and shown to be essential for viability (Phizicky et al., 1992). Sequence similarities between the yeast enzyme, T4 RNA ligase

and to a lesser extent with T4 polynucleotide kinase have been reported (Apostol et al., 1991)

A similar enzyme has also been purified from wheat germ (Pick and Hurwitz, 1986). The wheat germ RNA ligase has an intrinsic 5'-OH polynucleotide kinase activity which can phosphorylate RNA as well as DNA, although RNA substrates are preffered The polynucleotide kinase activity has a broad alkaline pH optimum around 7 9 and required dithiothreitol and divalent cation for activity This enzyme is also involved in tRNA splicing in wheat germ.

A similar tRNA processing pathway has been demonstrated in extracts from Hela cells (Zillmann et al , 1991), suggesting that the mechanism of tRNA splicing is conserved among eukaryotes The hallmarks of this pathway are the incorporation of an exogenous phosphate group from ATP into the splice junction, and the formation of a 2'-phosphate at the splice junction However, the major tRNA splicing pathway in vertebrates is somewhat different from the yeast pathway in that the origin of the splice junction phosphate is not exogenous but endogenous (Zillman et al , 1991) This pathway was reported in Xenopus oocyte nuclei (Nishikura and De Robertis, 1981) as well as in Hela cell extracts (Filipowicz and Shatkin, 1983). Therefore, it appears that there are two dinstinct pathways of tRNA splicing in Hela cells, one of them being similar to the yeast pathway.

Mammalian DNA kinases.

DNA kinase activities from mammalian sources have been detected in rat

liver (Ichimura and Tsukada, 1971, Levin and Zimmerman, 1976, Teraoka et al., 1975), rat testis (Bosdal and Lillehaug, 1985), calf thymus (Austin et al., 1978, Tamura et al , 1981) and in Xenopus oocytes (Saiga and Higashinagawa, 1979) The rat liver enzyme has a narrow substrate specificity, confined to oligonucleotides greater than 10 residues in length, and to 5'-OH termini of DNA (Levin and Zimmerman, 1976). It has a molecular weight of around 80 kDa (Teraoka et al., 1975, Levin and Zimmerman, 1976), has an acidic pH optimum of 5.5, and requires a divalent cation for activity The Km for ATP was 2 2 μM and for DNase digested, 5'-OH terminated DNA was 35 5 μM It is strongly inhibited by pyrophosphate and sulfate anions with an $I_{0\,5}$ of 0 2 mM and 0.5 mM respectively (Teraoka et al , 1975) The rat liver enzyme has also been reported to contain a 3'-phosphatase activity (Habraken and Verly, 1983). (For a summary of the different DNA kinases see also Table 1)

The enzyme isolated from rat testis differed from the liver enzyme in the molecular weight (about 38 kDa estimated from gel filtration (Bosdal and Lillehaug, 1985) It also had different kinetic properties. the K_M for ATP was 36 μM and K_M for DNA was 2 μM The enzyme had an acidic pH optimum of 5.3 and it displayed similar inhibition patterns to the liver kinase.

Two reports for the calf thymus enzyme had reported slightly different properties. Austin et al (1978) reported an enzyme with a molecular weight of about 70 kDa that had an acid pH optimum (5.5). required a divalent cation for activity and could phosphorylate RNA but to a much lesser extent than DNA The reported Km for ATP was 3.9 μM. Pyrophosphate and sulfate inhibited the

enzyme activity although sulfate was much less inhibitory than for the rat liver enzyme. Tamura et al. (1981) reported a 54 kDa enzyme estimated by gel electrophoresis, that was also acid-dependent and required a divalent cation for activity However, it could not phosphorylate RNA The K_M for ATP was 4 μM and the inhibition patterns by sulfate and pyrophosphate were similar to the enzyme reported by Austin et al. The DNA kinases from mammalian sources show markedly different properties in comparison with the phage-induced polynucleotide kinase, such as a lower Km for ATP (2-4 μM), an acidic pH optimum (around 5 5) and limited substrate specificity

Another enzyme designated as an RNA kinase was isolated from Hela cells (Shuman and Hurwitz, 1979) This kinase is active primarily on RNA and had little activity on DNA The kinase requires a divalent cation for activity, has an alkaline pH optimum between 7 9-8.9 and was inhibited by pyrophosphate In the same report there is evidence for a DNA kinase activity which is physically separable from the RNA kinase, suggesting that two different enzymes are responsible for the phosphorylation of DNA and RNA in Hela cells

Biological role

The biological function of mammalian DNA kinase is not known The enzyme has been designated to have a role in DNA repair processes (Zimmerman and Pheiffer, 1981) Single-strand breaks in DNA where 3'-phosphate and 5'-OH termini are created by enzymatic or environmental factors have been shown to exist When the DNA of irradiated thymocytes was analyzed, about 11% of the termini in single-stranded breaks were found to

bear a 5'-OH terminus (Lennartz et al., 1975) Henner et al. (Henner et al., 1982) found 3'-phosphate termini in the strand breaks of DNA irradiated in vitro. In addition, endonucleases that generate 5'-OH and 3'-phosphate termini have been described, such as DNase II isolated from calf spleen (Bernardi, 1971), which is also present in most mammalian cells (Slor and Lev, 1971), and the Drosophila apurinic / apyrimidinic endonuclease I (Spiering and Deutsch, 1986). These 5'-OH and 3'-phosphate termini are dead ends in DNA metabolism because they are not substrates for DNA ligases, polymerases, or many exonucleases and are potent inhibitors of several of these enzymes (Goulian et al., 1968, Kornberg and Baker, 1992). Therefore, there is a need for such an activity to exist in order to make these DNA ends ligatable. T4 polynucleotide kinase and the rat liver DNA kinase both posses an inherent 3'-phosphatase in addition to the kinase activity (Cameron and Uhlenbeck, 1977, Pheiffer and Zimmerman, 1982). In theory, the combined action of a 3'-phosphatase and a 5'-kinase could result in the concerted transfer of the phosphate group from the 3'-side to the 5'-side of such a break (Habraken and Verly, 1986) However, when this was investigated for the rat liver enzyme; the reaction was found to proceed in two steps instead (Habraken and Verly, 1986). This 3'-phosphatase activity has not yet been demonstrated in the calf thymus and human DNA kinase enzymes.

In addition, there is evidence for involvement of the T4 polynucleotide kinase, which has an inherent 3'-phosphatase activity, in T4 DNA metabolism. Furthermore, studies in T4 mutants in this activity indicate involvement of the

enzyme in T4 DNA synthesis (see above) A strong implication for the physiological role of the enzyme comes from the participation of the kinase in bacterial tRNA processing (Amitsur et al., 1987), possibly to adapt the host tRNA levels to the phage's codon usage requirements (Amitsur et al , 1987)

The ability to phosphorylate RNA by some enzymes may well turn out to be an important physiological reaction. One can envision that such phosphorylation may be involved in the sequence of reactions for splicing during RNA processing (Shuman and Hurwitz, 1979), especially since the discovery of a yeast-like tRNA splicing pathway in Hela cells (Zillmann et al., 1991).

Mammalian DNA kinase has not been purified to homogeneity, nor have antibodies or cDNAs been developed. The objective of this research was to purify a bovine enzyme capable of 5' phosporylation of DNA These experiments provide an extensive characterization of a novel mammalian polynucleotide kinase and will lead to development of molecular probes for isolation of a cDNA encoding the DNA kinase gene.

TABLE 1. Enzymes with DNA kinase activity.

	T4 PNK	Yeast tRNA Ligase	Rat liver	Calf thymus	RNA kinase Hela cells
M.W.(kDa)	33(monomer) 140(tetramer)[1]	90[5] (monomer)	80[7] (monomer)	54[9] (monomer)	nd
pH Optimum	7.6[2]	7.5 - 8.0[5]	5.5[7]	5.5	7.9 - 8.9
Substrates	DNA,RNA Oligos, 3'-mono-nucleotides[3]	tRNA half molecules[6]	DNA, oligos >10b[7]	DNA[9] RNA<DNA	RNA[10] DNA<RNA
3'-phospha-tase	yes[4]	nd	yes[8]	nd	no
2',3' cyclic phospho-diesterase	yes	yes[5]	nd	nd	nd

nd: not determined.

References. (see list of references at the back for full citation).

1 Lillehaug, 1977
2 Richardson, 1965
3. Novogrodsky et al , 1966
4 Cameron and Uhlenbeck, 1977.
5. Phizicky et al., 1986.
6. Apostol and Greer, 1991.
7 Levin and Zimmerman, 1976.
8 Pheiffer and Zimmerman, 1982.
9. Tamura et al., 1981
10 Shuman and Hurwitz, 1979.

MATERIALS AND METHODS

DNA kinase enzyme assays

DNA Kinase Assay A. Oligo dT_{25} was purchased from the Sheldon Biotechnology Centre (Montreal, Quebec) 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 (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 characterization experiments, 100 mM Tris-HCl, pH 7 5 was substituted for 100 mM MES, pH 5 5 The reactions were incubated at 37°C for 20 min, then 0 5 μL 0 5 M EDTA was added The samples were treated once with 3 μL of resuspended Strataclean Resin (PDI Biosciences), vortexed for 15 sec, incubated at room temperature for 1 min and then centrifuged in a microfuge at 6000 X g for 1 min, according to the manufacturer's instructions The supernatants were carefully transferred to new Eppendorf tubes and 5 μl DNA sequencing stop buffer (98% deionized formamide, 10 mM EDTA, pH 8 0, 0.025% xylene cyanol FF, 0 025% bromophenol blue) was added, and the tubes were heated to 90°C for 3 min The samples were loaded onto a 20% polyacrylamide/7 M urea gel in 1 X TBE (8 cm x 10 cm x 0 75 mm) prepared in a MiniProtean II apparatus (Bio-Rad) and 180 V was applied until the bromphenol blue dye marker reached the bottom of the gel The gel was placed on filter paper, covered with plastic wrap, and exposed to Fuji X-ray film

at -70°C with two intensifying screens for 18 to 90 hours Activity was quantified by liquid scintillation counting of excised bands. In experiments with a positive control, 2 mU of T4 PNK (New England Biolabs) was used

DNA kinase Assay B. Preparations of pUC19 DNA were made using a Magic Maxiprep kit (ProMega) 20 μg of the purified DNA was cleaved with 400 units EcoRI (Pharmacia) in 1X one phor all buffer (Pharmacia) The digestion was verified by withdrawing 1μl aliquots, followed by 0 8% agarose gel electrophoresis in 1 X TAE buffer after addition of 6 X loading buffer (0 25% Bromophenol blue, 0 25% Xylene Cyanol FF, 30% glycerol) (Sambrook et al., 1989) The linearized plasmid was dephosphorylated with 3 units calf intestinal phosphatase (Boehringer-Mannheim, molecular biology grade) in 1 X CIP buffer for 30 min at 37°C Residual protein was removed with Strataclean resin. The DNA was ethanol precipitated in 0.3 M sodium acetate with 2 volumes of ice-cold ethanol (Sambrook et al., 1989), dried under vacuum in a Speedvac apparatus and redissolved in sterile water at a concentration of 1 μg/μl. 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 [^{32}P] ATP EDTA was added to stop the reactions, and they were processed with Strataclean as described for assay A The reaction products were precipitated with ethanol, redissolved in 10 μL sterile H_2O, and electrophoresed through an 0 8% agarose gel in 1 X TAE buffer in a QSH model electrophoresis unit (IBI) at 100 V until the lower dye had just run off the bottom of the gel. In some experiments, the gel was stained with 0.5 μg/mL ethidium bromide and

photographed. The gels were dried and exposed to X-ray film for at least 24 hours

Preparation of substrate for 3'-phosphatase assay. 5'-[^{32}P]Tp was obtained by incubating 0 8 μmoles thymidine 3'-monophosphate (Tp) (final concentration 8 mM) with 10 μCi [γ-^{32}P]-ATP, 20 μM unlabelled ATP, 10 μg BSA, 50 mM Tris-HCl pH 7 5, 10 mM $MgCl_2$, 2 mM DTT with 10 units T4 PNK (New England Biolabs) at 37°C for 20 min (reaction volume 100 μl) Immediately after, T4 PNK was inactivated by heating at 70°C for 10 min and the sample was treated twice with 10 μl of resuspended Strataclean resin as described before

For DNA kinase assays on thymidine 3'-monophosphate (Tp), the reaction was essentially as described above, except that 1 mM $MgCl_2$ was used and the enzyme fraction employed was the Blue-Sepharose pooled fractions concentrated with a Microcon 30 ultrafiltration unit (Amicon) Reaction products were analysed on polyethylenimine thin layer chromatography plates (Sigma) as described subsequently

3'-phosphatase assays. The formation of [5'-^{32}P]T from thymidine 3'-monophosphate (Tp)(Sigma) was measured The 25 μl reaction mixture contained 100 pmoles [^{32}P]Tp (final concentration 4 μM), 50 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 2 mM DTT and 2 5 μg of BSA (Cameron and Uhlenbeck, 1977). In some reactions, the pH was 6 4 in 40 mM imidazole-HCl After incubation at 37°C for 20 min, 1 μl aliquots were withdrawn and spotted on 20 X

20 cm PEI thin-layer chromatography plates (Sigma). The plates were developed in 1 M LiCl (Culver et al., 1993) for about 1.5 hrs, dried and exposed to Fuji X-ray film for autoradiography. The radioactivity of the pT and pTp regions was determined by liquid scintillation counting.

Protein quantitation. Protein was determined in Bradford assays (Bradford, 1976) with Coomassie blue protein assay reagent (Pierce) using the micro assay procedure. A standard curve was run for each set of protein assays. Absorbance was determined at 595 nm in a Beckman DU-64 spectrophotometer.

Protein Purification

The following is the standard procedure used to prepare Blue-Sepharose fractions that were used for characterization experiments My thesis research involved developing conditions for the Polymin P precipitation and the Q-Sepharose (see pilot experiments below), Heparin agarose and FPLC MonoQ steps.

Buffer A was 50 mM Tris-HCl, pH 7.5, 30 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA. Thymus from calves about 6 months old was obtained from Aliments Danac (LaPlaine, Quebec) and processed within 2 hours. The crude extract was prepared 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. Essentially every step described was carried out in the cold room unless specified differently 100 g of tissue were ground with 200 ml of extraction buffer (buffer A+0 3 M NaCl) for 30 sec in a Waring blender. The mixture was chilled on ice for 30 sec and ground for another 30 sec. In this way 600 g of tissue was extracted in a total volume of 1.2 L. The extract was stirred for 1 hr in the cold room and subsequently centrifuged at 8000g 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 minus 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% (vol/vol) The mixture was stirred for 30 min at 4°C, and centrifuged at 8000 g for 20 min at 4°C The supernatant was removed and the precipitated material stored at 4°C overnight. The pellets were washed with 200 mL of buffer A containing no NaCl, then 200 mL of buffer A containing 0 6 M NaCl. Preliminary 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 typical preparation described in Table 1. 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 (buffer A), 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 (Bio-Rad, type HT; 2 mg protein/mL) column pre-equilibrated in the same buffer (1 mM potassium phosphate). 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 were dialyzed against buffer A containing 10 mM potassium phosphate and passed through a 1 mL (10 mg protein/mL) phosphocellulose column. The flow-through and 2 mL of wash from the phosphocellulose column was applied to a 2 5 mL column of Blue-Sepharose (Pharmacia) with 4 mg protein/mL resin. A gradient of 5 column volumes was applied ranging from 30 mM to 1 M NaCl in buffer A. Active fractions eluted at about 600 mM NaCl and were pooled. The activity in these fractions was stable for several months when stored on ice in a 4°C refrigerated cabinet. This protocol was carried out twice by myself together with Carolyn Slack (technician), and twice by Carolyn Slack to get purified protein for characterization experiments.

The Blue Sepharose pooled fractions were dialyzed against buffer A at pH 7.0 and loaded onto a MonoQ (size HR 5/5,Pharmacia) column connected to an HPLC system (Waters). The column was washed with the starting buffer (buffer A) and a linear salt gradient from 30 mM up to 1 M was applied. The flow rate was maintained at 0.5 ml/min and 48 fractions of 0.5 ml each were collected.

Protein was monitored through a Waters 484 absorbance detector tuned at 280 nm. DNA kinase activity eluted late in the gradient.

Pilot experiments. In a pilot experiment, the Q-Sepharose pooled fractions were dialyzed against buffer A and subsequently loaded onto a single-stranded DNA-agarose column (Gibco-BRL). The column was washed with the starting buffer and 4 increasing salt step elutions were applied : 0 1, 0 25, 0 5 and 1 M NaCl. In other resin investigations, the Blue Sepharose pooled fractions were dialyzed against buffer A and loaded onto an ATP-agarose column (Sigma). The resin was washed with 2 ml of the same buffer until the protein concentration reached backround levels in a Bradford assay, and the same increasing salt step elutions were applied. The collected fractions were concentrated 10-fold using Amicon 30 ultrafiltration units (Amicon) and then assayed for DNA kinase activity. Activity was detected in the wash fraction. Another resin tested was Heparin-agarose (Sigma) Again the Blue Sepharose pooled fractions were dialyzed and the same procedure as for the ATP-agarose was followed.

Experiments to optimize polymin P conditions. In preliminary experiments, the 0.5% Polymin P pellet was divided in 4 parts and protein was eluted with 200 ml of 4 different salt concentrations of buffer A : 0, 0.25, 0.5, 1 M NaCl. These experiments established the optimal salt concentration for extraction of the bulk of DNA kinase activity from the Polymin P pellet.

Protein characterization

pH optimum For pH profile experiments, assays were carried out at seven different pH pcints starting at pH 4.7 up to pH 10.0 : 4.7, 5.6, 7 0, 7.5, 8 7, 9.6, and 10 0 For the two lower points 100 mM MES buffer was used; for the rest 100 mM Tris base was used, with the pH appropriately adjusted with concentrated HCl. The values for pH are for 100 mM buffer at room temperature.

Identity of the phosphorylated product Two types of experiments established the nature of the phosphorylated oligonucleotide. In the first, T4-kinase phosphorylated oligo dT_{25} was prepared under standard conditions for Assay A at pH 7.5, with an excess of cold ATP (100 μM) and 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.). This 5'-phosphorylated oligonucleotide was then tested in assay A with the Blue-Sepharose fraction In addition, a tube with oligo dT_{25}, but without T4-PNK was carried along each step as a control. In other experiments, radiolabelled, phosphorylated product (1 μg) was incubated at 37°C for 16h with snake venom 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 the optimal pH of 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 μM up to 50 μM In ATP-dependence experiments, the ATP concentration was varied from 0 μM up to 500 μM, and the ^{32}P ATP from 0 01 μCi up to 3.72 μCi, in order for the specific activity of ATP in each reaction to remain constant. All inhibition experiments were carried out in 100 mM Tris-HCl, pH 7.5. In addition, the amount of ^{32}P ATP per reaction was doubled. To observe the effect of the magnesium concentration, the $MgCl_2$ concentration in the reactions was varied from 0 mM up to 100 mM; nine different concentrations were assayed. In salt inhibition experiments, six concentration points of NaCl or KCl were assayed, from 0 to 500 mM In other inhibitory assays, 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 In the EDTA inhibition experiment, the EDTA concentration was varied from 0 up to 100 mM.

Sedimentation Gradient Analysis. 5-20% linear sucrose gradients were prepared in thin walled polyallomer centrifuge tubes (Beckman), using a Hoefer SG 15 small gradient maker (Hoefer Scientific) as described by the manufacturer. Samples of Blue Sepharose-purified material (0 1 mL, 20 μg) were applied on top of 4.7 mL of a 5-20% sucrose gradient prepared in buffer A

plus 100 mM NaCl. The gradients were centrifuged for 20 h at 39,000 rpm in an SW55 rotor at 4°C in a Beckman L8-70 Ultracentrifuge The gradients were fractionated into 0.2 mL fractions by puncturing a hole at the bottom of the tube with a piercing unit (Kontron Instruments) and collecting the drops (Martin and Ames, 1961). 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 described by Martin and Ames (1961). Migration of PP-PNK was compared to standards purchased from Pharmacia (catalase (11.3 S), aldolase (7.8 S) bovine serum albumin (4.4 S), ovalbumin (3 5 S), and chymotrypsinogen (2.5 S)). Standards were detected by SDS-polyacrylamide gel electrophoresis followed by Coomassie blue staining.

Recovery Experiment - Microcon 100. 10 µl of the Blue-Sepharose pooled fractions were mixed with 90 µl of 20% sucrose solution and left overnight at 4°C Concurrently, a control tube was set up in 0% sucrose and carried along. The next day the tubes were centrifuged for 15 min at 3000g in a microfuge at 4°C using a Microcon 100 ultrafiltration unit (nominal molecular weight cut off : 100 kDa). The concentrated samples as well as the filtrates were assayed for DNA kinase activity using assay A.

SDS-PAGE Analysis

SDS-polyacrylamide gels (8 cm X 10 cm X 0.75 mm) were prepared in a Mini Protean II apparatus (Bio-Rad) as described in the manufacturer's instruction manual. The separating gel consisted of 10% acrylamide (37.5 : 1 acrylamide :

bis), 0.375 M Tris-HCl, pH 8.8, 0.1% SDS, 0.05% ammonium persulfate, 0.05% TEMED The gel was allowed to polymerize for 30-60 min at room temperature with a layer of water-saturated butanol on top. The stacking gel was 5% polyacrylamide (37.5 : 1 acrylamide : bis), 0 125 M Tris-HCl pH 6 8, 0 1% SDS, 0.05% ammonium persulfate, 0 05% TEMED The gel was allowed to polymerize for 1 hr at room temperature 8 μl of 3 X SDS reducing buffer (0.188 M Tris-HCl, pH 6.8, 6% (w/v) SDS, 0.1 M DTT, 3% glycerol, 0.0075% bromophenol blue) was added to 16 μl of sample The samples were heated at 95°C for 4 min and centrifuged at 6000Xg for 3min before loading onto the gel. Electrophoresis was carried out at 100 V in 25 mM Tris, 250 mM glycine, 0 1% SDS at pH 8 3 until the bromophenol blue dye had just run off the bottom of the gel. The gels were stained in Coomassie blue for at least 1 hr and destained in 40% methanol, 10% acetic acid until the background was clear. Then gels were kept in 5% methanol, 5% acetic acid until they were dried in a gel drier (Biodesign Inc.) The molecular weight standards used were broad range SDS-PAGE Standards (Bio-Rad), (14.5 kDa-200 kDa) or Rainbow markers (Amersham), (14.3 kDa-200 kDa) in renaturation experiments.

Renaturation Experiments after SDS-PAGE.

10% SDS-polyacrylamide gels were prepared as described above The Mono Q fraction 35 concentrated with a Microcon 30 unit was used in these experiments. In other experiments the Blue-Sepharose side fractions (the active fractions flanking the peak kinase activity) concentrated with Microcon 30

were used. Samples were prepared as described above, except that they were heated to 40°C for 10 min. 2 µl of Rainbow molecular weight markers (Amersham) were run on both sides of the samples. Immediately after electrophoresis, the band of interest (major band around 50 kDa) was excised from the gel and immediately washed in a 50 ml polypropylene conical tube (Diamed) with 20 ml of 50 mM Tris-HCl, 50 µg/ml gelatin with mild agitation at room temperature for 20 min. As a positive control, the T4-PNK band was excised and washed in the same way The wash was repeated 3 times, and the gel slices were stored in 50 mM Tris-HCl, pH 7 5, 0.5 mM DTT at 4°C overnight. The next day, the slices were incubated for 1 hr at room temperature in 50 mM Tris-HCl, pH 7 5, 0.5 mM DTT with mild agitation (Ohmura et al., 1986). After that, the gel slices were put in Eppendorf tubes together with 50 µl of DNA kinase assay mix and were crushed using a plastic pipette tip. The gel slices were assayed for DNA kinase activity in 50 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$ as described in assay A previously, except that the incubation time was increased to 1 hr at 37°C.

In another experiment, 4 gel slices were cut out that approximately covered the whole molecular weight range from 35-70 kDa, from the Blue-Sepharose pooled fractions Five lanes of the sample were run, for a total of 20 gel slices. Each slice of the same molecular range was washed in 5 different buffer conditions: a) 50 mM Tris-HCl, pH 7.5; b) 50 mM Tris-HCl, pH 7.5, 50 µg/ml gelatin, c) as in b + 0.5 mM DTT; d) as in b + 1 µM ATP; e) as in b + 1 mM $MgCl_2$. After the wash step, the gel slices were stored overnight in their

respective buffers, without gelatin. The next day, the slices were incubated for 1 hr at room temperature with the respective buffer without gelatin. Subsequently, they were assayed for DNA kinase activity as described above.

Assays for contaminating enzymatic activities.

Endonuclease activity was assayed by incubating 1 μg of pUC19 supercoiled DNA under assay A conditions for 4 hr followed by Strataclean treatment of the DNA and analysis on a 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. Assays were carried out as described in the manufacturer's instruction manual

RESULTS

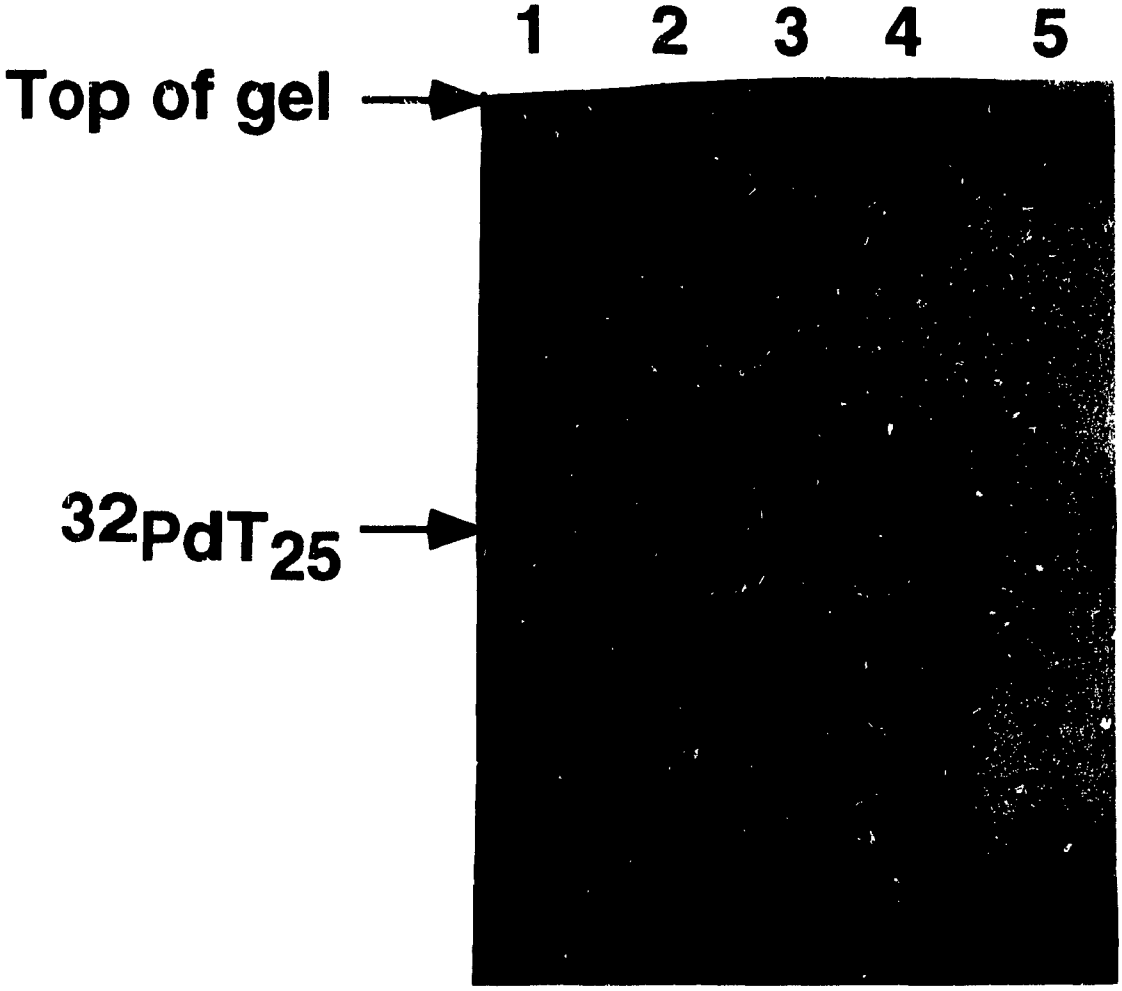
Identification of a Polymin P-precipitable polynucleotide kinase (PP-PNK) activity in calf thymus.

Whole cell extracts of calf thymus were fractionated by the addition of polymin P (polyethyleneimine) to 0 5% as described in Materials and Methods. In preliminary experiments protein was sequentially eluted from the Polymin P pellet with 4 increasing salt concentrations: 0, 0.25, 0.5 and 1 M NaCl. 0.03% of the total protein applied to the Polymin P came off in the 0 M eluate, 0 13% in the 0.25 M elution, 0.22% in the 0 5 M and 0.56% in the 1 M salt elution. No DNA kinase activity was detected in the 0, 0 25 M eluates, whereas substantial activity was detected in 8 μg of protein assayed from the 0 5 M elution and a small amount of activity was observed in the 1 M NaCl elution (Fig. 1) In subsequent preparations a 0 6 M elution was used. This concentration was used in the standard purification protocol.

In other experiments, calf liver extracts prepared the same way as the thymus, were precipitated with 0 5% Polymin P and protein was eluted from the pellet with the same salt concentrations. 0.57% of the total protein came off in the 0.25 M elution, 0.80% in the 0 5 M and 1.5% in the 1 M salt elution. No DNA kinase activity was detected in 10 μg of protein assayed from each salt elution from the Polymin P pellet of calf liver extract.

Figure 1. Optimization of Polymin P conditions.

Elution of DNA kinase activity from the 0.5% Polymin P pellet of the calf thymus crude extract. Lane 1: 3 units T4 Polynucleotide kinase; lane 2: 0.5 M NaCl eluate of the Polymin P pellet (8 μg); lane 3: 1 M NaCl eluate (8 μg); lane 4: 0.6 M eluate from another extract preparation (8 μg); lane 5: buffer control. No significant activity was detected after elution with 0 M or 0.25 M NaCl buffer. The radioactivity at the bottom of the gel is probably ATP that did not run out of the gel.


1
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3
4
5
Top of gel
$^{32}PdT_{25}$

Pilot purification experiments.

As part of the research toward this thesis, resins were tested that could be incorporated into a large scale purification protocol, such as Q-Sepharose, single stranded DNA-agarose, ATP-agarose, Heparin-agarose and FPLC MonoQ HR5/5. The PP-PNK activity bound to the Q sepharose column, eluting at about 0 5 M NaCl in step gradients or linear gradients The PP-PNK eluted after the protein peak, making this anion exchange resin a valuable purification step. PP-PNK activity did not bind tightly to single-stranded DNA agarose nor to ATP-agarose, in both cases it came out in the 30 mM NaCl wash steps In addition, these two resins did not retain any significant amount of protein, therefore it was concluded that they were not useful purification steps PP-PNK bound to Heparin-agarose, eluting in the 250 and 500 mM elution steps, while about 30% of the protein loaded came out in these fractions This resin was incorporated into the purification procedure for preparations IV and V (see fig 2). As expected from the Q-Sepharose results, all of the protein loaded bound to FPLC Mono Q column Four combinations of pH and salt gradient were tested The high resolution of the FPLC column resulted in samples that were not highly heterogeneous as assesed by SDS-PAGE (Fig. 3)

Figure 2. The standard purification procedure as described in the Results section.

PURIFICATION PROCEDURE

Calf thymus

(1) Crude Extract

(2) 0.5% Polymin P pellet

(3) Elute proteins with 0.6M NaCl

(4) Phosphocellulose at 1M NaCl

(5) Q - Sepharose (gradient)

(6) Hydroxyapatite (step elution)

(7) Phosphocellulose *

(8) Blue Sepharose

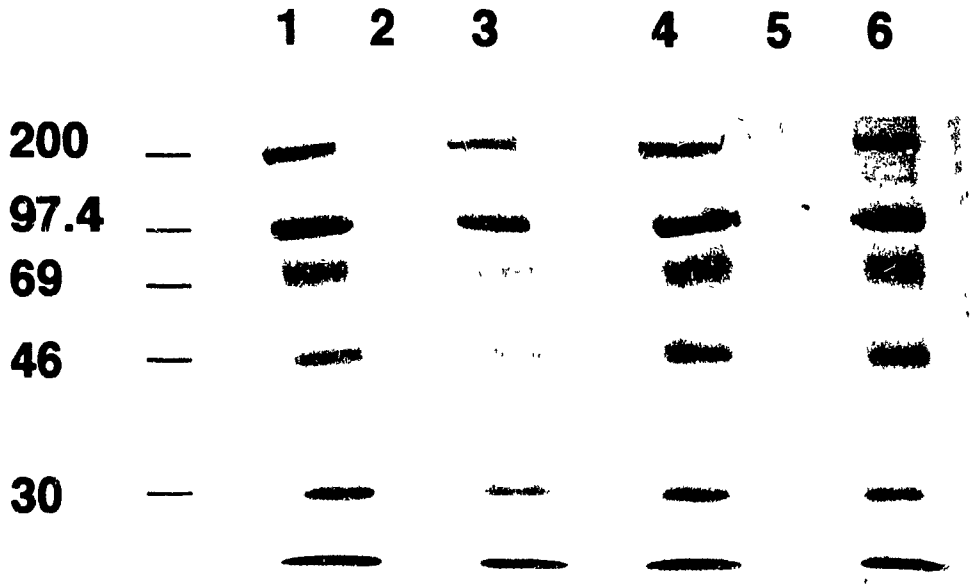
(9) Heparin - Agarose *

(10) HPLC Mono Q *

* Not done for some preparations

Figure 3. 10% SDS-PAGE analysis of the MonoQ fraction from preparation IV (see Table 2).

10% SDS-polyacrylamide gels were prepared as described in Materials and Methods. 16 μl of the MonoQ fraction were loaded on lane 5. Lane 1, 3, 4, 6 : Rainbow markers; lane 2 : 2 μl (20 units) T4-PNK. The gel was stained with Coomassie stain, destained and dried. The darker mark on the right-hand side of the gel is due to an air bubble that was caused by drying the gel.


1 2 3 4 5 6
200
97.4
69
46
30

Standard purification protocol of the Polymin-P precipitable enzyme.

After passage through a phosphocellulose column to remove residual Polymin P, the protein preparation was applied to a Q-sepharose column. PP-PNK activity eluted from the Q-sepharose column was applied to further columns by Carolyn Slack Elution from hydroxyapatite occurred in the 150 mM potassium phosphate step. The activity bound eluted as one peak late in the gradient on Blue-Sepharose chromatography. The activity did not bind (eluted in the flow-through) when applied to a phosphocellulose column preequilibrated with Buffer A. After the five purification steps, the specific activity increased 1500-fold above that in the polymin P pellet (a preparation typical of five purifications is summarized in Table 2). The yield was difficult to quantify precisely because it was not possible to measure activity in a crude extract. Samples were stable for at least 3 months when stored at 0°C with a protein concentration of at least 100 μg/mL. SDS-polyacrylamide gel analysis of the protein preparations revealed that the Blue-Sepharose fraction was heterogeneous, with more than 10 polypeptides present (data not shown). In preparations IV and V, the Blue-Sepharose fraction was further purified over Heparin agarose and MonoQ (HR5/5). The specific activity of these fractions appeared to increase but was not directly compared because Bradford assays would have required a significant proportion of the sample

The active MonoQ fraction from preparation IV was analyzed on SDS-PAGE and then stained with Coomassie stain; this showed a major protein

Table 2. PURIFICATION TABLE

Sample	Total Protein	Total Activity	Specific Activity	Yield (%)	Fold-Purification
(1) Crude Extract	33.5 g	--	--	--	--
(2) Polymin P Eluate/ Phosphocellulose Flow-through	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 4 determinations differing by less than 20%. When assayed at pH 7.5, the specific activity of the Blue Sepharose fraction was 40% higher.

band of about 50 kDa (see Fig. 3) and about 3-4 other faint bands. This major band was cut out and assayed for DNA-kinase activity in renaturation experiments (see below)

Substrate specificity of PP-PNK.

Other substrates in addition to dT_{25} were investigated. Another oligonucleotide (a 21mer of the following sequence: TTTATCCAGATTATAGGCCAT) was a substrate for PP-PNK. 5'-OH EcoRI-cleaved, dephosphorylated pUC 19 was also tested under assay B conditions. The double-stranded DNA molecule with 5'-OH 4-base overhangs was phosphorylated, although an increased amount of T4-PNK or PP-PNK was required for this reaction (fig 4)

A mononucleotide, thymidine 3'-monophosphate was also tested. This substrate was not efficiently phosphorylated under Assay A experimental conditions, with no product formed that was detectable when reaction products were analyzed on polyethyleneimine TLC plates (Fig. 5). The lower limit of detection of this analysis was 0.2 pmoles ^{32}P / 20min.

3'-Phosphatase assays.

The Blue-Sepharose fractions (2.2 μg) concentrated with a Microcon 30

Figure 4. Phosphorylation of an EcoRI digested, double-stranded DNA fragment.

EcoRI digested, dephosphorylated plasmid pUC19 as a substrate. DNA kinase assays were done as described in Materials and Methods (Assay B). Lane 1: buffer control; lane 2. 10 units T4-PNK; lane 3: 150 mM hydroxylapatite fraction (2.8 μg).

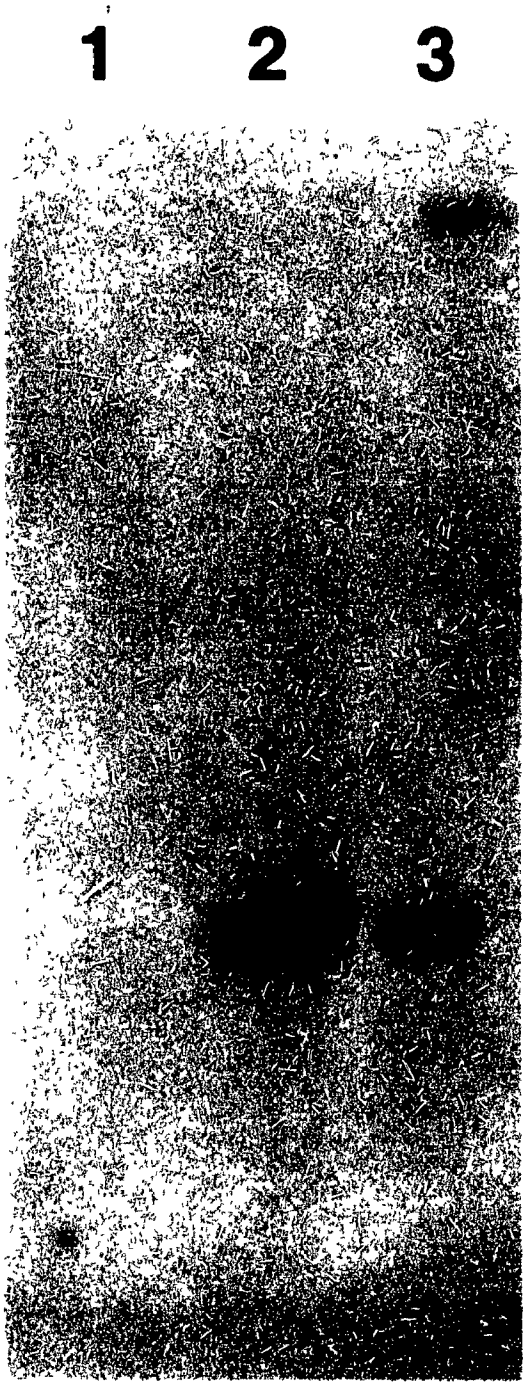
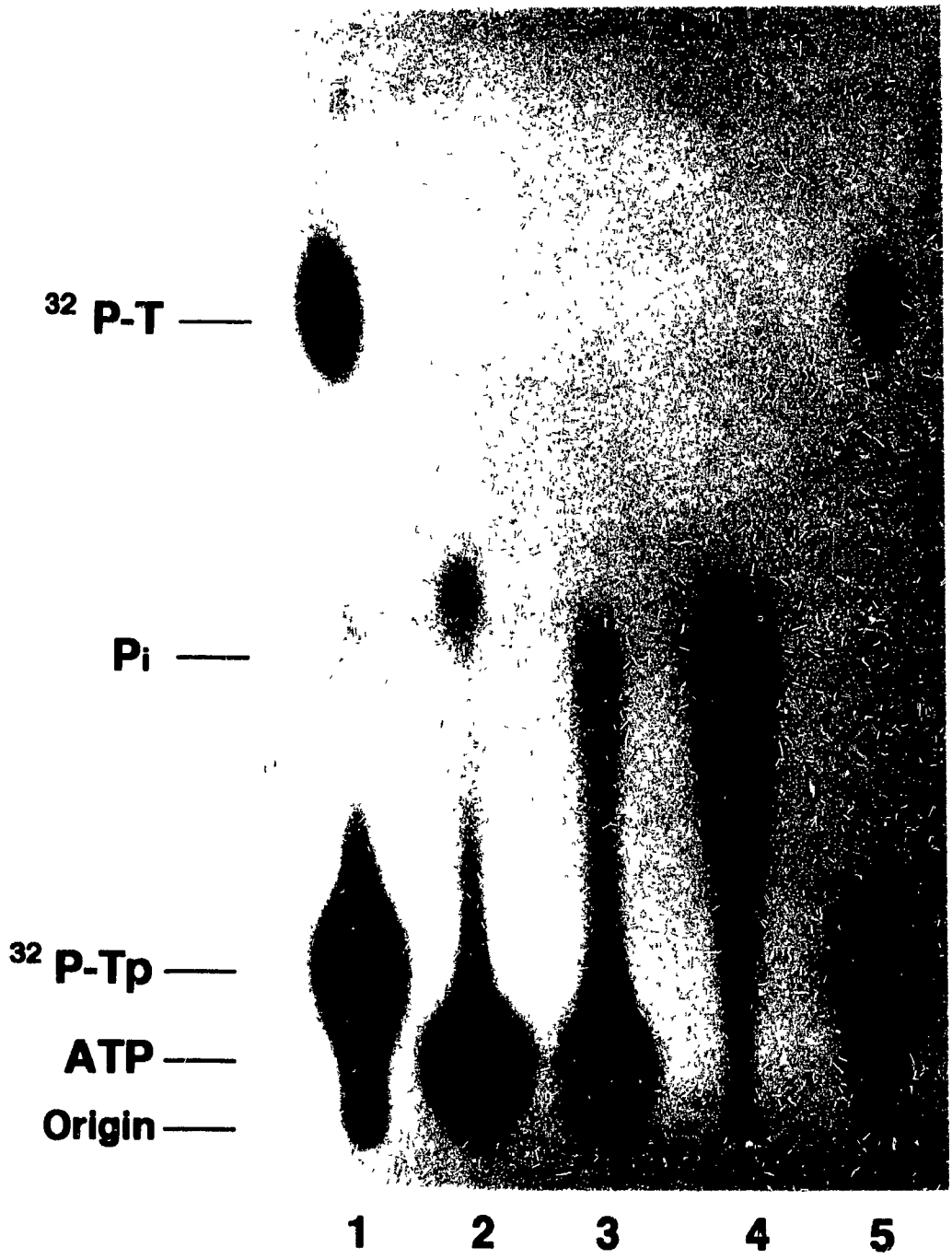

1
2
3

Figure 5. Kinase assay on Thymidine 3'-monophosphate (Tp).

The Blue-Sepharose fraction was tested for its ability to phosphorylate a nucleoside 3'-monophosphate (Tp). Reactions were as described in Materials and Methods. After 20 min at 37°C, 1 μl was withdrawn and spotted on a Polyethylenimine thin layer chromatography plate The plate was developed and exposed to film for autoradiography. Lane 1 : ^{32}PTp produced with 10 U T4-PNK; lane 2 : Tp incubated with the Blue-Sepharose fraction (36 mU, 4 4 μg) ; lane 3 : γ-^{32}P ATP; lane 4 : ^{32}Pi; lane 5 : ^{32}PTp
The upper spot in lanes 1 and 5 is ^{32}PT produced by the inherent 3'-phosphatase activity of T4-PNK on ^{32}PTp.


^{32}P-T
Pi
^{32}P-Tp
ATP
Origin
1
2
3
4
5

ultrafiltration unit were the fractions employed in these assays (18 mU). 3'-phosphatase assays were carried out at two different pH points 6.4 and 7.5 TLC analysis did not reveal any significant 3'-phosphatase activity (fig 6) on the particular substrate used (5'-^{32}PTp). (In any case, it was not higher than the limit of detection of the assay, which was 0.02 pmoles ^{32}P.) As a positive control, the same amount of units of T4-PNK showed a significant amount of 3'-phosphatase activity (0.45 pmoles ^{32}P released) Therefore, any 3'-phosphatase activity of this preparation was at least twenty fold lower than an equivalent amount of T4-PNK

Phosphorylated oligonucleotide product analysis.

^{32}P-phosphorylated oligonucleotide was produced using the Blue-Sepharose pooled fractions This was digested with snake venom phosphodiesterase (Dolapchiev et al., 1974) and the digestion product was analyzed on a 20% polyacrylamide/7 M urea gel (fig 7) In parallel, ^{32}P-phosphorylated oligo with T4-PNK was also digested under the same conditions. The digestion was complete in both cases (that is, to components that comigrated with ATP on gel electrophoresis) It is known that T4-PNK phosphorylates at the 5'-end of a polynucleotide (Novogrodsky and Hurwitz, 1966, Richardson, 1981). In addition, snake venom phosphodiesterase is known to attack preferentially 3'-OH terminated oligos, the presence of a 3'-monophosphate renders the oligonucleotide much less susceptible to hydrolysis by the phosphodiesterase (Laskowski, 1971). Therefore the added

Figure 6. 3'-Phosphatase assay on ^{32}PTp.

The substrate was incubated with 18 mU of the Blue-Sepharose fraction concentrated with a Microcon 30 ultrafiltration unit (One kinase unit is defined as the amount of enzyme needed to incorporate 1 nmol ^{32}P on the 5'-hydroxyl terminus of the oligonucleotide substrate in 20 min at 37°C). Reactions were as described in materials and methods Lane 1 · buffer control (no enzyme); lane 2· Blue-Sepharose at pH 6 4, lane 3 Blue-Sepharose at pH 7.5, lane 4 . T4-PNK (18 mU), pH 6.4; lane 5 ^{32}PT, lane 6 : ^{32}Pi T4-PNK is known to have 3'-phosphatase activity on nucleotides, hence it was used as a positive control. ^{32}PTp was prepared using T4-PNK under empirically determined conditions that minimized dephosphorylation by the inherent 3'-phosphatase. The substrate was deproteinized.

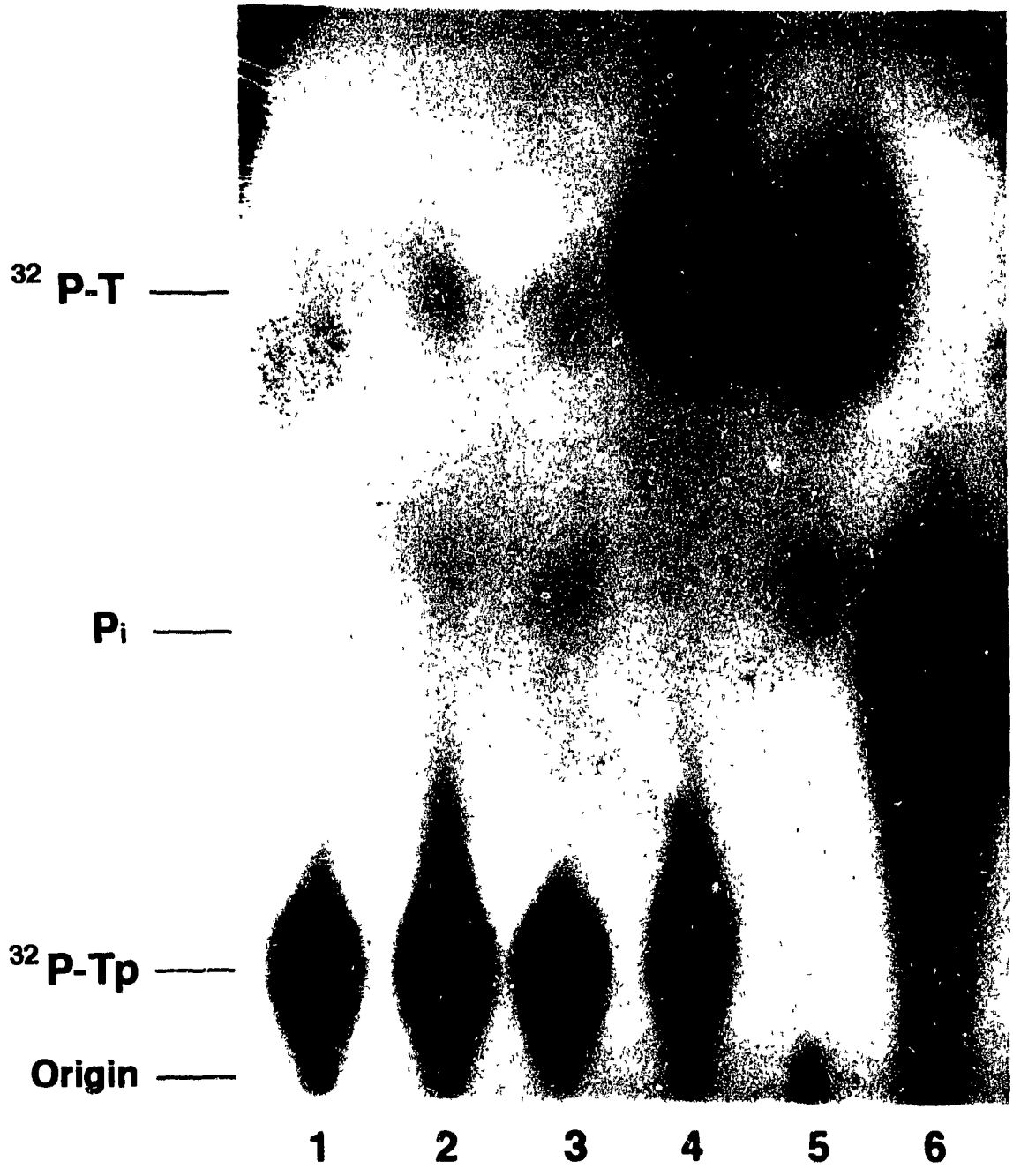

^{32}P-T
Pi
^{32}P-Tp
Origin
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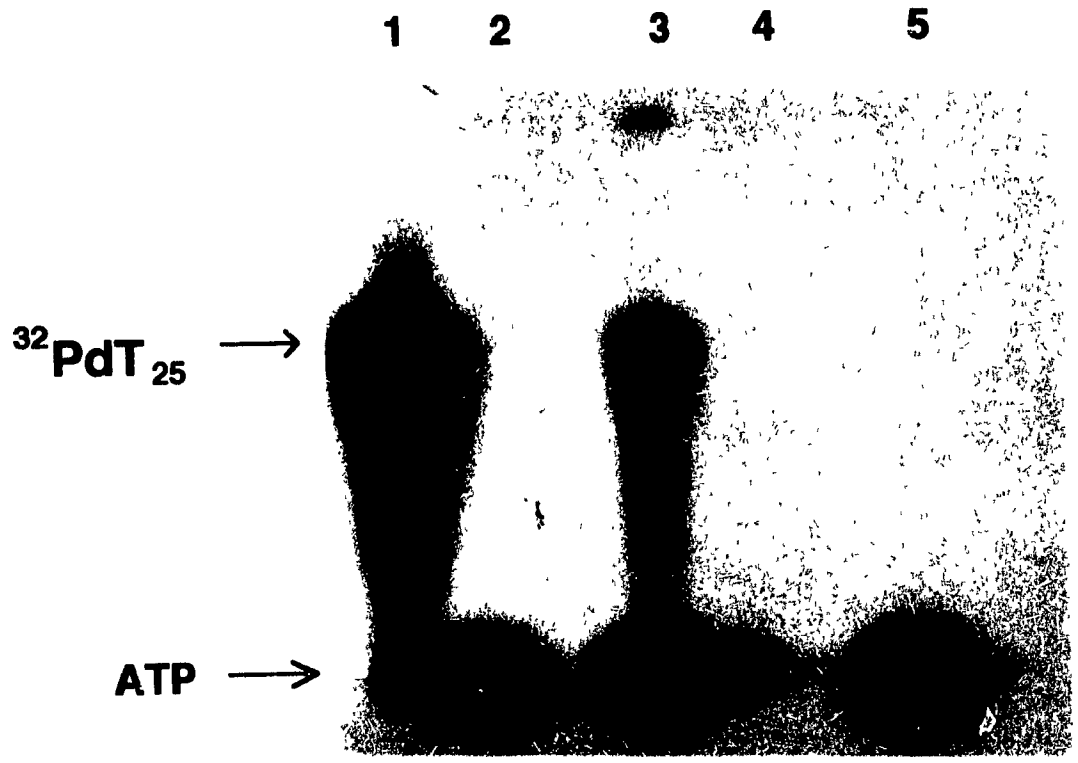
Figure 7. Snake Venom Phosphodiesterase digests of the phosphorylated product.

T4-PNK and PP-PNK phosphorylated oligo dT_{25} were prepared as described in Materials and Methods. Subsequently, they were subjected to digestion by snake venom phosphodiesterase, a 3'-exonuclease, and reaction products were analyzed on a 20% polyacrylamide/7 M urea gel Lane 1: T4-PNK phosphorylated oligonucleotide; lane 2: venom phosphodiesterase digested T4-PNK oligo, lane 3: oligo dT_{25} phosphorylated with the Blue-Sepharose fraction; lane 4: venom digest of lane 3, lane 5. an equal amount of T4-PNK-phosphorylated oligo dT_{25} and PP-PNK phosphorylated dT_{25} digested with snake venom phosphodiesterase. The radioactivity at the bottom of the gel are probably digestion products that can either be the monomer or slightly bigger size. Six times as many counts were subjected to digestion for the T4 phosphorylated oligo than for the PP-PNK oligo.


1
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$^{32}PdT_{25}$
ATP

phosphate is unlikely to be on the 3'-terminus of the oligo dT_{25}.

In order to confirm the nature of the phosphorylated oligonucleotide, another experiment was designed. Oligonucleotide 5'-phosphorylated by T4-PNK was then subjected to a kinase assay using the Blue-Sepharose fraction. The rationale behind this experiment was that if the 5'-OH terminus of the oligonucleotide is already occupied by a "cold" (non-radioactive) phosphoryl group transferred by T4-PNK, then one should not be able to see any radioactive incorporation on that terminus by the PP-PNK preparation Indeed, the previously 5'-phosphorylated oligonucleotide was not detectably phosphorylated by PP-PNK (fig. 8), although the untreated control was a substrate, indicating that the site of phosphorylation is indeed the 5'-terminus. The faint band that was visible when the phosphorylated oligonucleotide was incubated with T4-PNK (fig. 8, lane 5), may have been due to the phosphate exchange reaction catalyzed by T4-PNK, between the γ-phosphate group of ATP and the 5'-phosphoryl group of a polynucleotide (Van de Sande et al., 1973). In this experiment, PP-PNK and T4-PNK were indistinguishable in their ability to phosphorylate 5'-phosphorylated oligo dT_{25} Taken together, the above results provide strong evidence that PP-PNK is phosphorylating the 5'-OH terminus of oligo dT_{25}.

pH profile of PP-PNK.

When the pH of the buffer was varied (Fig. 9) using an oligonucleotide

Figure 8. PP-PNK tested for ability to phosphorylate 5'-PdT_{25}.

The T4-PNK phosphorylated oligonucleotide was tested in Assay A at pH 7.5 with the Blue-Sepharose fraction and also T4-PNK Lanes 1,2 and 3 . 5'-OH oligo dT_{25} was used; for lanes 4, 5 and 6 phosphorylated oligo dT_{25} was used as a substrate for the kinase reaction. 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.

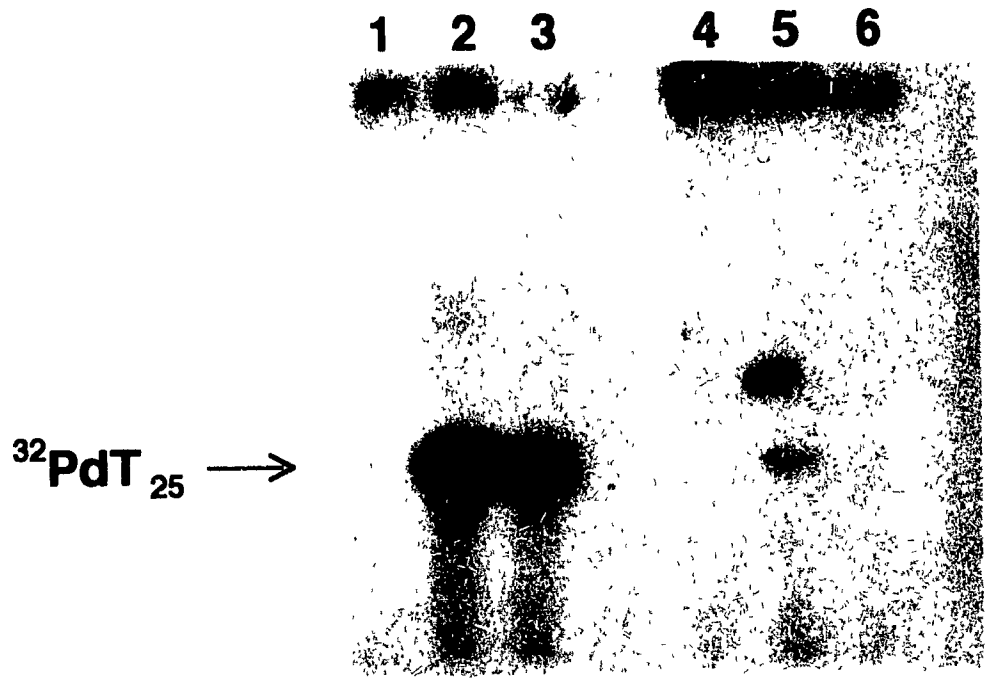
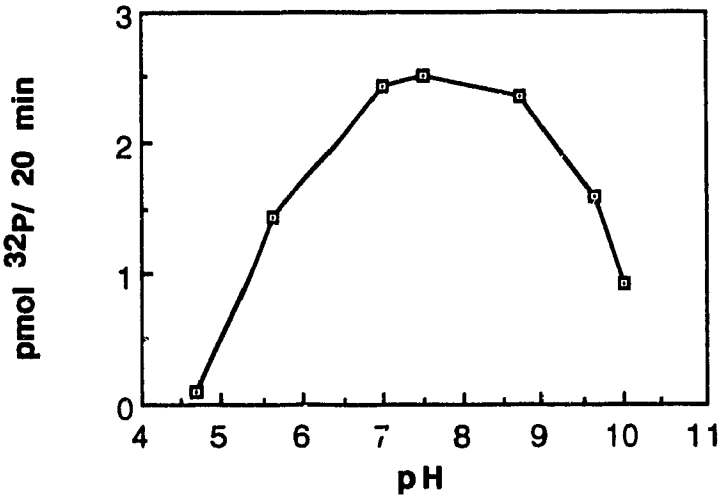

1 2 3
4 5 6
$^{32}PdT_{25}$

Figure 9. pH profile of PP-PNK.

The enzyme activity was determined using assay A. 7 different pH points were assayed : 4.7, 5.6, 7.0, 7.5, 8.7, 9.6 and 10 0. For the two lower points MES buffer was used; for the rest, Tris-HCl was used. The values given for pH are for 100 mM buffer at room temperature Each pH point was assayed in triplicate and the results were averaged The range variation between the three replicates was typically 15%. The graph was generated by connecting the mean of the replicates using the Cricket Graph program.


pmol 32P/ 20 min
0
1
2
3
4
5
6
7
8
9
10
11
pH

substrate, the enzyme displayed activity over a broad pH range, with greatest activity at neutral to alkaline pH (pH 7-9) The enzyme was active at all pH points tested, with the least amount of activity at pH 4.7 (4% of maximal activity) and the highest activity at pH 7.5. When assayed at pH 10, the enzyme displayed 40% maximal activity.

Requirements for activity.

The reaction components required for activity were studied, revealing an absolute requirement for ATP and 5' OH oligo dT_{25}. Omission of DTT resulted in 60% 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+} (Fig 10). Activity did not vary much at the concentration points assayed (from 0-10 mM), with 78% of the maximal activity detected at 0 mM and 80% at 10 mM [Mg^{+}] 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.

Kinetic studies.

Preliminary experiments were carried out in order to determine the linear range for assay A. As shown in figure 11, the assay is linear at the 20 min time point that was chosen for the kinetics. Kinetic experiments were carried out at pH 7.5, within the optimal range for this enzyme. When the concentration of ATP was varied, Lineweaver-Burk plots revealed an estimate of the apparent

Figure 10. $MgCl_2$ dependence.

DNA kinase assays were as described in assay A, except that reactions were in 100 mM Tris-HCl, pH 7.5 and the $MgCl_2$ concentration was varied from 0 mM up to 10 mM. Each concentration point was done in triplicate. The average of the replicates were used in the graph. Variation about the mean was less than 15%. The fraction employed was the Blue-Sepharose pool In another experiment, the $MgCl_2$ concentration was varied up to 100 mM.

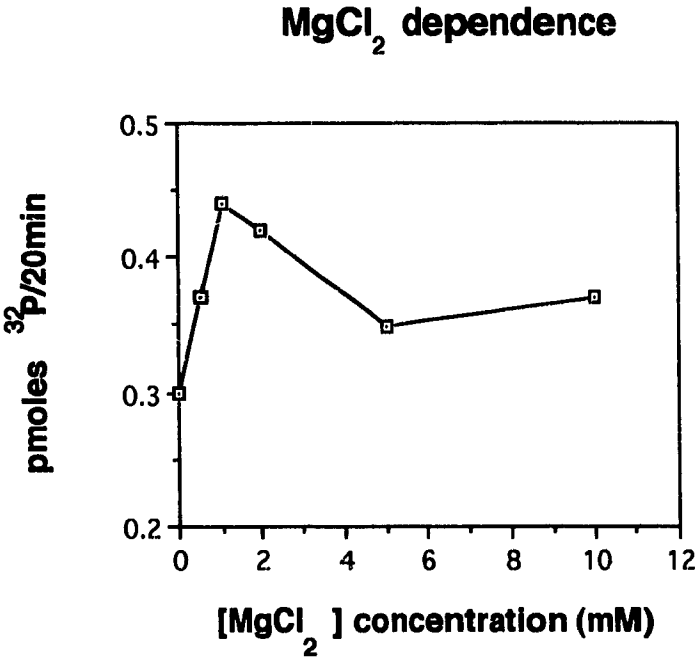
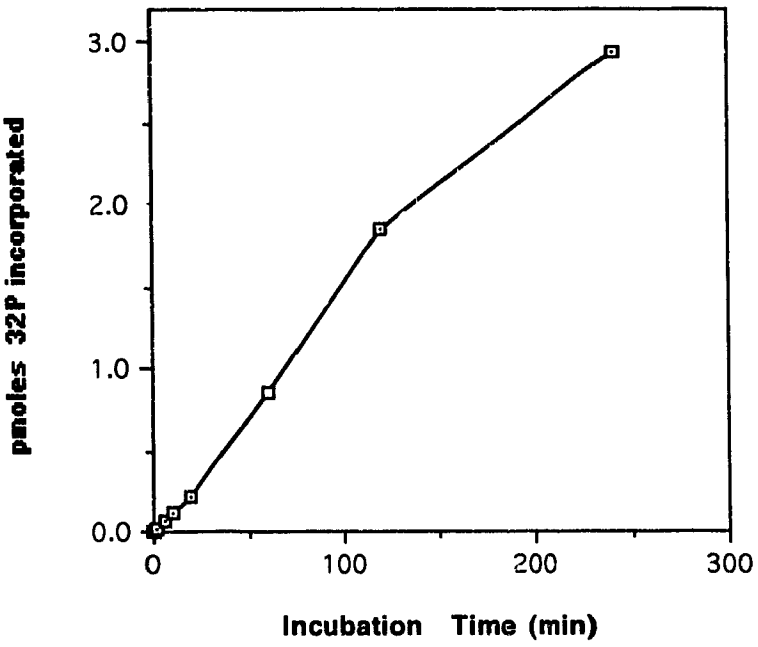

MgCl2 dependence
0.5
0.4
0.3
0.2
pmoles 32P/20min
0
2
4
6
8
10
12
[MgCl2] concentration (mM)

Figure 11. Time course of 5'-phosphorylation.

The linearity of the kinase reaction of PP-PNK was investigated using assay A at pH 7 5 in 100 mM Tris-HCl The enzyme fraction employed was the Blue-Sepharose pool. Seven time points were assayed in triplicate. Reactions were stopped by immediate addition of EDTA and 0.5 μl 10% SDS followed by heating at 75 C for 10 min. Results were quantitated by liquid scintillation counting of the excised bands. The mean of the triplicates was used in the graph. Variation about the mean was typically less than 8%.

Time course of 5'-phosphorylation



K_M of 52 $\pm$ 11 μM (Figs. 12A, 13A). An estimate of 8 $\pm$ 3 μM was obtained for the apparent K_M for oligo dT_{25} (Figs. 12B, 13B). The lines were determined by using the curve fit linear regression of the Cricket graph program (CA Associates) run on a Macintosh SE 30 computer.

Inhibition studies.

The effects of various anions and salts on enzyme activity were determined (Figs 14, 15) Pyrophosphate, phosphate and sulfate anions were added to the assay as described in Materials and Methods and quantitation of the results revealed a strong inhibition with pyrophosphate (50% reduction in activity at 2.2 mM NaPPi) (see Table 3) In contrast, phosphate and sulfate were less inhibitory: 11 and 8.5 mM for 50% inhibition respectively (Fig. 14 and Table 3). To confirm that the inhibition was specific for the sulfate anions and not due to the cation of the salt, two different sulfate salts were used ammonium sulfate and sodium sulfate. Indeed, sodium sulfate was as effective as ammonium sulfate (Table 3). In addition, activity was detectable in as much as 0.5 M salt, though greatly diminished (Fig.15).

Sedimentation Analysis.

Blue-Sepharose fraction PP-PNK was applied to a 5-20% linear sucrose gradient made in buffer A plus 0.1 M NaCl. The gradients were centrifuged and fractionated as described in Materials and Methods. A typical activity profile of the Blue-Sepharose fraction on a 5-20% sucrose gradient is shown in Fig. 16.

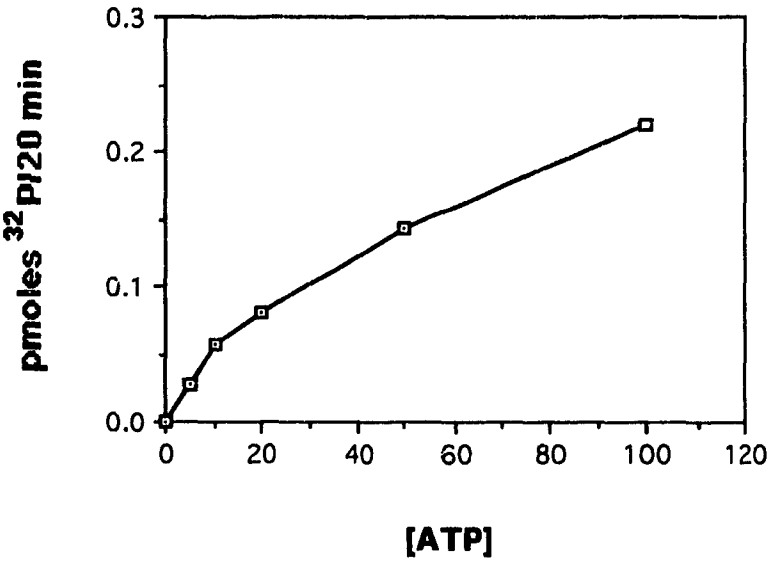
Figure 12. A. ATP dependence.

Assays were done as described in Materials and Methods. The ATP concentration was varied from 0 μM up to 100 μM. The amount of radioactivity was adjusted so that the specific activity of ATP remained constant for each reaction Each concentration point was done in triplicate. Variation about the mean was typically less than 20%

B. dT_{25} dependence.

Assays were done as before except that the ^{32}P-ATP per reaction was doubled, the $MgCl_2$ was 1 mM and the oligo dT_{25} concentration was varied from 0 μM up to 40 μM Each point was done in duplicate. Variation about the mean was typically less than 9%. The curves were generated by connecting the mean of the replicates using the Cricket graph program.

A. ATP dependence



B. Oligo dT25 dependence

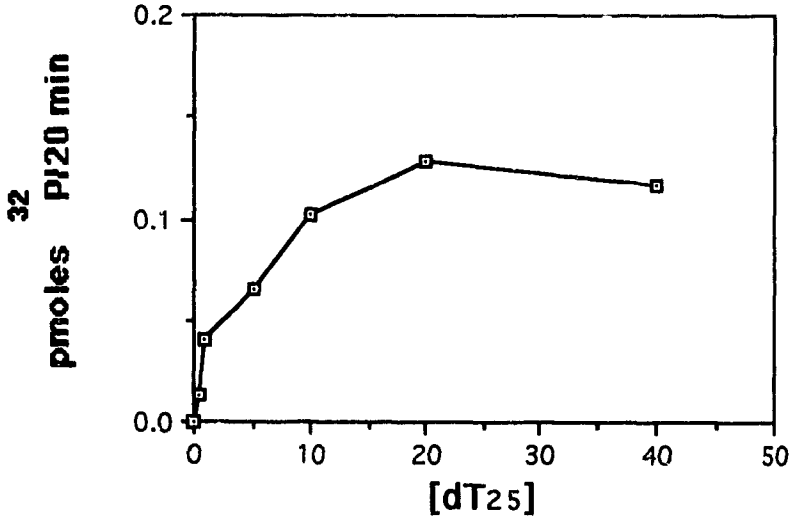


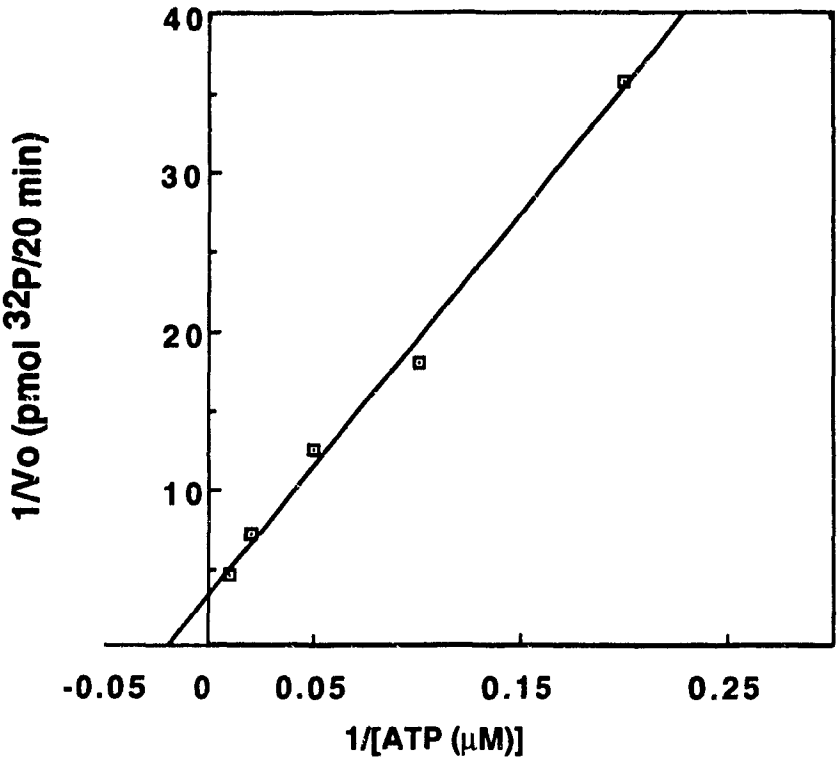
Figure 13. A. Lineweaver-Burk plot for ATP.

Data points were from figure 5A. Km for ATP calculated from the double reciprocal plot shown here was 50 μM. The mean of 3 determinations was 52 -- $\pm$ 11 μM.

B. Lineweaver-Burk plot for oligo dT_{25}.

Data points were from figure 5B. Km calculated from this plot was 6.8 μM. The mean of 3 determinations was 8.0 $\pm$ 3.7 μM. The lines were generated by linear regression, using the simple curve fit feature of Cricket graph.

A.


40
30
20
10
1/Vo (pmol 32P/20 min)
-0.05
0
0.05
0.15
0.25
1/[ATP (μM)]

B.

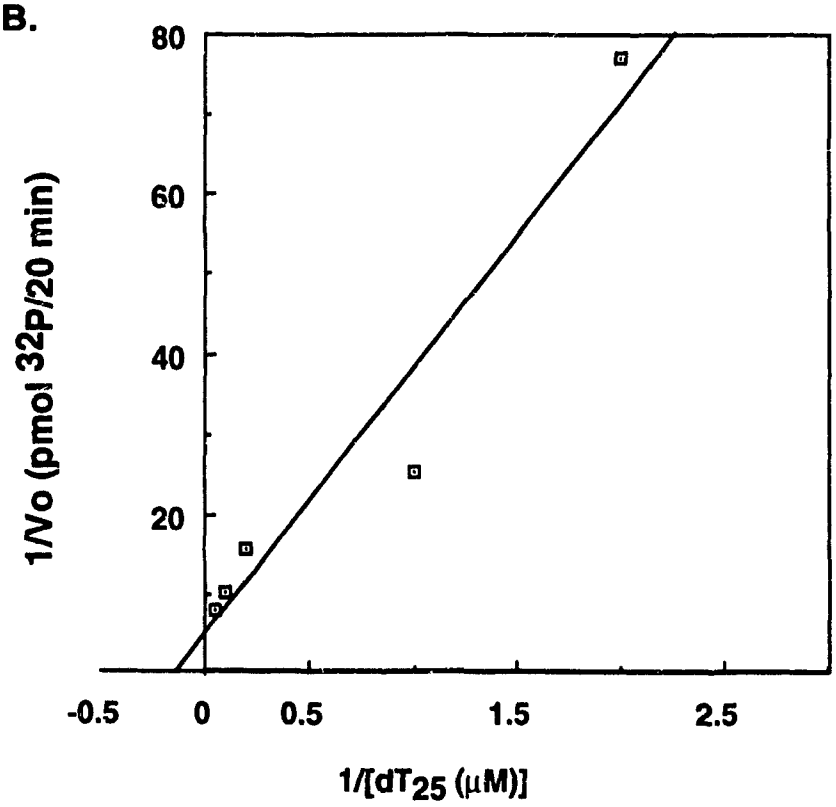
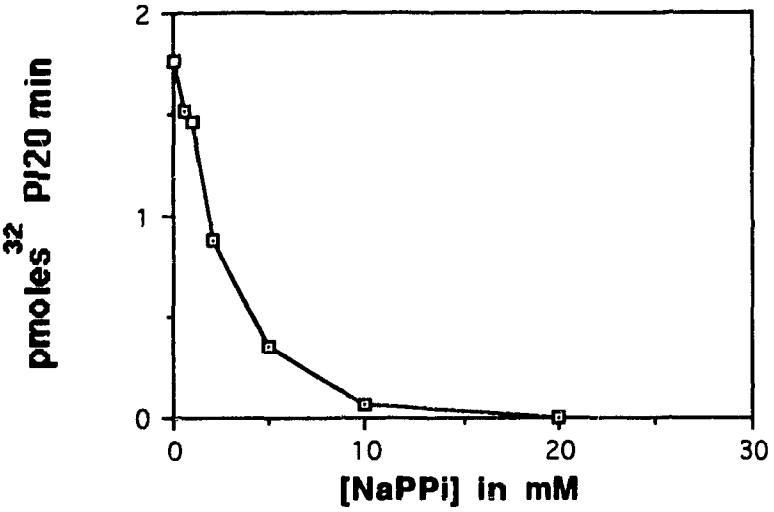

80
60
40
20
1/Vo (pmol 32P/20 min)
-0.5
0
0.5
1.5
2.5
1/[dT25 (μM)]

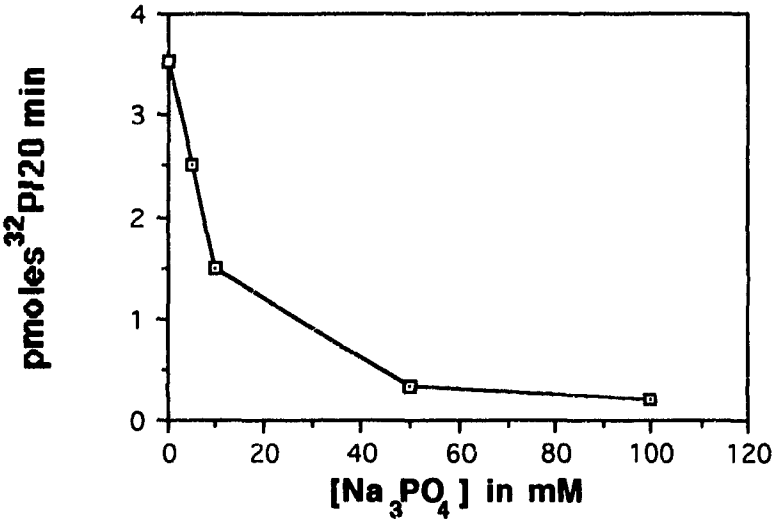
Figure 14. Inhibition of kinase activity by A. Sodium pyrophosphate, B. Sodium phosphate and C. Ammonium Sulfate.

The effect of certain anions on the DNA kinase activity in the Blue-Sepharose fraction was investigated using assay A. Reactions were as described in Materials and Methods except that increasing amounts of sodium pyrophosphate, sodium phosphate, ammonium sulfate was added to the reactions Each point was done in duplicate The products were quantitated by excision of the bands and liquid scintillation counting, and the average was used in the diagrams. Range variation between the replicates was typically less than 15%.

A. Sodium pyrophosphate course



B. Sodium Phosphate course



C. Ammonium Sulfate Course

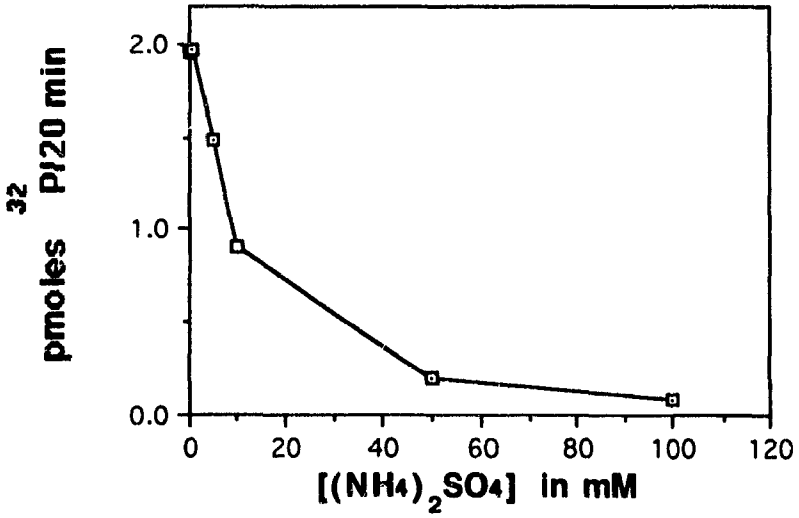
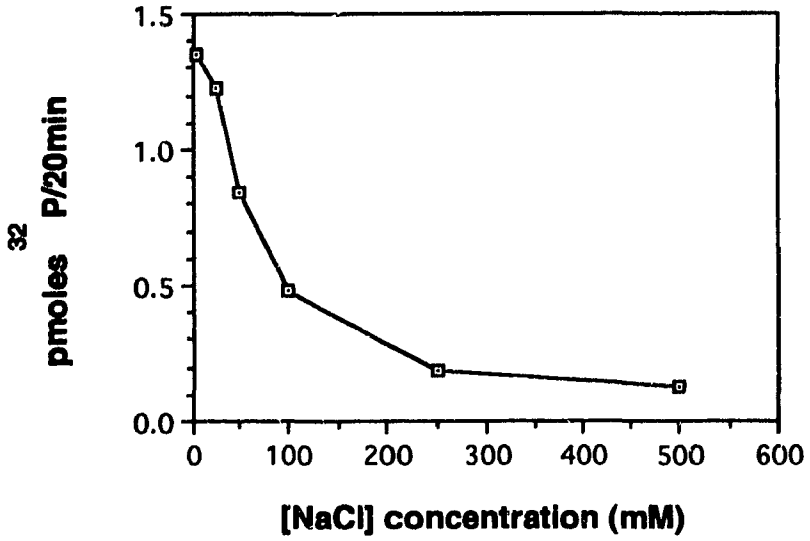


Figure 15. Effect of salt concentration on kinase activity.

The Blue-Sepharose fraction was employed. Assays were as described before except that increasing amounts of sodium chloride or potassium chloride was added to the reactions Each point was done in duplicate and the quantitated results were graphed. Variation about the mean was typically about 7%.

NaCl Course



KCl Course

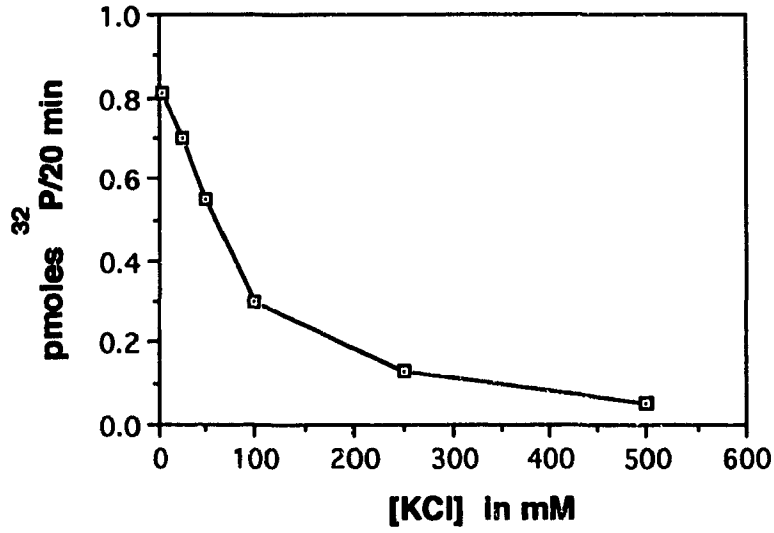


TABLE 3

INHIBITORS OF PP-PNK

Compound	Concentration for 50% inhibition of activity
Sodium Pyrophosphate	2.2 mM
Sodium Phosphate	11 mM
Ammonium Sulfate	8.5 mM
Sodium Sulfate	5.7 mM
Sodium Chloride	67 mM
Potassium Chloride	71 mM

The concentrations for 50% inhibition were obtained by interpolating the individual plots to 50% maximal activity. For the sodium pyrophosphate inhibition, the values from three separate experiments were averaged (2.2 mM, 2 mM and 2.5 mM). For the sodium phosphate, two separate experiments were averaged (8.6 mM, 13.8 mM).

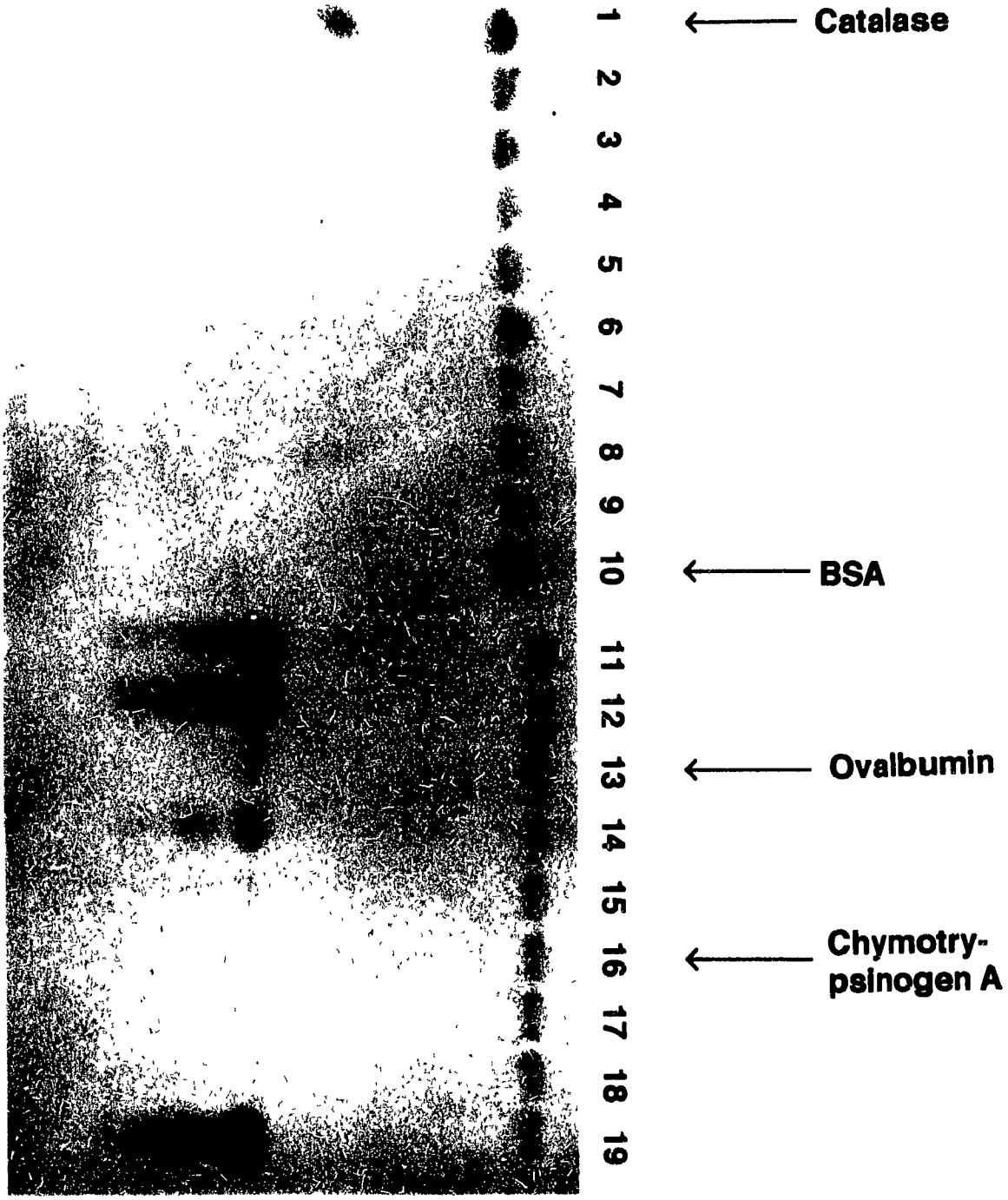
Four different standard proteins with known $S_{20,w}$ values were used as external markers: catalase, bovine serum albumin, ovalbumin and chymotrypsinogen A. A standard curve was constructed by plotting the distance migrated in the gradient by the standards versus their known $S_{20,w}$ values. PP-PNK migrated similarly to ovalbumin (molecular weight : 45 kDa) with an estimated sedimentation coefficient of 3.8 S by comparison with the standards (Martin and Ames, 1961). When analyzed by SDS-PAGE, the peak fractions from a sedimentation experiment contained ~10 polypeptides ranging in size from 35 to 70 kDa. Three independent experiments gave sedimentation values between 3.4-3.8 S. Although the activity in fraction 13 appears to be diminished (Fig. 16), possibly giving the appearance of another peak at fraction 14, two other experiments showed one clear peak at fraction 12. 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.

Recovery experiment with Microcon 100.

To investigate the possible structure of PP-PNK, 10 μl of the Blue-Sepharose fraction was incubated overnight with 0.1 M NaCl buffer at 4°C for 20 hr, in the presence or absence of 20% sucrose, and subsequently passed through a Microcon 100 ultrafiltration unit. About 10% of PP-PNK activity was retained by the membrane; however, the rest (90%) of the activity went through in the filtrate. In the presence of 20% sucrose, the distribution of DNA kinase activity in the retentate and eluate was the same.

Figure 16. PP-PNK activity profile in a sucrose density gradient.

The Blue-Sepharose fraction was layered on top of 5-20% sucrose gradients, then the gradients were centrifuged at 39,000 rpm for 20 hrs. The gradients were fractionated from the bottom of the tube and the fractions were concentrated with Microcon 30 ultrafiltration units (this step also permitted the removal of sucrose from the fractions). Subsequently the fractions were assayed for DNA kinase activity using assay A in 50 mM Tris-HCl, pH 7 5, 1 mM $MgCl_2$ with double the amount of radioactivity. 18 fractions were assayed, the numbers on the top of the lanes represent fraction numbers starting from the bottom of the gradient. Lane 19 is the load. The peak of PP-PNK activity came out in fraction 12.

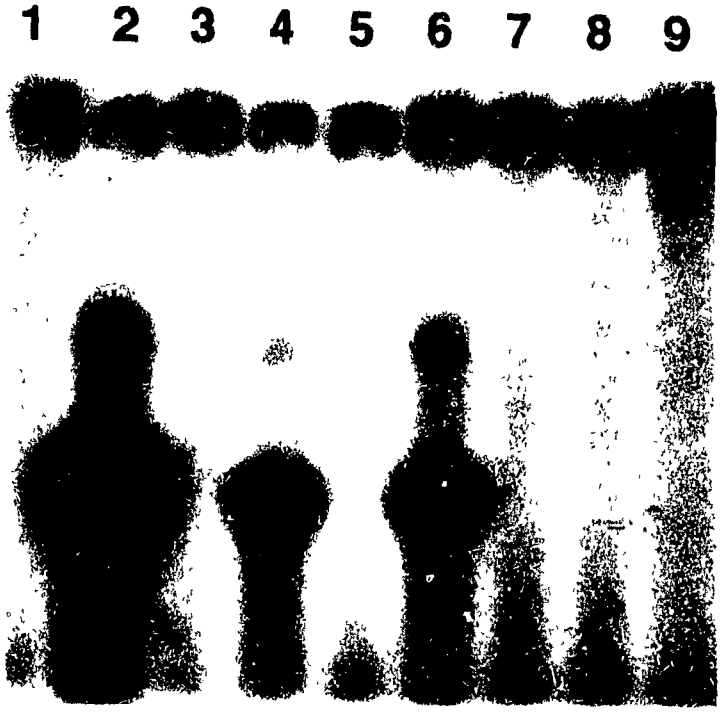

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Catalase
BSA
Ovalbumin
Chymotry-
psinogen A

Renaturation Experiments.

The concentrated MonoQ active fraction, which displayed a prominent Coomassie blue-stained band when analyzed on a SDS-protein gel, was used in these experiments. The Blue-Sepharose concentrated fraction was also used in some experiments (the same region around 45-55 kDa was excised) In addition, the same amount of units of T4-PNK was run on another lane as a positive control, the ~33 kDa band was cut out and tested These gel slices were renatured and tested for DNA kinase activity using the assay A as described in Materials and Methods T4-PNK did indeed renature under the conditions used, but only when an increased amount of T4-PNK was used (10 units). The recovery for T4-PNK after the renaturation step was only about 0 1% of the input amount of activity. Neither the MonoQ nor the Blue-Sepharose fraction showed any signal under the renaturation conditions used (Fig 17). When the same amount of units of T4-PNK was used, the corresponding band that was cut out and renatured did not show any activity. Another factor that could have possibly affected this experiment is the renaturation conditions. The conditions employed were the ones reported by Ohmura et al. that succesfully renatured the acidic DNA kinases from calf thymus and rat liver as well as T4-PNK in the activity gel approach. Furthermore, a set of 5 different renaturation buffer conditions were tried in another experiment without any detectable recovery of kinase activity The possibility that the band is not the right one was also tested in an experiment where 4 slices covering the whole 35-70 kDa

Figure 17. Activity renaturation experiments after SDS-PAGE.

16 µl (4 mU) of the MonoQ fraction concentrated with Microcon 30, were run on a 10% SDS-PAGE with Rainbow markers on both sides of the lane. In another lane 38 mU of the concentrated Blue-Sepharose fractions were loaded. The prominent protein band was excised from the MonoQ lane by comparison to the standards, washed and renatured as described in Materials and Methods Then the gel slice was tested in assay A with a 5-fold increase in the reaction volume As a positive control, the same amount of units of T4-PNK was run on another lane of the SDS gel, washed with the same conditions and carried along the assay Lane 1 buffer control, lane 2: Blue-Sepharose fraction (1 µl, 2.4 mU), lane 3 gel blank (a slice from the gel was cut and carried along the same steps as a control), lane 4. MonoQ fraction concentrated, lane 5· 38 mU of T4-PNK (after gel renaturation), lane 6 10 units of T4-PNK band after renaturation, lane 7. MonoQ band after renaturation, lane 8: Blue-Sepharose band (45-55kDa) after renaturation; lane 9: gel blank.


1 2 3 4 5 6 7 8 9

region of the SDS-polyacrylamide gel were assayed under 5 different renaturation conditions, though no signal was detected for any of the slices

Contaminating enzymatic activities.

Assays for alkaline and acid phosphatase activities did not reveal any significant activity. Endonuclease assays revealed no significant activity, either. The Blue-Sepharose fraction was used for these assays. There was no detectable AMP-labelling activity in a MonoQ fraction from preparation II that was active in assay A (D. Lasko, personal communication). Poly(ADP)-ribose polymerase was not detectable in the Blue-Sepharose fraction as determined by immunoblotting with a monoclonal antibody (J. Bramson, personal communication). Mammalian 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNPase) activity was at background levels and an antibody to CNPase did not cross-react with the Blue-Sepharose purified PP-PNK (Enoch Gao, personal communication).

DISCUSSION

In this thesis, the identification and characterization of a Polymin P-precipitable DNA kinase (PP-PNK) from calf thymus is described The Polymin P precipitation efficiently concentrated PP-PNK into the pellet. Polymin P has been used successfully for purifying a variety of DNA-binding proteins (Burgess, 1991).

A direct comparison was made between calf thymus and calf liver for presence of DNA kinase activity. Calf thymus glands were chosen as the source for Polymin P-precipitable DNA kinase, the Polymin P pellet from calf liver did not show any detectable DNA kinase activity Moreover, calf thymus is known to be an active site of DNA synthesis and recombination, therefore a good source of DNA metabolism enzymes. Thus, purification from this tissue was pursued

The polymin-P precipitable polynucleotide kinase (PP-PNK) identified in calf thymus extracts was the major activity in the Polymin P pellet Other DNA kinase activities were also detected in the Polymin P supernatant, after further processing of the protein preparation (Carolyn Slack, personal communication) One of these activities elutes differently from PP-PNK on Q-Sepharose and appears to have an acidic pH optimum. This may represent the previously described DNA kinase from mammalian sources.

PP-PNK displayed different chromatographic properties from the

previously described DNA kinases. First of all, it did not bind to phosphocellulose. All the reported DNA kinases as well as many other DNA-binding proteins bind strongly to phosphocellulose. Surprisingly, it was not retained on ATP-agarose, since the enzyme uses ATP as the phosphate donor, although only one type of ATP-agarose was tested. In addition, PP-PNK did not bind strongly to single-stranded DNA-agarose, another perhaps unexpected result for an enzyme that is able to phosphorylate DNA as a substrate in vitro. It is possible that other conditions such as the addition of $MgCl_2$ would have resulted in binding to these resins. Hydroxyapatite and Blue-Sepharose steps have been used for the purification of the DNA kinase from calf thymus previously (Tamura et al., 1981). However, their enzyme did not bind to hydroxyapatite in contrast to PP-PNK; it also eluted earlier in the salt gradient (at about 0 2 M) in Blue-Sepharose than PP-PNK. Taken together, the above results indicate that the enzyme described herein has different chromatographic properties than the previously reported DNA kinases.

In pilot studies, the Q-Sepharose resin was found to be a valuable purification step since PP-PNK bound to this resin, eluting after the protein peak The enzyme also bound strongly to Heparin-agarose, indicating an affinity for this particular polymer.

The enzyme was identified by its ability to phosphorylate a synthetic oligonucleotide dT_{25} When the substrate specificity of the enzyme was further investigated, a different synthetic oligonucleotide was also a substrate for PP-PNK. The enzyme was also able to phosphorylate a 2 7 kb, double-stranded

DNA molecule with 5'-overhangs. However, thymidine 3'-monophosphate was not detectably phosphorylated, thus making PP-PNK different from T4-PNK in this respect It is interesting to note that no mammalian polynucleotide kinase has been shown to phosphorylate nucleotide monomers Thus T4-PNK remains the only enzyme suitable for postlabelling analysis of carcinogen-damaged nucleic acids (Randerath et al., 1981)

Moreover, PP-PNK phosphorylated poly rA RNA about as well as oligo dT_{25} (Dana Lasko, personal communication)(3.2 pmoles ^{32}P/30 min/2 μl enzyme incorporated on poly rA, whereas 1 3 pmoles ^{32}P /20 min/1 μl enzyme incorporated on oligo dT_{25}). Both Blue-Sepharose and MonoQ fractions were able to phosphorylate poly rA. However, it should be stated that since the enzyme has not been purified to homogeneity, the DNA and RNA kinase activities cannot be conclusively shown to be contained within the same polypeptide. In this aspect, the enzyme reported here is distinct from the previously described mammalian DNA kinases that were specific for DNA, and is more similar to T4-PNK in its ability to phosphorylate RNA to the same extent as DNA.

In addition, the Polymin P-precipitable polynucleotide kinase does not have detectable 3'-phosphatase activity on a 5', 3' nucleotide diphosphate. Of the previously described enzymes, T4-PNK has such an activity (Cameron and Uhlenbeck, 1977), as does the rat liver DNA kinase (Pheiffer and Zimmerman, 1982) These two enzymes are also able to remove the 3'-phosphate terminus from a variety of deoxyribonucleotides and ribonucleotides This activity, in

combination with the 5' kinase has been suggested to catalyze the concerted transfer of a phosphate group from the 3'-side to the 5'-side of a DNA nick (Habraken and Verly, 1983) This reaction was shown to occur in two distinct steps (Habraken and Verly, 1986). Furthermore, no cyclic nucleotide phosphodiesterase (CNPase) activity was detectable in the purified preparation of PP-PNK (Enoch Gao, personal communication), in contrast to the T4-PNK enzyme, which has such an activity.

The nature of the phosphorylated oligonucleotide product was analyzed by two different methods First, snake venom phosphodiesterase digestion was complete, indicating that the phosphate group was not added at the 3'-terminus, since this particular phosphodiesterase attacks preferentially 3'-OH ended oligonucleotides In addition, no further incorporation of label was observed when the substrate was previously phosphorylated with T4-PNK using non-radioactive ATP, indicating that the phosphorylation site is the same as for T4-PNK, which is known to be the 5'-terminus.

Prompted by the fact that PP-PNK displayed distinct chromatographic properties from the previously described mammalian DNA kinases, the pH profile of the enzyme was investigated. This is a useful parameter to help distinguish between activities PP-PNK had a broad pH optimum, with a peak at neutral to alkaline pH in contrast with the previously described acid-dependent, DNA kinases from calf thymus (Tamura et al., 1981, Austin et al.,

1978) and rat liver (Ichimura and Tsukada, 1971, Levin and Zimmerman, 1976) which had a pH optimum of 5 5. As mentioned earlier, preliminary results on the Polymin P supernatant showed an activity which appears to have an acidic pH optimum (Carolyn Slack, personal communication) that could possibly be the previously described DNA kinase. Bosdal and Lillehaug (Bosdal and Lillehaug, 1985) have found an acidic pH optimum when using an oligonucleotide substrate for the rat testis enzyme, therefore the pH effect is not likely to be substrate dependent A better way of carrying out this experiment would be to have overlapping pH points in both buffers used, or use a broad range buffer throughout the pH range The approach reported here of using 2 buffers to cover the pH range was done for the mammalian DNA kinases described previously in the literature In conclusion, PP-PNK is similar to T4-polynucleotide kinase, the Hela cell RNA kinase and the yeast tRNA ligase and the wheat germ RNA ligase which also have a neutral to alkaline pH optimum

Since the pH profile indicated an activity distinct from the DNA kinases with acidic pH optimum, the need to further investigate the other reaction requirements arose. Activity was absolutely dependent on the presence of enzyme, oligonucleotide substrate and ATP Other phosphoryl donors were not investigated Kinase activity was reduced in the absence of a reducing agent, indicating that the enzyme might have essential sulfhydryl groups

The requirement for a divalent cation was not stringent for PP-PNK Activity was detected in the absence of added Mg^{2+}, and maximal activity occured at 1 mM Mg^{2+} However, EDTA abolished activity at a concentration of

25 mM, indicating that there may be a need for a divalent cation, that could be fulfilled by traces of metals that possibly exist in the buffers. In contrast, the previously reported DNA kinases have higher optimal Mg^{2+} concentrations.

The K_M for ATP (52 μM) was also different compared to the previously described enzymes The calf thymus enzyme had a much lower K_M, around 4 μM (Tamura et al , 1981), as did the rat liver kinase (2 μM, Levin and Zimmerman, 1976). T4-PNK has a K_M for ATP of 14 μM (Novogrodsky et al., 1966), whereas the Hela cell RNA kinase has a much higher value of 500 μM. The K_M for the oligo dT_{25} substrate was about 8 μM Since this is a new substrate used in a DNA kinase assay, no direct comparison can be made. However, the K_M value for micrococcal nuclease treated DNA for T4-PNK was 7.6 μM (Lillehaug and Kleppe, 1975) and for the rat liver enzyme for DNase I treated DNA was 35.5 μM (Teraoka et al., 1975).

Inhibition studies revealed that sulfate anions were not as strongly inhibitory as for the rat liver enzyme. The latter is remarkably inhibited by sulfate with an $I_{0.5}$ value of 0.5 mM and by pyrophosphate with an $I_{0.5}$ of 0.2 mM (Teraoka et al., 1975) In this aspect, PP-PNK, requiring 2.2 mM pyrophosphate or 8.5 mM ammonium sulfate for 50% inhibition of activity, resembles the enzyme isolated previously from calf thymus (showed 60% inhibition with 10 mM ammonium sulfate and complete inhibition of activity at 20 mM pyrophosphate) (Austin et al., 1978).

The sedimentation behaviour of PP-PNK was also different from every

reported DNA kinase; it eluted one fraction earlier than ovalbumin (sedimentation coefficient 3.5 S) with a sedimentation coefficient of 3.8 S, indicating a molecular size of about 50 kDa. An SDS-PAGE analysis of the peak activity fraction from a sucrose gradient revealed about 10 protein bands, all of them between 35 to 70 kDa. No protein bands bigger than 70 kDa and lower than 35 kDa were visible, indicating that the active polypeptide must be in that molecular range The enzyme from calf thymus reported by Austin et al. had a sedimentation coefficient of 4 3 S, whereas the rat liver kinase sedimented at 4.4 S (Teraoka et al., 1975) The HeLa cell RNA kinase gave a major component with a sedimentation coefficient of 5.8 S and a minor component sedimenting at 4.4 S. When T4-PNK was run under the same conditions, it displayed a different sedimentation behaviour than PP-PNK with the bulk of activity sedimenting at around 7.0 S

The estimated relative molecular mass compared to globular protein standards of Blue-Sepharose purified PP-PNK was 120-130 kDa from gel filtration data on Superose 12 and AcA 34 columns (Carolyn Slack, personal communication). This high molecular weight estimate is distinct from results reported for other eukaryotic kinases. For example, the gel filtration estimate for the rat liver kinase was 80 kDa (Teraoka et al., 1975), and for the calf thymus enzyme reported by Tamura et al 54 kDa, whereas Austin et al reported a molecular weight of about 70 kDa. It is therefore possible that PP-PNK has a subunit structure, which dissociates under sedimentation analysis while remains intact under gel filtration conditions Another possibility could be that

the enzyme forms aggregates under certain conditions. To combine the data from gel filtration and sedimentation, a Siegal / Monty calculation can sometimes be done, but not in this case since the salt concentrations were different 0.1 M for sedimentation and 0.3 M for gel filtration The experiments were attempted with the reciprocal salt conditions, but without detection of activity. Recovery of activity was measured after passing the sample through a Microcon 100 ultrafiltration unit with a nominal molecular weight cut off of 100 kDa under the same salt conditions used for the sedimentation analysis. The bulk of PP-PNK activity was present in the filtrate whereas about 10% was retained on the membrane. This behaviour is consistent with the results of the sedimentation analysis.

To further investigate the size of the active polypeptide, renaturation experiments were carried out after SDS-polyacrylamide gel electrophoresis of the active, purified fraction. The major protein band was excised, but no renaturable activity was detected, even after extensive exposure of the assay autoradiographs. However T4-PNK, the positive control, did renature when a greatly increased amount of units was used, although with a low recovery of activity after the renaturation step (less than 0 1 % of the input units were recovered) This low percentage of recovery might be the reason why activity was not detected after renaturation for the PP-PNK To strengthen this hypothesis, 38 mU of PP-PNK or T4-PNK showed no detectable activity when renatured, suggesting that there is a threshold in the amount of activity needed for detection of activity after renaturation. Since the renaturation buffer

conditions may not have been optimal, a set of five different buffers was also used; no detectable DNA kinase activity was recovered. Finally, there was also no activity detectable when several bands covering the whole region from 35 up to 70 kDa of the gel were assayed

An alternative approach to correlate DNA kinase activity with a polypeptide is the activity gel method (Ohmura et al., 1987). This is an *in situ* assay of DNA kinase enzyme after SDS-PAGE analysis. The SDS-polyacrylamide gel contains 5'-OH nicked DNA which serves as a DNA substrate. After renaturation of polypeptides, the gel is incubated with γ-^{32}P ATP in the presence of Mg^{2+} and the active polypeptides can be visualized as radioactive bands. This method has been successful with T4-PNK and the rat liver DNA kinase (Ohmura et al., 1987) Several trials have been made by myself using T4-PNK, but they were unsuccessful.

Lacks and Springhorn (Lacks and Springhorn, 1980) have reported that oligomeric enzymes composed of identical subunits were poorly renaturable after polyacrylamide gel electrophoresis in the presence of SDS. This might be one reason why the renaturation experiments did not work As mentioned earlier, it is possible that PP-PNK has a subunit structure If it is a dimer of identical subunits of about 50 kDa each, then renaturation may be more difficult than if the enzyme was a monomer In the case of a heterodimer, renaturation would require the assembly of the individual subunits to make the active enzyme. This would be highly unlikely to occur, since the two different subunits might be expected to separate on the gel, thereby making their interaction

impossible. In addition, PP-PNK seems to have essential sulfhydryl groups for activity (40% loss of activity when DTT is ommited from the reaction). It is known that SDS-electrophoresis in the presence of a reducing agent can disrupt disulfide bridges Therefore, PP-PNK may not be able to refold correctly when its disulfide bridges are broken Future experiments with a higher amount of activity might reveal the active polypeptide, and subsequently resolve some of the questions left unanswered in this thesis. For the present, the maximum possible amount of activity was used for these experiments.

In conclusion, novel information has been reported in this thesis concerning DNA kinases. First, the use of Polymin P to purify such an activity is a new step. The enzyme purified with this method can be distinguished from the previously described kinases, in terms of its distinct properties PP-PNK differs from the rat liver DNA kinase in terms of its pH optimum, action on RNA, lack of binding to phosphocellulose, lack of 3'-phosphatase and different sensitivity to sulfate inhibition. Comparison with the previously reported calf thymus enzymes reveals differences in pH profile, phosphocellulose binding and high activity on RNA The HeLa cell RNA kinase has a much higher K_M for ATP, requires Mg^{2+} ions for activity and has different sedimentation behaviour, with two distinct components sedimenting at 4 4 S and 5 8 S, although the possibility of an active proteolytic fragment of these faster sedimenting activities cannot be ruled out

Furthermore, PP-PNK can be distinguished from the yeast and wheat germ tRNA ligases in that these activities sediment faster and contain RNA

ligase and 2', 3'-cyclic phosphodiesterase activities PP-PNK did not contain any cyclic nucleotide phosphodiesterase activity (Enoch Gao and Peter Braun, personal communication) Despite this fact, it would be difficult to rule out the possibility of a proteolytic fragment of tRNA ligase, that might be devoid of CNPase activity However, the polynucleotide kinase and RNA ligase activities of tRNA ligases have not been reported to be dissociated in controlled proteolysis or in genetic engineering experiments (Xu et al , 1990, Pick and Hurwitz, 1986). In addition, AMP-binding assays showed no AMP-labelled bands in an active Mono Q fraction (Dana Lasko, personal communication) An experiment that could address the possibility of a proteolytic fragment of tRNA ligase would be an AMP-binding experiment across a sucrose gradient

Although the physiological role of DNA kinases is still unknown, inferences have been made for the involvement of the enzyme in DNA metabolism, on the basis of the reactions catalyzed in vitro As mentioned before, 5'-OH ends in DNA can be generated in vivo, these termini need to be phosphorylated in order to become functional.

The fact that PP-PNK can phosphorylate RNA might be another important physiological role for this enzyme Previous reports on the yeast tRNA ligase that possesses a polynucleotide kinase activity have shown that the enzyme has an active role in a tRNA splicing pathway (Greer et al , 1983) Moreover, the processing of RNA chains in vivo can lead to the generation of 3'-phosphate and 5'-hydroxyl terminated RNAs The presence of an RNA kinase activity would be important in phosphorylating the 5'- hydroxyl RNAs, thus

rendering them susceptible to further processing events (Shuman and Hurwitz, 1979).

In conclusion, Polymin-P precipitable polynucleotide kinase was isolated by a new technique, has distinct properties and it seems to be a novel mammalian DNA kinase However, final determination of its distinct character must wait until antibodies and cDNAs for all the mammalian polynucleotide kinases become available. These studies may help in the development of molecular probes against this enzyme that could lead to the isolation of the gene encoding PP-PNK. Antibodies will be developed that could provide a powerful tool in intracellular localization studies. Oligonucleotide probes will be designed from peptide sequence information and will be used for screening cDNA libraries Once the gene is known, experiments such as gene knockouts and overexpression studies could indicate the function of this enzyme in nucleic acid metabolism Furthermore, the expression levels could be subsequently studied and localization of the gene to a chromosome may be attempted. These would be important steps in characterization of the biological role of this activity.

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