AN ABSTRACT OF THE THESIS OF

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Title : Nucleoside Diphosphokinase of *Eschericia coli* and its Interactions with Bacteriophage T4 proteins of DNA synthesis

Abstract approved: Redacted for privacy

Escherichia coli nucleoside diphosphokinase (NDPK), the product of gene *ndk,* synthesizes nucleoside triphosphates from the corresponding diphosphates. This bacterial enzyme is an integral component of the T4 bacteriophage dNTP synthetase complex, a multienzyme complex for deoxyribonucleotide biosynthesis, and it plays an indispensable role in T4 DNA replication. A goal was established to locate and clone *ndk,* in order to overexpress the gene and obtain large enough quantities of the enzyme for the analysis of protein interactions, involving NDPK and proteins of both the T4 dNTP synthetase complex and the T4 DNA replication complex. NDPK was first purified 5000-fold from crude extracts of *E. coli* B cells for N-terminal amino acid sequencing. Over forty residues of N-terminal sequence were determined, providing information used to design mixed oligonucleotide probes, designed to search for the *ndk* gene in the Clarke and Carbon *E. coli* ColE1 plasmid library. A 3.2-kb Pst I fragment from the Clarke and Carbon plasmid, pLC34-9, hybridized specifically to one of the probes and was subcloned into pUC19. Six-fold higher NDPK enzyme activity, over host NDPK enzyme activity, was generated by the recombinant pUC19 plasmid in JM83 cells. A 6.0-kb EcoRI fragment from the Kohara *E. coli* lambda library, mapping to approximately the

same area, was also cloned into pUC18 based on the elevated, overlapping NDPK enzyme activity of two lambda clones, 2D5 and 7F8. NDPK was overexpressed from a pUC9 recombinant plasmid obtained from Masayori Inouye, and purified to homogeneity for protein-protein interaction studies with T4 early proteins (proteins primarily involved in T4 dNTP metabolism and DNA replication). NDPK was then immobilized onto an affinity column and extracts of [^{35}S]-methionine-labeled, T4-infected and uninfected *E. coli* B cells were applied to the column. Labeled proteins were then eluted from the column with increasing concentrations of salt and analyzed by both one and two-dimensional polyacrylamide gel electrophoresis. Several T4 dNTP biosynthesis enzymes, including dihydrofolate reductase, the large and small subunits of ribonucleotide reductase, and dCMP hydroxymethylase, as well as several T4 DNA replication proteins, were bound to the column at 0.2 M NaCl, and eluted at 0.6 M NaCl. Protein-protein interactions involving NDPK and T4 early proteins have also been investigated by NDPK-anti-idiotypic antibody immunoprecipitation analysis. One T4 early protein has been found to be specifically immunoprecipitated by NDPK-anti-idiotypic antibody. This protein is not among those so far identified by two-dimensional polyacrylamide gel analysis, and further identification is underway. The specific interaction of NDPK and T4 DNA polymerase has also been investigated through the use of fluorescence anisotropy.

Nucleoside Diphosphokinase of *Escherichia coli* and its Interactions with Bacteriophage T4 Proteins of DNA Synthesis

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Nancy Bisset Ray

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Head of department of Biochemistry and Biophysics

Redacted for privacy

Dean of Graduate School

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I. Introduction

A. General Introduction

Nucleoside diphosphokinase (NDPK) is an important enzyme, since it is responsible for synthesizing all but one of the eight common ribo- and deoxyribonucleoside triphosphates. NDPK catalyzes the nonspecific transfer of the γ-phosphate group of 5'-triphosphate nucleotides to 5'-diphosphate nucleotides as seen below:

$$N_1TP + N_2DP \rightleftharpoons N_1DP + N_2TP$$

Aerobically, ATP is synthesized by oxidative phosphorylation and is the most abundant nucleotide in most cells. Therefore, the γ-phosphate from ATP is most commonly transferred towards the synthesis of GTP, UTP, CTP, dATP, dGTP, dTTP, and dCTP, from the respective diphosphates. In this way, NDPK forms a functional link between the intermediary metabolism of small molecules and the macromolecular metabolism of nucleic acids (Parks and Agarwal, 1973).

Escherichia coli nucleoside diphosphokinase is of interest because it is an enzyme that is involved in deoxyribonucleotide biosynthesis, whose activity has been detected in a multienzyme deoxyribonucleotide synthesis complex found in

E. coli after infection with bacteriophage T4. This complex, referred to as the T4 dNTP synthetase complex, is composed of several T4-encoded enzymes and two bacterial-encoded enzymes, one of which is NDPK. NDPK represents a link between T4 precursor metabolism and T4 replication, since it catalyzes the last reaction step towards the production of deoxynucleoside triphosphates (dNTPs) and since replication begins with the incorporation of dNTPs into nascent DNA. Indeed it has been shown that T4 DNA synthesis cannot occur at an appreciable rate without NDPK.

Bacteriophage T4 has served as a model system for studying molecular biology and genetics since it was first described in 1946 (For review, see Stent,1963). It has also served as an excellent model system for studying DNA precursor metabolism. After infection of the host, *E.coli,* the rate of DNA synthesis increases up to tenfold over the rate of DNA synthesis before infection. However, the pools of dNTPs do not increase substantially (Mathews, 1972). A tenfold increase in the rate of DNA synthesis is necessary for rapid reproduction of the virus, and is sustained by producing enhanced enzyme activities of several of the dNTP biosynthetic enzymes present in uninfected bacteria (see fig. I-1). These enzymes, such as thymidylate synthase and dUTPase, duplicate pre-existing bacterial enzyme activities, and are encoded by T4 with enhanced activities. T4 also induces the production of unique enzyme activities, such as dCMP hydroxymethylase, which catalyzes the production of 5-hydroxymethylated deoxycytidylate. However, two host enzyme activities are not duplicated by T4. These are adenylate kinase, which is responsible for dAMP phosphorylation, and nucleoside diphosphokinase. Both of these enzymes have high activities in the host and are utilized by T4 after infection.

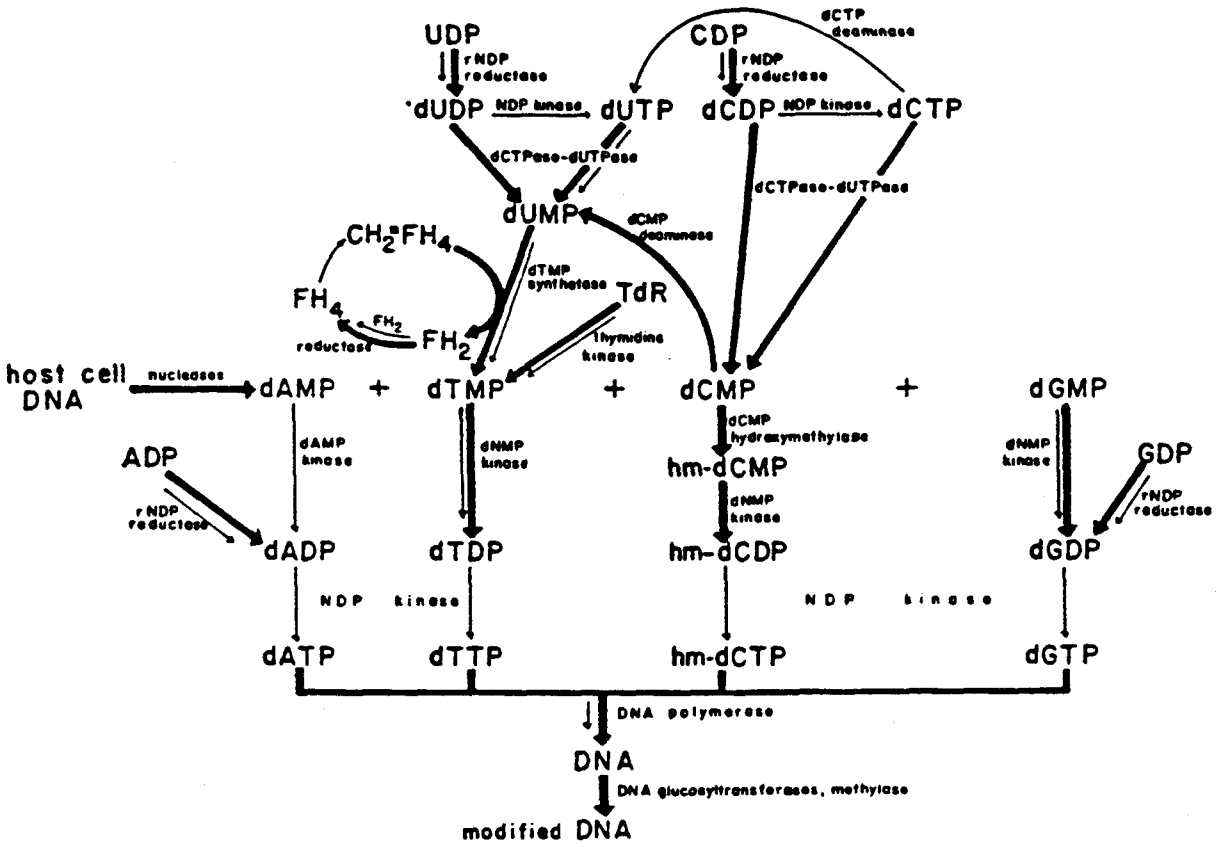


Figure I-1. T4 dNTP Metabolism Pathway. Dark arrows represent T4 bacteriophage-encoded enzyme reactions; lighter arrows represent host-encoded enzyme reactions (Mathews et al, 1979).

T4 has been used as a model system for studying enzymes involved in dNTP biosynthesis and their relationship to DNA replication and fidelity. It serves as a unique system for studying both virally-encoded and host-encoded dNTP biosynthesis enzymes, such as NDPK, which interact as a multienzyme complex in such a way as to produce a substantial increase in the rate of DNA replication without loss of fidelity.

B. General Description of Bacteriophage T4

The T4 genome is composed of 166 kb (or approximately 200 genes) of linear, circularly permuted, double-stranded DNA. T4 DNA contains 5-hydroxymethylcytosine, synthesized at the nucleotide level by deoxycytidylate (dCMP) hydroxymethylase, instead of cytosine. The DNA hydroxymethylation at the dCMP level protects it from T4-encoded nucleases, which degrade the host chromosome within minutes after infection. After the DNA is synthesized, it is also glucosylated at the hydroxymethyl groups by α and β-glucosyl transferase, which protect the DNA from host nucleases. In the first few minutes after infection, all synthesis of host proteins and mRNA stops and the host RNA polymerase begins transcribing early genes, including the genes encoding deoxyribonucleotide biosynthesis enzymes and DNA metabolism and replication proteins. The synthesis of early proteins stops at approximately 12 minutes after infection, when the cells are incubated at 37°C. DNA replication begins about 5 minutes after infection along with transcription of the late genes, which code mostly for structural proteins. T4 encodes its own DNA polymerase, which was the first T4 replication enzyme isolated, and which is known to exist in a complex

of at least six replication proteins (Alberts and Nossal, 1983) of the eleven replication proteins that have been studied extensively (see Table I-1).

By 25 to 30 minutes after infection, approximately 200 phage particles are released from the host by lysis. This represents the time it takes for phage reproduction to occur, beginning with insertion of the phage DNA into the host and the production of early proteins, followed by replication of the phage DNA and the production of late proteins, and finally concluding with phage assembly.

C. The T4 dNTP Synthetase Complex and its Relationship to T4 DNA Replication

As mentioned previously, the rate of DNA synthesis, after infection of *E. coli* with T4, increases up to ten-fold relative to the rate of synthesis in the uninfected host. Deoxyribonucleotides must be delivered to replication forks at a rate of approximately 1700 nucleotides per second per fork, but average dNTP concentrations are in the range of 50-60 μM. This concentration range cannot account for such a replication rate, which requires a dNTP concentration range of 200-250 μM of each nucleotide for saturation *in vitro* (Mathews et al, 1988). Therefore, concentration gradients probably exist at replication sites. These gradients can be understood if one considers the possibility of channeling of deoxynucleotides directly to replication forks through a localized organization of dNTP biosynthesis enzymes. Several other lines of evidence, including kinetic enzyme coupling and genetic data, point to the existence of a multienzyme deoxynucleotide metabolism complex, known as the T4 dNTP synthetase complex.

TABLE I-1

Proteins of Bacteriophage T4 DNA Replication

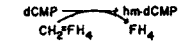
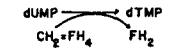
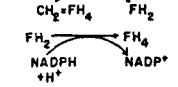
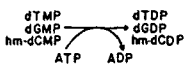
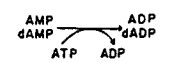
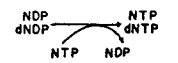
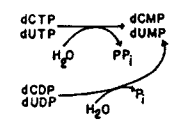
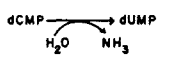
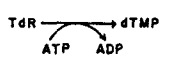
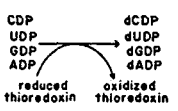
Designation	T4 gene	Activities of protein alone[b]	Role in DNA replication		
			RNA primer synthesis	Synthesis on single-stranded templates	Strand displacement synthesis
DNA polymerase	43	5′-to-3′ polymerase on ssDNA templates only; 3′-to-5′ exonuclease	—[c]	Required	Required
Helix-destabilizing protein (gene 32 protein)	32	Cooperative binding to ssDNA; helix destabilization	—	Stimulates	Required
Polymerase accessory proteins					
Gene 44/62 protein	44/62	ssDNA termini-dependent ATPase (dATPase)	—	Stimulates	Required
Gene 45 protein	45	Stimulates gene 44/62 protein ATPase	—	Stimulates	Required
Priming proteins					
Gene 61 protein	61	Binds ssDNA	Required	—	—
Gene 41 protein	41	Long ssDNA-dependent GTPase and ATPase; DNA unwinding (5′-to-3′ helicase)[d]	Required	—	Stimulates
T4 DNA helicase	*dda*	ssDNA-dependent ATPase (dATPase); DNA unwinding (5′-to-3′ helicase)[d]	—	—	Stimulates
T4 DNA topoisomerase	39/52/60	dsDNA-dependent ATPase; type II topoisomerase (dsDNA strand passage); dsDNA cleavage when inhibitors added	—	—	—

[a] References and further details are given in the text.
[b] Abbreviations: ss, single stranded; ds, double stranded.
[c] —, No role has been demonstrated.
[d] Helicase direction is indicated as the direction of movement on the strand that is not displaced.

(source: Alberts and Nossal, 1983)

TABLE I-2

Enzymes of Bacteriophage T4 dNTP Biosynthesis

Enzyme	Reaction(s) catalyzed	Structural gene	Mutants available	Mol wt (×103)	
				Native	Subunit
dCMP hydroxymethylase		42	Amber, *ts*	60	27, 25[b]
dTMP synthetase		*td*	Nonlethal amber, missense, deletions	58	29
Dihydrofolate reductase		*frd*	Nonlethal amber, deletions, missense	44.5[c]	23[c]
dNMP kinase		1	Amber, *ts*	Unknown	22
(d)AMP kinase		Host gene		Unknown	Unknown
NDP kinase		Host gene	One *cs* in *Salmonella*	110[d]	15.5[e]
dCTPase-dUTPase		56	Amber, *ts*	59	15
dCMP deaminase		*cd*	Nonlethal missense	124, 129[f]	20.2
Thymidine kinase		*tk*	Nonlethal missense; BUdR resistant	86	28
rNDP reductase		*nrdA*, *nrdB*	Nonlethal missense, amber, deletions, folate analog resistant[g]	225	85 (*nrdA*), 35 (*nrdB*)

(source: Mathews and Allen, 1983)

This complex is composed of at least ten enzymes (see Table I-2) involved in T4 dNTP biosynthesis, and has been isolated by sucrose gradient centrifugation (Reddy et al, 1977; Chui et al, 1982; Allen et al, 1983) as well as chromatographically, with an apparent molecular weight of 1500 kDa (Moen et al, 1988). The enzyme components of the complex are believed to be held together by weak interactions (Chiu et al, 1982; Allen et al, 1983), and have been isolated in the absence of DNA or membrane contamination. It has been shown that the complex can be disrupted by mutations affecting its enzyme components, including deoxycytidylate deaminase, ribonucleotide reductase, and deoxycytidylate hydroxymethylase (Moen et al, 1988; Thylén and Mathews, 1989). Kinetic coupling, which can be observed as a reduction of transient times for passage of molecules through a multi-step sequence and a reduction in steady-state levels of intermediates (caused by their limited diffusion away from enzyme active sites), has also been demonstrated among several enzymes in the complex as further evidence for a multienzyme association (Reddy et al, 1977; Reddy and Mathews, 1978; Allen et al, 1980; Chiu et al, 1982; Allen et al, 1983).

An important question is whether the T4 dNTP synthetase complex is physically linked to the replication complex. Evidence for a direct coupling of the T4 dNTP synthetase complex and T4 DNA replication (represented in Figure I-2) has been provided by nucleotide incorporation experiments in which it was found that distal precursors of DNA such as dNMPs and rNDPs were actually incorporated into DNA much more efficiently than dNTPs (Reddy and Mathews, 1978). This helps confirm the belief that nucleotides are channeled through an interacting association of deoxynucleotide-synthesizing enzymes directly to replication sites, maintaining a higher concentration of dNTPs at replication

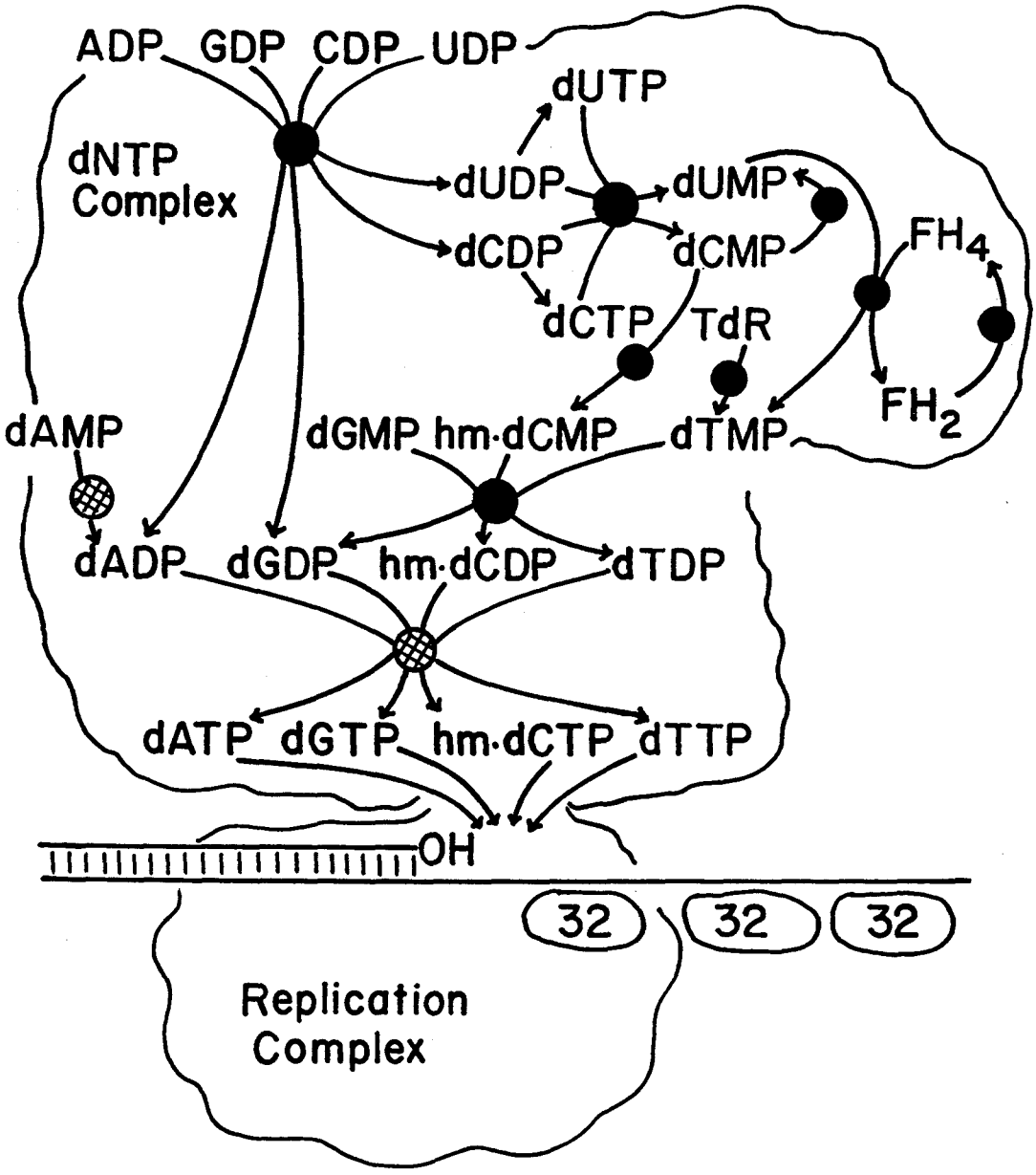


Figure I-2. Putative Coupling of the T4 dNTP Synthetase Complex and the T4 DNA Replication Complex (Mathews and Allen, 1983).

forks. Other evidence for an interaction between proteins found in the T4 dNTP synthetase complex and proteins involved in T4 replication includes genetic evidence, such as a T4 DNA polymerase mutation that suppresses a gene 42 (dCMP hydroxymethylase) mutation (Chao et al, 1977) and the expression of *nrdB* (small subunit of ribonucleotide reductase) being controlled by gp39 (subunit of topoisomerase) (Cook et al, 1988). Physical evidence for an interaction between proteins involved in the T4 dNTP synthetase complex and replication proteins has been obtained in the Greenberg laboratory at the University of Michigan by sucrose gradient sedimentation (Chiu et al, 1982) as well as by an affinity chromatography technique applied in the Mathews laboratory at Oregon State University (Wheeler et al, 1992). Chiu et al used sucrose step gradient centrifugation to isolate a deoxyribonucleotide synthetase complex that contained several replication proteins, including subunits of topoisomerase (gp39, gp60, and gp52), T4 DNA polymerase (gp43), and single strand binding protein (gp 32). However, this isolated association of deoxynucleotide synthesizing proteins included the two subunits of ribonucleotide reductase (NrdA and NrdB), dCMP hydroxymethylase (gp42), dCTPase-dUTPase (gp56), and thymidylate synthetase (gp*td*), whereas others have detected the presence of at least ten enzymes involved in dNTP synthesis cosedimenting through sucrose gradients (Reddy et al, 1977; Reddy and Mathews, 1978; and Allen et al, 1980). Perhaps the isolation conditions used by Chiu et al were more favorable towards preserving interactions between certain dNTP biosynthesis enzymes and replication proteins rather than among the enzymes involved in the T4 dNTP synthetase complex itself. Another possibility is that their preparations were not free of DNA or membrane contaminants, possibly causing some artifactual aggregations. In any case, other groups from the Mathews laboratory have detected the presence of at least ten dNTP

biosynthesis enzymes in an isolated dNTP synthetase complex and have not detected the presence of any replication proteins, except for one unconfirmed report of a cosedimenting T4 DNA polymerase activity (Allen et al, 1980).

As mentioned previously, other evidence for a physical interaction between enzyme components of the T4 dNTP synthetase complex and T4 replication proteins has been obtained in the Mathews laboratory through the use of an affinity chromatography technique first used with immobilized gp32 (Formosa et al, 1983), where the T4 protein of interest is immobilized onto an affinity gel matrix and T4-infected *E. coli* proteins (radiolabeled from 3 to 8 minutes) are passed through the column. Early T4 proteins which bind to the protein of interest are then eluted from the column with increasing concentrations of salt. This method has been used successfully with immobilized dCMP hydroxymethylase, where it was found that several T4 replication proteins as well as dNTP biosynthesis enzymes apparently interact with this protein of interest (see Table I-3). Apparent specific interactions of thymidylate synthase and gp32 with dCMP hydroxymethylase were investigated by using amber mutants of the genes coding for these proteins (Wheeler et al, 1992).

D. NDPK: Historical Background

The first NDPKs that were identified were reported in 1953 for yeast (Berg and Joklik, 1953) and for pigeon muscle (Krebs and Hems, 1953).

TABLE I-3

Retention of T4 Proteins by dCMP Hydroxymethylase Column

Bound specifically at 0.2 M NaCl
Eluted at 0.6 M NaCl

Gene	Gene product	Function	Phage Strain		
			T4D	T432^{am}	T4td^{am}
42	dCMP HMase	dNTP	+	+	+
td	dTMP synthase	dNTP	+	+/-	-
frd	DHF reductase	dNTP	+	+/-	+
56	dCTPase/dUTPase	dNTP	+	+	+/-
nrdA	rNDP reductase R1	dNTP	+	-	-
nrdB	rNDP reductase R2	dNTP	+	-	-
32	ss DNA binding	DNA repl.	+	-	-
43	DNA polymerase	DNA repl.	+/-	+/-	+/-
44	pol. accessory	DNA repl.	-	+	+
45	pol. accessory	DNA repl.	+	+/-	+/-
61	primase	DNA repl.	+	+	+
62	pol. accessory	DNA repl.	-	+	+
46	nuclease	recomb.	+	+	+
uvsX	RecA analog	DNA repair	+	-	-
uvsY	repair protein	DNA repair	+	+	+
pseT	phosphatase/kinase	?	+	+	+
ßgt	glucosyltrans-ferase	modifica-tion	-	+	+

(source: Wheeler et al, 1992)

Since then NDPK has been found to be a ubiquitous enzyme that has been studied in many systems (reviewed in Parks and Agarwal, 1973), including plants, animals, and microorganisms.

In the early 1960s Bello and Bessman identified a T-even phage-coded deoxyribonucleoside monophosphokinase (MPK or gp1 in T4), which phosphorylates dGMP, dTMP, and 5-hydroxymethyl dCMP to the corresponding diphosphates (Bello and Bessman, 1963). When they asked whether T4 encodes a nucleoside diphosphokinase, they found no increase in NDPK activity after T4 infection, but they did find a 20-fold higher NDPK activity in uninfected *E. coli* over MPK activity in T4 phage-infected *E. coli* extracts (Bello and Bessman, 1963). They concluded that the NDPK activity found in uninfected cells was already sufficient to match the increased rate of dNTP synthesis in phage-infected cells. Also, because NDPK is nonspecific, it can phosphorylate hydroxymethyl-dCDP. Therefore, T4 has no need to encode its own NDPK.

In the 1970s a group in Japan partially purified NDPK from *E. coli* B cells and found that it was specifically and irreversibly inhibited by the antibiotic desdanine (Saeki et al, 1974). In another study they showed that pyruvate kinase (see below) can substitute for NDPK by synthesizing NTPs under anaerobic conditions (Saeki et al, 1974).

NDPK has also been purified and characterized from the periplasmic space of *E. coli* (Roisin and Kepes, 1978), from *Bacillus subtilis* (Sedmak

and Ramaley, 1971), and from *Salmonella typhimurium* (Ginther and Ingraham, 1974). Later the *ndk* gene location was mapped in *Salmonella* (Rodriguez and Ingraham, 1983), which is 80% homologous with *E. coli* at the DNA level.

E. NDPKs Reaction Mechanism and Physical Properties

As mentioned previously, NDPK nonspecifically transfers phosphates from any ribo- or deoxyribonucleoside triphosphate to diphospho- (ribo- or deoxyribo) nucleosides. All NDPKs studied so far react through a phosphoenzyme intermediate in which a histidine is phosphorylated, and follow a ping-pong reaction mechanism (Parks and Agarwal, 1973):

$$N_1TP + E \rightleftharpoons N_1DP + E\sim P$$
$$N_2DP + E\sim P \rightleftharpoons N_2TP + E$$

The enzyme activity in most cells is very high and the equilibrium constant is essentially unity. No allosteric effectors have been found and the enzyme has been found to require Mg^{++}. The enzyme is composed of up to six (15-18 kDa) identical subunits; in yeast NDPK exists as a hexamer. In most higher eukaryotes, the enzyme has been found to exist as isozymes.

In *E. coli*, NDPK has an isoelectric point of approximately 4.75 and an apparent subunit molecular weight of approximately 15,000 Da. It has a specific activity of about 2400 µmol/min/mg, which corresponds to a turnover number of about 2.4 x 10^5 µmol/min/µmol based on an apparent holoenzyme molecular weight of 100,000 Da (found during purification of the complex), leading to the conclusion that it is a hexamer. In crude extracts NDPK has been estimated to represent 0.08% of the total soluble *E.coli* protein (Ray and Mathews, 1992). As mentioned previously, NDPK activity has been detected in both the cytoplasm and periplasmic space in *E. coli,* and it has also been detected in the cytoplasm, cellular membrane, and nucleus (Rosengard et al, 1989) in eukaryotic cells, as well as in mitochondria (Glaze and Wadkins, 1967).

F. The Role of NDPK in the T4 dNTP Synthetase Complex and in T4 DNA Replication

The first evidence that indicated an involvement of NDPK in the T4 dNTP synthetase complex came from experiments on nucleotide pool dynamics (Mathews and Sinha, 1982; Mathews, 1976). T4 DNA replication was reversibly blocked by shifting an *E. coli* cell culture, infected with a temperature-sensitive phage mutant defective in gene 44 (encodes an essential DNA polymerase accessory protein), to a nonpermissive temperature. When the pool sizes of thymidine nucleotides were measured after such an upshift, it was found that thymidine diphosphate accumulated as rapidly as the corresponding triphosphate (see Fig. I-3). Since the activity of NDPK is much greater than the activity of monophosphokinase, as noted above, accumulation of any nucleoside

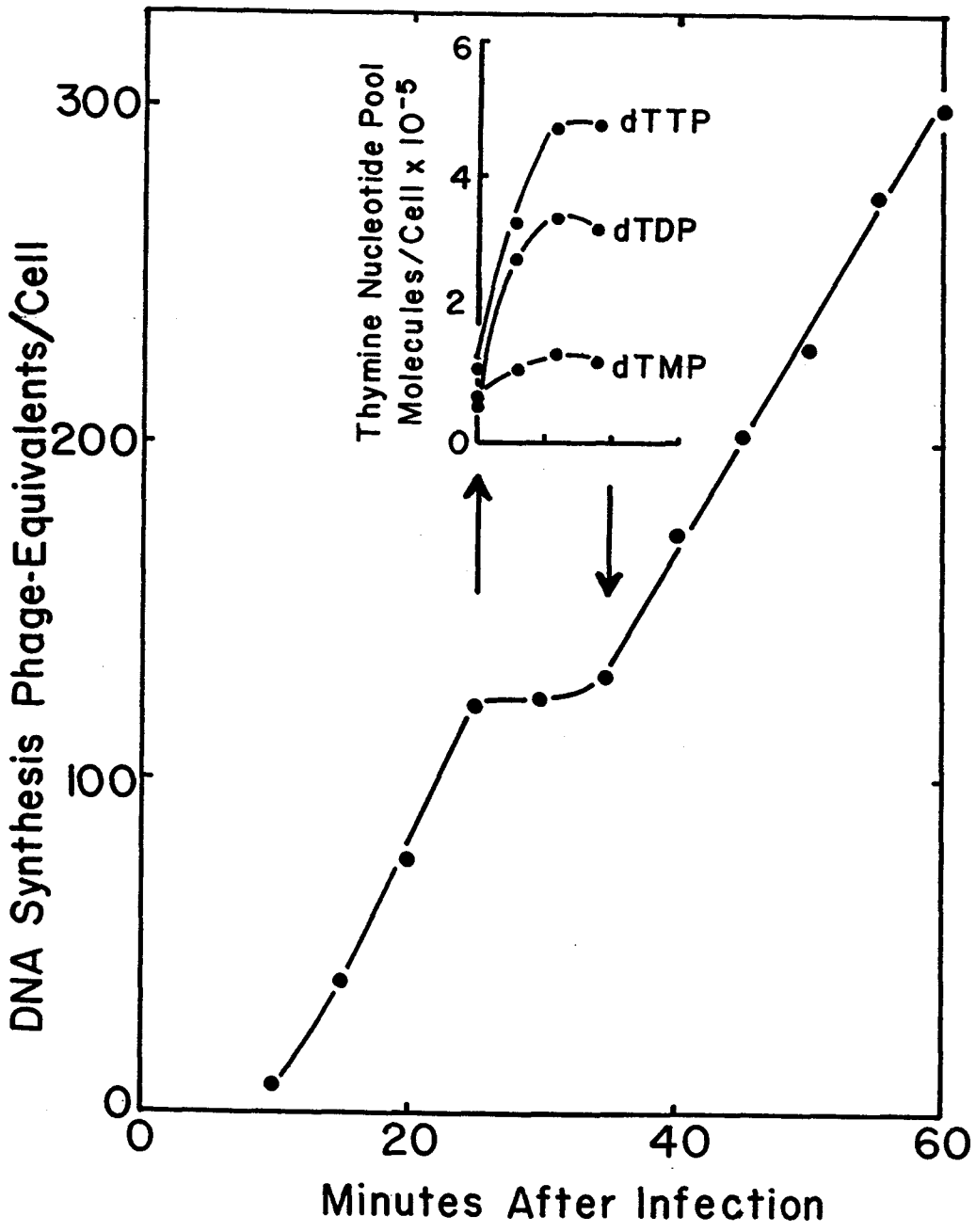


Figure I-3. Kinetics of Thymidine Nucleotide Accumulation After Inhibition of T4 DNA Replication (Mathews and Sinha, 1982).

diphosphate was not expected. Therefore, it was proposed that only a small proportion of the total NDPK content of the cell is available to participate in dNTP synthesis. This could occur if a small fraction of the cellular NDPK is sequestered for this purpose, as an integral component of the T4 dNTP synthetase complex. Indeed, it has been found that the activity of NDPK detected in preparations of the complex represents approximately 5% of the total cellular NDPK activity found in infected extracts.

Another way the presence of NDPK has been detected in the complex has been through kinetic coupling experiments (Mathews et al, 1979; Allen et al, 1980; Chiu et al, 1982; and Moen et al, 1988), in which NDPK catalyzes one of the steps in coupled reaction sequences , such as:

dUMP ⟶ dTMP ⟶ dTDP ⟶ dTTP

where rapid attainment of synthesis of the final product is seen, with little or no lag before the final product is detectable.

It has also been shown that NDPK plays an indispensable role in T4 DNA replication. As mentioned previously, a group in Japan, Saeki et al (Saeki et al, 1974), found that pyruvate kinase can substitute for NDPK anaerobically, and that desdanine is a specific and irreversible inhibitor of NDPK. Therefore, Reddy and Mathews asked whether T4 DNA synthesis could occur in anaerobically grown T4-infected *E. coli* cultures treated with desdanine. They found that T4 DNA synthesis was acutely sensitive to inhibition by desdanine in cultures grown aerobically or anaerobically (see Fig. I-4), indicating that NDPK plays an indispensable role that cannot be filled by pyruvate kinase

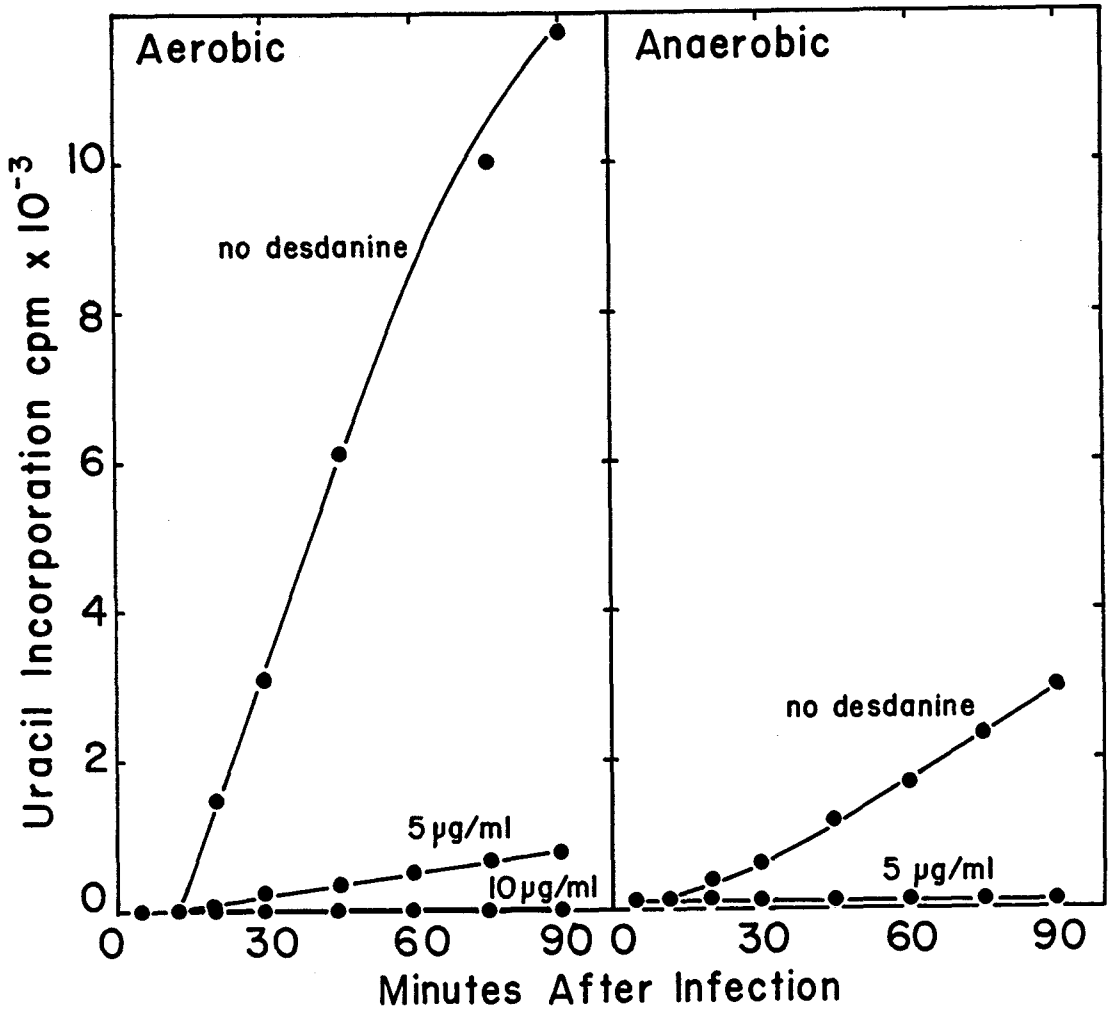


Figure I-4. Sensitivity of T4 DNA Synthesis to Inhibition by Desdanine, an Inhibitor of NDPK (Reddy and Mathews, 1978).

(Reddy and Mathews, 1978). One interpretation is that the indispensable role involves its interaction with proteins involved in the T4 dNTP synthetase complex and proteins involved in T4 replication.

G. Other Detected Protein Interactions Involving NDPK

In 1971, it was reported that NDPK activity was found associated with several highly purified DNA polymerase preparations, including DNA polymerase from *Micrococcus luteus, E. coli,* and avian myeloblastosis virus (Miller and Wells, 1971). Several attempts at separating the NDPK and DNA activities were not successful, suggesting a fairly strong interaction or a tenacious contaminant.

In 1976, von Döbeln also reported NDPK activity associated with highly purified preparations of the large and small subunits of *E. coli* ribonucleotide reductase. Attempts at separating the two activities chromatographically were also unsuccessful. However, it was possible to separate NDPK activity from the small subunit, B2, by preparative gel electrophoresis. Therefore, it was concluded that the NDPK activity was not catalyzed by ribonucleotide reductase and that the tight association of the activities might represent an association between the two enzymes *in vivo.*

Recently, an increasing amount of interest has been generated over interactions involving NDPK and G proteins. More and more evidence is accumulating that eukaryotic NDPKs interact with G proteins including the reports of NDPK associating with the following: (a) G proteins, called G_1 and G_2, in Ehrlich ascites tumor cells (Ohtsuki et al, 1987); (b) G_s protein in rat liver cells (Kimura and Shimada, 1988); (c) elongation factor eIF2 (Walton and Gill, 1975); (d) microtubules (Nickerson and Wells, 1984); (e) ras-like 21,000 kDa G

proteins in tumor cells (Ohtsuki et al, 1984; Ohtsuki et al, 1986); and (f) G_s protein in the membranes of human platelets (Wieland and Jakobs, 1989). In these studies and others NDPK has been proposed to play a major role in the processes of GTP channeling, regulation of adenylate cyclase, signal transduction, and cell proliferation induced by growth factors. A question of great interest and debate is whether NDPK can actually activate G proteins by direct interaction and channeling of GTPs to G proteins (Randazzo et al, 1991; Kikkawa et al, 1990).

H. Strategy for Cloning and Overexpressing the *ndk* Gene

As mentioned previously, Rodriguez and Ingraham mapped the location of the *ndk* gene in *Salmonella* (Rodriguez and Ingraham, 1983). They mapped the *ndk* gene location near the location of the *Salmonella his S* gene. Since *Salmonella* is 80% homologous with *E. coli* at the DNA level, we made the assumption that the *E. coli ndk* gene would also be located near the *E. coli his S* gene at approximately 54 minutes. At this time a Clarke and Carbon colE1 plasmid *E. coli* library (Clarke and Carbon, 1976) was available and we obtained five clones: pLC34-9, pLC34-10, pLC32-25, pLC15-12, and pLC1-41, containing DNA from the region of 54 minutes near *his S* (see Fig. I-5). *E. coli* NDPK had been purified to homogeneity previously in our laboratory and we were confident that we could obtain highly purified NDPK that we could use for N-terminal

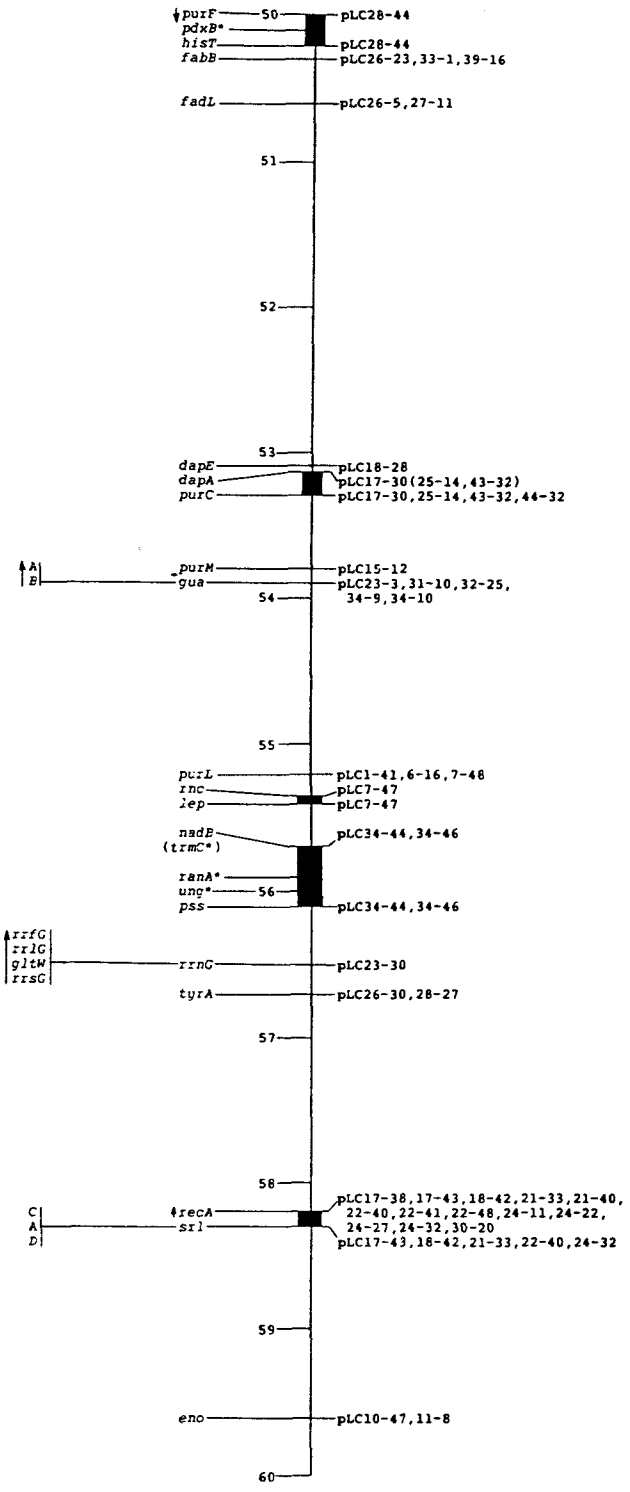


Figure I-5. *Escherichia coli* Chromosomal Map of Clarke and Carbon Plasmids (Bachmann, 1990).

amino acid sequencing. Once N-terminal amino acid sequence data were obtained, we could design mixed oligonucleotide probes that could be used to search for the *ndk* gene among the five Clarke and Carbon clones we had selected. Unfortunately, at this time there were no other amino acid or DNA sequence data available for comparison or homology studies. Therefore, I was not able to select conserved regions for the probes. I designed the probes based on low degeneracy and high uniqueness as compared to other known *E. coli* gene sequences. I also did enzyme activity assays of the Clarke and Carbon clones to select the one or ones (since they were likely to contain overlapping regions of DNA) most likely to contain the *ndk* gene. However, there was no mutant available and the background activity of NDPK was fairly high.

Later, when another *E. coli* library became available, the Kohara lambda library (Kohara et al, 1987), we chose five clones: 2D5, 7F8, 5E10, 6F10, and 8E12, again near *his S* (see Fig. I-6). Although this was not an expression library, it was found with another *E. coli* protein, thymidine kinase, that enzyme activity could be detected by assaying individual lysates from the lambda clones (Black and Hruby, 1991). I therefore assayed lysates prepared from the five clones we selected, for NDPK activity.

Since I had no reason to suspect that overexpression of the *ndk* gene would be deleterious to the cells containing the cloned gene, I planned to clone DNA fragments containing the *ndk* gene into pUC18 and pUC19 vectors. These vectors contain the *lac* gene and expression of cloned genes, although not tightly controlled, is induced by IPTG. Also, since I did not know whether or not the *ndk* gene had its own promoter, I could use these vectors in either case.

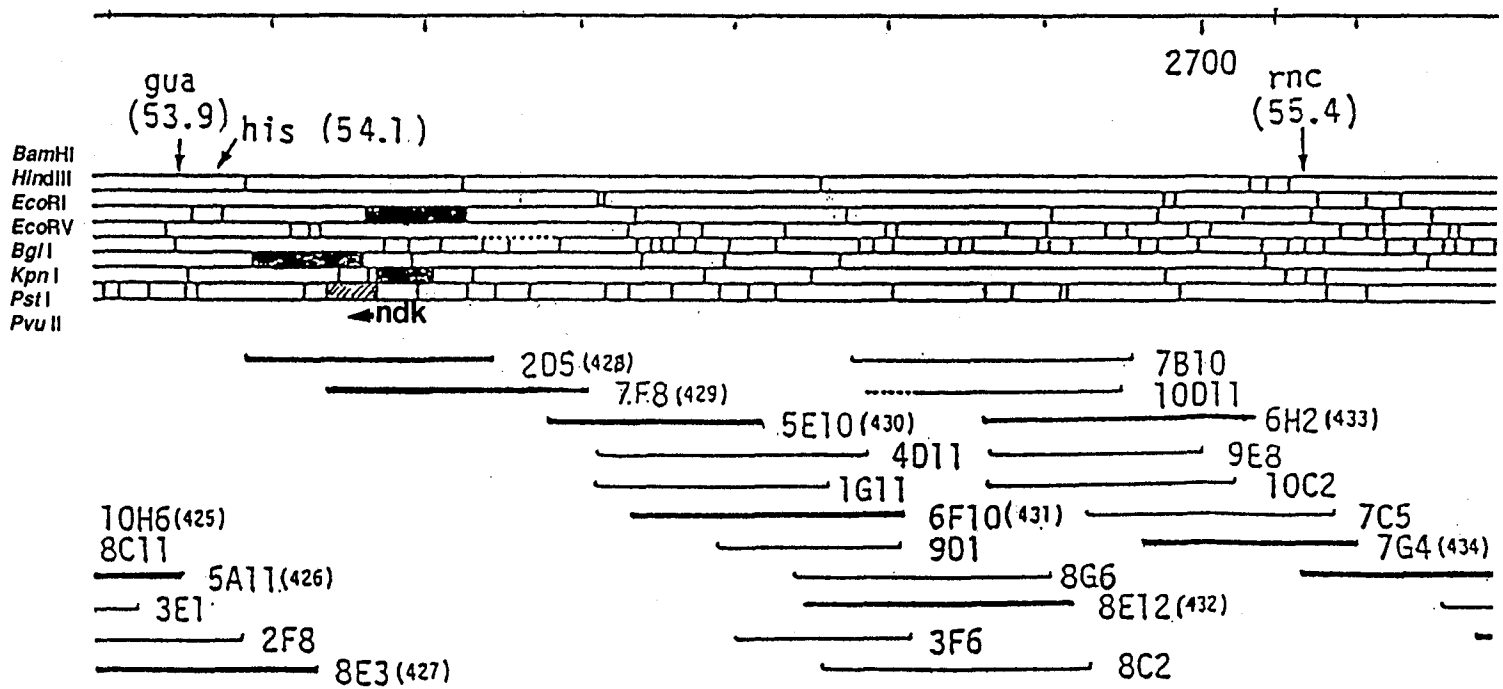


Figure I-6. *Escherichia coli* Chromosomal Map of Kohara Clones (modified from Kohara et al, 1987).

These vectors could also be used for plasmid sequencing to determine how much if any of the gene had actually been cloned.

I. Significance of Cloning and Overexpressing the *ndk* Gene

The purpose for cloning and overexpressing the *ndk* gene was to obtain large enough amounts of protein for interaction studies with NDPK and other enzymes involved in the T4 dNTP synthetase complex and with proteins involved in T4 replication. Since NDPK represents only about 0.08% of the total soluble protein in *E. coli* (Ray and Mathews, 1992), and its purification from crude extracts is fairly difficult to achieve, it would be much easier to obtain large amounts of pure protein from an over-expression system, in which it could represent at least 20% of the total soluble protein. Once large amounts of pure protein are obtained, antibodies can be made, characterization studies can be done, and protein-protein interaction experiments can be performed. Also, significant amounts of pure protein are needed for future reconstitution of the T4 dNTP synthetase complex. Once all the proteins involved in the complex are available in large enough quantities, crucial reconstitution experiments can begin.

Protein-protein interaction experiments involving NDPK and early T4 proteins are important because it is of interest to know whether this bacterial protein can be shown to interact specifically with viral proteins. In this way, host-viral interactions can be explored. Also, because NDPK catalyzes the last reaction step towards the production of dNTPs, it may be the most likely to interact with T4 replication proteins since DNA synthesis begins with incorporation of dNTPs into DNA. Therefore, NDPK may help answer the

question of whether the T4 dNTP synthetase complex is linked *in vivo* with proteins of the T4 replication apparatus.

NDPK is also of interest because it is a GTP-binding protein and as mentioned previously, there is growing evidence for NDPK-G protein associations in eukaryotic systems. An important question is whether NDPK interacts with other GTP-binding or "G-like" proteins in *E. coli.* Perhaps *E. coli* could serve as a model system for studying NDPK-"G-like" protein interactions, which could help elucidate the processes involving these interactions in eukaryotes.

J. Other *E. coli* GTP-binding Proteins

In *E.coli*, at least five other GTP-binding proteins have been described, including three proteins involved in protein translation: IF2, EF-Tu, and EF-G. IF-2 (M_r=90,000-118,000) is a translation initiation factor which binds fMet-tRNA to form the initiation complex, EF-Tu (M_r=45,000) is a translation elongation factor which binds aminoacyl-tRNA to the site of the 30s ribosome, and EF-G (M_r=80,000) is an elongation factor that mediates the translocation step during translation. Translation factors EF-Tu and EF-G are very abundant proteins in *E. coli,* accounting for 10^5 and 10^4 molecules per cell respectively (Gouy and Goutier, 1982).

Two other GTP-binding proteins not involved in protein translation have been described: LepA and Era. LepA is a cytoplasmic membrane protein with an apparent molecular weight of 76,000. LepA is believed to be involved in protein secretion due to the fact that it is cotranscribed with signal peptidase I (gp *lep*), which is responsible for endoproteolytic removal of signal peptides from

precursor proteins during the process of secretion (March and Inouye, 1985). Era is a membrane-associated protein with a estimated molecular weight of 35,232 (based on amino acid sequence), that has greater than 42% homology to the yeast RAS proteins (Ahnn et al, 1986). The two genes (*lepA* and *era*) encoding these proteins map at 55 minutes on the *E. coli* chromosome flanking the *lep* gene and the *rnc* (encoding RNase III) gene respectively (Inada et al, 1989). The cellular function of Era is unknown, but studies involving conditional mutants have shown that it is essential for cell growth (March et al, 1988; Inada et al, 1989). There is also evidence indicating that it plays a role in regulating cell division (Gollop and March, 1991).

All of these GTP-binding proteins contain three conserved regions of high homology that are known to be involved in phosphoryl binding (see region 1 and 2 in Fig. I-7) and guanine specificity (see region 3 in Fig. I-7), based on x-ray crystallography analysis of EF-Tu and computer studies (Jurnak, 1985; McCormick et al, 1985; and Dever et al, 1987). The consensus amino acid sequences of the three regions are GXXXGK, DXXG, and NKXD, respectively. It has also been shown that several ras proteins and other G proteins contain these three consensus sequences (McCormick et al, 1985).

K. Homology Among NDPK Sequences

As mentioned previously, when this thesis project began, there were no known or deduced amino acid sequences for NDPK in any organism.

	Region 1
E. coli EF-Tu	-Gly-His-Val-Asp-His-Gly-Lys-Thr- (18–25)
E. coli EF-G	-Ala-His-Ile-Asp-Ala-Gly-Lys-Thr- (17–24)
E. coli LepA	-Ala-His-Ile-Asp-His-Gly-Lys-Ser- (11–18)
E. coli IF2	-Gly-His-Val-Asp-His-Gly-Lys-Thr- (398–405)
S. cerevisiae RAS1	-Gly-Gly-Gly-Gly-Val-Gly-Lys-Ser- (17–24)
S. cerevisiae RAS2	-Gly-Gly-Gly-Gly-Val-Gly-Lys-Ser- (17–24)
Human Ha-ras, N-ras, Ki-ras	-Gly-Ala-Gly-Gly-Val-Gly-Lys-Ser- (10–17)
E. coli Era	-Gly-Arg-Pro-Asn-Val-Gly-Lys-Ser- (15–22)
	Region 2
E. coli EF-Tu	-Asp-Cys-Pro-Gly-His- (80–84)
E. coli EF-G	-Asp-Thr-Pro-Gly-His- (88–92)
E. coli LepA	-Asp-Thr-Pro-Gly-His- (77–81)
E. coli IF2	-Asp-Thr-Pro-Gly-His- (444–448)
S. cerevisiae RAS1	-Asp-Thr-Ala-Gly-Gln- (64–68)
S. cerevisiae RAS2	-Asp-Thr-Ala-Gly-Gln- (64–68)
Human Ha-ras, N-ras, Ki-ras	-Asp-Thr-Ala-Gly-Gln- (57–61)
E. coli Era	-Asp-Thr-Pro-Gly-Leu- (62–66)
	Region 3
E. coli EF-Tu	-Asn-Lys-Cys-Asp- (135–138)
E. coli EF-G	-Asn-Lys-Met-Asp- (142–145)
E. coli LepA	-Asn-Lys-Ile-Asp- (131–134)
E. coli IF2	-Asn-Lys-Ile-Asp- (498–501)
S. cerevisiae RAS1	-Asn-Lys-Leu-Asp- (123–126)
S. cerevisiae RAS2	-Asn-Lys-Ser-Asp- (123–126)
Human Ha-ras, N-ras, Ki-ras	-Asn-Lys-Cys-Asp- (116–119)
E. coli Era	-Asn-Lys-Val-Asp- (124–127)

Figure I-7. Three Consensus Amino Acid Sequences of GTP-binding Proteins (Ahnn et al, 1986).

The first amino acid sequence, deduced from DNA sequence, came from the Gram-negative, "slime-mold-like" bacterium, *Myxococcus xanthus*, where it was discovered by photoaffinity labeling that NDPK was one of five proteins to bind GTP (Munoz-Dorado et al, 1990). Soon after, an NDPK amino acid sequence was also deduced for *Dictyostelium discoideum*, a slime mold that has been used as a model system for studying signal transduction and differentiation (Lacombe et al, 1990), and later the gene encoding NDPK was cloned from rat cDNA, and an NDPK amino acid sequence was deduced (Kimura et al, 1990). Recently, the NDPK polypeptide chains, A and B, from human erythrocytes have been sequenced at the amino acid level. It has been found that the differences in these two chains and their possible combinations can account for the isozymic heterogeneity that has previously been described for this hexameric enzyme. It was also found that the A and B chain amino acid sequences were completely identical to the deduced amino acid sequences for the human cancer suppressor genes *nm23H1* and *nm23H2,* respectively (Gilles et al, 1991), which along with a mouse suppressor gene (*nm23-1*) and an abnormal wing disc gene (*awd*) in *Drosophila*, were known to have high homology with other NDPKs (Wallet et al, 1990; Biggs et al, 1990).

By comparing NDPK amino acid sequences from *M. xanthus, D. discoideum*, *Drosophila* (*awd*), rat , mouse, and human (*nm23*) systems, conserved regions were identified, mixed oligonucleotide probes were designed, and PCR amplification of genomic DNA was achieved. The *E. coli ndk* gene was located at 54.1 minutes (Hama et al, 1991) , near the *his S* gene, cloned and sequenced. *E. coli* NDPK was found to be 57% homologous to *M. xanthus* NDPK, 45% homologous to *D. discoideum* NDPK, 45% homologous to Drosophila (*awd*), 42% homologous to mouse (*nm23*), 43% homologous to the rat NDPK, and 43% homologous to human (*nm23*) at the amino acid level (see

Fig. I-8). The (deduced) N-terminal 46 amino acids agreed completely with the amino acid sequence determined directly from purified *E. coli* NDPK (Ray and Mathews, 1992), confirming that the *E. coli ndk* gene had been cloned (Hama et al, 1991).

L. NDPK: Its Relationship to the Tumor Suppressor *Nm23* Gene and to *Drosophila* Development

Nm23 was discovered as a potential cancer suppressor gene by differential colony hybridization between related low and high metastatic murine K-1735 melanoma cell lines (Steeg et al, 1988). They found that the *nm23* RNA levels were approximately ten-fold higher in cells and tumors of relatively low metastatic potential in two experimental systems: (1) murine K-1735 melanoma cell lines, and (2) N-nitroso-N-methylurea-induced rat mammary carcinomas. Quantitative reductions in *nm23* RNA and/or protein levels have also been observed in mouse mammary tumor virus induced tumor cells (Bevilacqua et al, 1989) and in ras ± adenovirus 2 Ela-cotransfected rat embryo fibroblasts (Steeg et al, 1988). Transfection of murine *nm23-H1* cDNA into highly metastatic K-1735 TK murine melanoma cells resulted in a decreased incidence of primary tumor formation and significant reductions in tumor metastatic potential (Leone et al, 1991a).

It was also found that a high metastatic phenotype in human infiltrating ductal breast carcinomas was accompanied by reduced levels of

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E. coli              MAIERTFSIIKPNAVAKNVIGNIFARFEAAGFKIVGTKMLHLTVEQARGF  50
M. xanthus           MAIERTLSIIKPDGLEKGVIGKIISRFEEKGLKPVAIRLQHLSQAQAEGF  50
Dictyostelium  MSTNKVNKERTFLAVKPDGVARGLVGEIIARYEKKGFVLVGLKQLVPTKDLAESH  55
Drosophila(awd)   MAANKERTFIMVKPDGVQRGLVGKIIERFEQKGFKLVALKFTWASKELLEKH  52
Mouse(nm23)         MANSERTFIAIKPDGVQRGLVGEIIKRFEQKGFRLVGLKFLQASEDLLKEH  51
Rat                 MANLERTFIAIKPDGVQRGLVGEIIKRFEQKGFRLVAMKFLRASEEHLKQH  51
Human(nm23)         MANCERTFIAIKPDGVQRGLVGEIIKRFEQKGFRLVGLKFMQASEDLLKEH  51

E. coli              YAEHDGKPFFDGLVEFMTSGPIVVSVLEGENAVQRHRDLLGATNPANALA  100
M. xanthus           YAVHAARPFFKDLVQFMISGPVVLMVLEGENAVLANRDIMGATNPAQAAE  100
Dictyostelium        YAEHKERPFFGGLVSFITSGPVVAMVFEGKGVVASARLMIGVTNPLASAP  105
Drosophila(awd)      YADLSARPFFPGLVNYMNSGPVVPMVWEGLNVVKTGRQMLGATNPADSLP  102
Mouse(nm23)          YTDLKDRPFFTGLVKYMHSGPVVAMVWEGLNVVKTGRVMLGETNPADSKP  101
Rat                  YIDLKDRPFFPGLVKYMNSGPVVAMVWEGLNVVKTGRVMLGETNPADSKP  101
Human(nm23)          YVDLKDRPFFAGLVKYMHSGPVVAMVWEGLNVVKTGRVMLGETNPADSKP  101

E. coli              GTLRADYADSLTENGTHGSDSVESAAREIAYFFGEGEVCPRTR          143
M. xanthus           GTIRKDFATSIDKNTVHGSDSLENAKIEIAYFFRETEIHSYPYQK        145
Dictyostelium        GSIRGDFGVDVGRNIIHGSDSVESANREIALWFKPEELLTEVKPNPN-LYE  156
Drosophila(awd)      GTIRGDFCIQVGRNIIHGSDAVESAEKEIALWFNEKELVTWTPAAKDWIYE  153
Mouse(nm23)          GTIRGDFCIQVGRNIIHGSDSVKSAEKEISLWFQPEELVEYKSCAQNWIYE  152
Rat                  GTIRGDFCIQVGRNIIHGSDSVESAEKEIGLWFKPEELIDYKSCAHDWVY   151
Human(nm23)          GTIRGDFCIQVGRNIIHGSDSVESAEKEIGLWFHPEELVDYTSCAQNWIYE  152
```

Figure I-8. NDPK Amino Acid Sequence Homology.

nm23 RNA (Bevilacqua et al, 1989), and subsequently that *nm23* expression in human breast cancer is associated with a good prognosis and a lack of lymph node metastasis (Hennessey et al, 1991).

Somatic allelic deletion of one or both alleles from tumor chromosomal DNA is a hallmark of suppressor genes identified to date, and somatic allelic deletion of *nm23-H1* has been observed in human breast, renal, colorectal, and lung carcinoma DNA samples (Leone et al, 1991b).

Until recently, the function of *nm23* was unknown, but it was found that Nm23 exhibited 78% identity to the *Drosophila* Awd protein, at the amino acid level (Rosengard et al, 1989). The *awd* (abnormal wing discs) gene was identified in a screen for hybrid-dysgenic late larval/early pupal lethal mutations. Besides abnormal wing discs, the mutant *awd* phenotype includes: (1) abnormal morphology of the larval brain and proventriculus; (2) aberrant differentiation of the wing, leg, and eye-antenna imaginal discs and ovaries; (3) cell necrosis, predominantly in the wing discs; and (4) heterogeneity in the pattern of abnormal morphology and cell death (Dearolf et al, 1988a). Transformation of the mutant *Drosophila* germ line with wild-type *awd* DNA resulted in normal morphology and development (Dearolf et al, 1988b).

Later it was found that the *awd* gene did indeed encode NDPK. This was shown by antibody cross-reactivity experiments via Western blotting and by measuring the loss of NDPK activity in an *awd* null mutation. It was also shown that the Awd/NDP kinase was associated with microtubules and that neuroblasts in *Drosophila* larvae homozygous for the null mutation in the *awd* gene are arrested in metaphase, indicating a critical role played by Awd in spindle microtubule polymerization (Biggs et al, 1990).

As mentioned previously, the human Nm23-H1 was found to be completely identical to the human erythrocyte NDPK A chain, and the Nm23-H2,

encoded by a separate gene which was discovered later (Stahl et al, 1991), was found to be completely identical to the human erythrocyte NDPK B chain. Therefore, it can be concluded that the *nm23* genes and the *awd* gene encode NDPKs. NDPKs are known to participate in at least two major functions that could play a role in cancer and development: microtubule assembly/disassembly and signal transduction through G proteins. Microtubule assembly requires the conversion of GDP to GTP. A microtubule-associated NDPK has been isolated that may catalyze the direct phosphorylation of bound GDP thereby activating assembly (Nickerson and Wells, 1984). As mentioned previously, there is also evidence for NDPK-G protein interactions and direct activation of G proteins by phosphorylation of bound GDP on G proteins or regulatory GTP-binding proteins that modulate the activity of G proteins (Randazzo et al, 1991; Kikkawa et al, 1990; Teng et al, 1991). In this way NDPK could regulate a range of cell signal responses that involve G proteins (see Fig. I-9) including development, oncogenic transformation, and metastasis.

M. Current Work

NDPK has been overexpressed and purified to homogeneity. The purified protein was immobilized onto an affinity column and has been found to selectively retain several early T4 proteins, which are currently being identified by two dimensional electrophoresis. An extract of *E. coli* cells infected with T4 phage containing a deletion mutation of the recombination and repair protein,

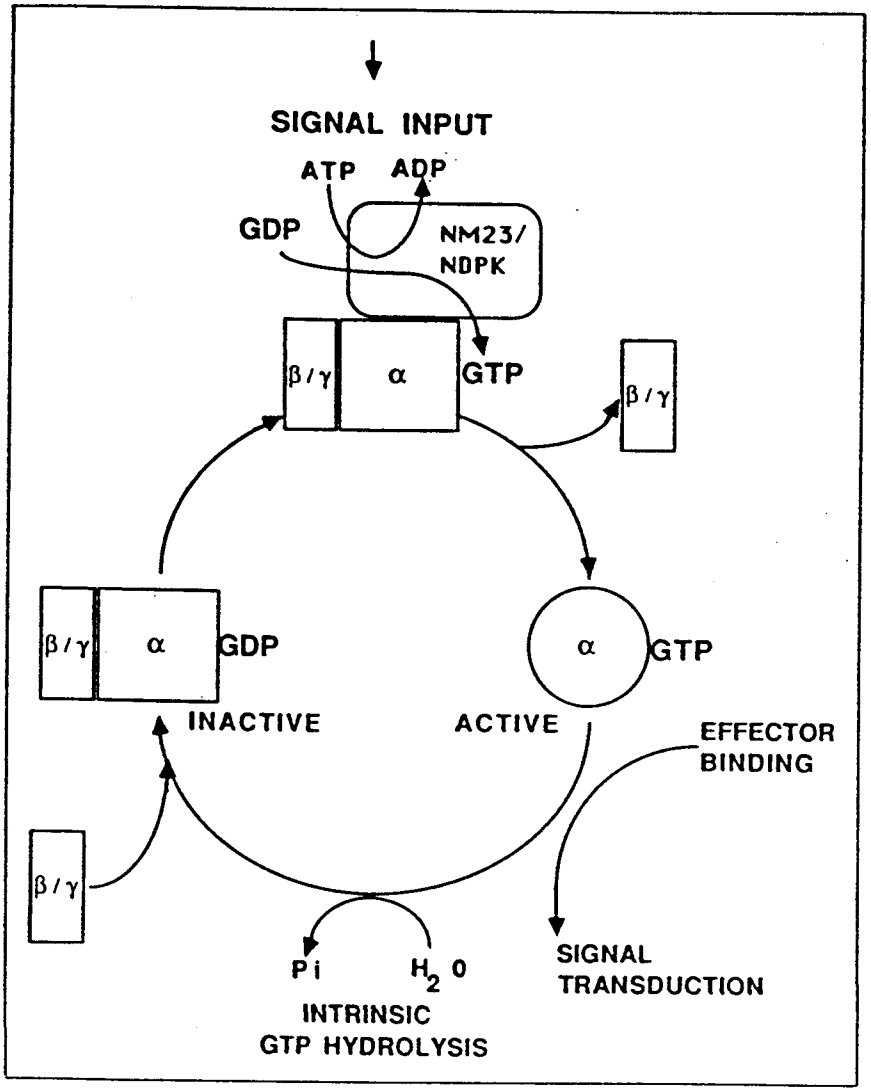

Figure I-9. Putative Role of NDPK/NM23 in the Activation of G Proteins (Liotta and Steeg, 1990).

UvsY, is being applied to the column to test the potentially strong interaction of UvsY with NDPK. Antibodies to NDPK have been generated, and used to generate anti-idiotypic antibodies, which mimic the original antigen, NDPK. Immunoprecipitations are currently being done with anti-idiotypic antibodies in order to probe for protein-protein interactions involving NDPK. Early T4 proteins which precipitate specifically with anti-idiotypic antibodies are being identified by two-dimensional electrophoresis, and by competition experiments. NDPK has also been fluorescently labeled in order to probe for protein-protein interactions through changes in fluorescence anisotropy. The potential interaction of NDPK and T4 DNA polymerase has been investigated by this method.

II. Materials and Methods

A. Materials

Restriction enzymes and reaction buffers were purchased from Bethesda Research Laboratories (BRL), Promega, or New England Biolabs. All radioisotopes were purchased from New England Nuclear (NEN). Antibiotics and DNA modifying enzymes were purchased from Sigma, and premade, ultrapure acrylamide for SDS polyacrylamide gel electrophoresis (Protogel) and DNA sequencing (Accugel-40), was purchased from National Diagnostics.

B. Media, Antibiotics and Buffers

1. Media

M9

1 g NH_4Cl, 6 g Na_2HPO_4, 3g KH_2PO_4, 1 g NaCl 0.01 g $CaCl_2$, 0.2 g Mg_2SO_4, and 3 g glucose per liter

SM9

M9 and 2 g casamino acids per liter

Nutrient Broth

8 g Difco nutrient broth, and 5 g NaCl per liter

Nutrient Agar

4 g Difco nutrient broth, 5 g NaCl, and 20 g Difco nutrient agar per liter

L-Broth

10 g Difco Bactotryptone, 5 g Difco Bactoyeast extract, 10 g NaCl, and 1g glucose per liter (adjusted to pH 7.2 before autoclaving)

L-Agar

10 g Difco Bactotryptone, 5 g Bactoyeast extract, 10 g NaCl, and 15 g Difco Bactoagar per liter

SOB

20 g Bactotryptone, 5 g Bactoyeast extract, 0.6 g NaCl, 0.18 g KCl, 2.03 g $MgSO_4$, and 2.47 g $MgCl_2$, add purest water available to 1 liter

SOC

SOB and 20 mM sterile filtered glucose

Terrific Broth

12 g Bactotryptone, 24 g Bactoyeast extract, bring up to 896 ml with sterile distilled water, autoclave then add 100 ml of a .17 M KH_2PO_4, 0.72 M K_2HPO_4 sterile solution and 4 ml of sterile glycerol

Lambda Soft Agar

5 g Difco Bactoagar, 10 g Difco Bactotryptone, 8 g NaCl, 2.46 g $MgSO_4$, and 1.3 g maltose per liter

NZCYM Medium

10 g NZamine (ICN), 5 g Bactoyeast extract, 5 g NaCl, 1 g casamino acids, and 2 g $MgSO_4$ per liter

SM Medium

5.8 g NaCl, 2 g $MgSO_4$, 25 ml 2M Tris pH 7.5, and 5 ml 2% gelatin per liter

2. Antibiotics

Ampicillin

25 mg/ml ampicillin (sodium salt) in distilled water, filter sterilize, store in aliquots at -20°C, add to media to a final concentration of 100 µg/ml.

Tetracycline

12.5 mg/ml of tetracycline in ethanol, filter sterilize, store in aliquots -20°C in the dark, add to media to a final concentration of 12.5 µg/ml.

Kanamycin

25 mg/ml in distilled water, filter sterilize, store in aliquots at -20°C, add to media to a final concentration of 30 µg/ml.

Colicin E1

3000 units/ml in distilled water, filter sterilize, store in aliquots at -20°C, add to media to a final concentration of 30 units/ml.

3. Buffers

Buffer A

20 mM Tris pH 7.4, 10 mM $MgCl_2$, 10% glycerol, and 10 mM β–mercaptoethanol

Buffer B

20 mM Tris pH 8.0, 10 mM $MgCl_2$, 50 mM KCl, 10% glycerol, and 1 mM β–mercaptoethanol

Holmes and Quigley Buffer

8% sucrose, 50 mM EDTA, 10 mM Tris pH 8.0, 0.5% Triton X-100, add lysozyme freshly before use.

Lysis Buffer

50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, and 4 mg/ml lysozyme, store in aliquots at -20°C.

TE Buffer

10 mM Tris-HCl (pH 8.0), 1 mM EDTA, autoclave.

10X TBE Buffer

108 g Tris base (pH 8.0), 55 g boric acid, and 7.4 g EDTA per liter

10X TBBE

54 g Tris base (pH 8.0), 55 g boric acid, and 4.65 g EDTA per liter

NDPK Enzyme Assay Buffer

1.58 g Tris-HCl (pH 7.1), 0.41 g $MgCl_2$, 0.14 ml β-mercaptoethanol, and 1.50 g KCl per 100 ml

NP-40 Lysis Buffer

150 mM NaCl, 1% NP-40, and 50 mM Tris base (pH 8.0)

NEPHGE Buffer

5.52 g ultrapure urea, 3.2 ml 25% NP-40, 0.5 ml β-mercaptoethanol, 0.5 ml 2% ampholines (pH 5-7), and 0.5 ml 2% ampholines (pH 3-10) per 10 ml

SDS PAGE Sample Buffer

0.76 g Tris base, 10 ml glycerol, 1 g SDS, 1 ml β–mercaptoethanol, and 0.5 mg bromophenol blue (adjust to pH 6.8 with 1 N HCl) per 50 ml

2D Sample Buffer

12.5 ml 0.5 M Tris pH 6.8, 5 ml β–mercaptoethanol, 20 ml 10% SDS, and 10 ml glycerol per 100 ml

Western Transfer Buffer

14.5 g Tris base, 67 g glycine, 4.8 L distilled water, and 1.2 L Methanol

TBST Buffer

30.5 g Tris base, 41 g NaCl, 2 g EDTA, and 5 ml Tween 20 (Sigma)

C. Cells, Plasmids and Phage

See Table II-1.

D. Purification of NDPK from Crude Extracts of *E. coli* B Cells

1. Preparation of Extract and Salt Precipitations

E. coli B cells were grown in 10 liter cultures in a New Brunswick Microferm fermentor in nutrient broth overnite. The cultures were centrifuged at 5000 rpm for 10 minutes in a Sorvall GSA rotor. Approximately 300 grams of cells were resuspended in 600 ml of Buffer A and sonicated on ice with a Branson sonicator at a setting of 8, four times for 30 seconds each. The sonicated suspension was centrifuged at 7500 rpm in a GS3 Sorvall rotor for 15 minutes. Solid ammonium sulfate was added to a final concentration of 45%, the solution was stirred for a period of 30 minutes on ice, and centrifuged at 7500 rpm for 15 minutes. The pellet was resuspended in 200 ml of Buffer A, and 0.3 volumes of 8% streptomycin sulfate was added to the supernatant over a period of 30 minutes while the solution was stirred on ice. The solution was centrifuged again at 7500 rpm for 15 minutes and the supernatant was dialyzed overnight in Buffer A.

TABLE II-1

Cells, Plasmids, and Phage

Cells	Phenotype/genotype	Source
E. coli B	wild-type	our collection
XL-1 Blue	recA1, endA1, gyrA96, thi-1, hsdR17, SupE44, relA1, lac F', pro AB, lacZΔM15, Tn10	Stratagene
DH5α	F^-ϕ80dlacZΔM15Δ(lacZYA-argF) U169, recA1, endA1, hsdR17, supE44λ^-, thi-1, gyrA, relA1	our collection
JM83	F^-, ara,Δ(lac-proAB) rspSL,ϕ80dlacZΔM	our collection
JA200	thr-1, leuB6, trpE63, recA56, thi-1, ara-14, lacY1, galK2, galT22, xyl-5mH-1^-, supE44	Barbara Bachmann
LE 392	hsdR514, supE44, supF58, lacY, galK2, galT22, metB1, trp55	Margaret Black
JM101	supE, thiΔ(lac-proAB) F' [traD36, $proA^+$, $proB^+$, $lacI^q$ lacZΔM15]	our collection
Plasmids	**Phenotype/genotype/description**	**Source**
pUC18, pUC19	$lacZ^+$, Amp resistance, pBR322 derivative	our collection
pIBI30	F1 $origin^+$, $lacZ^+$, Amp res., T7 and T3 promoter	IBI
pKT8P3	$lacZ^+$, Amp res., *ndk* gene and promoter, pUC9 der.	Masayori Inouye
pLC34-9, pLC34-10 pLC32-25, pLC15-12 pLC1-41	Clarke and Carbon plasmids, ColicinE1 res., colE1 der., 12-15 Kb sheared *E. coli* DNA inserts	Barbara Bachmann
Phage	**Phenotype/genotype/description**	**Source**
T4D	wild-type	our collection
M13K07	M13 helper phage, Kan res.	our collection
2D5, 7F8, 5E10, 6F10, 8E12	lambda phage clones, EMBL4 der.	Yugi Kohara
T4 $UvsY^-$	*uvsY* promoter deleted T4 phage	Ken Kruezer

2. DEAE Sephadex and Hydroxyapatite Fractionations

The resuspended, dialyzed ammonium sulfate precipitate was applied to a 4 cm x 44 cm DEAE Sephadex column equilibrated in Buffer A, and a 0-300 mM KCl gradient was applied to the column. Eluted fractions with NDPK activity, as determined spectrophotometrically (see below), were pooled and applied to a 2.5 cm x 10.5 cm hydroxyapatite column equilibrated in Buffer A, and eluted with a 0-0.5 M ammonium sulfate gradient.

3. Blue Sepharose (Matrex Blue) Fractionation

Fractions eluted from the hydroxyapatite column which had NDPK activity were pooled and dialyzed with Buffer B, loaded onto a 1.5 cm x 7 cm Blue Sepharose (Pharmacia) column equilibrated in Buffer B, and NDPK was eluted with either 2 mM UDP, 2 mM GDP, or 2 mM dTDP (Sigma).

4. Chromatofocusing Fractionation

Fractions eluted from the Blue Sepharose column having NDPK activity were pooled, concentrated, applied to a 24 cm x 1 cm PBE94 Chromatofocusing column, equilibrated in Buffer B, and eluted with Polybuffer 74 adjusted to pH 4.0.

5. Molecular Sieve Fractionation

Fractions eluted from the chromatofocusing column with NDPK activity were pooled and concentrated. Sodium dodecyl sulfate (SDS) was added to a concentration of 1%, and the solution was applied to a 1 cm x 50 cm G200 (Pharmacia) molecular sieve column.

6. HPLC Fractionation

Fractions eluted from the Blue Sepharose column with 2 mM GDP were combined and loaded onto an LKB DEAE FPLC column, which was run at room temperature and 2-10 atmospheres on a Varian 5000 HPLC. A 0.375 M phosphate buffer was run continuously through the column and a 0-0.3 M KCl gradient was applied to elute NDPK, which was detected by absorbance at 280 nm. A Labtronix autosampler was used for multiple injections of enzyme solutions and data was collected and processed by a Varian CDS 401 computer.

E. Enzyme Assays

NDPK enzyme activity was measured spectrophotometrically at 340 nm by a coupled enzyme assay (Bello and Bessman, 1963), in which the oxidation of NADH is followed (see Fig. II-1). 18 mM dTDP (Sigma) was used as the substrate and water was used to calibrate the Beckman spectrophotometer.

F. Protein Determinations

Protein concentrations were determined by the Coomassie Blue dye binding quantitation method (Peterson, 1983). Coomassie Brilliant Blue G-250 (Eastman Kodak) stock solution was prepared by dissolving 0.923 g of dye powder in 200 ml of 95% ethanol, which was then filtered. 150 ml of this stock solution was diluted to 600 ml with distilled water. 10 µl of sample or protein standard (BSA) was added to 1.0 ml of distilled water and 1.5 ml of the dye solution. The absorbance of the samples was measured at 595 nm after 5 to 10 minutes incubation at room temperature.

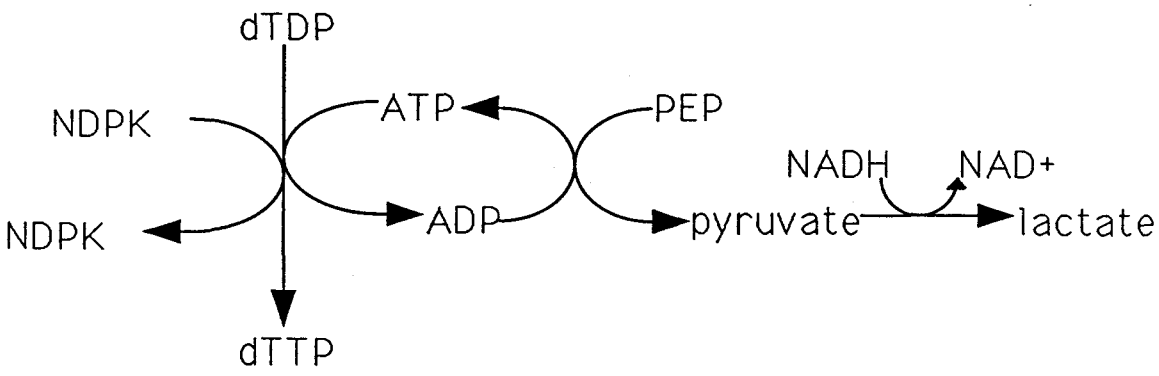

Figure II-1. Coupled Enzyme Reactions of the Spectrophotometric Assay of NDPK.

G. Gel Electrophoresis

1. One-dimensional SDS PAGE

Mini gels and larger slab gels were made according to the formulation given in Table II-2. Acrylamide concentrations were chosen according to the size of the protein to be electrophoresed. Low molecular weight and high molecular weight markers were purchased from Bio-Rad. 10% ammonium persulfate (APS) in distilled water was made at least weekly and kept at room temperature. Water-saturated 2-butanol was used as an overlay for running gels, which were either used immediately after polymerization or stored in the refrigerator (with water as an overlay) for future use. Gels were run at 100-200 volts.

2. Two-dimensional PAGE

Isoelectric focusing, nonequilibrium pH gradient electrophoresis (NEPHGE) tube gels used for the first dimension of two-dimensional analysis of proteins, were composed of 3.1 ml distilled water, 1.50 ml of 30% acrylamide: 1.6% bis acrylamide, 0.8 ml 25% NP-40, 0.5 ml ampholines pH 5-7 (40%), 0.5% ampholines pH 3-10 (40%), 5.2 g ultrapure urea (IBI), 10 µl 10% APS, and 7 µl TEMED (Bio-Rad). The tube gels were poured in 2.2 mm (internal diameter) glass tubing up to 11 cm from the bottom of the parafilm-sealed ends.

TABLE II-2

Formulations for SDS Polyacrylamide Gel Electrophoresis

Running Gel

(Volume in milliliters)

Percent Acrylamide:	7%	8%	9%	10%	12%	12.5%	15%
Composition							
Distilled water	15.0	14.0	13.0	12.0	10.0	9.5	7
1.5 M Tris pH 8.8	7.5	7.5	7.5	7.5	7.5	7.5	7.5
10% SDS	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Acylamide:Bis	7.0	8.0	9.0	10.0	12.0	12.5	15.0
10% APS	0.1	0.1	0.1	0.1	0.1	0.1	0.1
TEMED	.01	.01	.01	.01	.01	.01	.01

4% Stacking Gel Compostion	Volume in milliliters
Distilled water	6.3
0.5 M Tris pH 6.8	2.5
10% SDS	0.1
Acrylamide:Bis	1.5
10% APS	0.1
TEMED	.02

Water-saturated 2-butanol was used as an overlay, which was removed after the tubes were allowed to polymerize for one hour. It was replaced with 25 µl of NEPHGE buffer and 25 µl of distilled water, and the gels were allowed to equilibrate for 30 to 60 minutes before use.

3. DNA Agarose Electrophoresis

Plasmid, phage, or single-standed DNA was electrophoresed through 0.6-1% ultrapure agarose (BRL) in TBE buffer. A 1-kb ladder DNA marker (BRL) was used for molecular weight (kb) determinations, and the gels were run at 100 volts.

4. DNA Acrylamide Electrophoresis

The purity of mixed oligonucleotide probes and sequencing primers was determined by electrophoresis through 20% acrylamide large slab gels composed of 25.2 g of ultrapure urea, 30 ml 40% acrylamide:2% bis acrylamide, and 6 ml 10X TBE buffer brought up to a total volume of 60 ml with distilled water. The solution was degassed and 35 µl of TEMED and 500 µl of APS were added.

6 or 8% acrylamide gels were used for DNA sequencing depending upon which bands needed to be visualized. 8% gels were used to visualize bands closer to the sequencing primer at the bottom of the gel, and 6% gels were used to visualize bands further from the sequencing primer. 8% gels were composed of 460 g of ultrapure urea, 100 ml of 10X TBE buffer, and 200 ml of 40%

acrylamide: 2% bis acrylamide brought up to a total volume of one liter, filtered and degassed. 6% gels were composed of the same mixture except 150 ml of 40% acrylamide: 2% bis acrylamide was used. The gels were run at 25 milliamps.

H. N-terminal Amino Acid Sequencing

N-terminal amino acid sequencing was done in the Center for Gene Research and Biotechnology by Dr. Reg McParland and by Barbara Robbins on an Applied Biosystems 475 Gas Phase automated protein sequencer. One nanomole of lyophilized NDPK was brought up in 30% acetonitrile for sequencing.

I. Growth and Amplification of Clarke and Carbon ColE1 and Kohara Lambda Clones

1. Clarke and Carbon Clones

Clarke and Carbon ColE1 plasmid clones pLC34-9, pLC34-10, pLC32-25, pLC15-12, and pLC1-41 in JA200 *E. coli* cells obtained from Barbara Bachmann (*E. coli* Genetic Stock Center, Yale Univ.) were grown in LB medium containing 30 units/ml of colicin E1 and amplified in the presence of chloramphenicol as described in Maniatis (Maniatis et al, 1982).

2. Kohara Lambda Clones

Kohara lambda clones 2D5, 7F8, 5E10, 6F10, and 8E12 were obtained from Dr. Yugi Kohara (National Institute of Genetics, Japan) and were spotted onto nutrient agar plates covered with lambda top agar containing LE392 *E. coli* (obtained from Margaret Black, Microbiol. Dept., O.S.U.). The plaque that formed was then picked with the tip of a sterile Pasteur pipette and placed in 1 ml of SM media containing a drop of chloroform, and allowed to sit at room temperature for 1-2 hours. The solution was then titered and lysates with higher titers were prepared by using the titering plates to make plate lysate stocks as described in Maniatis (Maniatis et al, 1982). This was repeated until lysates of 1×10^{10} or greater titers were achieved.

J. Plasmid, Phage, and Single-stranded DNA Preparation and Analysis

1. Plasmid DNA

Clarke and Carbon ColE1 plasmid DNA was prepared on a large scale by the method of Holmes and Quigley (Maniatis et al, 1982) and the DNA was analyzed for purity by electrophoresis through 0.6% and 0.8% agarose, and by performing restriction analyses with several restriction enzymes. Other plasmid DNA, such as pUC plasmid DNA, was prepared either by a small scale Holmes and Quigley preparation or by an alkaline lysis miniprep method in which 1 ml of an overnight culture grown in the presence of ampicillin was placed in a

microfuge tube and centrifuged at 8000 rpm for one minute then resuspended in 200 µl of lysis buffer. After incubation for 5 minutes at room temperature, 400 µl of freshly prepared 0.2N NaOH, 1% SDS solution was added and the solution was mixed by inverting the tube 3 to 6 times. After 5 minutes on ice, 300 µl of a 7.5 M ammonium acetate solution was added and the solution was vortexed and placed on ice. After 10 minutes on ice, the solution was centrifuged at 10,000 rpm for 3 minutes and the clear supernatant was transferred to another tube. 0.6 volumes of isopropanol was added to the supernatant, which was incubated at room temperature for 10 minutes, then centrifuged at 15,000 rpm for 10 minutes. The pellet was resuspended in TE buffer, and the DNA was analyzed for purity by electrophoresis and by performing restriction enzyme digests.

2. Phage DNA

Lambda DNA was prepared by a miniprep method in which a 20-ml lysate, prepared as described above, was cleared of debris by centrifuging at 10,000 rpm for 10 minutes and 400 µl of DNase I (1 mg/ml) and 20 µl of RNase A (10 mg/ml) were added to the lysate, which was incubated at 37°C for 15 to 30 minutes. 0.2 volumes of 20% PEG (8000), 2.5 M NaCl was then added and the solution was centrifuged at 10,000 rpm for 15 minutes. The pellet was resuspended in TE buffer and transferred to a microfuge tube. A phenol:chloroform extraction was done, the aqueous layer was transferred to another microfuge tube, and 1/2 volume of 7.5 M ammonium acetate and 2 volumes of ethanol were added. The microfuge tube was then placed in the -80°C freezer for 1 hour or more, removed and centrifuged at 15,000 rpm for 10 minutes, and the pellet was resuspended in TE buffer.

3. Single-stranded DNA

An overnight culture of JM101 cells containing the recombinant pIBI30 plasmid was grown in the presence of 100 µg/ml of ampicillin and used to inoculate a fresh 50-ml culture, which was allowed to grow to a density of approximately 40 Klett. 35 to 70 µl of M13K07 helper phage with a titer of 1×10^{11} pfu/ml was added to the 50 ml culture, which was placed in the shaking incubator set at 37°C. After 30 minutes, 70 µl of a 25 mg/ml solution of kanamycin was added to the culture, which was allowed to incubate for another 5.5-7.5 hours, then centrifuged at 10,000 rpm for 10 minutes. The supernatant was sterile filtered, transferred to two 30-ml Corex tubes, and 15% PEG (8000), 3 M NaCl was added to the supernatant at a 1/5 dilution. The supernatant was left on ice for 30 minutes or more, centrifuged at 11,000 rpm for 15 minutes, and the pellet was resuspended in TE buffer. Phenol:chloroform extraction was done until no interface remained and the DNA was precipitated with 1/2 volume of 7.5 M ammonium acetate and 2 volumes of ethanol, at -80°C for 30 minutes or more. The DNA was pelleted at 15,000 rpm for 10 minutes and resuspended in TE buffer.

K. Mixed Oligonucleotide Probes

1. Design and Synthesis

Mixed oligonucleotide probes were designed based on the N-terminal amino acid sequence that I obtained for *E. coli* NDPK since no other known NDPK DNA or amino acid sequence data (i.e. for other organisms) was available. Oligos of at least 18 nucleotides in length were designed for specificity based on uniqueness and low degeneracy. Uniqueness was determined by a computer homology search performed by Jack Kramer of each oligo sequence compared against other bacterial DNA sequences entered in GenBank. Completely mixed oligo probes were made by including all possible combinations of bases. An Applied Biosystems automated DNA synthesizer located in the Center for Gene Research and Biotechnology was operated by Dr. Reg McParland.

2. Determination of Purity

The purity of each probe was determined by electrophoresis through 20% acrylamide gel (described previously), where the major DNA products synthesized were visualized by UV shadowing or by exposure to film if the DNA was end-labeled.

L. Southern Blotting

1. Southern Transfer

0.6% to 0.8% agarose DNA gels were stained with ethidium bromide after electrophoresis and photographed with a fluorescent ruler placed on the edge of the gel. The DNA was then transferred onto a Zeta-probe membrane (Bio-Rad) overnight as described in Maniatis (Maniatis et al, 1982). Gels with restriction fragments greater than 8 kb were acid depurinated (Wahl et al, 1979) to partially hydrolyze the DNA. The gel was then stained with ethidium bromide again to determine the efficiency of DNA transfer.

2. End-labeling of Oligonucleotide Probe

10 µl of oligonucleotide (20 pmoles) was added to 2.5 µl of 10X polynucleotide kinase along with 2 µl (20 units) of polynucleotide kinase (purchased from New England BioLabs), and approximately 20 pmoles of [$^{32}\gamma$P]-ATP (specific activity of approximately 3000 Ci/mmol) purchased from New England Nuclear. This mixture was incubated in a water-filled heating block set at 37°C for 30 to 45 minutes and the labeling reaction was stopped by incubation for 2 minutes in a heating block set at 95°C.

3. Prehybridization

After DNA transfer, the Zeta-probe membrane was placed in a plastic bag containing a prehybridization solution composed of 7.75 ml of distilled water, 2.5 ml of 20X SSPE (3.6 M NaCl, 0.2 M sodium phosphate pH 7.0, 0.2 M EDTA), 0.5 ml 20% SDS, 0.5 ml 10% Blotto (10 g of Carnation nonfat powdered milk, 0.2% sodium azide in 100 ml sterile distilled water), and 0.5 sheared salmon sperm DNA (Sigma), which was denatured by heating to 100°C for 5 minutes. The membrane and solution were then carefully sealed in a plastic bag, placed in a plastic box containing 200 to 500 ml of distilled water (preincubated to the correct hybridization temperature), and placed in a Grant shaking water bath incubator set at the correct hybridization temperature for 1 to 2 hours. The shaking incubator was set at a medium setting.

4. Hybridization

The membrane was placed in a fresh plastic bag containing the same solution used for prehybridization except that the end-labeled oligonucleotide probe was included in the solution. The bag was then sealed and placed in preincubated water in a plastic box and allowed to incubate as before at the hybridization temperature overnight.

5. Wash

After hybridizing overnight, the membrane was washed three times in a plastic box containing at least 200 ml of 2X SSC (0.3 M NaCl, 0.3 M sodium citrate) and 0.1% SDS. The box was rocked on a Reliable Scientific platform rocker set at a medium setting. The membrane was then washed once for 5 minutes at the hybridization temperature with the same wash solution except that it was preincubated at the hybridization temperature. This last wash was done in the Grant shaking water bath incubator set at the hybridization temperature.

6. Development

The membrane was removed from the wash solution, allowed to dry somewhat (not completely), wrapped in Saran wrap, and placed in a film holder with Kodak X-OMAT film on top of the membrane. The film holder was then placed in the -80°C freezer.

M. Cloning

1. DNA Isolation from Agarose Gels

After digestion of the DNA with restriction endonucleases generally for 2 hours at 37°C and electrophoresis through agarose, restriction fragments were isolated from the agarose by a freeze/phenol method. The fragment was

excised with a clean razor blade and placed in a sterile 1.5-ml microfuge tube, which was then placed in the -80°C freezer. After 5 minutes, the microfuge tube was removed and the contents were allowed to thaw. The semi-frozen agarose was mashed thoroughly with a cone-shaped agarose masher. An equal volume of phenol was added, mashing was continued, and the mixture was vortexed for 20 minutes. The microfuge tube was then placed in the -80°C freezer again for 10 to 15 minutes, then removed and centrifuged at 15,000 rpm for 10 minutes. The supernatant was transferred to a new tube, an equal volume of a 1:1 phenol/chloroform (24 chloroform:1 iodoacetic acid) was added, the solution was vortexed for 20 minutes, and the mixture was centrifuged as before. The supernatant was transferred to a new sterile tube and the phenol/chloroform extraction was repeated twice more. Finally the supernatant was transferred to a new tube and the DNA was precipitated with 1/2 volume of ammonium acetate and 2 volumes of ethanol. The microfuge tube was placed in the -80°C freezer and after 1 or more hours was removed and centrifuged immediately at 15,000 rpm for 10 minutes. The supernatant was decanted and the DNA pellet was air dried and resuspended in TE buffer. Recovery was estimated by electrophoresis of the DNA through an agarose gel and staining with ethidium bromide.

2. Ligation

After freeze/phenol recovery from agarose, the restriction fragments were diluted to a concentration of approximately 10 ng/µl in TE buffer and at least three ligation reactions were set up with ratio of insert (restriction fragment) to linearized vector being 1:1, 2:1, 3:1 etc. for "sticky"-end ligations and 3:1, 4:1,

5:1, etc. for "blunt"-end ligations. For example, a 1:1 ratio would be 10 ng insert to 10 ng of linearized vector. 1 unit of ligase (BRL) was used for "sticky"-end ligations and 2 units were used for "blunt"-end ligations. With "blunt"-end cloning, the linearized vector was dephosphorylated with calf-intestinal phosphatase (BRL) as described in Maniatis (Maniatis et al, 1982) to eliminate the possibility of the vector self-ligating. To the insert, ligase, and vector mixture 5X ligation buffer (BRL) and sterile distilled water were added in an amount resulting in a final concentration of 1X ligation buffer. The final ligation mixtures were then incubated at either 14°C overnight for "sticky"-end cloning or at 25°C overnight for "blunt"-end cloning, and the ligation reaction mixtures were subsequently transformed into competent cells.

3. DNA Transformation

Competent cells were either made by the calcium chloride method (Maniatis et al, 1982) or for higher efficiencies by a method using frozen storage buffer, DMSO, and SOC media described by Hanahan (Hanahan, 1985). Ligation reaction mixtures along with a positive control of 10 ng of plasmid DNA were mixed separately with the competent cells and incubated on ice followed by heat shock treatment at 45°C for 90 seconds, incubation with SOC media, and moderate shaking in a water bath set at 37°C for 30 minutes to one hour.

N. DNA Sequencing

1. Plasmid Sequencing (double-strand)

Plasmid DNA was prepared by the alkaline lysis described previously without further purification. Sequenase version 2.0 was purchased from USB in a kit including 5X annealing buffer, 0.1 M DTT, labeling nucleotide mixtures, termination nucleotide mixtures, stop solution, and universal M13 forward primer. Reverse primer was purchased from BRL. Sequencing was done by the Sanger dideoxy sequencing method with ^{35}S-labeled dATP thiophosphate purchased from NEN having a specific activity of 1000-1500 Ci/mmol.

Approximately 1 µg or more of plasmid DNA was brought up to a volume of 24 µl and 6 µl of NaOH was added. The solution was allowed to sit at room temperature for 5 minutes, then transferred to ice where 3 µl of 3 M ammonium acetate (pH 4.5) and 75 µl of ethanol were added. The solution was then placed in the -80°C freezer for at least 30 minutes, then microfuged for 20 minutes at 15,000 rpm. The DNA pellet was then brought up in 6 µl of sterile distilled water, and 2 µl of primer (1-2 pmol) plus 2 µl of 5X annealing buffer were added. The mixture was heated to 65°C in a heating block filled with distilled water for 2 minutes and allowed to cool to room temperature for 30 minutes. To the annealed template-primer mixture, 1 µl of 0.1 M DTT, 2 µl of labeling nucleotide mix, 1 µl of [^{35}S]dATP and 2 µl of Sequenase version 2.0 (3 units) were added, mixed, and incubated for 2 to 5 minutes. After the labeling reaction was completed, 3.5 µl of the labeling reaction was transferred to four separate

microfuge tubes labeled A, T, G, and C respectively, containing 2.5 µl of the appropriate termination mixture. Each tube was incubated for 2 to 5 minutes in a 37°C water bath and 4 µl of stop solution was added to each termination mixture, which were then stored in a -20°C freezer or heated to 80°C for 5 minutes, and loaded (2-5 µl) onto a sequencing gel.

2. M13 Sequencing (single-strand)

M13 sequencing was done by the same method as plasmid sequencing except the single-stranded DNA was prepared as described previously and 1 µg of single-stranded DNA was added to 1 µl of primer and 2 µl of 5X annealing buffer, brought up to a final volume of 10 µl with sterile distilled water.

O. Overexpression of Recombinant NDPK

pKT8P3 plasmid DNA containing the *ndk* gene and its promoter (kindly provided by Dr. Masayori Inouye, Rutgers Univ.) was freshly transformed into DH5α cells, which were spread onto a nutrient agar plate containing 100 µg/ml of ampicillin, and incubated overnight at 37°C. Colonies were picked and used to inoculate 10 ml of nutrient broth containing the same concentration of ampicillin, which was then incubated overnight in a New Brunswick Scientific shaking incubator. 10 ml overnight cultures were used to start large cultures (composed of 600-800 ml of nutrient broth plus ampicillin in 2-liter flat-bottomed flasks), which were incubated overnight at 37°C in the shaking incubator set at 300 rpm.

P. Purification of Overexpressed NDPK

1. Preparation of Extract and Salt Precipitations

Large overnight cultures were centrifuged at 5000 rpm for 10 minutes. The cell pellets were resuspended in 10 ml of Buffer A and sonicated on ice four times for 30 seconds with a Branson sonicator at a setting of 8. Soluble proteins were fractionated from insoluble proteins by centrifugation at 10,000 rpm for 10 minutes. The total soluble protein concentration was determined by Coomassie dye binding quantitation, and the extent of overexpression was analyzed via SDS PAGE. Soluble protein suspensions exhibiting optimal overexpression were fractionated by ammonium sulfate precipitation on ice for 30 minutes, where NDPK was specifically precipitated in the concentration range between 45% and 60% ammonium sulfate. 0.3 volumes of 8% streptomycin sulfate was then added to the resuspended pellet (Buffer A) over a period of 30 minutes while the solution was kept on ice. The protein concentration of the ammonium sulfate precipitates were determined and the precipitated protein was analyzed by SDS gel electrophoresis.

Immunoprecipitation experiments were also done with sera that included anti-idiotypic antibody generated from antibody to native, active NDPK that had been fractionated through a protein A Sepharose column, as well as eluted specifically from an immobilized NDPK affinity column. This was done to obtain an IgG fraction specific for native, active NDPK.

2. Blue Sepharose Fractionation

Ammonium sulfate; streptomycin sulfate-precipitated protein was dialyzed overnight in Buffer B and loaded onto a 12-ml Blue Sepharose (Pharmacia) column equilibrated in Buffer B. NDPK was specifically eluted with 2 mM dTDP (Sigma) in distilled water. Eluted fractions were analyzed by SDS gel electrophoresis, combined and concentrated by centrifugation at 5,000 rpm in Centricon 30 concentrators (Amicon).

3. MonoQ Fractionation

Concentrated NDPK was loaded onto an FPLC MonoQ column (Pharmacia) equilibrated with filtered, degassed Buffer B and eluted with a 0-0.5 M KCl gradient. Eluted fractions were analyzed for purity and concentrated as described above.

Q. Characterization of NDPK

1. UV Scan

A UV absorbance scan was done of highly purified (Blue Sepharose and FPLC MonoQ fractionated) recombinant NDPK in 20 mM MOPS (Sigma) buffer (pH 7). The scan was done from 230 nm to 300 nm on a Beckman DU-64 spectrophotometer in UV scan mode.

2. Quantitation of Amino Acids

The quantitation of several amino acid components of NDPK was done by a single-column reverse-phase HPLC method, in which 4'-dimethylamino-azobenzene-4-sulfonyl (dabsyl) chloride derivatives can be detected down to 1 picomol (Malencik et al, 1990). Sixteen amino acids were analyzed by comparison to standards and a total molar determination was used to define the extinction coefficient (absorptivity) of a 1g/liter solution of purified NDPK at 280 nm. A 1 mg/ml solution of NDPK in 20 mM MOPS buffer (pH 7) was used for the analysis.

3. Intrinsic Fluorescence Scan

An emission scan of the intrinsic fluorescence of purified NDPK was done to determine whether the intrinsic fluorescence could be used for anisotropy measurements, and as a reference to detect any shift in emission or change in intensity of fluorescence upon the addition of T4 DNA polymerase. A 1 mg/ml solution of NDPK in 20 mM MOPS buffer (pH 7) was excited at 280 nm and the scan was performed on a Perkin Elmer MPF2A fluorometer.

R. Immobilized NDPK Affinity Chromatography

1. Immobilization of NDPK

Approximately 8 mg of highly purified NDPK dialyzed in coupling buffer (see below) was coupled to 2 to 3 ml of Bio-Rad affigel 10 matrix that had been washed with distilled water, and 0.1 M MOPS (pH 7.5), 80 mM $CaCl_2$ coupling buffer. Coupling of NDPK to the matrix was done in a 15-ml Falcon tube at 4°C on a rotating platform overnight. The degree of coupling was determined by sampling coupling buffer (100 µg or more) and performing a Coomassie binding protein assay. 0.1 M ethanolamine-HCl (pH 8) was then added to block any remaining active esters on the matrix. The matrix was packed in a 3-ml syringe and the "column" was equilibrated with affinity column buffer, washed with 4 M NaCl, and re-equilibrated with affinity column buffer.

2. Preparation of Extract

500-ml cultures of *E. coli* B cells in M9 media were grown to a density of approximately 5 X 10^8 cells per milliliter (80 Klett). 2.5 ml of L-tryptophan (5 mg/ml) was added to cultures that were infected with T4 dialyzed in M9. Cultures infected with T4 were infected at a multiplicity of infection of 10, and 10 µl (100 µCi) of ^{35}S-labeled methionine was added at 3, 4, 5, 6, and 7 minutes post infection. At 8 minutes post infection, 2 ml of unlabeled methionine

(5 mg/ml) was added and the cultures were immediately chilled on ice, split into 2 large centrifuge bottles, and centrifuged at 5000 rpm for 20 minutes. Uninfected *E. coli* B cell cultures were also grown to 80 Klett and 50 µl of ^{35}S-labeled methionine was added. After 30 minutes, 2 ml of unlabeled methionine was added and the cultures were centrifuged as described above. Cell pellets were stored in the -80°C freezer, resuspended in 5 ml of affinity column buffer, and sonicated on ice with a Branson sonicator at a setting of 8, four times for 30 seconds each. Incorporation of label was measured by adding 5 µl of sonicate to 5 ml of Ecolite scintillation fluid and counts per minute (cpm) were measured for two minutes on channel C of a Beckman scintillation counter. DNA and RNA were eliminated from the extracts by adding 5 mM $CaCl_2$, 5 mM $MgCl_2$, 10 µg/ml DNase I and 3 µg/ml micrococcal nuclease and incubating on ice for 2 hours. Extracts were then centrifuged for 15 minutes at 10,000 rpm in an SS34 Sorvall rotor.

3. Affinity Column Fractionation

Approximately 5 ml of labeled soluble extract was loaded onto the NDPK affinity column and allowed to circulate through the column overnight at 4°C. A sample of the flow-through fraction (FT-1) of labeled proteins that did not bind to the column was counted, and 100-µl samples of the fraction were also used for one-dimensional and two-dimensional polyacrylamide gel analysis. The remaining portion of the fraction was discarded. The column was then washed with approximately 15 ml of affinity column buffer, a 5 µl sample of the flow-through fraction (FT-2) was measured for radioactivity, and the rest was discarded. The column was then allowed to equilibrate in affinity column buffer

for 30 minutes, washed with another 15 ml of affinity column buffer, and a sample of the flow-through fraction (FT-3) was measured for radioactivity with the rest discarded. The column was washed with 10 more ml of affinity column buffer and the fraction (FT-4) was sampled for radioactivity and discarded. 3 ml of the same affinity column buffer as used previously was passed through the column, except the concentration of salt (NaCl) was increased to 200 mM from 25 mM. A sample of the fraction (A-1) was measured for radioactivity with the rest discarded. An additional 2 ml of the 200 mM NaCl affinity column buffer was passed through the column and the eluted fraction (A-2) was sampled for radioactivity and saved. The column was then allowed to equilibrate in the 200 mM NaCl affinity column buffer for 30 minutes, after which 8 more ml were eluted and saved. Protein eluted in the 10 ml total A-2 fraction was immediately precipitated with 100 µl of 1.5% sodium deoxycholate and 1 ml of 75% trichloroacetic acid, kept at 4°C overnight, and pelleted at 2000 rpm, for 15 minutes in a GLC Sorvall centrifuge. The protein pellet was then resuspended in 100 µl of affinity column buffer and 10 µl of 1 N NaOH, 60 µl of which was added to 60 mg of ultrapure urea and 120 µl of NEPHGE buffer for two-dimensional polyacrylamide gel analysis. The rest was added to 2X sample buffer for one dimensional SDS PAGE analysis. The column was then washed with 5 ml of 200 mM NaCl affinity column buffer, and the fraction (A-3) was sampled for radioactivity and discarded. The same steps were used to elute proteins from the column with 600 mM NaCl and 2 M NaCl affinity column buffer and the fractions containing eluted proteins (B-2 and C-2 respectively) were precipitated as before and analyzed on one and two-dimensional gels.

4. Two Dimensional Gel Analysis

Total labeled extract and fractions FT-1, A-2, B-2, and C-2 were prepared for two dimensional NEPHGE analysis as described above, loaded onto NEPHGE tube gels overlaid with 8 M urea and 1% ampholines (pH 5-7 and pH 3-10), and electrophoresed for 4 hours at a constant voltage of 400 volts. The tube gels were then blown or squirted out of the tubes, placed in 2D sample buffer, in which they were allowed to equilibrate while rocking for 30 minutes, then placed in 15-ml Falcon tubes and stored in the -80°C freezer. 12.5% SDS polyacrylamide, thick (1.5 mm) slab gels were used for the second dimension. Thawed tube gels were placed directly on top of the stacking gel and sealed with bromophenol blue-dyed agarose. The slab gels were then run at 30 milliamps each for approximately 6 hours, placed in 50% methanol, 10% acetic acid for 15 minutes, 5%, methanol, 10% acetic acid for another 15 minutes, allowed to sit in distilled water for 15 minutes, and finally placed in 1 M sodium salicylate for another 15 minutes. The slab gels were then dried and Kodak X-OMAT film was placed over the gel and allowed to expose at -80°C. The film was then developed and the subsequent autoradiogram was compared to autoradiograms of total labeled extract for the identification of specifically eluted proteins.

S. Antibody Generation and Fractionation

1. Antibody to Denatured NDPK

Total insoluble protein from an extract of DH5α cells containing pKT8P3, in which NDPK represented approximately 50% of the total protein, was run on a 15% polyacrylamide gel. The band corresponding to NDPK was excised from the gel and electroeluted into SDS running buffer in a Schleiger and Schuell Elutrap run at 100 volts overnight. A Coomassie protein determination was performed to estimate the amount of eluted NDPK, which was electrophoresed through another 15% SDS polyacrylamide gel to estimate purity. Approximately 200 µg of denatured NDPK was mixed with Freund's Complete adjuvant (Sigma) in a 1:1 ratio, and the solution was injected into two white New Zealand rabbits subcutaneously. After one month, the rabbits were boosted intramuscularly with approximately 100 µg of denatured NDPK mixed with Freund's Incomplete adjuvant (Sigma) in a 1:1 ratio. Sera were collected after approximately one week and stored in the -20°C freezer. Subsequent boosts were done similarly.

2. Antibody to Native, Active NDPK

Overexpressed NDPK was purified as described previously and assayed for enzyme activity. Approximately 200 µg of highly pure, active NDPK (in Buffer B) mixed with Freund's Complete adjuvant was injected subcutaneously into two

white New Zealand rabbits. The rabbits were boosted after one month and sera were collected and stored as described above.

3. Protein A Fractionation of Antibody to Denatured NDPK

Serum containing antibody generated from denatured NDPK was adjusted to pH 8 by adding 1/10 volume of 1 M Tris (pH 8) and loaded onto a protein A Sepharose (Sigma) minicolumn equilibrated in the same buffer. The column was then washed with 10 column volumes of 100 mM Tris (pH 8) and 10 column volumes of 10 mM Tris (pH 8). The IgG fraction was then eluted with 100 mM glycine (pH 3) into microfuge tubes containing 1/10 volume of 1 M Tris (pH 8) and the eluted samples were mixed immediately to bring the solutions to neutral pH. Fraction containing IgG were identified by absorbance at 280 nm, pooled, and concentrated.

4. Fractionation of Antibody to Native, Active NDPK

Serum containing antibody generated from native, active NDPK was fractionated on a protein A column as described above and the pooled, concentrated IgG fraction was loaded onto an affigel NDPK affinity column (described previously) equilibrated in 10 mM Tris (pH 8). The column was then washed with 20 column volumes of 10 mM Tris (pH 8) and 20 column volumes of 500 mM NaCl, 10 mM Tris (pH 8). The NDPK-specific antibody was then eluted with 10 column volumes of 100 mM glycine (pH 3) into microfuge tubes containing 1/10 volumes of 1 M Tris (pH 8), and the solutions were mixed

immediately. The specificity of the eluted antibody was then tested by doing a Western blot with *E.coli* extracts and different dilutions of the antibody.

T. Western Blotting

SDS polyacrylamide gels were run as described and the protein was transferred immediately onto an Immobilon-P (Millipore) membrane in an Idea Scientific transfer apparatus in Western Transfer buffer overnight at approximately 100 milliamps. The membrane (blot) was then incubated in TBST buffer containing 1% gelatin (Bio-Rad) for 30 minutes and washed three times for 10 minutes each with TBST buffer. The blot was then incubated in a solution of antiserum diluted in TBST for 2 hours, then washed with TBST three times for 15 minutes each to remove nonspecifically binding antibody. The blot was then incubated in a solution of secondary antibody (α-rabbit IgG) conjugated to alkaline phosphatase (Promega) diluted 1/7000 in TBST, and washed three times for 10 minutes with TBST. The blot was next washed for 10 minutes with 30 ml of a 40 ml staining solution containing 36 ml 0.1 M ethanolamine (pH 9), 2 ml 2M NaCl, and 2 ml 1 M MgCl . 44 µl of Immunoselect (BRL) nitroblue tetrazolium and 33 µl of BCIP was added to the remaining 10 ml of stain solution, which was applied immediately to the blot. After proper development, the blot was washed with TBST and distilled water and allowed to dry.

U. Immunoprecipitations

1. Preparation of Extract and Precipitation

125-ml cultures of *E.coli* B cells were grown to approximately 80 Klett and either labeled for 30 minutes with ^{35}S-methionine or infected with T4 bacteriophage and labeled as described for NDPK affinity chromatography, except that one-fourth the amount of tryptophan, label, and unlabeled methionine were added. The cells were pelleted and resuspended in 2 ml of either NP-40 lysis buffer or affinity column buffer, and sonicated as described previously. The soluble protein fraction was separated from the insoluble protein fraction by centrifugation at 10,000 rpm for 10 minutes and a 5-µl sample was used to determine the efficiency of incorporation (as described for affinity fractionation). 10 to 20 µl of labeled extract was then added to 500 µl of NP-40 lysis buffer in microfuge tubes placed on ice, and 50 µl of antiserum was added. The solution was allowed to incubate on ice for one hour. 100 µl of 10% protein A Sepharose (Sigma) in NP-40 lysis buffer was added and the microfuge tubes were placed on an Orbitron orbiting platform at 4°C for one hour. The microfuge tubes were then centrifuged at 15,000 rpm for 15 seconds, the supernatant was removed with a 1-ml syringe and the protein A pellet was resuspended in 500 µl of NP-40 lysis buffer. The protein A pellets were washed with NP-40 lysis buffer in a similar manner three more times, and the final protein A pellet was resuspended in 2X sample buffer, boiled for 5 minutes, and loaded onto an SDS polyacrylamide gel. The gel was then soaked in high methanol and low

methanol solutions, distilled water, and 1 M sodium salicylate as described previously. The gel was then dried and Kodak X-OMAT film was allowed to expose at -80°C.

2. Identification of Immunoprecipitating Protein

Competition experiments were done to help identify immunoprecipitating labeled proteins by adding approximately 20-40 µg of unlabeled suspect protein to approximately 300 µl of NP-40 lysis buffer and 50 µl of antisera. The solution was allowed to incubate on ice for one hour before labeled extract was added and treated as described above.

Immunoprecipitated protein was "spiked" with pure unlabeled reference protein and loaded onto NEPHGE tube gels, and two dimensional analysis was performed. The second dimension gel was stained to identify known reference proteins and film was placed on the gel, after drying, to visualize the labeled protein, which was identified by its migration relative to the unlabeled, stained proteins.

V. Fluorescence Anisotropy Interaction Studies

1. Labeling of NDPK with 1,5-IAEDANS

3 mM 1,5-IAEDANS (5-(2-(iodoacetyl)amino)ethyl)amino)nap-thalene-1-sulfonic acid)(purchased from Molecular Probes, Inc.) was added to a 1 mg/ml solution of purified NDPK in 20 mM MOPS buffer (pH 7), and allowed to react in

the dark at 4°C overnight. This was done to covalently label (through the cysteine residue) NDPK with a highly fluorescent probe, so that the fluorescence intensity would be great enough to detect even small changes in anisotropy. NDPK fluorescence and covalent labeling was then tested by electrophoresis through a 15% SDS polyacrylamide gel, and the specific fluorescence of NDPK was visualized with a model UVG-54 (Ultra-Violet Prod., Inc.) UV lamp. A spectrophotometric NDPK enzyme assay was also done with fluorescently labeled NDPK and unlabeled NDPK.

2. Anisotropy Measurements

Fluorescence emission and excitation spectra of 1,5-IAEDANS-labeled NDPK were obtained with a Perkin-Elmer LS50 fluorometer. A 1 mg/ml solution of fluorescently labeled NDPK in 20 mM MOPS buffer (pH 7) was excited at 365 nm to obtain the emission spectrum, and an intrinsic fluorescence emission scan was done of T4 DNA polymerase to determine background fluorescence, and so that optimal emission wavelengths could be determined for anisotropy measurements. The 1 mg/ml solution of NDPK was then diluted with MOPS buffer to give a final concentration of 3 μM of NDPK (based on the molecular weight of the hexamer) and purified T4 DNA polymerase (obtained from Dr. William Konigsberg's laboratory, Yale University) was added at a final concentration of 3 μM polymerase. An equimolar ratio of both enzymes was used to optimize any potential interaction by mimicking putative *in vivo* ratios. Intensity and anisotropy measurements were done with each enzyme solution alone and with an enzyme solution containing both enzymes together, and were processed by an IBM compatible 386 computer.

III. Results

A. Purification of NDPK from Crude Extracts

1. First Purification

In the first purification of NDPK from crude extracts of *E. coli* B cells, a purification scheme was followed (see Fig. III-1, left-hand side) that had been used previously with success in our laboratory by Gerry Lasser. However, during this purification two other proteins cofractionated with NDPK, having molecular weights of approximately 27,000 daltons and 45,000 daltons respectively. These two proteins were specifically eluted, along with NDPK, from the Matrex blue (Blue Sepharose) column with 2 mM dTDP as well as 2 mM UDP. Since the isoelectric point (pI) of NDPK had been determined previously to be approximately 4.7, a chromatofocusing fractionation was done in hopes that the other two proteins would not have similar pI values. However, chromatofocusing did not fractionate these proteins from NDPK, and all three proteins coeluted at a pH of approximately 4.3 (see Figs. III-2 and III-3) A final concentration of 1% SDS was then added to the protein solution , and it was loaded onto a G200 molecular sieve column. The proteins eluted in three separate fractions and the fraction containing NDPK was detected by gel electrophoresis. An attempt was made at N-terminal amino acid sequencing of

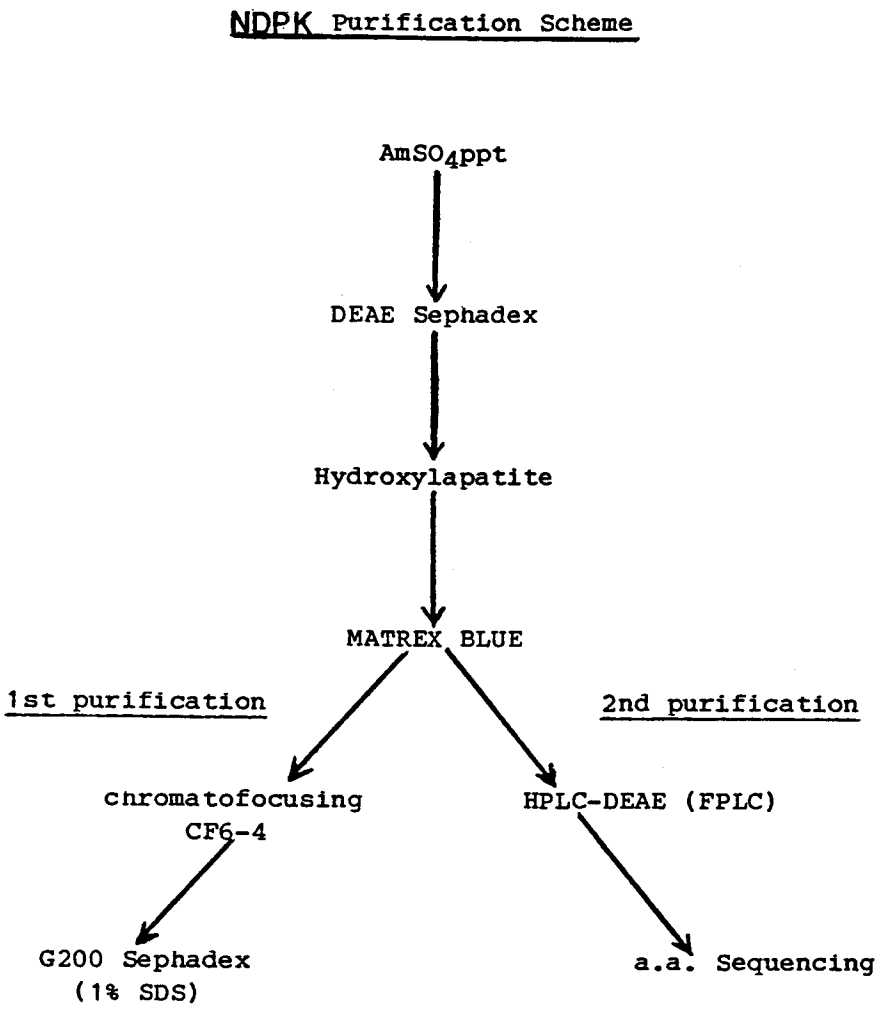

Figure III-1. Purification Scheme Used in the Purification of NDPK from Crude Extracts of *E. coli* B Cells.

the eluted NDPK, which had been dialyzed to remove as much SDS as possible. However, the remaining SDS, which could not be removed, did not allow successful sequencing.

2. Second Purification

In the second purification of NDPK from crude extracts, a modified purification scheme was followed (see Fig. III-1, right-hand side). Instead of chromatofocusing or molecular sieve fractionation following Blue Sepharose fractionation, high performance liquid chromatography (HPLC) was used to fractionate NDPK from the copurifying proteins. HPLC fractionation was necessary because it was again found that the same two proteins coeluted with NDPK from the Blue Sepharose column when 2 mM dTDP was applied. 2 mM GDP was then used in hopes that it would elute NDPK more specifically. However, all three proteins again coeluted, but the elution of the 45-kDa protein was greatly enhanced (see Fig. III-4).

After elution from the Blue Sepharose column the protein was applied to a DEAE Cellulose FPLC column attached to an HPLC. The column was run at room temperature and low pressure conditions. In this way NDPK was successfully fractionated as could be seen by the elution of a well-defined peak, which corresponded to the fraction containing NDPK enzyme activity (see Fig. III-5). A 5000-fold purification of NDPK was achieved (see Table III-1), and this highly purified NDPK was successfully used to obtain the N-terminal amino acid sequence of *E. coli* NDPK.

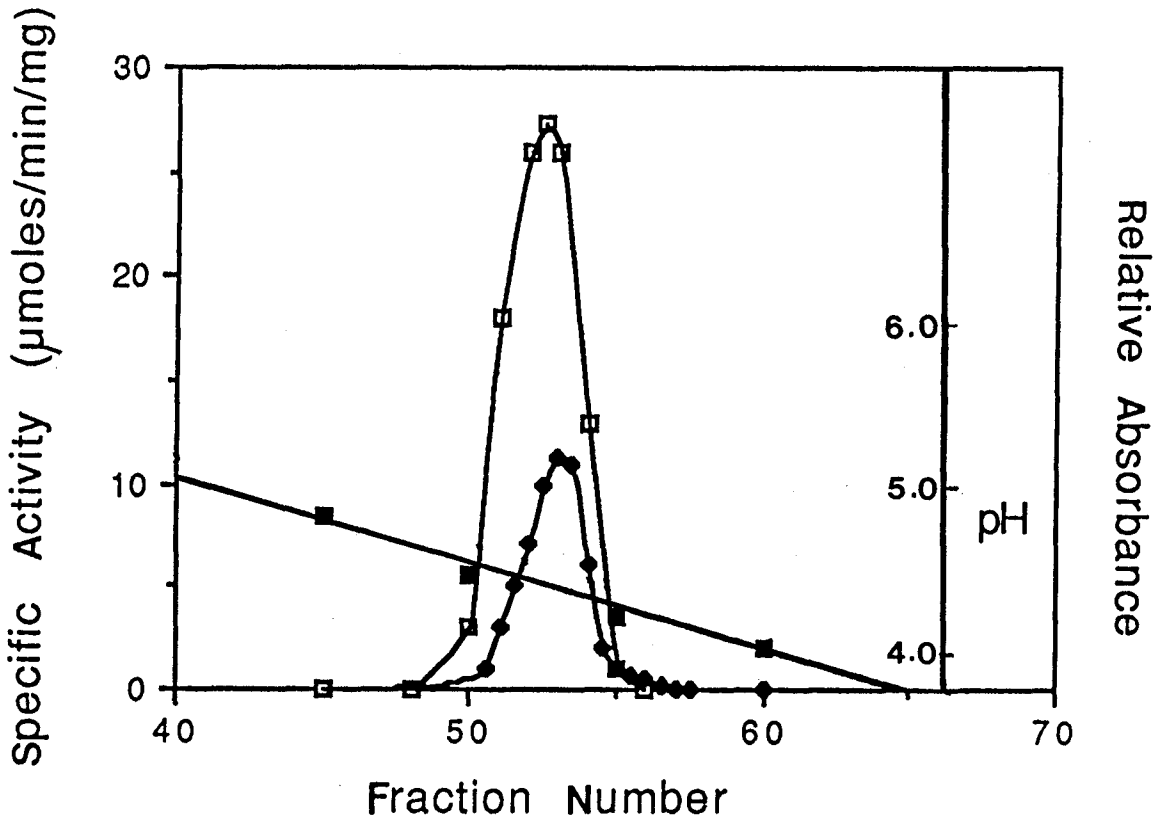

Figure III-2. Chromatofocusing Elution Profile. Diamonds represent relative absorbance at 280 nm, open squares represent the corresponding activity values, and filled squares represent the chromatographic pH gradient.

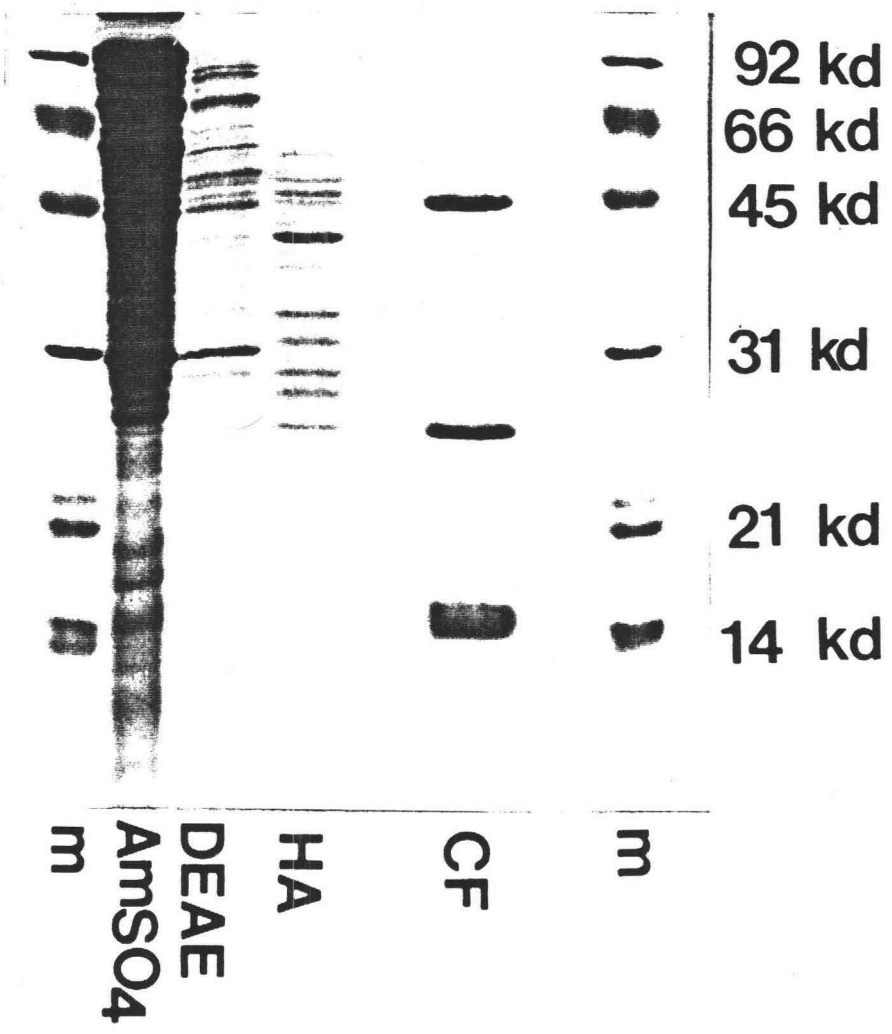

Figure III-3. 15% SDS Polyacrylamide Gel of the First Purification of NDPK from Crude Extracts of *E. coli* B Cells. $AmSO_4$ represents 0-45% ammonium sulfate precipitated proteins, DEAE represents DEAE Sepharose fractionated proteins, HA represents hydroxyapatite fractionated proteins, and CF represents chromatofocusing fractionated proteins.

B. NDPK N-terminal Amino Acid Sequence

1. Use in Design of Mixed Oligonucleotide Probes

When the first N-terminal amino acid sequencing of NDPK was done, about forty residues of sequence were obtained. This sequence was used to design mixed oligonucleotide probes (see Fig. III-6) that were used to search for the *ndk* gene in an *E.coli* DNA library. Only the sequence determined with 100% certainty (amino acid residues not in parentheses) was used, and the probes were designed based on uniqueness and low degeneracy. The probe NR2 was predicted to be the most unique due to the fact that it was based on a sequence beginning with two phenylalanines, which is quite distinctive and which only have a degeneracy of two. Therefore, it was expected that NR2 would prove to be the most specific and useful probe in locating the *ndk* gene.

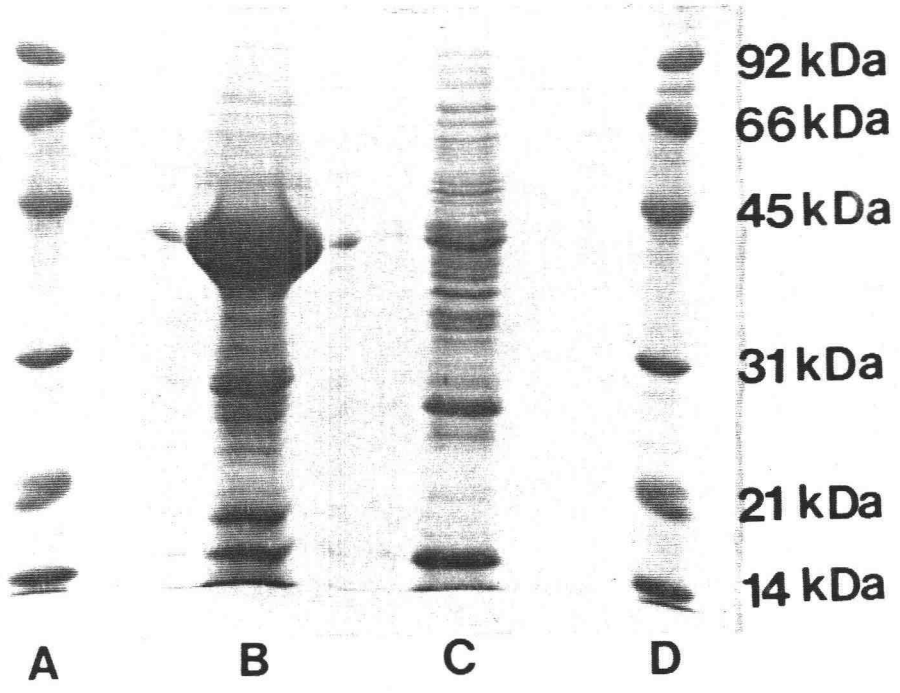

Figure III-4. 15% SDS Polyacrylamide Gel of Proteins Eluted from the Blue Sepharose Column. Lanes A and D, molecular weight markers; B, proteins eluted with 2 mM GDP; C, proteins eluted with 2 mM dTDP.

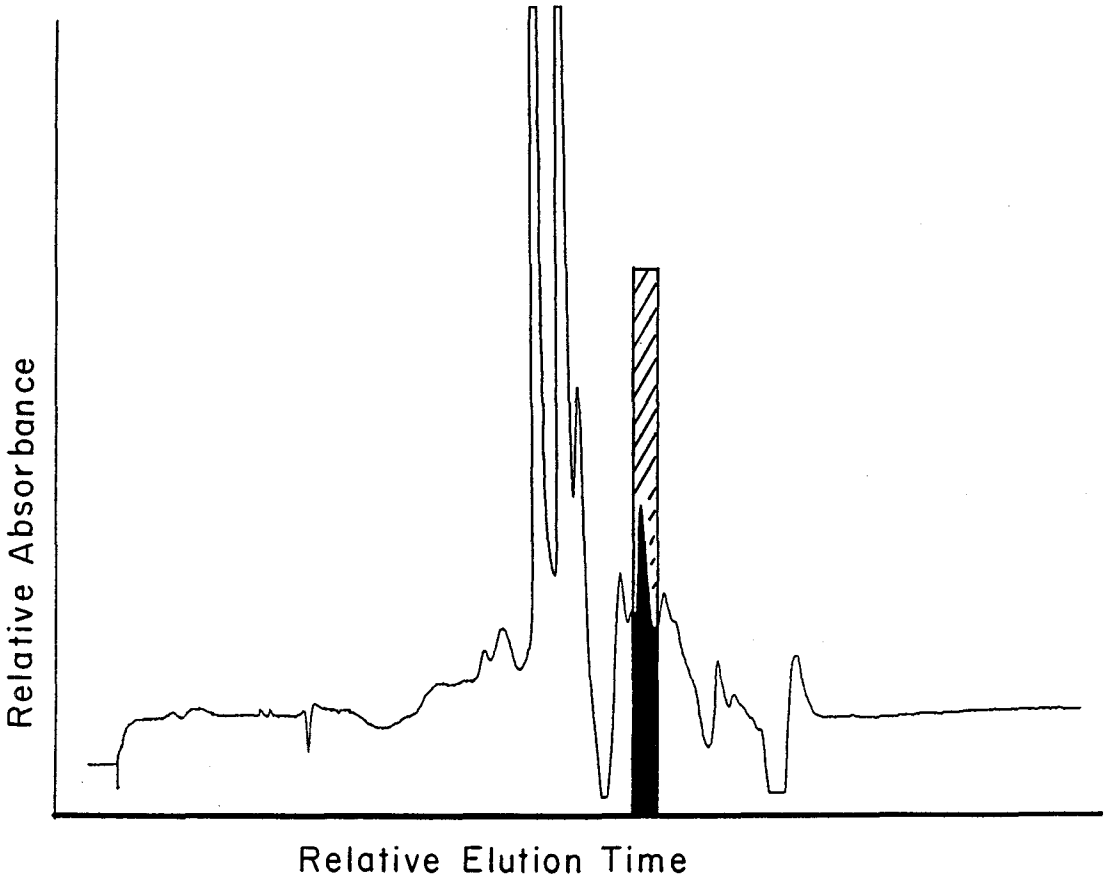

Figure III-5. HPLC Elution Profile of GDP Eluate from the Blue Sepharose Column. Fractions from the peak identified with the bar were found to contain enzyme activity.

TABLE III-1

NDPK Purification from Crude Extracts of *E. Coli* B Cells

purification step	Specific activity (μ mol/min/mg)	Estimated NDPK (mg)	Purification increase (X fold)
Total Sonicate	0.4	1.9	——
0-45% AmSo4 ppt.	1.3	1.6	3.3
DEAE Sephadex	2.1	1.6	5.3
Hydroxylapatite	7.2	1.5	18
Blue Sepharose (TDP Elution)	476	0.6	1190
HPLC (DEAE)	2423	0.052	6057

DPK Amino Acid Sequence

A I (E)(R) T F S I I K (P) N A V A K N V I G N I F A (R) F F A A G F K I V G (K) (K) (A) (L) (A) (L)

Figure III-6. NDPK N-terminal Amino Acid Sequence Used to Design Mixed Oligonucleotide Probes.

2. Comparison to the *M. xanthus* N-terminal Amino Acid Sequence

The same preparation of highly pure NDPK was later resequenced and forty-six residues of amino acid sequence were obtained with 100% certainty, except that the second phenylalanine at the beginning of the region of sequence used to design NR2, was determined to be an amino acid other than phenylalanine (labeled X in Fig. III-7). The identity of this amino acid could not be determined, and it was predicted to be a modified amino acid residue. At this time, the *Myxococcus xanthus* NDPK deduced amino acid sequence was known so that a comparison could be made of its N-terminal forty-six residues with the forty-six residues of N-terminal sequence obtained for *E. coli* NDPK (Ray and Mathews, 1992). It was found that the first twelve amino acids matched perfectly except for the seventh residue from the N-terminus, where there is a conservative difference (see Fig. III-7). Later when the *E.coli ndk* gene was located, cloned, and sequenced (Hama et al, 1991), it was found that the deduced N-terminal forty-six amino acid residues corresponded exactly to the N-terminal amino acid sequence obtained from purified NDPK, except the amino acid labeled X in Fig. III-7 was deduced as a glutamate.

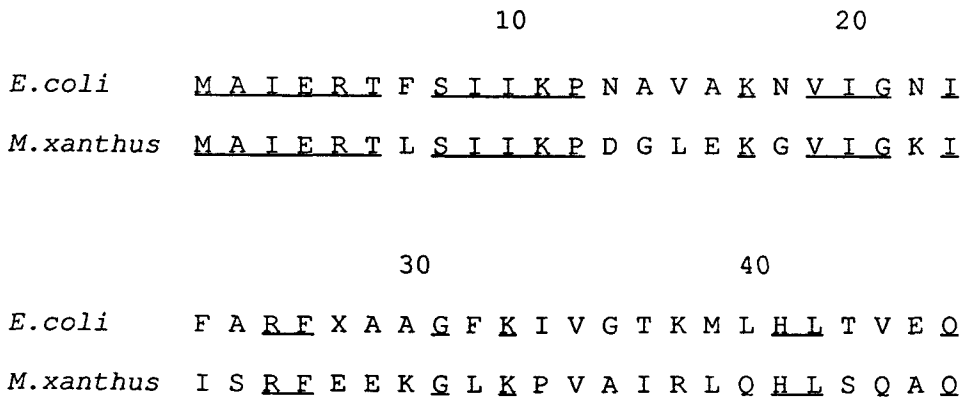

Figure III-7. Comparison of the N-terminal Amino Acid Sequences of *E. coli* and *M. xanthus* NDPK. Numbers represent residue positions past the N-terminus, defined as 1. X represents an amino acid that could not be identified.

C. Southern Blotting and Activity Assays with Clarke and Carbon Plasmids

A blot containing DNA from the Clarke and Carbon plasmids pLC34-9, pLC34-10, pLC32-25, pLC1-41, and pLC15-12 was probed with the mixed oligonucleotide NR2, and it was found that pLC34-9, pLC34-10, and pLC32-25 hybridized specifically to NR2 and did not hybridize to the negative control DNA (colE1 plasmid DNA).

NDPK enzyme activity assays were also done with sonicated JA200 *E. coli* cells, grown to the same density, containing the five selected Clarke and Carbon plasmids. PLC34-9, pLC34-10, and pLC32-25 were found to have higher activities, with pLC34-9 having the highest activity. Therefore these three plasmids were chosen for restriction analyses and further Southern blotting analysis with NR1, NR2, and NR3. NR2 was found to be the most specific probe, and upon probing the plasmid DNA of pLC34-9, pLC34-10, and pLC32-25, a 3.2-kb Pst I fragment from pLC34-9 specifically hybridized to NR2 (see Fig. III-8). This Pst I fragment was then subcloned into pUC19, and restriction analysis was done with the recombinant pUC19 plasmid. Southern blotting was also done using NR2 (see Fig. III-9) to map and align the restriction fragments from the recombinant plasmid that specifically hybridized to NR2 (see Fig. III-10). Aligning fragments which overlapped, were believed to contain the *ndk* gene.

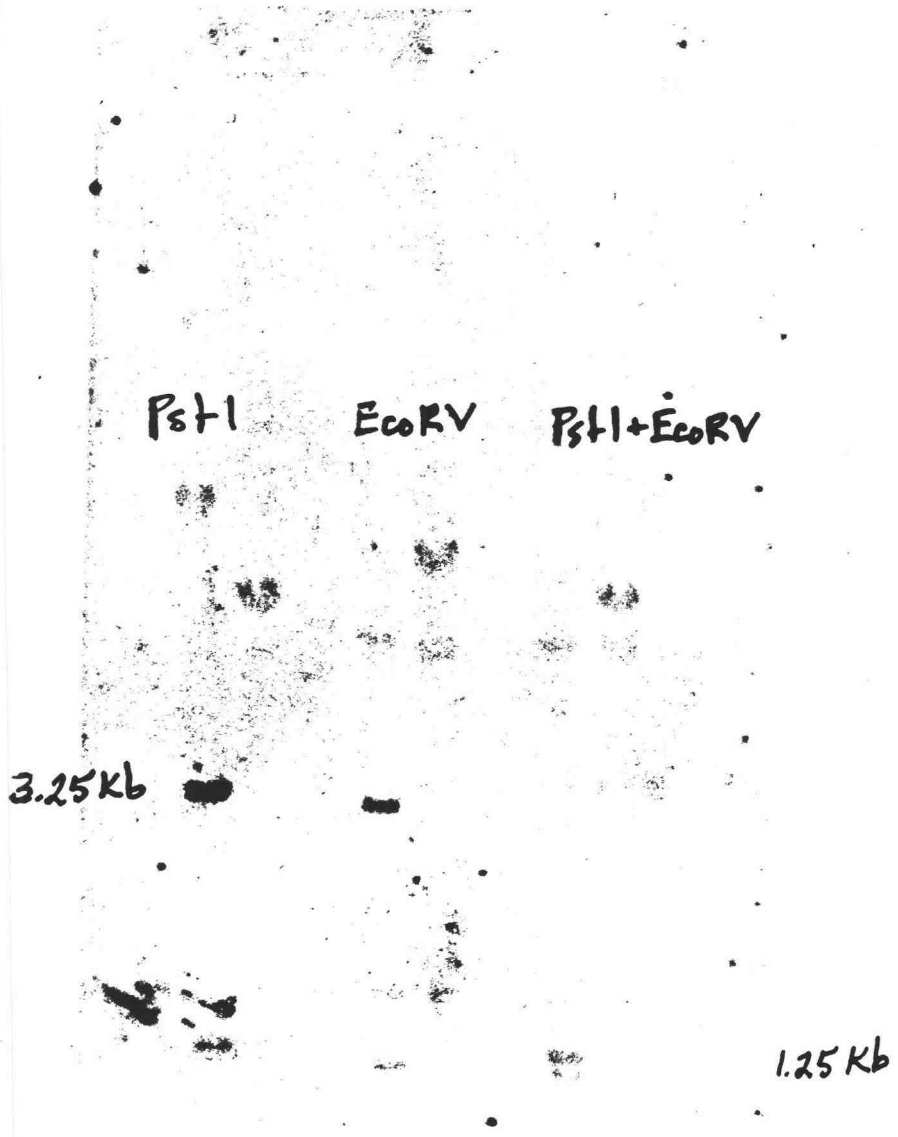

Figure III-8. Southern Blot of pLC34-9, pLC34-10, and pLC32-25 Restriction Fragments Probed with NR2.

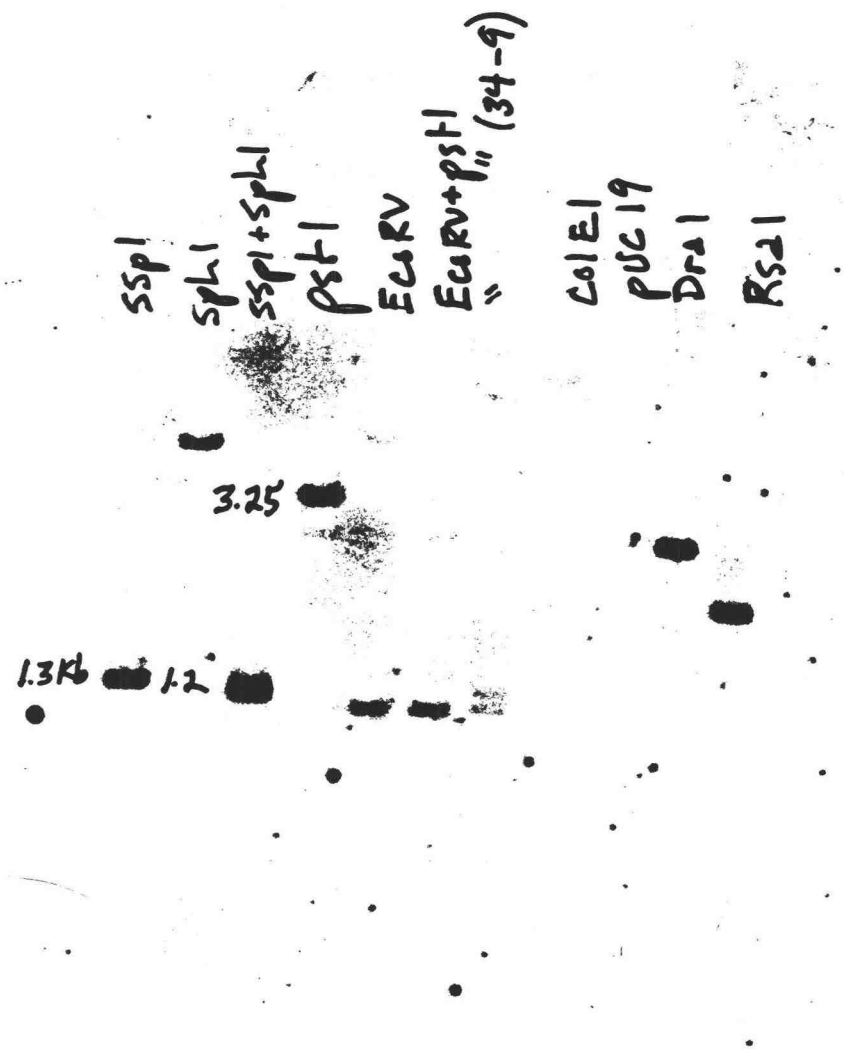

Figure III-9. Southern Blot of pLC34-9 and 3-11 Subclone Restriction Fragments Probed with NR2. Also included are control ColE1 and pUC19 DNA.

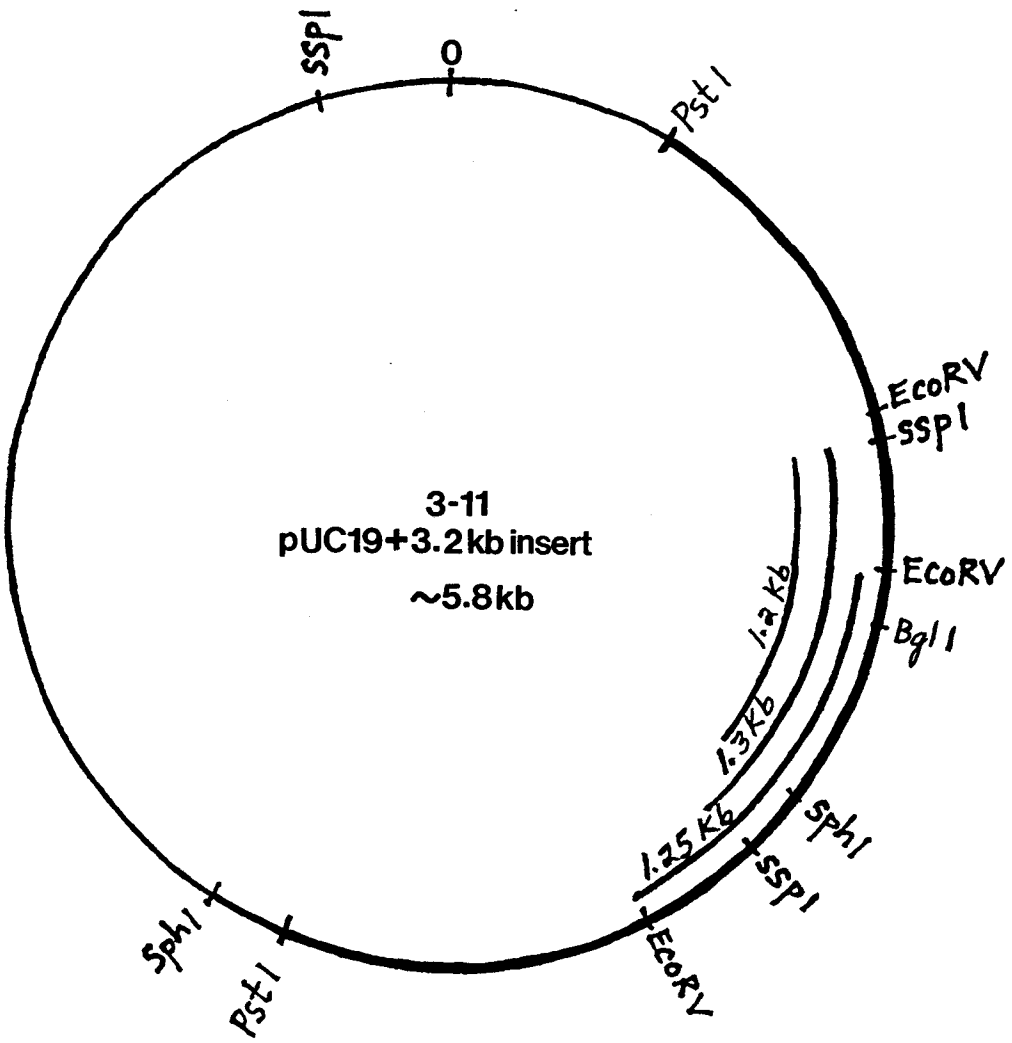

Figure III-10. Map of Alignment of 3-11 Subclone Restriction Fragments Hybridizing to NR2.

D. Subcloning of the 3.2-kb Pst I Fragment and Six-fold Higher Activity

As noted above, the 3.2-kb Pst I fragment, which specifically hybridized to the NR2 probe, was subcloned into pUC19, and this recombinant plasmid was mapped by restriction analysis. This 5.8-kb recombinant clone (3-11) was transformed into JM83 cells, which constitutively express *lacZ*, and it was found that JM83 cells containing the 3-11 clone had six-fold higher NDPK activity over host (JM83) NDPK activity. Therefore, it was believed that the Pst I fragment and 3-11 clone did contain the *ndk* gene in the region where the restriction fragments that hybridized to NR2 overlapped. Sequencing was then done to locate the *ndk* gene in this region.

E. DNA Sequencing

Four subclones of the recombinant plasmid 3-11 were made, and plasmid (double-strand) as well as single-strand sequencing was done such that the sequence overlapped and could be aligned. Over 1 kb of DNA was sequenced (see Fig. III-11) in the region where NR2 hybridized and the *ndk* gene was not located. This sequence was then used to search for open reading frames using Intelligenetix, and a homology search was done to find homology with any DNA sequence that had been entered into GenBank. The DNA was translated into six potential reading frames; however, no potential open reading frames were recognized. The most interesting homology was found between the DNA sequence and part of the *str* operon, which includes the genes encoding

TTTTTCGCCGCTAAAATATCCCGCCACGCTTTTCCTGCCAGCTTAAGGTCATTGCTCCCAT

CGCTTTGGCGTTCATCGCCCCGCGCCAGTGACTCTCTTTACTGACACCGTAGCTCTCTTCA

TAGACAAAGAGCTTGATATCATCAATGTCATCAATATCTTCGCCGCCGGTGGTGTTGATTT

EcoRV

GCAGAAACTCATCGCCTGAAGTGTAGTAGCGGAAAATCTGACTACCGCCGCCCAGATCGAT

GTGGCTGACGGCGCCTACCGTAAATTCCTCACGCGGCGGCGCAATCAGCAATTCATCTTCC

AGCAAACGAAGCGCTAACGTGTCGAGCGTAAAGCACTATGAGATGAAGTCCAGCACCACGA

GCGATAGCTGGCTTATTATCCTTGCCAAACAGACGCTGAAAAACCAGACATATTTTATCCT

TAAAATAGTTGCCAGCCTTTTAGCGAGGCTGCAAAAAAATTACTCGCCCTGTTGGCGTTGC

Bgl I

AGTCTTGCTAATACATCCTGGGCGCTACTTTTATTGCTACCGCCAATTCCGGCTTCCGCCA

GCTTTTCGTCAAGGTCGCGACCGTCTGCGACTTTCTCCAACTGTGCGGCAGCATCCAGGCG

AGCATGACGTTCGGCCTGACGCGTTTGCAGGCGTTTTAAGGATTCTGCCGCGGTCGAAACG

CTGGAAGATGCGCCAACGGTAGAGGTTGTTACAGCCTGTTGTGCACGCTGCATGGCTTCAG

TGGCTTTAACGACTTCCATTTGCTGTCAAACTGAGCGAATACGTGGCTGTCGCTGTAACTG

CTTTTTCCACGCCATCACGGAGACTTCCAGTTCGACAACACTTGCTCTTCAGCGGTAATGA

GATTCTCAAGGCGCGCGATTTCTTCAGCAATTCGTTAATCAACGACGGATTAACGCTTCAG

CGCACGGGCTTCCAGACTGGCTTTGCGCTCACGTAAATCTTTCAGCTTATCGTGACTTAAT

TTCACCCGCGCCAGCAGATCAACGCGAGATTTTCCGGCTTCATCGAGTTCAGCTTTAGCGT

CACGAATATGCTGTTCCAGCATGCGCACGCCCTGGGCTTCTTCAATGGATTCCTCCGCCTG

Sph I

GGAGATAAACGATTTCCCCAGCGGAAATCAACTTTTTAAAATTCCCATAAATACCCGTCCT

TGTAATTAGTATGAATGGG

SmaI-SspI

Figure III-11. *E. coli* DNA Sequence Obtained from 3-11 Subclone.

EF-G (*fus A*) and EF-Tu (*tuf A*). A homology search was also done between the NR2 sequence and the approximately 1 kb of *E. coli* DNA sequence obtained from the 3-11 clone. It was found that the NR2 oligonucleotide sequence had approximately 50% homology with a Bgl I-Sph I fragment contained in the *E. coli* DNA sequence, which would allow it to hybridize specifically to NR2 at the hybridization temperature that was used (53°C). Therefore, since the *ndk* gene was not found in the region of the Bgl I-Sph I fragment, and a Southern blot confirmed the location of hybridization to the Bgl I-Sph I fragment, it was concluded that the probe sequence was not specific for the *ndk* gene and the amino acid sequence from which it was derived was incorrect. As noted previously, upon resequencing the N-terminus, this was found to be the case.

F. NDPK Activity Assays of the Kohara Lambda Clones

NDPK activity assays were done with the selected lambda clones 2D5, 7F8, 5E10, 6F10, and 8E12, in sonicated LE392 *E.coli* cells, and the highest overlapping activity was found for the clones 2D5 and 7F8. Based on restriction mapping data published for these clones (Kohara et al, 1987), it was deduced that 2D5 and 7F8 were located in approximately the same region as the Pst I fragment, originally subcloned from the Clarke and Carbon plasmid pLC34-9. Therefore, it was concluded that the *ndk* gene was located in this region and would be found in either an approximately 6.0-kb EcoR1 fragment or an approximately 6.0-kb Kpn I fragment, together spanning the overlapping region of the 2D5 and 7F8 clones (see Fig. I-6).

G. Subcloning of the 6.0-kb EcoR1 Fragment

The first fragment which potentially contained the *ndk* gene to be considered was the 6.0-kb EcoR1 fragment, which was subcloned into pUC18. The recombinant pUC18 plasmid was being prepared for plasmid sequencing when it was learned that the *E. coli ndk* gene had recently been cloned and sequenced in the laboratory of Dr. Masayori Inouye. It had been located in the same region, where the Kohara clones 2D5 and 7F8 overlap (Hama et al, 1991). The beginning of the gene was located at one end of the EcoR1 fragment extending into the Kpn I fragment (see Fig. I-6).

H. Improved Overexpression of NDPK from pKT8P3

Dr. Inouye was kind enough to agree to send the NDPK overexpression plasmid pKT8P3 to facilitate protein-protein interaction studies of *E. coli* NDPK and T4 bacteriophage early proteins. With their clone, NDPK had been overexpressed in JM83 cells to a level of 25% of the total soluble protein. However, they believed the clone was unstable in cultures grown overnight and recommended using freshly transformed cells to start cultures grown to log phase for harvesting. PKT8P3 was made by inserting a Pvu II fragment (located in the region between the 6.0-kb EcoRI fragment and the 6.0-kb Kpn I fragment, see Fig. I-6) into pUC9. The *ndk* gene and its promoter were actually cloned in the opposite orientation to the *lacZ* gene and promoter. Therefore, I asked whether this might somehow interfere with the expression of NDPK, since *lacZ* is constitutively expressed in JM83 cells. I also tested the stability of the pKT8P3

plasmid by isolating the plasmid from cells grown to log phase versus cells grown overnight, and could find no evidence for the plasmid itself becoming unstable in cells grown overnight. Therefore, I transformed the pKT8P3 recombinant plasmid into DH5α cells, in which *lacZ* is not constitutively expressed, and found approximately 2-fold higher expression of NDPK (see Fig. III-12). I also found that the overexpression of NDPK was stabilized in cultures of DH5α cells grown overnight.

I. Purification of Overexpressed NDPK

1. Salt Precipitations

Because the possibility was considered that overexpressed NDPK could precipitate differently than NDPK from crude extracts, a range of saturating ammonium sulfate concentrations were tested, and it was found that overexpressed NDPK precipitated most specifically and to the greatest degree in the concentration range of 45-60% ammonium sulfate. During the purification of NDPK from crude extracts of *E. coli* B cells, NDPK had been precipitated in the concentration range of 0-45% ammonium sulfate. A streptomycin sulfate precipitation was done subsequent to the ammonium sulfate precipitation, as in the case of the purification of NDPK from crude extracts, due to the consideration that the order of precipitation (ammonium sulfate then streptomycin sulfate) could be important in maintaining interactions of NDPK with other *E.coli* proteins. However, the two *E. coli* proteins, which had

previously cofractionated with NDPK during its purification from B cells, did not cofractionate with overexpressed NDPK.

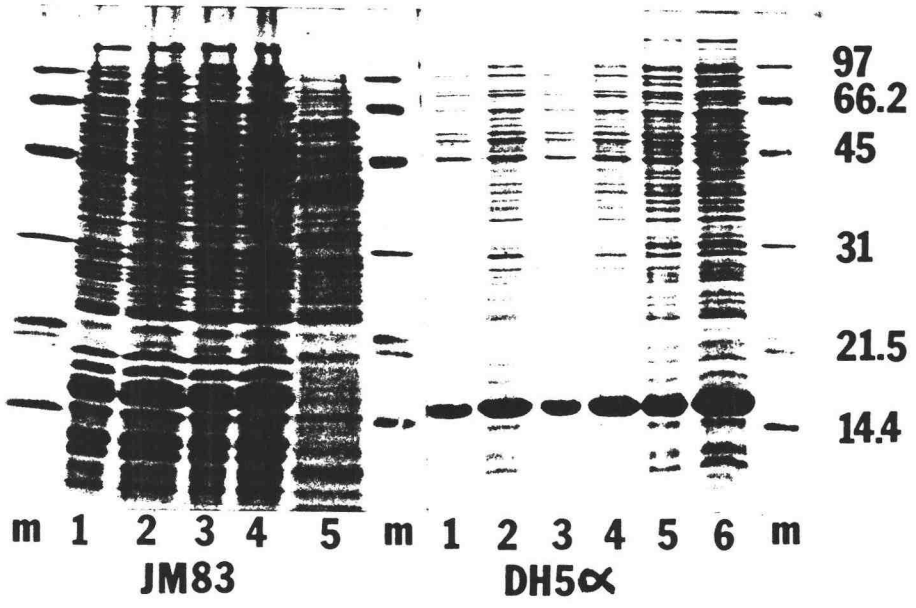

Figure III-12. Comparison of the Overexpression of NDPK from pKT8P3 in JM83 Cells versus DH5α Cells.

2. Blue Sepharose Fractionation

Blue Sepharose has been used as an affinity fractionation step for the purification of nucleotide-binding proteins, and as can be seen in Table III-1, it was the most important fractionation step during the purification of NDPK from crude extracts of *E. coli* B cells, in terms of giving the greatest enrichment during purification. Since the overexpressed NDPK in DH5α cells already represented approximately 50% of the total soluble protein, Blue Sepharose fractionation was the next logical purification step after ammonium sulfate and streptomycin sulfate precipitation. As can be seen in Fig. III-13, approximately 90% pure NDPK eluted from the Blue Sepharose column with 2 mM dTDP.

3. MonoQ Fractionation

To further purify NDPK, it was loaded onto an FPLC MonoQ column and eluted with a 0-0.5 M KCl gradient. NDPK eluted from the MonoQ column at approximately 0.3 M KCl, an increase in the purification of NDPK was observed (see Fig. III-13), and the dTDP which coeluted with NDPK from the Blue Sepharose column was fractionated from NDPK in the first peak (see Fig. III-14).

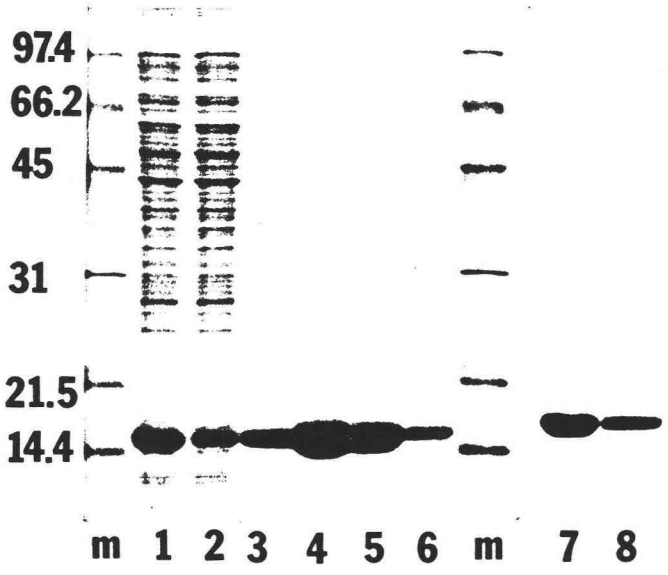

Figure III-13. Purification of Overexpressed NDPK. Lane 1, total soluble protein (pKT8P3 in DH5α Cells); lane 2, ammonium sulfate precipitated protein; lane 3, 4, 5, and 6, Blue Sepharose fractionated NDPK; lanes 7 and 8, MonoQ fractionated NDPK.

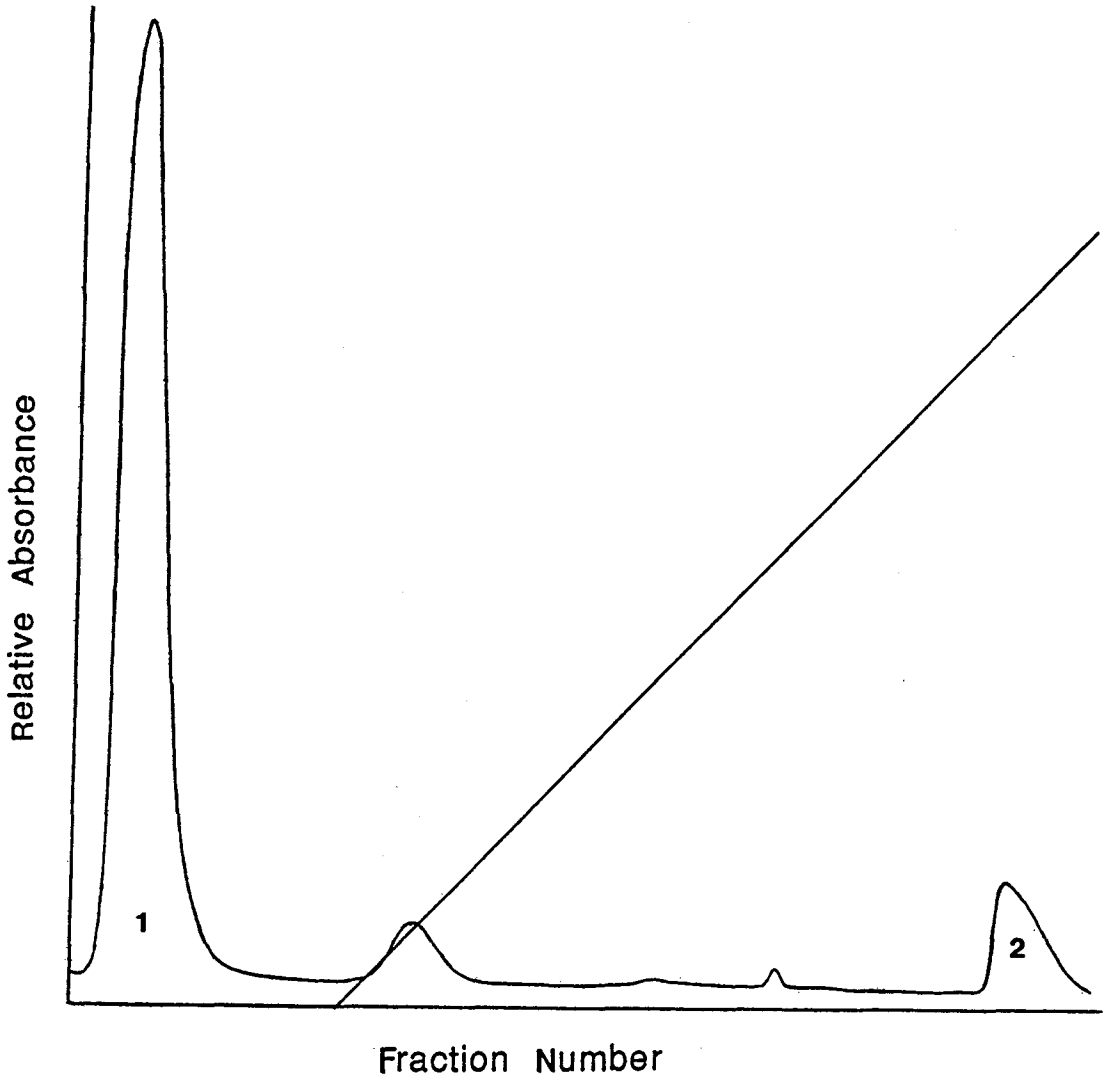

Figure III-14. MonoQ FPLC Elution Profile. 0-0.5 KCl gradient applied. Peak 1, dTDP; peak 2, NDPK.

J. Characterization Studies

1. UV Scan

A UV scan of NDPK from 230-300 nm (see Fig. III-15) was done to estimate the purity of NDPK and to investigate the possibility of cofactors associating with NDPK. A cofactor would be identified by an additional absorption peak. However, no potential cofactor peaks were observed, and the protein was estimated to be greater than 95% pure based on the fact that the protein does not contain tryptophan, such that well-defined phenylalanine peaks could be observed in the scan. Contaminating protein would be very likely to contain some tryptophan, which would appear as a distinctive peak even at low concentrations (tryptophan strongly absorbs within this region), and which would interfere with the detection of the phenylalanine peaks. The UV scan of NDPK resembles the UV scan of calmodulin, in which tryptophan is also absent.

2. Quantitation of Amino Acids

The exact amount of amino acids in NDPK, as compared to standards, was measured in order to determine the absorptivity of NDPK, as defined by the UV aborption value at 280 nm of a 1 mg/ml solution of pure protein. In this way, the exact amount of amino acids was totaled to give the corresponding

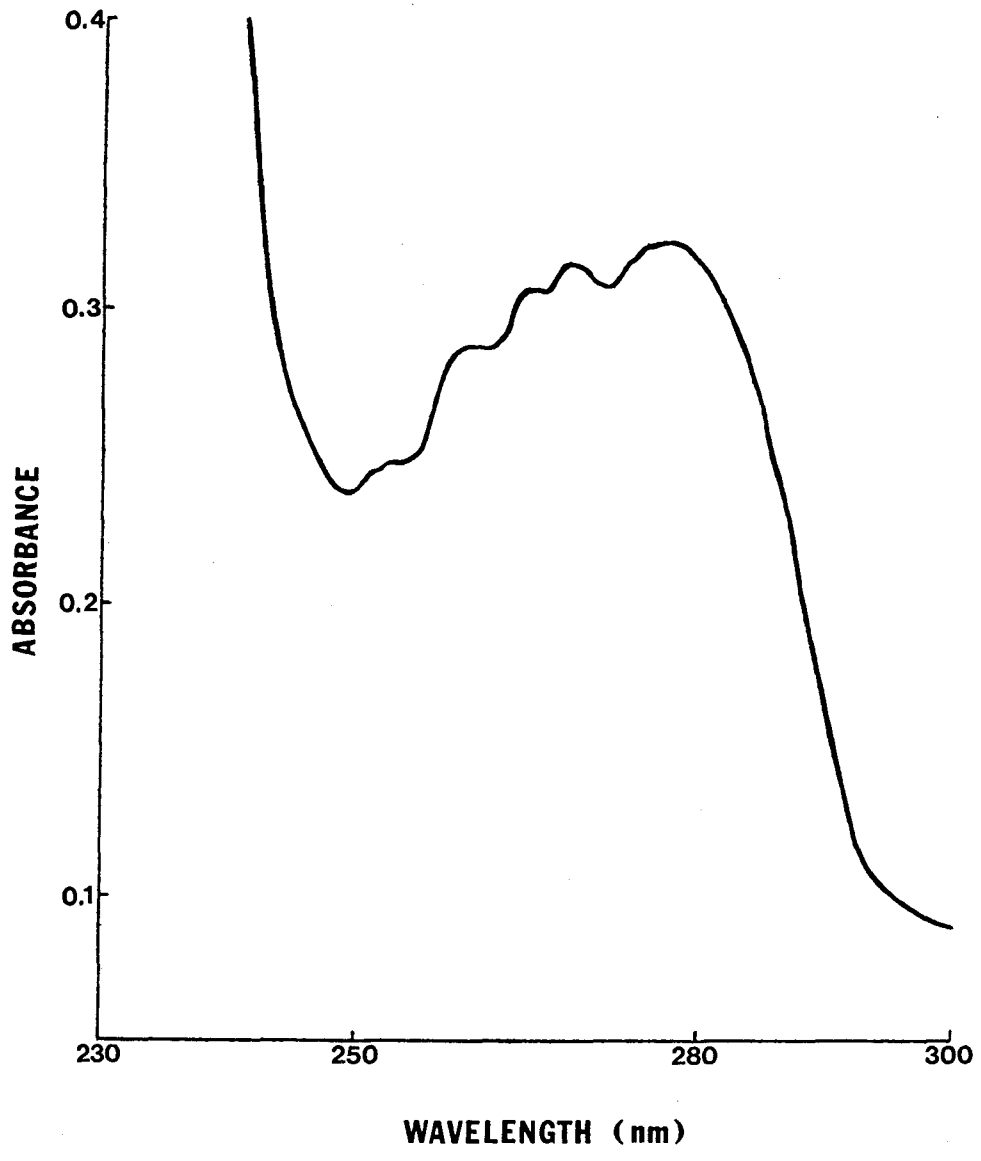

Figure III-15. UV Scan of NDPK (1 mg/ml).

concentration of protein at the given absorbance. A direct correlation between the concentration of protein in a solution and its absorbance at 280 nm was then used. The absorptivity value for *E. coli* NDPK was determined to be:

$$A^{280\ nm}_{1\ cm}(1\%) = 3.00$$

This matched very closely to the absorption value measured for a 1 mg/ml solution (multiplied by a factor of 10) measured at 280 nm prior to this calculation (0.305), again indicating a high degree of purity of NDPK.

3. Fluorescence

A scan of the intrinsic emission fluorescence of purified NDPK was done (see Fig. III-16) to see if its intrinsic fluorescence could be used to detect any change in the fluorescence of NDPK upon the addition of T4 DNA polymerase. However, it was determined that the level of intensity of its fluorescence emission was too low to detect small changes in fluorescence that could occur upon the addition of T4 DNA polymerase. The fluorescence of the latter, which contains 12 tryptophan residues, would totally obscure the relatively low tyrosyl fluorescence of NDPK.

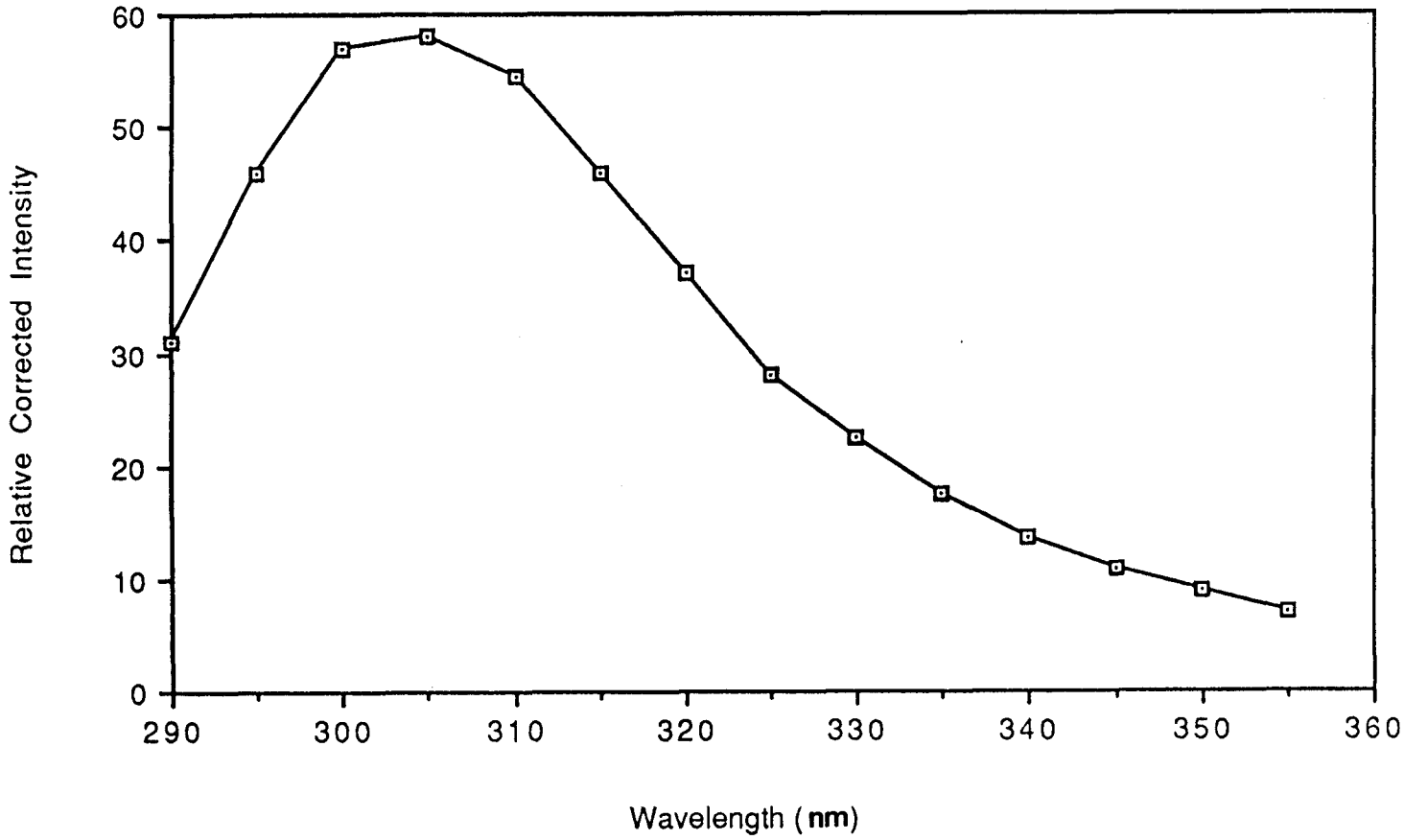

Figure III-16. Scan of NDPK Intrinsic Emission Fluorescence. Excitation wavelength, 280 nm.

K. Immobilized NDPK Affinity Chromatography

1. Elution of Radioactivity

As can be seen in Fig. III-17, approximately 5-10 fold more ^{35}S-labeled T4 phage protein (based on incorporation of label and subsequent trichloroacetic acid/sodium deoxycholate precipitation), specifically eluted from immobilized NDPK than did ^{35}S-labeled *E. coli* proteins. Many more T4 early proteins from both the T4-infected *E coli* B cell extract and the *uvsY*$^-$T4-infected *E coli* B cell extract eluted from NDPK than did proteins from the *E. coli* B uninfected extract with increasing concentration of salt. Also, as can be seen, much less labeled protein from both T4-infected and uninfected extracts, eluted from the control BSA column. BSA (bovine serum albumin) was chosen for a control column because it is a relatively "sticky" protein that binds to other proteins nonspecifically. Therefore, proteins that bind to NDPK, but not BSA are likely to interact with NDPK specifically.

2. One-dimensional Analysis of Eluted Proteins

The one-dimensional analysis of proteins eluted from the immobilized NDPK affinity column with increasing concentrations of salt (see Fig. III-18 A) shows the same result. Eluted proteins were loaded in proportion to the total amount of incorporated ^{35}S methionine label in each extract. Therefore, the

E. coli protein lanes are not as well exposed in comparison to the *uvsY*$^-$ or wild-type T4 protein lanes. As can be seen, the pattern of protein bands seen for *uvsY*$^-$T4-infected E.coli B extract is very similar to the pattern of

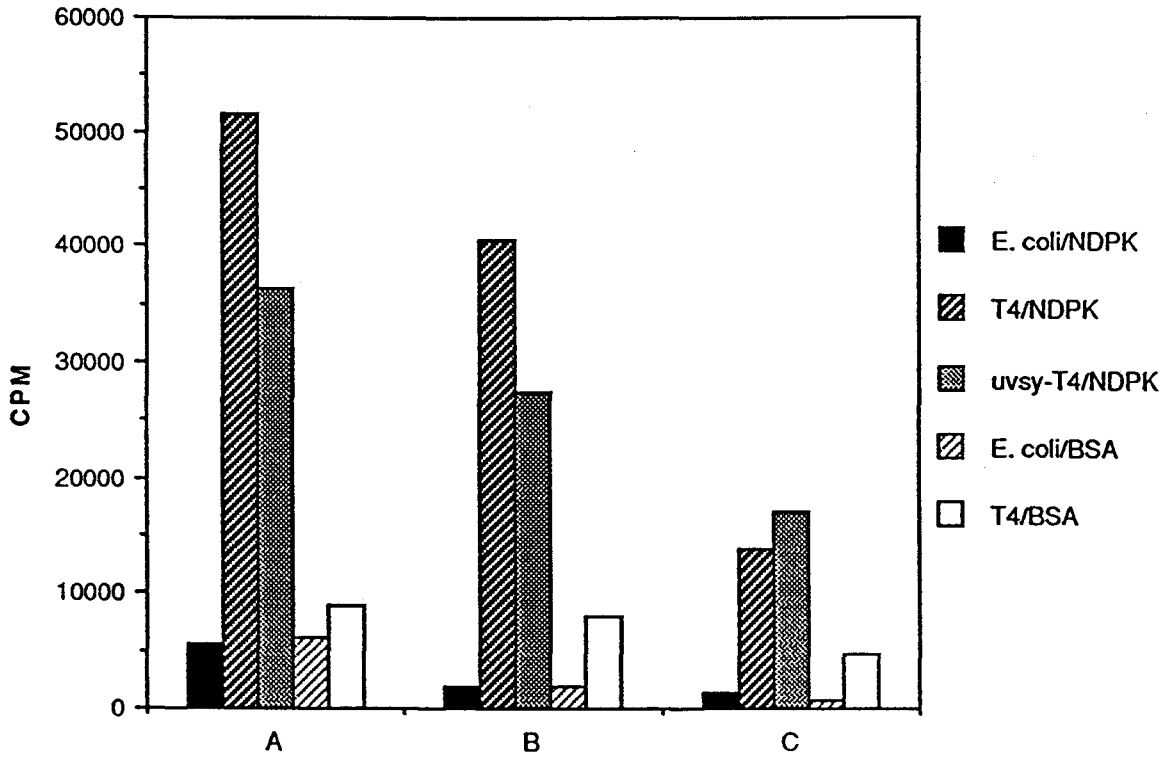

Figure III-17. Relative Elution of Radioactivity (cpm) from the NDPK and BSA Affinity Columns.

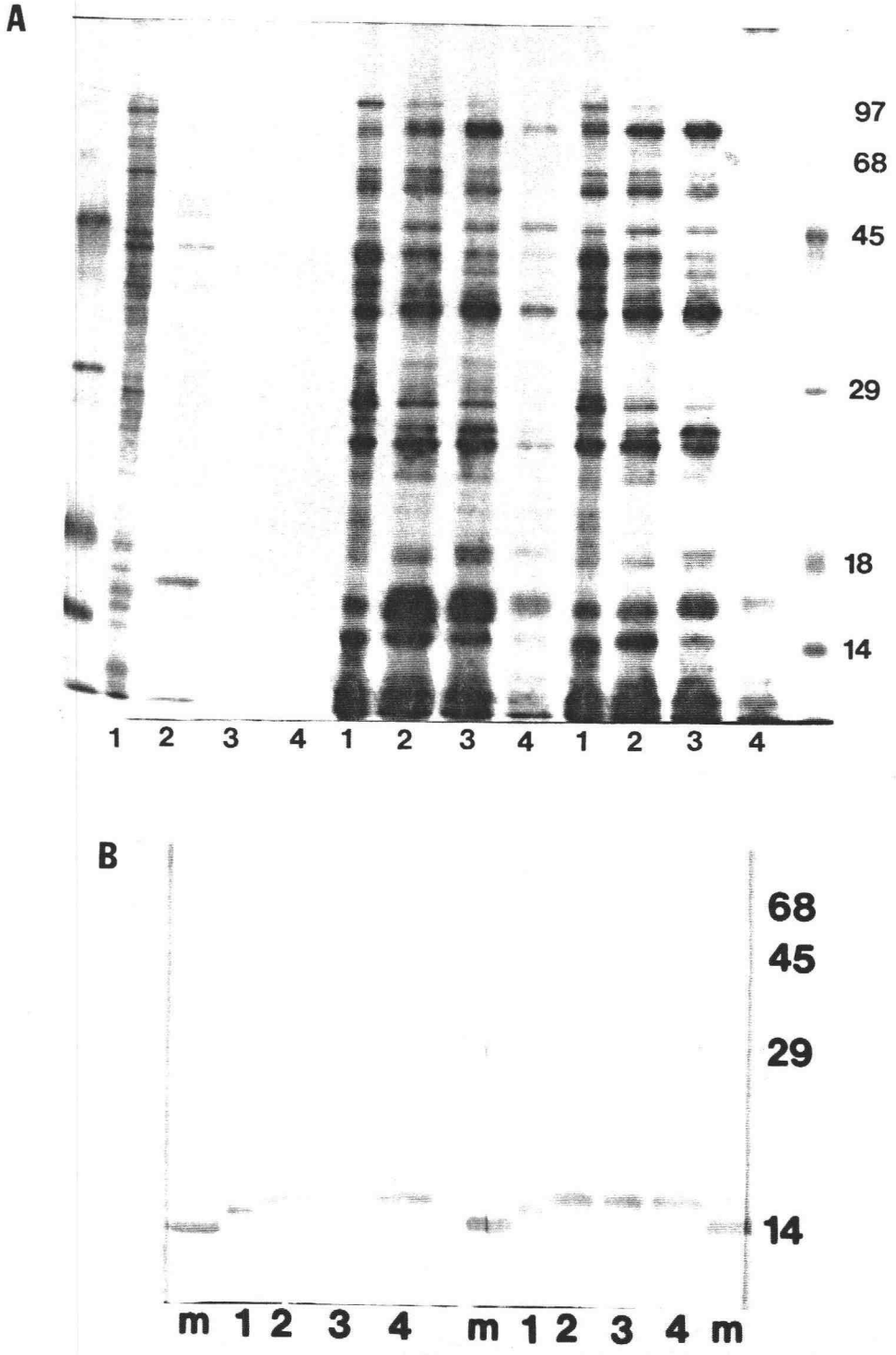

Figure III-18. **A**. Autoradiogram of a 15% SDS Polyacrylamide Electrophoresis Gel of Uninfected, T4, and *uvsY*-T4-infected *E. coli* Extract Proteins. **1**, flowthrough proteins; **2**, proteins eluted at 0.2 M NaCl; **3**, proteins eluted at 0.6 M NaCl; **4**, proteins eluted at 2 M NaCl. **B**. Western Blot of *E. coli* extract proteins probed with affinity column purified anti-NDPK (left side) and anti-NDPK generated from denatured NDPK.

protein bands seen for wild-type T4-infected *E.coli* B extract. Because early NDPK affinity chromatography experiments gave an indication of a potentially strong interaction between NDPK and UvsY, a *uvsY*$^-$T4 strain (obtained from Dr. Ken Kreuzer, Duke Univ.) was used to infect *E. coli* B cells, the extract was applied to the column, and salt eluted proteins were analyzed. It was expected that the labeled protein spot representing UvsY would disappear, and some difference in the specific proteins eluted at different salt concentrations would be seen, indicating a loss of interactions. By examining the one-dimensional 15% polyacrylamide gel closely (see Fig. III-18 A), one can see a greater concentration of labeled protein migrating at approximately 16 kDa (M_r of UvsY) in the wild-type T4 protein lanes. Therefore, it seems likely that less UvsY is expressed by *uvsY*$^-$T4-infected *E. coli* B cells. Although the UvsY mutant is described as a "*uvsY*$^-$ mutant" (Kreuzer et al, 1988), the *uvsY* promoter was actually deleted, not the *uvsY* gene. Perhaps some read-through transcription can occur, which could account for the similarity between T4 wild-type and T4 *uvsY*$^-$ salt eluted proteins. The possibility also exists that UvsY does not directly interact with NDPK, and only binds to immobilized NDPK through some distant interaction.

Although the *E. coli* protein lanes from the autoradiogram of the one-dimensional gel are not as well exposed, one can see that an approximately 16-kDa protein (see Fig. III-18 A) was eluted from the NDPK affinity column at 0.2 M NaCl. The fact that a much greater amount of this protein was eluted than any other *E. coli* protein may be significant. Also, an *E. coli* protein that migrated very similarly has been seen to elute from a dCMP hydroxymethylase column at the same salt concentration (Wheeler et al, 1992). NDPK itself has an apparent molecular weight of approximately 15 kDa. Therefore, a Western blot was done with the same extracts and anti-NDPK antibody to see if the 16-kDa protein was

NDPK. Since NDPK is a hexamer, it is possible that some subunit association-disassociation would occur on the Affi-gel matrix. The Western blot (see Fig. III-18 B) shows a band lighting up fairly equally in every lane (flow through, 0.2 M NaCl elution, 0.6 M NaCl elution, and 2 M NaCl elution) that corresponds to a slightly lower molecular weight. Since the 16 kDa band was seen on the one-dimensional gel primarily in the 0.2 M NaCl elution lane, one would expect that this lane would show a more darkly stained band on the Western blot.

3. Two-dimensional Analysis of Eluted Proteins

Total ^{35}S-methionine-labeled *E. coli* protein extract was analyzed by two-dimensional polyacrylamide electrophoresis (see Fig. III-19), although no identifications of proteins could be made since our protocol is not designed for *E. coli* protein identification. *E. coli* proteins eluted from the NDPK affinity column at 0.2 M NaCl were also analyzed by two-dimensional electrophoresis (see Fig III-20), and the relative pI value of the approximately 16-kDa protein (see above) was determined. It was found to be approximately 10, which is much more basic than that of NDPK (pI=4.76). Total ^{35}S-methionine-labeled T4D(wild-type)-infected *E. coli* protein extract (see Fig. III-21) and *uvsY*$^{-}$T4-infected *E. coli* protein extract were also analyzed by two-dimensional polyacrylamide electrophoresis. A labeled protein spot corresponding to the migration of UvsY was detected running with the dye-front. *UvsY*$^{-}$T4 early proteins eluted from the NDPK affinity column at 0.6 M NaCl were also analyzed by two-dimensional gel electrophoresis, and no significant difference was seen

Figure III-19. Autoradiogram of a Two-dimensional NEPHGE Gel of Total *E. coli* B Extract Applied to the NDPK Affinity Column.

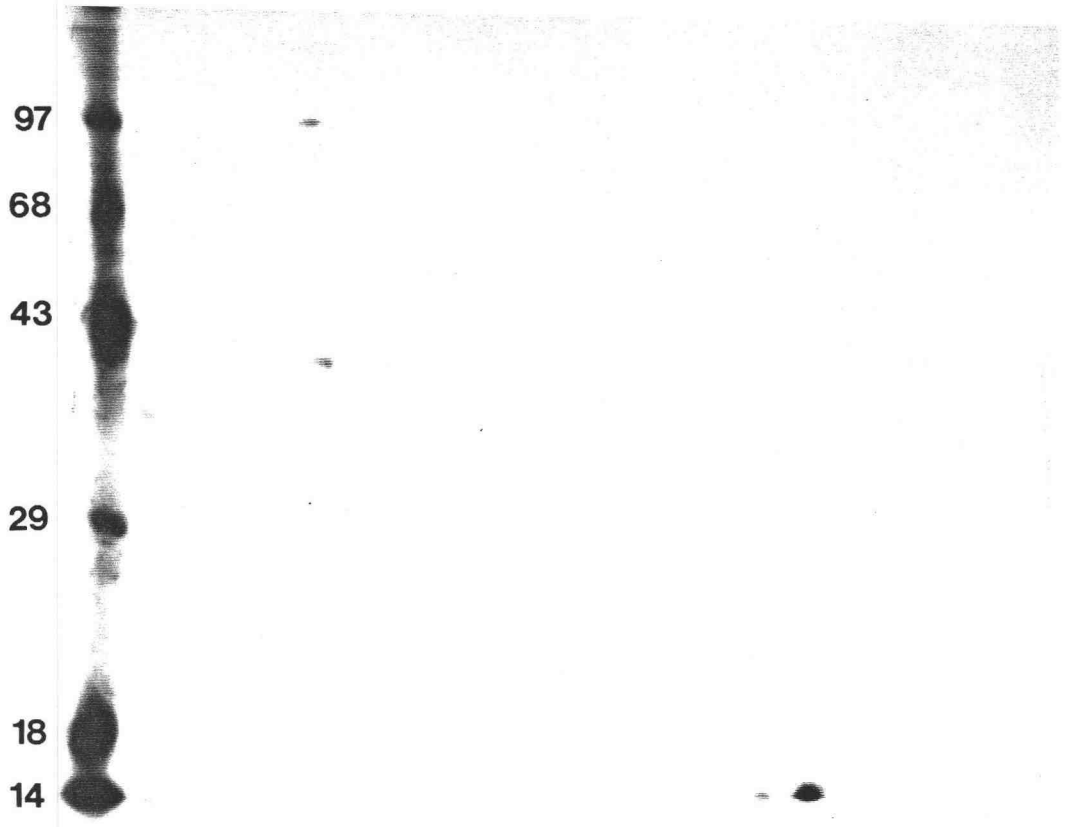

Figure III-20. Autoradiogram of a Two-dimensional NEPHGE Gel of *E. coli* B Extract Proteins Eluted from the NDPK Affinity Column at 0.2 M NaCl.

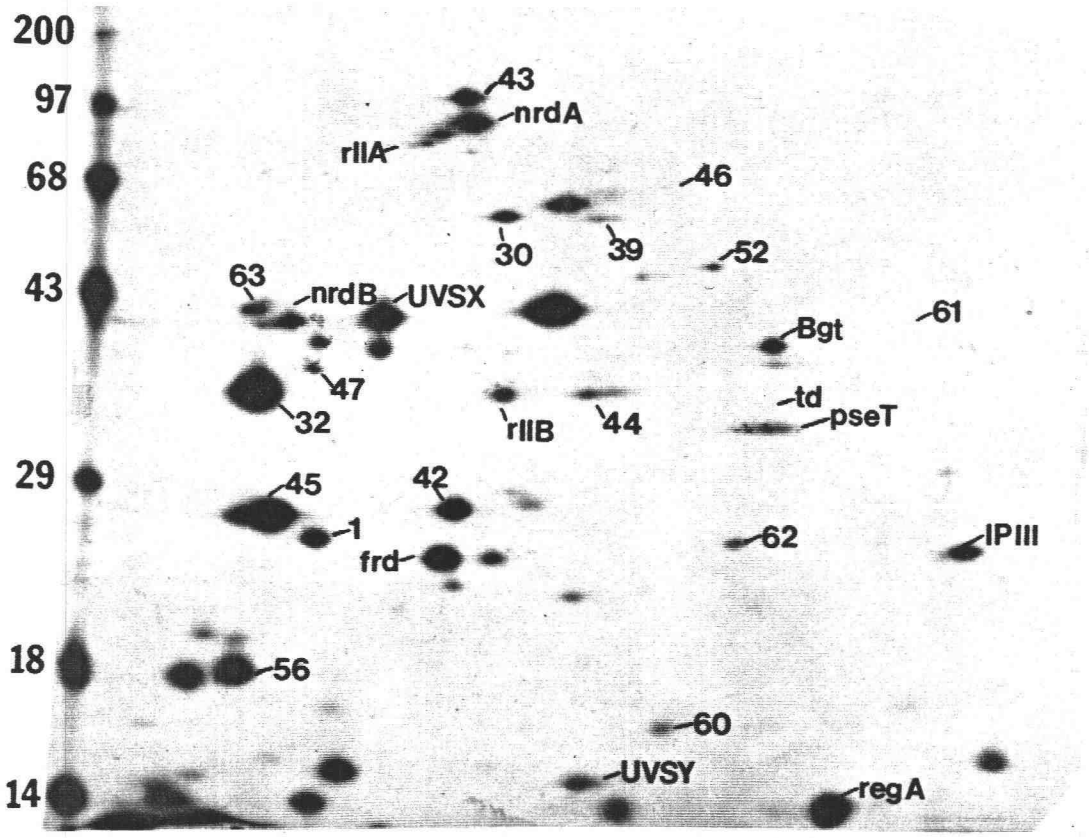

Figure III-21. Autoradiogram of a Two-dimensional NEPHGE Gel of Total T4-infected *E. coli* Extract.

as compared to the two-dimensional polyacrylamide gel of T4D early proteins eluted at 0.6 M NaCl. Both 0.2 M (see Fig. III-22) and 0.6 M NaCl (see Fig. III-23) eluted T4D early proteins were analyzed and, as can be seen (see Fig. III-23 and Table III-2), many T4 early proteins appear to interact with NDPK even at fairly high concentrations of salt (up to 0.6 M NaCl). However, some of these proteins have also been found to bind BSA (used as a control) at the same concentrations of salt, and therefore may not actually represent specific interactions with NDPK (see Table III-2). At 2 M NaCl, several T4 early proteins (see Fig. III-24) were eluted from the NDPK affinity column. Some of these also bind to BSA, including gp*rIIA*, gp*rIIB*, and gp52; however, the elution of UvsY, dihydrofolate reductase, gp32, and gp45 can also be detected and seems significant. Apparently, these four proteins bind to NDPK more tightly than other T4 early proteins.

L. Anti-idiotypic Antibody Interaction Studies

1. Immunoprecipitation with Anti-idiotypic Antibody to Denatured NDPK and Analysis

Antibody generated from insoluble, overexpressed NDPK that had been electroeluted from an SDS polyacrylamide gel was fractionated for IgG specifically, on a protein A Sepharose column and used to generate NDPK anti-idiotypic antibody. This was done to produce a secondary antibody that would mimic the original antigen, NDPK, and hopefully its interactions with T4 early proteins.

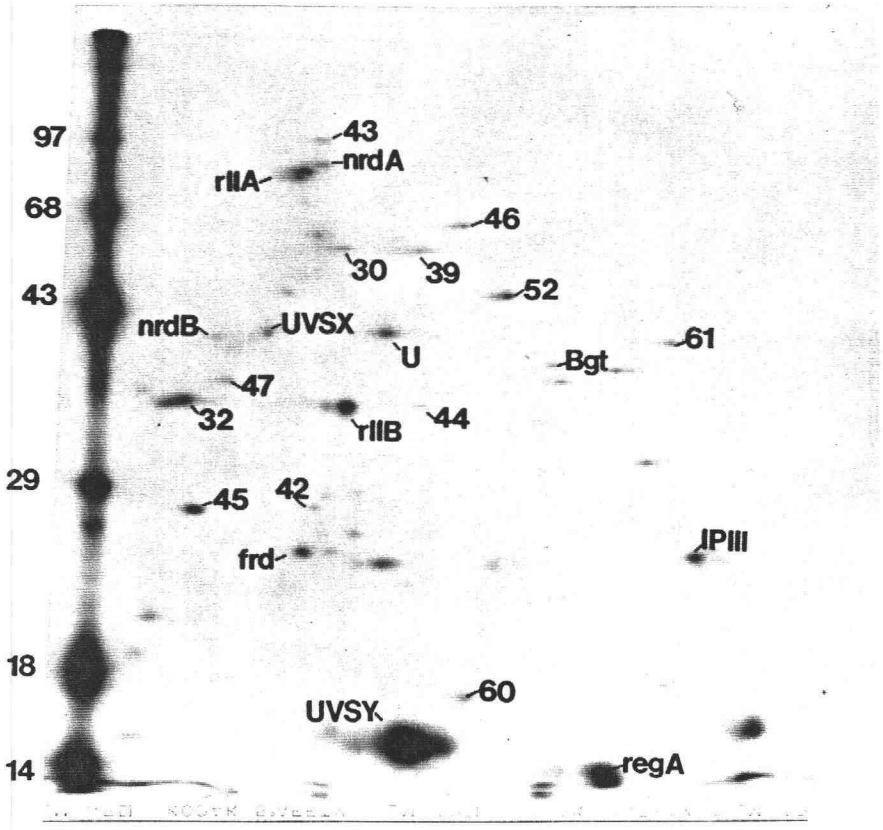

Figure III-22. Autoradiogram of a Two-dimensional NEPHGE Gel of T4-infected *E. coli* Extract Proteins Eluted from the NDPK Affinity Column at 0.2 M NaCl.

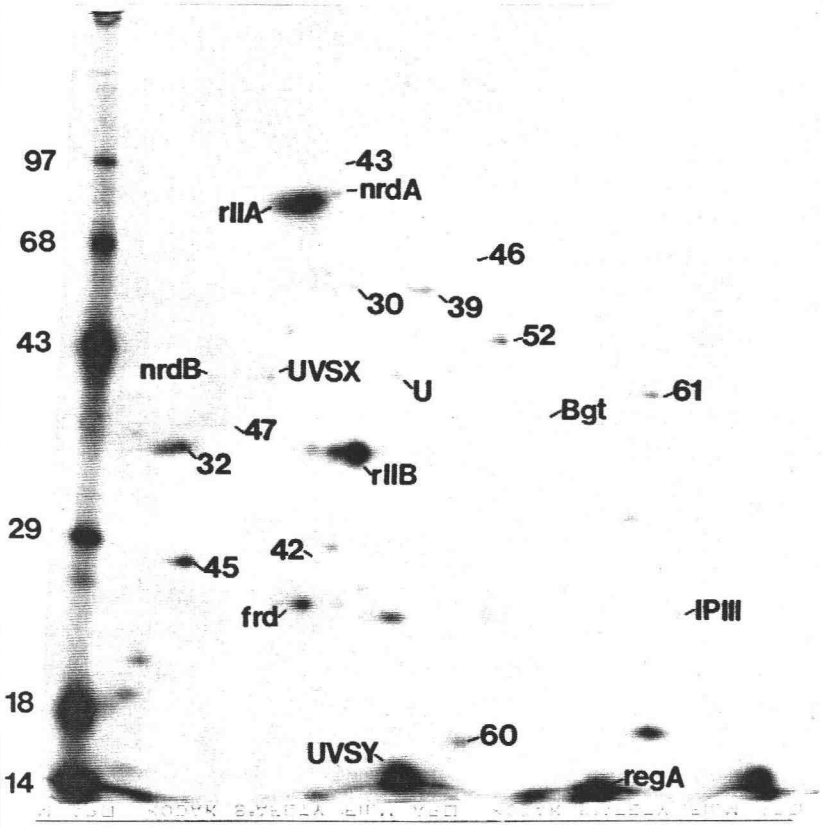

Figure III-23. Autoradiogram of a Two-dimensional NEPHGE Gel of T4-infected *E. coli* Extract Proteins Eluted from the NDPK Affinity Column at 0.6 M NaCl.

TABLE III-2

Table of T4 Early Proteins Bound at 0.2 M NaCl

and Eluted at 0.6 M NaCl

Gene	Gene Product	Function	NDPK Column	BSA Column
42	dCMP HMase	dNTP met.	+	-
frd	dihydrofolate red.	dNTP met.	+	-
nrdA	rNDP red. R1	dNTP met.	+	-
nrdB	rNDP red. R2	dNTP met.	+	-
32	SSB protein	DNA repl.	+	-
43	DNA pol.	DNA repl.	+	-
45	DNA pol. access.	DNA repl.	+	-
61	DNA primase	DNA repl	+	-
46	Nuclease	recomb.	+	-
47	Nuclease	recomb.	+	-
52	DNA topo. subunit	DNA repl.	+	+
39	DNA topo. subunit	DNA repl.	+	+
60	DNA topo. subunit	DNA repl.	+	+
61	DNA primase	DNA repl.	+	-
30	DNA ligase	DNA repl.	+	-
uvsX	RecA analog	DNA repair	+	-
uvsY	repair protein	DNA repair	+	-
ßgt	ß-glucosyltransferase	DNA mod.	+	-
regA	translation regulator	transcript.	+	-
IPIII	internal protein III	struct. prot.	+	+
rIIA	rapid lysis prot. A	membr. prot.	+	+
rIIB	rapid lysis prot. B	membr. prot.	+	+

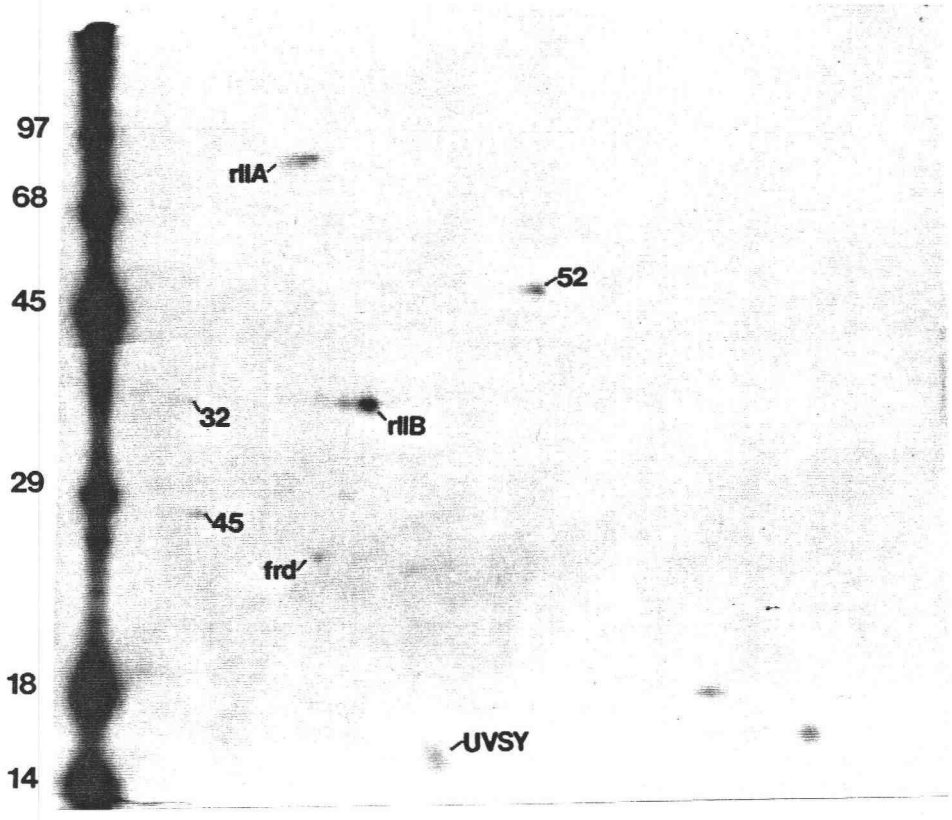

Figure III-24. Autoradiogram of a Two-dimensional NEPHGE Gel of T4-infected *E. coli* Extract Proteins Eluted from the NDPK Affinity Column at 2 M NaCl.

Sera from two rabbits that should include anti-idiotypic antibody, were used to immunoprecipitate ^{35}S-labeled *E.coli* and T4-infected *E. coli* extracts as described previously. It was found that a T4 early protein of approximately 43 kDa (see Fig. III-25) was specifically immunoprecipitated by the second rabbit (rabbit 2), and was not immunoprecipitated by preimmune serum (serum collected from the rabbit previous to the immunization with antibody to denatured NDPK). An *E. coli* protein of approximately 60 kDa was immunoprecipitated with the same serum (rabbit 2), but this 60-kDa protein was also immunoprecipitated by the preimmune serum, leading to the conclusion that an antibody present in the serum of the second rabbit before immunization with anti-denatured NDPK antibody, caused the immunoprecipitation of this *E. coli* protein. The first rabbit (rabbit 1), immunized with the same amount of anti-denatured NDPK antibody, showed no specific response to the antigen, as judged by the absence of any significantly immunoprecipitated protein (see Fig. III-25). It was concluded that the immunoprecipitated 43-kDa protein is a T4 early protein that interacts with NDPK *in vivo*. Since several T4 early proteins are known to have molecular weights close to 43 kDa, it was necessary to attempt to identify the immunoprecipitating protein by other criteria.

2. Two-dimensional Analysis of Immunoprecipitated Protein

The 43-kDa immunoprecipitated protein along with purified gp32 and gp61 reference proteins, were then treated with urea and NEPHGE buffer (as described previously for the preparation of samples for two-dimensional analysis). The 43-kDa protein, which migrated to the same molecular weight position (second dimension) as seen on SDS polyacrylamide gels, migrated to a

position halfway between gp32 and gp61 in the first dimension (relative to pI values). When a comparison was done between the migration of the 43-kDa protein in both dimensions and a two-dimensional gel of total T4 early proteins (run under the exact same conditions), it was found that the 43-kDa immunoprecipitated protein corresponded to an unidentified early T4 protein that migrates just to the basic side of UvsX (see spot labeled U in Fig. III-20). Although this protein has not been identified on two-dimensional gels, it most certainly is involved in either T4 dNTP metabolism or DNA replication, since it is definitely an early protein, and is not labeled along with late proteins (Kutter et al, 1992).

Competition experiments were also done with purified NrdB (from Eric Hanson) and gp61 (from Dr. Von Hippel's laboratory) proteins, which were readily available and have molecular weights of approximately 43 kDa each. Since no competition was seen to interfere with the immunoprecipitation of the 43-kDa protein, it was concluded that the 43-kDa protein is not NrdB or gp61 (see Table III-3 for a list of T4 proteins having molecular weights and/or pI values in the range of those values observed for the immunoprecipitated protein). Competition experiments will continue as more purified proteins become available, and attempts at identifying the immunoprecipitated protein will continue through the use of amber mutants of specific proteins. Purification of the immunoprecipitated protein in large enough quantities for N-terminal amino acid sequencing will also be attempted in order to identify the protein. It is very interesting to note that the same unidentified protein has been seen to elute from the NDPK affinity column at 0.2 M NaCl and more significantly at 0.6 M NaCl (see Fig. III-23 and Fig. III-24), lending support to the conclusion that the 43-kDa protein is a T4 early protein that interacts with NDPK *in vivo*.

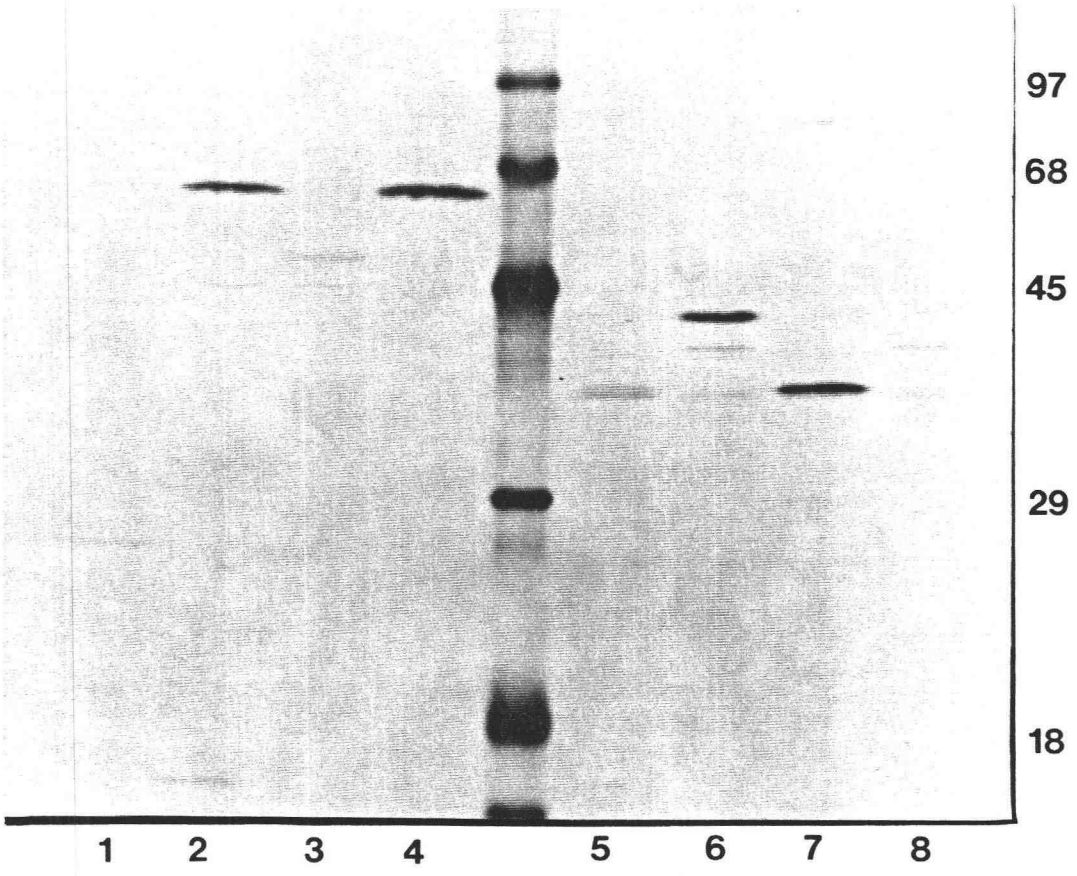

Figure III-25. Autoradiogram of the Immunoprecipitation of Uninfected and T4-infected *E. coli* B Extract with Anti-anti NDPK Antibody and Preimmune Sera. Lane **1** and **2**, *E. coli* extract immunoprecipitated with anti-anti NDPK from rabbit 1 and 2 (respectively);**3** and **4**, *E. coli* extract immunoprecipitated with preimmune from rabbit 1 and 2; **5** and **6**, T4-infected *E. coli* extract immunoprecipitated anti-anti NDPK from rabbit 1 and 2; **7** and **8**, *E. coli* extract immunoprecipitated with preimmune from rabbit 1 and 2.

TABLE III-3

T4 Early Proteins Having pI Values and/or M_r Values in the Range of the Unidentified Anti-idiotypic Antibody-immunoprecipitated 43-kDa Protein

Protein	Gene	M_r Value	Calculated pI Value
DNA priming protein	61	39,780	10.05
RNA ligase	63	44,944	4.96
DNA αglucosyltransferase[a]	*αgt*	46,707	6.09
DNA βglucosyltransferase	*βgt*	40,668	10.01
ribonucleotide reductase small subunit (Nrd B)	*nrdB*	45,338	4.77
UvsX recombination and repair protein	*uvsx*	43,338	5.10

[a] not yet identified on two-dimensional polyacrylamide gels

3. Immunoprecipitation with Anti-idiotypic Antibody to Native, Active NDPK

Immunoprecipitation experiments were also done with sera that included anti-idiotypic antibody generated from antibody to native, active NDPK that had been fractionated through a protein A Sepharose column, as well as eluted specifically from an immobilized NDPK affinity column. This was done to obtain an IgG fraction specific for native, active NDPK. Several *E. coli* proteins and T4 early proteins were immunoprecipitated, even after extensive washings, by both rabbits that were injected with anti-(native, active) NDPK (data not shown). Therefore, it was concluded that a weak and nonspecific antiidiotypic response had been generated.

M. Fluorescence Anisotropy Studies

1. Labeling with 1,5-IAEDANS

Efficient, covalent labeling of NDPK with 1,5-IAEDANS was achieved as judged by gel electrophoresis and fluorescence intensity measurements (see Fig. III-26 and III-27 for fluorescence excitation and emission spectra). An enzyme activity assay with fluorescently labeled NDPK was compared with an enzyme activity assay of unlabeled NDPK done at the same time with the same amount of NDPK. It was found that the covalent labeling of NDPK by 1,5-IAEDANS did not effect the enzyme activity of NDPK. Therefore, it was

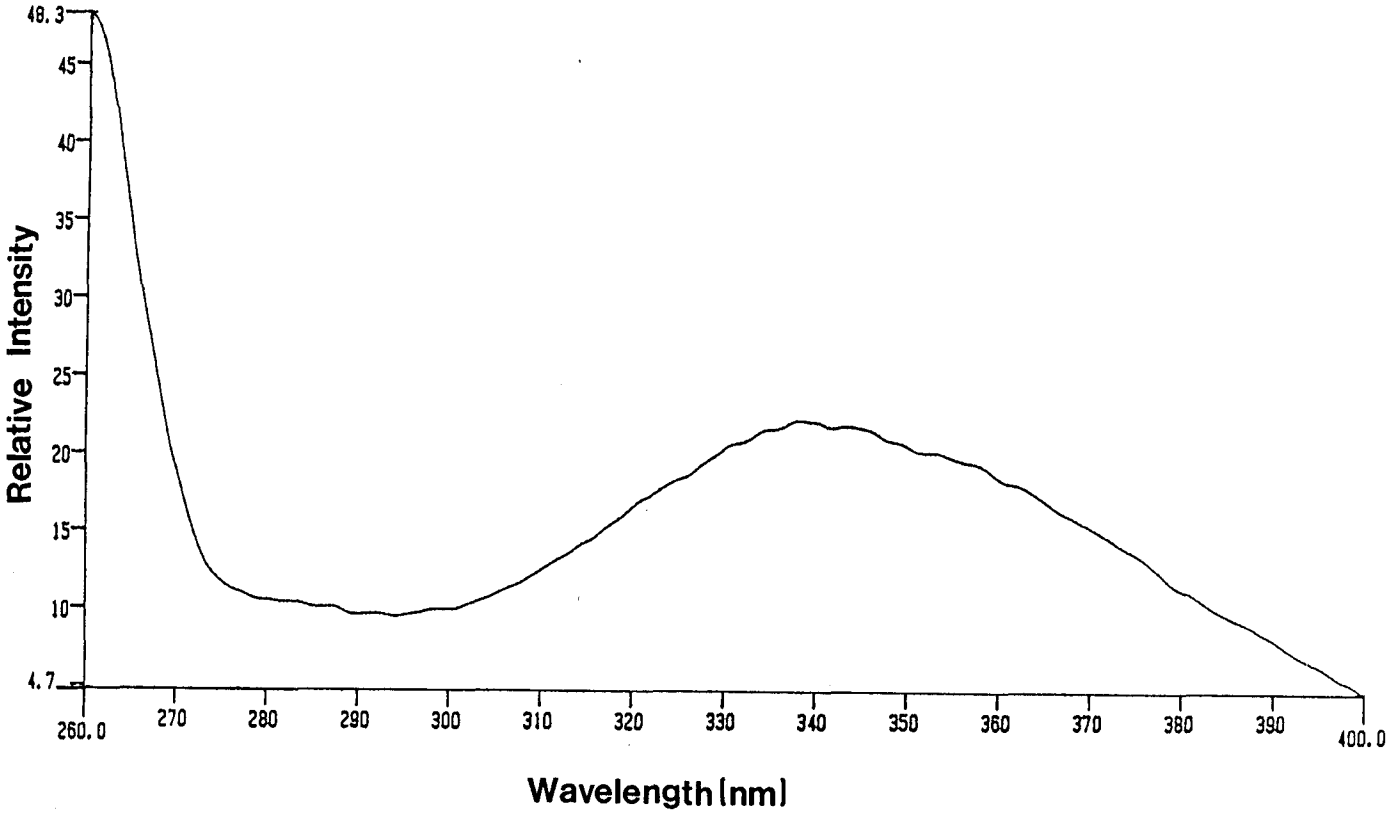

Figure III-26. Fluorescence Excitation Spectrum of 1,5-IAEDANS-labeled NDPK.

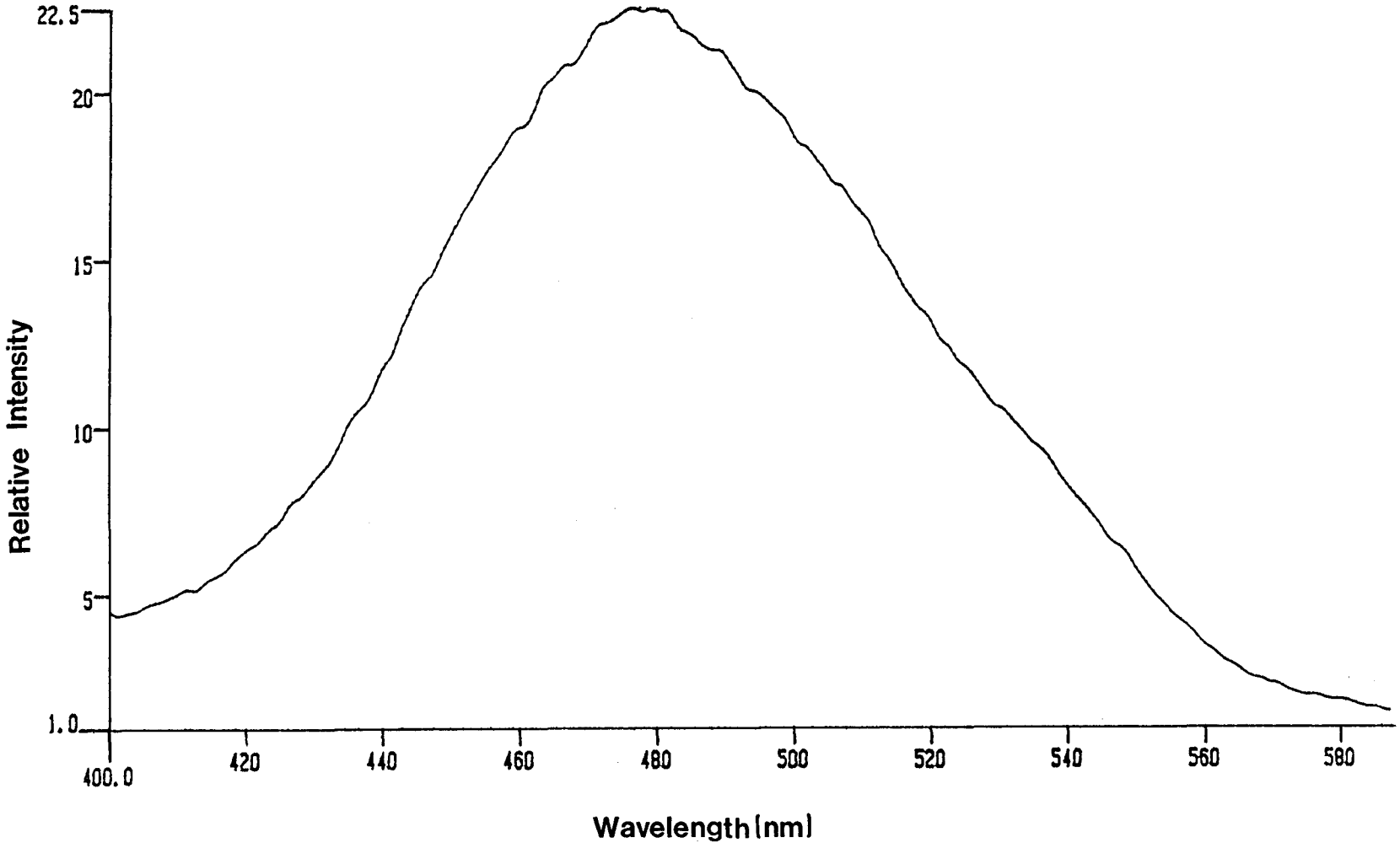

Figure III-27. Fluorescence Emission Spectrum of 1,5-IAEDANS-labeled NDPK excited at 365 nm.

concluded that the labeling reaction did not change the conformation of NDPK, at least near the activity site, and that the cysteine residue (site of labeling) was not important for enzyme activity.

2. Anisotropy Measurements of NDPK and T4 DNA Polymerase

An approximately two-fold increase in anisotropy was seen upon the addition of T4 DNA polymerase (from Dr. Konigsberg's laboratory) to NDPK. However, when the experiment was repeated with T4 DNA polymerase from a different source (Dr. Von Hippel's laboratory) the same result was not seen. It was concluded that much more enzyme was needed to repeat these experiments, and that it would be necessary to use purified T4 DNA polymerase from one source for a more controlled experiment. Even small differences in purification or conformation of the enzyme could affect the detection of small changes in anisotropy values, which might occur if the interaction of NDPK and T4 DNA polymerase is relatively weak.

IV. Discussion

A. NDPK and Other *E.coli* GTP-binding Proteins

The 45-kDa protein that cofractionated with NDPK during the purification from crude extracts of *E. coli* B cells, was seen to specifically elute from Blue Sepharose with 2 mM GDP in significantly larger quantities than with 2 mM dTDP (see Fig. III-4). Since G proteins are known to bind to GTP as well as GDP, I asked whether NDPK might interact with G proteins, possibly regenerating bound GTP from GDP. I then discovered a few reports describing potential interactions between NDPK and G proteins in eukaryotic systems (see Introduction), which became more numerous and frequent as more interest in NDPK evolved. Because the 45-kDa GDP-binding protein was also eluted with 2 mM dTDP (to a lesser degree, see Fig. III-4), and was difficult to fractionate from NDPK, it is possible that it interacts fairly tightly with NDPK given the correct conditions. This potential interaction may represent a physiologically relevant association that could occur *in vivo.* It was hoped that an indication of this potential interaction would again be observed with overexpressed NDPK, especially in association with the immobilized NDPK affinity chromatography and antiidiotypic antibody studies, in which labeled *E. coli* extracts were used for comparison with labeled T4-infected *E. coli* extracts. However, evidence for this potential interaction was not seen with overexpressed NDPK, which could mean: a) overexpressed NDPK is somehow different from NDPK in *E.coli* B cells, b) overexpressed NDPK is present in greater than stoichiometric amounts required

for the interaction, c) the conditions were not optimal for the interaction, or d) the GDP-binding protein association with NDPK was artifactual and does not represent a physiologically relevant interaction that occurs *in vivo.* Further investigation of this potential interaction was not directly pursued because Dr. Inouye's laboratory specifically cloned the *ndk* gene and overexpressed NDPK as a GTP-binding protein, which could potentially interact with other GTP-binding proteins found in *E. coli.* We had agreed to focus upon the interactions of the recombinant NDPK and T4 proteins when we gratefully accepted their clone.

It is interesting to note that the *era* and *lepA* genes (see introduction) have been mapped in fairly close proximity to the *ndk* gene at 55 minutes (Inada et al, 1989). Perhaps this represents a functional organization of these genes on the *E. coli* chromosome, since they all code for GTP-binding proteins. The possibility also exists that NDPK does not interact with GTP-binding or "G-like" proteins in *E.coli,* and that this interaction has only evolved in eukaryotes for the specific purpose of true signal transduction. If one examines the NDPK amino acid sequence, one will find the absence of two known conserved sequences found for many GTP-binding proteins, including G-proteins. However, a conserved sequence believed to be involved in phosphoryl binding (see Introduction), DXXG, based on x-ray crystallography analysis of EF-Tu, is found in the *E. coli* NDPK amino acid sequence (residues 88-91).

It has been noted that mutations in the *ndk* gene have never been isolated, which may be due to pleiotropic effects of *ndk* mutations caused by the associations of NDPK with other cellular proteins (Hama et al, 1992). Perhaps NDPK has several roles in *E. coli* cells, which may be related to its different locations in the cell (cytoplasm vs. periplasmic space). It is surprising that the results from the affinity chromatography and anti-idiotypic antibody studies show

evidence for little interaction between NDPK and other *E. coli* proteins. Many more T4 proteins actually appear to interact with *E. coli* NDPK, which is quite interesting. It is possible that the coevolution of *E. coli* and bacteriophage T4 has promoted the evolution of NDPK towards a protein that can interact more specifically with T4 dNTP biosynthesis proteins. This may help maintain the association of these proteins as a complex and also increase their interactions with T4 DNA replication proteins. A small portion (approximately 5% based on activity assays) of the total cellular NDPK is sequestered in the T4 dNTP synthetase complex (see Introduction). Perhaps the interactions of NDPK with T4 dNTP biosynthesis proteins must necessarily be stronger than the interactions of NDPK with other cellular proteins, for this sequestering to occur.

B. Potential Regulation of NDPK

There is no evidence for any enzymatic regulation (i.e. allosteric) regulation of NDPK in any system. NDPK seems to be a highly active and stable housekeeping enzyme. However, the fact that it appears to play a role in tumor suppression involving elevated levels of NDPK mRNA in mammalian systems has opened the possibility of genetic or transcriptional regulation. It is interesting to note that the NM23 amino acid sequences contain periodic leucine repeats in an α-helical region, preceded by a region containing basic amino acids. This is suggestive of a leucine zipper motif, which could indicate involvement in transcriptional regulation (Leone et al , 1991), through its interaction with DNA. In this way it may regulate the expression of proteins such as G-proteins involved in signal transduction, and perhaps its own expression.

Mutations in NDPK could cause a loss of such regulation manifested as oncogenesis.

Although the *E. coli* NDPK amino acid sequence does not contain an obvious leucine zipper motif, it may still regulate its own expression or the expression of other GTP-binding proteins at a genetic or transcriptional level. The fact that approximately two-fold higher expression of NDPK was seen in DH5α cells (see Fig. III-12), in which *lacZ* is not constitutively expressed is quite intriguing. How *lacZ* expression interferes with the expression of *ndk* is unknown. Since the *ndk* gene was cloned with its promoter in the opposite orientation to *lacZ,* it is possible that the RNA polymerases could collide during transcription. Another interpretation of the significantly higher expression is antisense mRNA regulation. Since the *lac* promoter would drive transcription in the opposite direction, antisense *ndk* mRNA could bind the sense mRNA, inhibiting its own expression. This could be a physiologically relevant process of regulation that could occur *in vivo* under certain conditions. In any case, the increase in expression in DH5α cells is significant and seems to indicate that *ndk* has a strong promoter, allowing the expression of NDPK to a level of approximately 50% of the total soluble protein. If *E. coli* NDPK does play a role in the regulation of other cellular GTP-binding proteins, it is surprising that the overexpression and increased overexpression of NDPK does not appear to affect the growth of the cells in which it is overexpressed. The possibility exists that there is another gene coding for NDPK in *E. coli* paralleling eukaryotic systems, such as human erythrocytes, in which NDPK is composed of A and B chains encoded by two separate genes. Some evidence for this possibility is accumulating (personal communication with Dr. Inouye's laboratory).

C. NDPK's Role in T4 DNA Replication

There are several lines of evidence that strongly indicate *E. coli* NDPK is an integral component of the T4 dNTP synthetase complex, and plays an indispensable role in T4 DNA replication (for review, see introduction). In the presence of desdanine, the specific inhibitor of *E. coli* NDPK, little T4 DNA synthesis occurs (see Fig. I-4). The relationship of NDPK to DNA replication could be correlated to the relationship of the T4 dNTP synthetase complex to the T4 DNA replication complex. In this way, inhibition of NDPK would cause a disruption of the channeling of dNTPs to T4 replication forks, thereby inhibiting DNA synthesis. The possibility also exists that NDPK may itself directly interact with one or more replication proteins (such as T4 DNA polymerase), as well as with proteins involved in the T4 dNTP synthetase complex. It is interesting to note that several proteins involved in the dNTP synthetase complex, as well as several proteins involved in DNA replication (see Fig III-23 and Table III-2), were eluted from the NDPK affinity column at a fairly high concentration of salt (0.6 M NaCl), suggesting a physiologically relevant interaction between NDPK and these proteins. Some of the interactions may occur through a "piggyback" effect in which certain proteins are actually piggybacking on other proteins that directly interact with NDPK. Hydroxymethylase appears to interact with many of the same dNTP metabolism and DNA replication proteins (Wheeler et al, 1992) (see Table III-2 and Table I-3). It was observed that many of the same replication proteins that were eluted from the hydroxymethylase affinity column at 0.6 M NaCl, had also been seen to elute from a gp32 affinity column at 0.6 M NaCl (Formosa et al, 1983), such as gp32 itself, gp43 (actually eluted at 0.2 M NaCl

from both columns), gp45, gp*uvsX*, gp*uvsY* and gp46. The possibility that these replication proteins were piggybacking on gp32 was considered and a 32$^-$T4 amber mutant extract was applied to the hydroxymethylase column. T4 ^{35}S-labeled proteins were again eluted from the column at different concentrations of salt and identified by two-dimensional gel analysis. There is also strong evidence for a direct interaction between hydroxymethylase and thymidylate synthase (gp*td*) (Young and Mathews, 1992). Therefore, a *td*$^-$T4 amber mutant extract was also applied to the hydroxymethylase column, and the premise that gp32 binds to hydroxymethylase through its interaction with thymidylate synthase was tested. An important result of these experiments was that gp32 was found to be absent in the 0.6M NaCl elution when both amber mutant extracts were applied, indicating that thymidylate synthase is required for the interaction of gp32 with hydroxymethylase. Another important result was that the large and small subunits (NrdA and NrdB) of ribonucleotide reductase disappeared from among the proteins eluted from the hydroxymethylase column at 0.6 M NaCl, when both mutant extracts were applied. Since ribonucleotide reductase is a central component of the T4 dNTP synthetase complex, this supports the premise that the specificity of the binding of T4 proteins to immobilized hydroxymethylase corresponds to the specificity of interactions which must occur in the assembly of a functional T4 dNTP synthetase complex (Wheeler et al, 1992).

As noted above, many of the same DNA replication proteins were observed to elute from the NDPK affinity column at 0.6 M NaCl as were found eluting from the hydroxymethylase column at the same concentration of salt, including gp32, gp43, gp45, gp*uvsX*, gp*uvsY* and gp46. Therefore, one could again conclude that these are interacting with NDPK through a gp32 piggyback interaction. It also interesting to note that some of the same dNTP metabolism

proteins eluted from the NDPK affinity column at 0.6 M NaCl were also eluted from the hydroxymethylase affinity column at the same concentration of salt, including dihydrofolate reductase, hydroxymethylase itself, and the large and small subunits of ribonucleotide reductase. One interpretation is that the dNTP synthetase complex is being assembled in both cases due to the interactions that are occurring among enzyme components of the complex, which are disrupted between 0.2 M and 0.6 M NaCl. Perhaps this assembly is necessary for interactions to occur with proteins involved in the replication complex, or multiple interactions are occurring between the dNTP biosynthesis enzymes and replication proteins. It is unfortunate that the retention of NDPK on the hydroxymethylase column has not been detected. However, at the time that the hydroxymethylase affinity studies were being done, antibodies to NDPK had not yet been generated. Since NDPK runs very close to the dye front on 12.5% SDS polyacrylamide gels, and since the two-dimensional analysis of proteins used in our laboratory is optimized for the detection of T4 early protein separation (many of which are basic, unlike *E. coli* proteins), it would be difficult to detect the specific elution of NDPK without doing a Western blot. In the future, this experiment should be done. Further protein-protein interaction experiments employing anti-idiotypic antibody, affinity chromatography, and fluorescence anisotropy analysis, as well as reconstitution experiments, will help elucidate which dNTP biosynthesis and DNA replication proteins interact directly and in what order these interactions occur.

D. Potential Interaction of NDPK and T4 DNA Polymerase

One likely direct interaction that may occur *in vivo* between an enzyme component of the T4 dNTP synthetase complex and T4 replication protein, is that of NDPK and T4 DNA polymerase. Since these enzymes catalyze sequential reactions towards DNA synthesis, NDPK may channel dNTPs directly to T4 DNA polymerase, and thereby the replication apparatus. For this reason, and because T4 DNA polymerase had been seen to specifically elute from an NDPK affinity column in early experiments, a direct interaction was investigated by fluorescence. Fluorescence analysis was done because relatively small amounts of protein (μM range) could be used to detect small changes in intensity. Even though a conclusive indication of a direct interaction between NDPK and T4 DNA polymerase was not found, these experiments could be repeated with greater amounts of purified protein. Also, a range of conditions should be tested more thoroughly (i.e. different concentrations of salt, magnesium, and nucleotides). It is possible that certain nucleotides must be present perhaps in specific combinations before "active" conformations of the enzymes would exist favoring the interaction. The fact that T4 DNA polymerase eluted from the NDPK column at 0.2 M NaCl and at 0.6 M NaCl is promising. As noted above, T4 DNA polymerase eluted from a gp32 affinity column at 0.2 M NaCl (Formosa et al, 1983). Therefore, the elution of T4 DNA polymerase from the NDPK affinity column at 0.6 M NaCl seems significant.

E. Unidentified Immunoprecipitated Protein

As noted, the 43-kDa T4 early protein, specifically immunoprecipitated by the anti-idiotypic antibody generated from denatured NDPK, could not be identified by two-dimensional gel analysis, since its migration corresponded to an unidentified protein. Competition experiments with unlabeled NrdB and gp61 have eliminated these proteins from the list of proteins having pI values and/or apparent molecular weights in the range of the immunoprecipitated protein (see Table III-3). It is interesting to note that a labeled protein spot representing α-glucosyltransferase has not yet been identified on two-dimensional gels of total T4 early proteins. It also has a M_r of approximately 46 kDa and a calculated pI value of 6.09, which is approximately halfway between the calculated pI values of gp32 (4.5) and gp61 (10.01), corresponding to the migration of the unidentified protein. Therefore, α-glucosyltransferase is a likely candidate for the unidentified immunoprecipitating protein. Further experiments will be done to investigate this possibility, including the use of an extract of T4 α*gt*(⁻)-infected *E.coli* for immunoprecipitation experiments. The absence of a 43-kDa immunopreciptating protein would then be a good indication that the unidentified protein is α-glucosyltransferase. It is very interesting to note that β-glucosyltransferase has been observed to elute from both the immobilized hydroxymethylase and NDPK affinity columns, and could be involved in the T4 DNA replication complex. It also seems significant that a labeled-protein (spot), corresponding to the unidentified protein (labeled U in Fig. III-22 and Fig. III-23), was eluted from the NDPK affinity column at 0.2 M NaCl and 0.6 M NaCl. One possible conclusion is that both analyses (affinity chromatography and anti-

idiotypic antibody immunoprecipitation) provide evidence for a physiologically relevant interaction between NDPK and the unidentified T4 early protein. It is interesting that anti-idiotypic antibody to native, active NDPK did not also immunoprecipitate the 43-kDa unidentified protein. However, this anti-idiotypic antibody was generated from NDPK affinity-fractionated antibody, which would seem to select for antibody that binds fairly strongly to NDPK. Weakly binding antibody would wash off the column and tightly binding antibody may not elute from the column even at low or high pH. Therefore, the range of epitopes may have been too limited to generate a good response. One would expect that anti-idiotypic antibody generated from antibody to active, native protein would be more likely to immunoprecipitate a protein potentially interacting with the protein of interest *in vivo,* since it would be more likely to mimic a surface epitope. However, strong evidence for an interaction between the vaccinia small subunit of ribonucleotide reductase and a single-strand binding protein has been obtained from antibody to the denatured protein of interest (Davis et al, 1992). It seems possible that many factors determine the likelihood of finding a specific immunoprecipitation with anti-idiotypic antibody, including range of epitopes, antigenicity, conformation, solubility, and luck.

V. Bibliography

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