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Title: Enzyme Associations in Deoxyribonucleotide Biosynthesis: Anti-idiotypic Antibodies as Probes for Direct Protein-Protein Interactions.

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Christopher K. Mathews, Ph.D.

The ability to faithfully replicate DNA is dependent upon the maintenance and regulation of its precursors, the deoxyribonucleoside triphosphates. Enzymes encoded by the bacteriophage T4 have been widely used as models of biochemical processes. A body of evidence supports the concept that the bacteriophage T4 enzymes involved in deoxyribonucleotide biosynthesis are associated as a complex within the infected *Escherichia coli*. This dissertation describes the continued examination of the protein-protein interactions involved in deoxynucleotide biosynthesis of bacteriophage T4.

My studies on the protein-protein interactions involved in deoxyribonucleotide biosynthesis focused on two unique phage proteins, the dCMP hydroxymethylase enzyme and the translational regulator RegA. An initial study was undertaken to determine if the generation of anti-idiotypic antibodies would prove useful in the identification of direct interactions between dCMP hydroxymethylase and other proteins of the

deoxyribonucleotide synthetase complex.

For the initial generation of anti-idiotypic antibodies, polyclonal rabbit antibodies were generated to affinity purified anti-dCMP hydroxymethylase polyclonal rabbit IgG. The anti-anti-dCMP hydroxymethylase antibody was found to be specific in binding to the bacteriophage T4 dTMP synthase.

A second method to generate anti-idiotypic antibodies was attempted with the translational regulator RegA. The generation of anti-idiotypic antibodies to the RegA protein involved the purification of anti-RegA rabbit Fab fragments and the generation of anti-anti-RegA polyclonal antibodies within mice. This alternative method was determined to be inferior to the initial method for generating anti-idiotypic antibodies. Additional studies involved the examination of RegA protein-protein interactions using affinity chromatography. A number of bacteriophage T4 early proteins were determined to associate with an immobilized RegA column.

Enzyme Associations in Deoxyribonucleotide Biosynthesis: Anti-idiotypic Antibodies as Probes for Direct Protein-Protein Interactions

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CHAPTER 1

Introduction

Background

Bacteriophage T4 has been an essential element in the birth and development of molecular biology. The knowledge gained during this infancy and adolesence has brought a wealth of information about the biochemistry involved in bacteriophage T4 replication within the host, *Escherichia coli*. More importantly research dealing with bacteriophage T4 has given insights into fundamental processes of all living systems. A number of current biochemical paradigms were first investigated and described by using bacteriophages. Examples include the discoveries of genetic information being encoded in DNA, the genetic code being read from a fixed starting point in triplets, and the identification of messenger RNA. These basic processes and many others were revealed through the model system bacteriophage T4.

The enzymology of deoxyribonucleotide biosynthesis represents one of the classical and best studied areas of T4 biochemistry. In general all organisms that contain DNA are required to provide more than just an adequate supply of the four deoxynucleotides. To prevent misincorporation during DNA replication the supply of each deoxyribonucleoside triphosphate must also be balanced relative to the levels of the other three deoxyribonucleoside triphosphates. The bacteriophage T4 as a model system provides a powerful tool to further investigate the properties and interactions in this delicately balanced system.

This dissertation describes several approaches to understanding the interactions of the enzymes involved in a deoxynucleotide biosynthesis complex. The first chapter is a review of bacteriophage T4 and the enzymes

that are involved in deoxynucleotide biosynthesis. This will be followed by two manuscripts. The first, chapter 2, describes the initial identification of an interaction between bacteriophage T4 thymidylate synthase and dCMP hydroxymethylase using an anti-idiotypic antibody as an immunological probe. Chapter 3 describes the purification of the phage thymidylate synthase and dCMP hydroxymethylase enzymes. Chapter 4 describes the investigation to understand the interactions between a bacteriophage T4 translational regulator, RegA, and the enzymes involved in dNTP biosynthesis. The final section will discuss the implication of the work described in the manuscripts and their general importance.

Introduction to Bacteriophage T4:

Bacteriophage T4 History and its Replication Machinery

The original foundation for the study of bacteriophages started with Frederick Twort's discovery in 1915 of a new phenomenon, the lysis of bacteria (Twort, 1915). By 1917 Felix d'Herelle postulated the concept that bacteriophagy was caused by an organism parasitizing bacteria (d'Herelle, 1917). Continued research by d'Herelle led to his quantitation of his findings and the development of the plaque method, which became a powerful tool both in bacteriophage and animal virus research (d'Herelle, 1922).

The early research that followed these initial findings was spurred on by the belief that bacteriophages would prove to be powerful therapeutic agents in the fight against bacterial infection. As early as 1926, articles describing the attempt to control a variety of epidemics with bacteriophages were being published in journals (Schumm et al., 1926). While bacteriophages did fail in their role as therapeutic agents, the research on the phages' unique properties

and general biology continued.

Bacteriophage T4 itself was not isolated until 1945 by Milislav Demerec, and by this time a bewildering variety of bacteriophages had been identified with a wide range of characteristics. Max Delbrück selected a small number of phages from the Demerec collection that he hoped would be a foundation for future research (Waterson and Wilkinson, 1978). Delbrück believed that the seven isolates were distinct types of bacteriophages and designated them T1 through T7, the "T" standing for "type" (Calendar, 1988). Later it was recognized that Delbrück had not selected seven distinct phages but that the T-even phages, T2, T4, and T6, are a related clan, while T3 and T7 form a second distinct group.

In the past decade the study of bacteriophage T4 has revealed a number of surprises about this classic laboratory organism. Bacteriophage T4, supposedly an entrenched representative of the prokaryotic kingdom, has demonstrated several biochemical characteristics that had once been thought to be unique to the eukaryotic world.

One of the recent surprises is the demonstration that three bacteriophage T4 genes: *td*, thymidylate synthase; *nrdB*, the small subunit of ribonucleotide reductase; and *sunY*, a putative anaerobic ribonucleotide reductase, were demonstrated to contain group I introns that are also found in certain eukaryotic RNAs (Shub and Belfort, 1988). The biological role of the introns is currently unknown. Also, the significance of the fact that the bacteriophage T4 introns all likely reside within deoxynucleotide biosynthesis enzyme genes is an unanswered question.

Another surprise recognized within the last decade involved the T4 DNA replication enzymes. Amino acid sequence analysis (Spicer and Konigsberg,

1988) demonstrated that the T4 DNA polymerase contains the six conserved functional domains that characterize the eukaryotic DNA replication polymerases (Wong and Wang, 1988). A striking observation is that while the T4 DNA polymerase shows homologous domains with the eukaryotic polymerases the *E. coli* polymerases do not contain these functional domains and have low sequence similarity.

Besides the polymerases' similarities the human replication factor C and proliferating-cell nuclear antigen, PCNA, have been shown to be "completely analogous" in function to the T4 DNA polymerase accessory proteins (Tsurimoto and Stillman, 1990). The human proliferating-cell nuclear antigen has 31-50% homology with gene 45, a DNA polymerase accessory protein in bacteriophage T4 that stimulates a DNA-dependent ATPase activity. PCNA can also stimulate the human replication factor C ATPase activity, and can be functionally replaced *in vitro* with the T4 gene product 45. As in the case with the DNA polymerases the ß subunit of *E. coli*, which has been proposed to have a function similar to PCNA (Tan and Downey, 1986), shows almost no amino acid homology.

While bacteriophage T4 represents one of the best understood organisms in its biochemistry, very little is known about its evolution. Are modern day T-even bacteriophages ancient organisms that arose with the first bacteria, or is the recruitment of host genes to generate new viruses a frequent ongoing process, with the T-even bacteriophages being the latest development?

Tsurimoto and Stillman postulate that during the development of eukaryotes an ancestral bacteriophage T4 type polymerase gene as well its accessory genes duplicated and diverged to function in the eukaryotic cells. This model that proposes a vertical acquisition of genetic information to explain the similarity between T4 and eukaryotic genes is but one of several possible

scenarios to explain the homology.

While bacteriophage T4 is well known for its powerful ability for DNA recombination, little is currently known about the interactions of phages in the wild with other organisms. It has been proposed that the mammalian gut is the major natural environment of T-even phages (Kutter, 1992), and a horizontal transmission of genetic information between T4 and eukaryotes after the divergence of prokaryotes and eukaryotes could be an alternate explanation for the homology.

I would propose that without any conclusive evidence on the antiquity of T-even bacteriophages, even the direction of horizontal transmission of genetic information cannot be made. An unlikely but still possible explanation of the T4 and eukaryotic homology is that the T-even bacteriophages are evolutionary recent chimeras of prokaryotic and eukaryotic information.

Composition of Bacteriophage T4

Bacteriophage T4, along with its clan members, T2 and T6, is the most structurally complex of the T phages. Bacteriophage T4 is among the largest viruses ever isolated, with a DNA content of around 171 kb containing 3 % terminal redundancy. The T4 genome contains approximately 200 open reading frames that are often organized into groups of related gene products or "constellations" (Figure I-1). The complete sequence for bacteriophage T4 has yet to be determined. At the current time more than 90% of the genome has been sequenced and the remaining unread areas often represent the connections between gene constellations.

Bacteriophage T4 DNA is quite low in the percentage of GC basepairs, with only 36% of the basepairs being GC. As will be discussed later in more detail the T4 DNA contains not C, but hydroxymethylated deoxycytidine that is glycosylated.

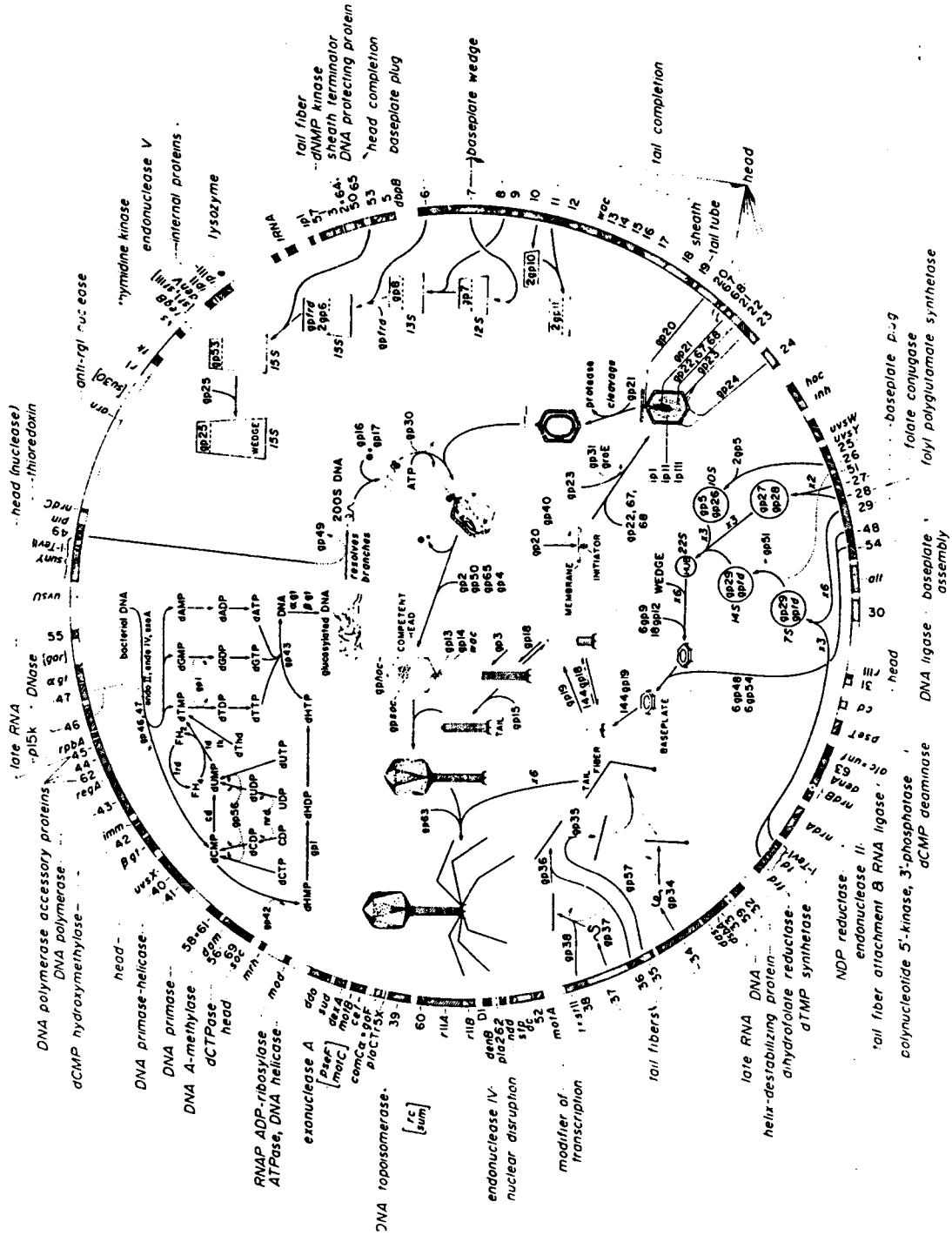


Figure I-1. Genomic Map of Bacteriophage T4 from B. Guttman and E. Kutter, 1991.

The large size of the bacteriophage T4 genome relative to its host *E. coli* is quite remarkable. By comparing either the complexity of gene regulation or the relative amount of DNA, bacteriophage T4 has a size and complexity that is one of the closest to its host's of any virus.

Bacteriophage T4 Infection Cycle

After the initial injection of T4 DNA into the *E. coli* cell, the host RNA polymerase transcribes a set of phage genes encoding nucleases, which cleave the host DNA for later salvage of deoxynucleoside triphosphates, dNTPs (Kutter and Wiberg, 1968). The host DNA is thought to receive a large number of single strand breaks, which become gaps and finally develop into double strand cleavages (Warner and Snustad, 1983). While the viral nucleases are being made the synthesis of all host proteins and mRNA ceases (Cohen, 1968). The shutdown of the host synthesis is dependent upon an initial ADP-ribosylation of the host's RNA polymerase α subunits (Goff and Weber, 1970) and a number of T4 gene products (Goff and Selzer, 1980).

Additional phage gene products are made within the first 12 minutes after infection and are classified as either immediate early or delayed early genes (Brody et al., 1983). One group of immediate early genes is transcribed only for the first 4 minutes, while the rest are made throughout the 12 minutes of early gene expression (Figure I-2). The delayed early genes commence transcription 2 minutes after infection and are dependent upon T4 proteins for their synthesis. These genes allow *de novo* synthesis of deoxynucleotides and T4 DNA replication.

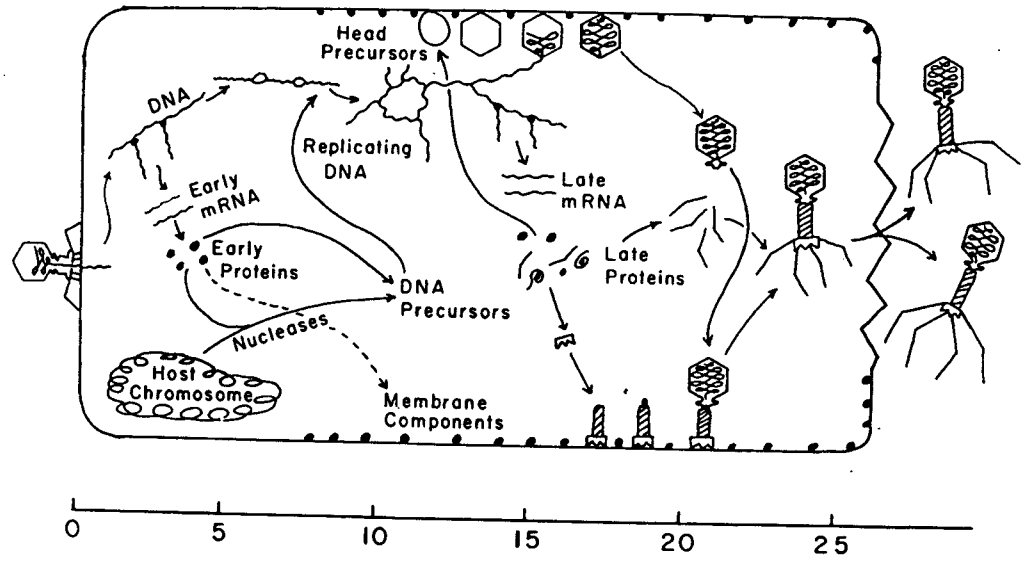


Figure I-2. Bacteriophage T4 Life Cycle from Mathews, 1977

The replication of T4 DNA usually begins 5 minutes after infection at 37°C as does additional ADP-ribosylation of the host RNA polymerase (Rabussay, 1982). T4 late transcription is dependent upon concurrent DNA replication (Lembach et al., 1969). The signal for expression of the late genes in bacteriophage T4 is also dependent upon the presence of hydroxymethylated dCMP sequences and the interaction of at least four virally encoded subunits that interact with the host RNA polymerase (Wu and Geiduschek, 1975). The late gene products code for structural and packaging proteins that create the well known icosahedral shaped phage head and contractile tail.

T4 DNA Replication, Recognition of Self Versus Host

The rate of T4 DNA synthesis is 10- fold higher than that in the uninfected *E. coli* (Mathews, 1972). It takes the host, *E. coli*, 40 minutes to replicate its 4000-kbp genome using 2 replication forks at a rate of 850 nucleotides/second. However, *E. coli* is able to divide every 20 minutes under optimal conditions by starting a second round of DNA replication while the parental genomes are only only half replicated. Under these conditions 6 replication forks can be active at any one time in a given *E. coli* cell. An average T4 infection contains 60 replication forks per host cell, and up to 200 phage particles can be synthesized from one infected *E. coli* within 25 minutes (Mathews, 1993).

The large size of T4 relative to its host means that only 10% of the required deoxynucleotides can be salvaged from the host DNA, while the rest must come from *de novo* synthesis. The *E. coli* chromosome, in other words, is only 20 times larger than each individual phage genome. The T4 replicative chain growth of 700 to 800 nucleotides per second demonstrates that the T4

DNA polymerase is operating under what appear to be ideal conditions and is not limited by lack of dNTPs (Mathews, 1988). This high rate of synthesis does not fit with the observed concentrations of deoxyribonucleoside triphosphates seen in the infected *E. coli*. The average concentration over the entire cell is only about 100 μM, while the replication forks are not saturated *in vitro* until a concentration of 250 μM for each of the four dNTPs (Mathews and Sinha, 1982). It has been proposed and will be discussed in more detail latter in this chapter that the actual localized concentration at the replication forks is higher than the overall cell concentration.

As stated above, the synthesis of the viral nucleases which break down the *E. coli* chromosome can augment the supply of deoxynucleotides for T4 DNA synthesis. However, the T4 DNA replication machinery is then required to operate in a very inhospitable environment for the synthesis of its own DNA. To be able to have intact phage DNA under these conditions the T4 nucleases have to be able to distinguish between information that is coding for self versus information that was part of the host's chromosome. This selectivity is made possible by the unique features of T4 deoxyribonucleotide biosynthesis. Specifically, the synthesis of 5-hydroxymethyldeoxycytidylate (hmdCMP) by the enzyme dCMP hydroxymethylase allows salvage and phage DNA replication to coexist. T4 DNA containing hydroxymethylated cytosine is protected from its own cleavage system.

An additional modification of the phage DNA, the glucosylation of the hydroxymethyl groups of the modified cytosine, occurs after dNTPs have been incorporated into DNA. The glucosylation confers resistance to host restriction systems (Hewlett and Mathews, 1975). In T4 DNA 70% of the hydroxymethyl cytosine residues are α-glucosylated, and 30% are modified in the β

configuration (Mathews and Allen, 1983). The two different glycosylation reactions are catalyzed by two phage-encoded glucosyltransferases, encoded by genes α-gt and β-gt. Phages are viable as long as at least one functional gene is present. Computer modeling of glucosylated bacteriophage T4 DNA suggests that the glucose residues can occupy the major groove and thereby block the recognition sites of the *E. coli* restriction enzyme systems (Figure I-3).

A critical step for T4 DNA replication is limiting the insertion of unmodified cytosine into DNA. Even a very low insertion frequency of unmodified cytosine could make a 166-kbp T4 genome, which is surrounded in a milieu of viral nucleases, damaged and nonviable for further infections. This makes it somewhat surprising that the T4 DNA polymerase does not distinguish between dCTP and hmdCTP. It can be assumed that under these *in vivo* conditions the T4 DNA polymerase does not come in contact with unmodified dCTP. In fact the unmodified dCTP pool is below measurable levels by five minutes post infection (Mathews, 1972).

What structures or enzymes allow this to take place inside the infected *E. coli*? An erroneous initial assumption would be that the dCMP hydroxymethylase is a very efficient enzyme where it rapidly modifies the dCMP even under conditions where the dCMP is at very low concentrations. This turns out to not be the case *in vitro*. The purified dCMP hydroxymethylase has a high Km of 600 μM (Pizer and Cohen, 1962) and a slow turnover of 276 moles hmdCMP/ mole dCMP hydroxymethylase/minute (North and Mathews, 1977) .

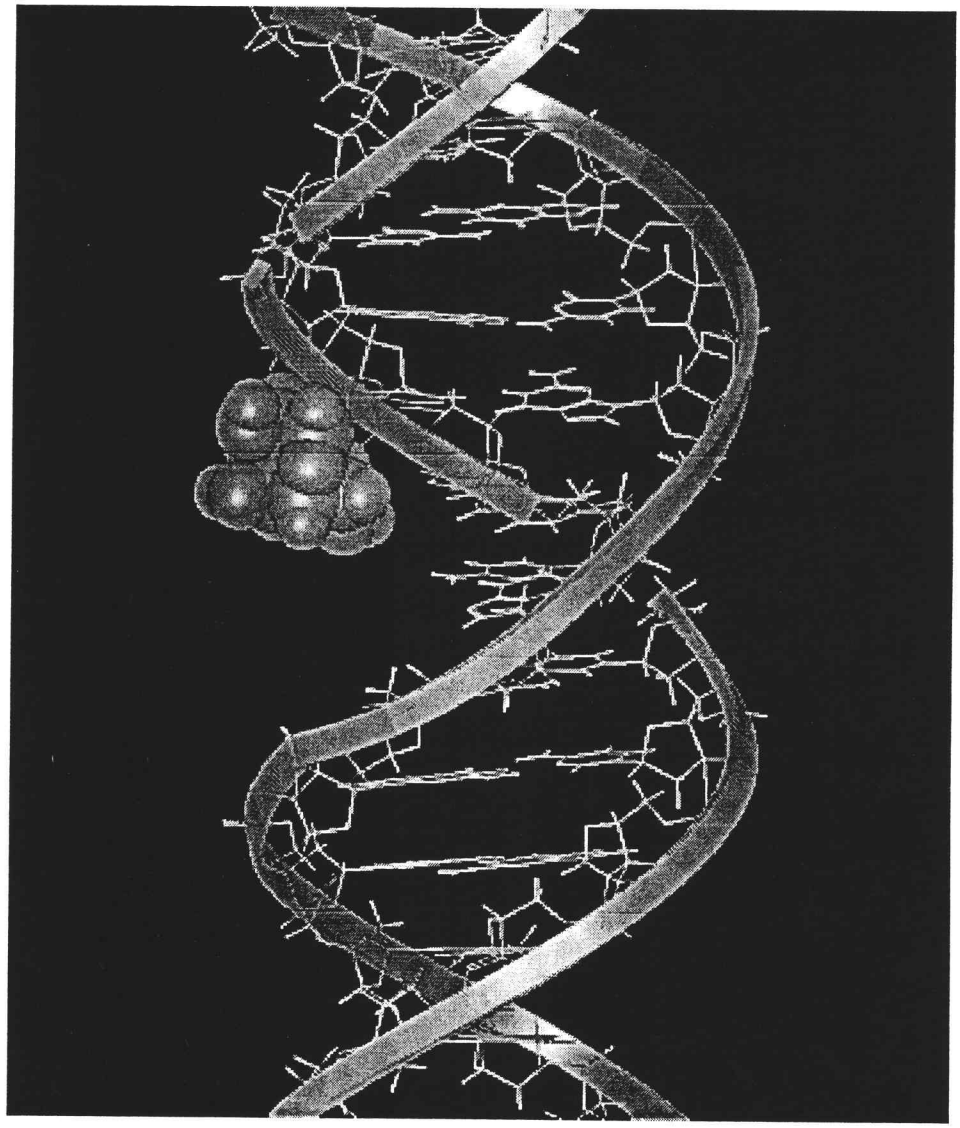


Figure I-3. The Structure of Bacteriophage T4 Glucosylated DNA. A model of DNA containing a space filled α-glucosylated hydroxymethyl cytosine residue. The glucose molecule was allowed to rotate to the lowest minimum energy for the model. (model was created by Dr. Shing Ho)

How is unmodified dCTP prevented from being incorporated into T4 DNA when the final enzyme in the biosynthetic pathway, DNA polymerase, does not distinguish between hmdCTP and dCTP, and the modifying enzyme, dCMP hydroxymethylase, functions slowly and requires high substrate concentrations? The answer in part lies with recognizing the mistake of examining individual enzymes separate from the context of the overall pathway. Assumptions of what specific catalytic properties an individual enzyme should have to make an overall pathway the most efficient cannot be made without understanding the properties and interactions of all the enzymes involved in the pathway.

For bacteriophage T4 the overall deoxypyrimidine pathway appears to have been modified by evolution to prevent the incorporation of dCTP by the T4 polymerase. One important modification of the T even bacteriophage deoxynucleotide biosynthesis pathway is the expansion of the viral dUTPase substrate specificity to include dCTP and dCDP. The bacteriophage dCTPase/dUTPase, gene product 56, has a turnover of approximately 8,000 moles product/mole enzyme/minute for dCTP and 1,600 for dCDP (Zimmerman and Kornberg, 1961) with K_m values of 4.3 μM and 2.4 μM, respectively. These values for dCTP are close to the dUTP turnover and Km values for the enzyme.

While it appears that natural selection has made the T4 dUTPase less specific in its choice of substrate, another enzyme, deoxynucleoside monophosphate kinase, has apparently undergone a much more complicated substrate specificity evolution.

The T4 deoxyribonucleotide biosynthesis pathway contains a

deoxynucleoside monophosphate kinase, gene product 1, which can transfer a phosphate from ATP to either dGMP, dTMP, or hmdCMP (Brush and Bessman, 1990). The dNMP kinase is a small homodimer (Sakiyama and Buchanan, 1973) with a subunit molecular weight of 27,300. All three deoxynucleoside monophosphates have been shown to be competitive inhibitors of each other (Bello and Bessman, 1963). The competition for catalysis is a strong indication that there is one active site that is shared by the three deoxynucleoside monophosphates. However, the possibility that there are three active sites which overlap or that a substrate-induced conformational change takes place cannot be conclusively ruled out.

What is surprising for an enzyme that recognizes three structurally dissimilar nucleotides is the exclusion of unmodified dCMP as a suitable substrate. The T4 dNMP kinase has not only evolved the ability to accommodate hmdCMP in the active site but can recognize the absence of the hydroxymethyl group and exclude deoxycytidine that is unmodified.

The mechanism that requires dCMP to contain a hydroxymethyl group at the 5 position to be a substrate for dNMP kinase is still not understood and probably will stay an enigma until the structure of dNMP kinase with hmdCMP bound in its active site is solved.

T4 Deoxyribonucleotide Biosynthesis:

Enzymes Involved in T4 Deoxyribonucleotide Biosynthesis

In general ribonucleotides serve a multitude of functions within organisms. They serve as the precursor for RNA, as carriers of energy metabolism, as components of coenzymes, and as metabolic regulators. On the other hand, deoxyribonucleotides are used almost exclusively for the synthesis of DNA, and their concentrations found in living systems represent a small fraction the

size of the ribonucleotide pools. While the size of the deoxynucleotide pools may be in the minority their proper regulation is vital for all living organisms that contain DNA (Reichard,1988)

The enzymes involved in T4 deoxyribonucleotide biosynthesis have been recognized for the past 30 years, with the exception of the T4 gene *sunY* product, which is currently being investigated by Britt-Marie Sjöberg as a possible anaerobic ribonucleotide reductase. Table I-1 lists the enzymes involved in T4 deoxynucleotide biosynthesis with their reported apparent K_m and turnover values. Several of the reported values were determined for enzymes encoded in related T-even phages other than bacteriophage T4. Various kinetic values have been published for the T4 thymidylate synthase in the past. The K_m and turnover values for T4 thymidylate synthase shown in Table I-1 were values derived from experiments described in Chapter 4 using the cloned enzyme. Figure I-4 diagrams the deoxynucleotide biosynthesis pathway for bacteriophage T4.

Ribonucleotide Reductase

The committed step in the specialization and removal of deoxy-ribonucleotides as a substrate for other pathways involves the replacement of the 2' hydroxyl moiety of the ribose sugar by a hydride ion. This first unique step in the flow of precursors to DNA is catalyzed by the enzyme ribonucleotide reductase (Reichard and Ehrenberg, 1983). In most organisms, including bacteriophage T4, ribonucleotide reductase carries out the reduction at the ribonucleoside diphosphate level. However, in a minority of organisms, *Euglena*, cyanobacteria, and certain bacteria, the reduction is carried out at the ribonucleoside triphosphate level (Mathews and van Holde, 1990). The *E.coli* anaerobic ribonucleotide reductase also carries out the reduction at the triphosphate level. All ribonucleotide reductases that have been studied likely use a free radical mechanism to extract the 2' hydroxyl moiety (Stubbe, 1990).

Table I-1. Survey of T4 Enzymes Involved in Deoxyribonucleotide Biosynthesis

Enzyme	Gene	Substrate		Product	Km[1]	Turnover[2]	Allosteric Regulation	
							stimulation	inhibition
Ribonucleotide reductase[3]	*nrdA/nrdB*	CDP	-	dCDP	43 μM	245	dATP/ATP/hm-dCTP	-
		UDP	-	dUDP	100 μM		dATP/ATP/hm-dCTP	-
		GDP	-	dGDP	45 μM		dTTP	dATP
		ADP	-	dADP	48 μM		dGTP	-
	glutaredoxin-$(SH)_2$		-	glutaredoxin-$(S)_2$				
dCTPase/dUTPase[4]	56	dCTP	-	dCMP	4.2 μM	8,357		
		dCDP	-	dCMP	2.2 μM	1,671		
		dUTP	-	dUMP		11,143		
		dUDP	-	dUMP		2,229		
dCMP deaminase[5]	*cd*	dCMP	-	dUMP	100 μM	31,605	hm-dCTP	dTTP
Dihydrofolate reductase[6]	*frd*	FH_2	-	FH_4	2.3 μM	4,350		
		NADPH	-	$NADP^+$	18 μM			
Thymidylate synthase[7]	*td*	dUMP	-	dTMP	7 μM	607		
		CH_2FH_4	-	CH_2FH_2	43 μM	912		
dCMP hydroxymethylase[8]	42	dCMP	-	hm-dCMP	600 μM	276		
		CH_2FH_4	-	CH_2FH_4	100 μM			
dNMP kinase[9]	1	hm-dCMP	-	hm-dCDP	56 μM	250		
		dTMP	-	dTDP	300 μM	500		
		dGMP	-	dGDP	85 μM	500		
		ATP	-	ADP	800 μM			

1) Apparent K_m
2) moles product/moles enzyme/minute
3) K_m, Berglund, (1972A); turnover and regulation, Berglund (1972B)
4) Zimmerman and Kornberg, (1960)
5) K_m, turnover, Scocca, et al., (1969); regulation data from T2 dCMP deaminase Maley and Maley (1982)
6) Erickson and Mathews, (1972)
7) Determination from experiment done by this author discussed in Chapter 3.
8) K_m For T6 enzyme, Pizer and Cohen, (1962); turnover T4 enzyme, North and Mathews, (1977)
9) T2 enzyme, Bello and Bessman, (1963)

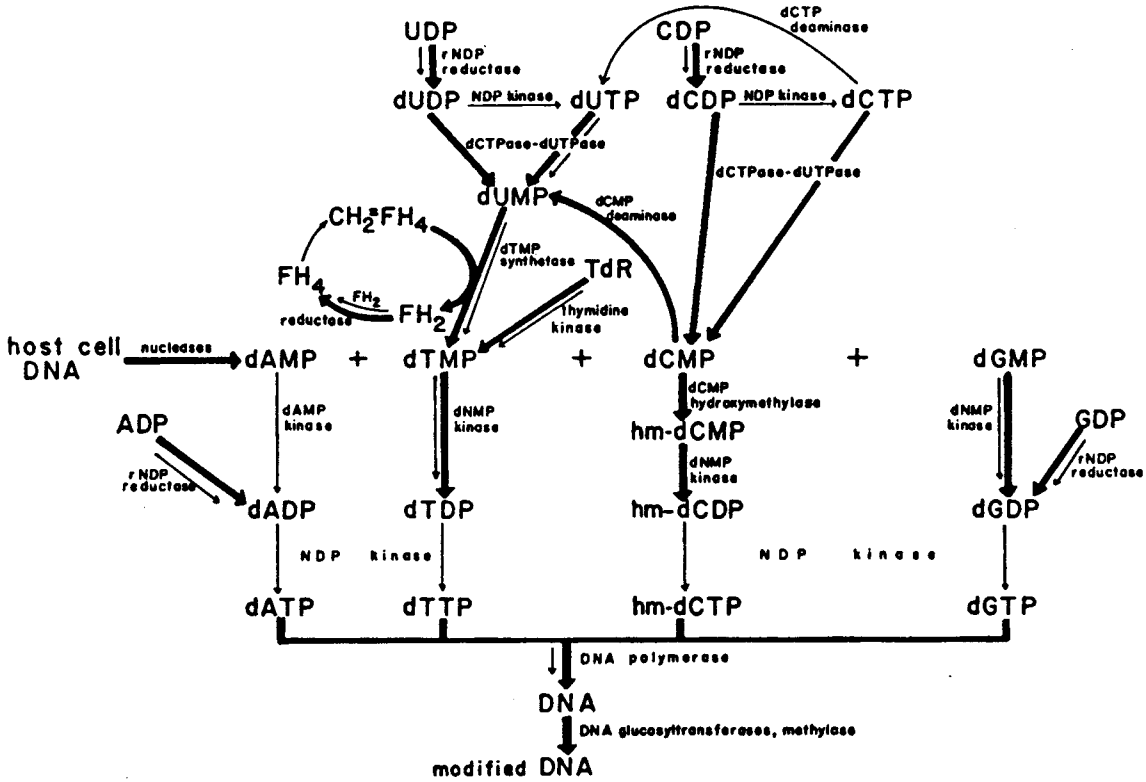


Figure I-4. Deoxyribonucleotide Biosynthesis Pathway from Mathews and Allen, 1983. Bold arrows correspond to enzymes encoded by the bacteriophage T4.

The bacteriophage T4 and the *E. coli* ribonucleotide reductases are $\alpha_2\beta_2$ tetramers. This structure is analogous to the ribonucleotide reductase found in mammalian cells. The bacteriophage T4 and *E. coli* ribonucleotide reductase also resemble the higher eukaryotic form in containing two unusual characteristics present in the small subunits of all $\alpha_2\beta_2$ reductases, a tyrosine radical and an oxygen atom bridging two ferrous ions. The function of the binuclear iron center is believed to involve stabilization of the tyrosine radical. The source of the electrons for the reduction of the ribose originates from NADPH, with the reducing power usually transferred by redox-active proteins to thiol groups on the large subunits (Figure I-5).

The bacteriophage T4 ribonucleotide reductase regulation has certain similarities with the host enzyme's regulation. Both enzymes are allosterically regulated by deoxynucleoside triphosphates, which determine the substrate specificity by changing both the K_m and the V_{max} (Berglund, 1972). Low levels of ATP and dATP stimulate the reduction of pyrimidine ribonucleotides. Increased levels of dTTP stimulate the reduction of dGTP, while dGTP itself stimulates the synthesis of dATP.

A major difference between the bacteriophage and host reductases is that the purified T4 reductase has no feedback regulation, in terms of pyrimidine reduction. The *E. coli* enzyme in the presence of high levels of dATP, $> 10^{-5}$ M, is inhibited in reduction of all four substrates. The bacteriophage T4 reductase is not feedback regulated by high levels of dATP, and in fact dATP stimulates pyrimidine reduction (Berglund, 1972). Recent work in our lab suggests that the T4 ribonucleotide reductase is feedback sensitive in vivo and that protein-protein interactions may play a role in the regulation (Ji et al., 1991).

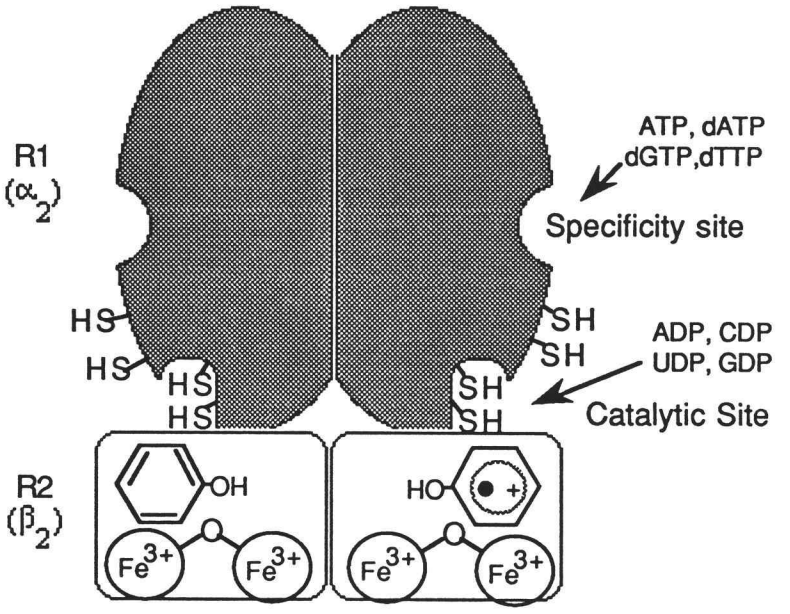


Figure I-5. Bacteriophage T4 Ribonucleotide Reductase. The large subunits, R1 contain both a specifity site and a catalytic site. The small subunit contains at least one tyrosine radical which is required for catalysis. The tyrosine radical is buried within the small subunit and not on the surface.

dCMP Hydroxymethylase and Thymidylate Synthase

The second unique feature of deoxynucleotides is the modification of the uracil base to thymine. In bacteriophage T4 an additional modification takes place where cytosine is also hydroxymethylated. These modifications are carried out by the related enzymes thymidylate synthase and the enzyme unique to T-even phages, dCMP hydroxymethylase The majority of my work described in this thesis involves the investigation and understanding of a protein-protein interaction of the T4 thymidylate synthase with the dCMP hydroxymethylase.

Both enzymes catalyse similar reactions, with thymidylate synthase catalyzing the reductive methylation of deoxyuridine monophosphate (dUMP) by 5,10-methylenetetrahydrofolate (CH_2-H_4folate) to form thymidine monophosphate (dTMP) and dihydrofolate (H_2folate). The dCMP hydroxymethylase, gene product 42, catalyses the non-reductive hydroxymethylation of deoxycytidylate (dCMP) by CH_2-H_4folate to hydroxymethyldeoxyctidylate (hmdCMP) and H_4folate (Figure I-6). The dCMP hydroxymethylase was in fact the first DNA precursor biosynthesis enzyme identified to be induced by the bacteriophage T4 (Flaks and Cohen, 1957).

The mechanism for catalysis for both dCMP hydroxymethylase and thymidylate synthase has been proposed to involve the formation of a covalent bond between a nucleophilic cysteine thiol side chain and the C-6 of the dNMP (Figure I-7). A second covalent bond is postulated to form between the C-5 position of the dNMP and the 5,10-methylenetetrahydrofolate (Moore et al., 1986).

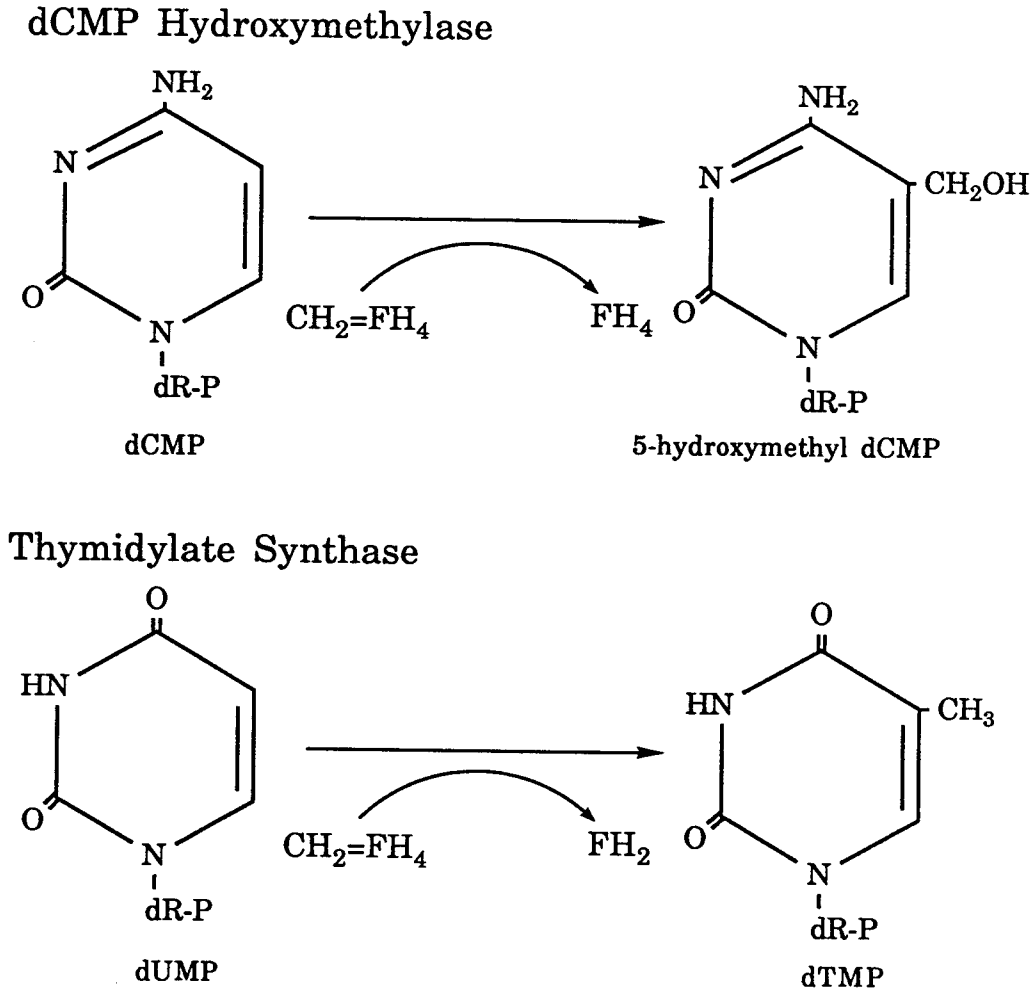


Figure I-6. Thymidylate Synthase and dCMP Hydroxymethylase Reactions

Figure I-7. Proposed Mechanism for Thymidylate Synthase

Formation of a covalent bond between a catalytic thiol and dUMP generates a resonance-stabilized anion. The C-5 atom on dUMP becomes a nucleophile and carries out a condensation reaction with the activated form of the cofactor, 5-CH_2=H_4. A basic group on the enzyme had been postulated to abstract a proton from C-5. However, no such basic group has been identified in X-ray crystallography. Elimination and hydride transfer from C-6 of the tetrahydrofolate cofactor produces dTMP and the oxidized cofactor, dihydrofolate (Finer-Moore et al, 1990).

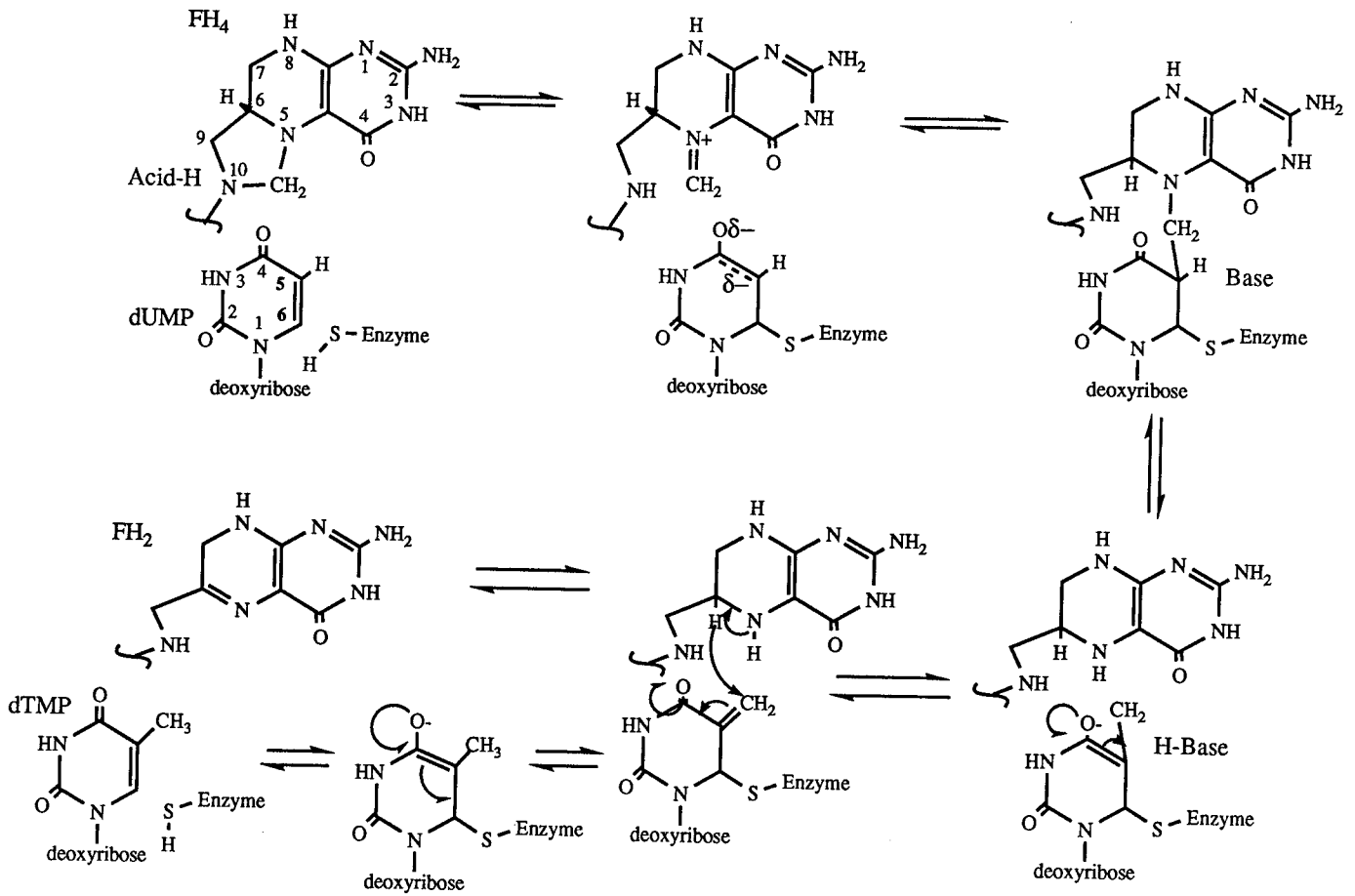


Figure I-7.

Because thymidylate synthase represents the only *de novo* pathway for the synthesis of dTMP the enzyme mechanism has been studied in great detail in hopes of developing potent anticancer drugs. Thymidylate synthase is an obligate dimer, with components of each monomer contributing to each of the two active sites (Montfort et al., 1990). T4 dCMP hydroxymethylase is also is a dimer in its native conformation. For thymidylate synthase, the inhibitor FdUMP has been used to understand the mechanism of binding of substrates for the enzyme. A recent and unexpected observation is the ability of FdUMP, 5-fluoro-2' deoxyuridine-5'-monophosphate, to be an inhibitor of dCMP hydroxymethylase, while FdCMP the (the dCMP analog of FdUMP) is not (Graves and Hardy, 1992).

For thymidylate synthase early studies demonstrated that when monoglutamylated CH_2-H_4folate was used, the ligands bound in an ordered fashion in which dUMP binds first followed by CH_2-H_4folate and that the H_2folate leaves before dTMP in a bi-bi reaction sequence (Lorenson and Maley, 1967). However, it had also been observed that a random component to the kinetics of ligand binding could take place when a polyglutamylated CH_2-H_4folate was used (Lu et al., 1984). The binding sites for substrate and cofactor were demonstrated to be independent, as long as the CH_2-H_4folate is polyglutamylated, by the ability to modify the enzyme to prevent binding of either dUMP or polyglutamylated CH_2-H_4folate analogues without interfering with binding of the other ligand (Galivan and Baugh, 1976; Danenberg and Danenberg, 1978).

Some evidence suggests that in thymidylate synthase the two active sites in thymidylate synthase react sequentially (Santi and Danenberg, 1984; Danenberg and Danenberg, 1979). These early findings implied an

asymmetry during the binding of substrates with enzymes. Comparison between the unliganded and substrate bound crystal structures of *E. coli* thymidylate synthase revealed a large conformational change in the enzyme when substrate bound (Montfort et al., 1990). The binding site for CH_2-H_4 folate is formed in large part by the nucleotide, and the dUMP binding site is somewhat inaccessible once cofactor is bound.

The thymidylate synthases have been sequenced from a variety of organisms, free living and parasitic, and have been shown to have a family of the most highly conserved sequences for any known group of enzymes (Perry et al., 1990). The functions for these highly conserved regions in the thymidylate synthases, which appear not to be directly involved in catalysis, have not been identified, but Montfort et al. (1990) postulate that they may be involved in protein-protein interactions with other enzymes. The T4 dCMP hydroxymethylase shares homology with the thymidylate synthases in its active site region (Graves and Hardy, 1992).

An unusual feature is seen with the thymidylate synthases from parasitic organisms where the thymidylate synthases are bifunctional enzymes linked to dihydrofolate reductase in the same gene. This bifunctional nature has been identified in seventeen different parasites and its function may reside in protecting the labile cofactor. Why this bifunctionality is exclusive, and so far universal, to parasitic organisms is still unknown.

Protein-Protein Interactions, Enzyme Complexes and Metabolons: Proteins Reside in a Crowded Environment

The basic goal of enzymology is to purify an enzyme to homogeneity and study it in an isolated environment where the factors affecting its ability to catalyze a reaction are identified and understood. Enzymes involved in intermediary metabolism have usually been found to be globular proteins which can be easily solubilized, isolated and characterized away from other enzymes of the cell. Studying an enzyme in vitro has been and will continue to be an immensely powerful tool in determining the specific reaction being catalyzed, determination of the reaction mechanism, and identification of the metabolic role of the enzyme. Yet purification and isolation will always carry the risk that important interactions that occur within the cell are missed by studying the isolated enzymes.

How different is the environment between a purified enzyme and an enzyme in vivo? The activity of an enzyme is not only dependent on its own kinetic parameters but also by the concentration of protein that surrounds it. *In vivo* the protein concentration is very high, where often the amount of water is nearly equal to the minimum amount needed to solubilize the protein present. 200 mg/ml of protein is a rough estimate of what an enzyme is surrounded by in a typical living cell. It has been demonstrated that the volume occupied by proteins affects the activity of other proteins without directly interacting with them (Fulton, 1982). Proteins are more likely to self-associate and hetro-associations are more likely to take place in an environment crowded with other proteins. An example has been shown with myoglobin being driven to form dimers when in the presence of other proteins. However, high protein concentrations do not always stimulate catalysis. For example,

glyceraldehyde-3-phosphate dehydrogenase is less active in the presence of other proteins which tend to drive the formation of low activity tetramers (Minton and Wilf, 1981)

At these high protein concentrations the ability to understand and model metabolic flux rates of given pathways becomes extremely difficult. The high concentration may allow direct exchange of the intermediate between enzymes of a pathway without having to allow diffusion of the substrate into the bulk water. The possible advantages of direct transfer could include the protection of labile metabolites, the maintenance of metabolite concentration gradients, and faster responses to environmental changes. The prevention of accumulation of intermediates may be of particular significance in pathways where the rate-limiting reaction is not the first committed step in the sequence (Friedrich, 1984).

Different Classes of Enzymatic Associations

There are many examples where catalytic sites of a pathway are physically associated. Having two or multiple catalytic sites of a pathway physically juxtaposed occurs in several different patterns of associations. These patterns of enzymatic organization can be divided into three distinct groups.

The first group, of which DNA polymerase I is an appropriate example, is multifunctional proteins where a single polypeptide has multiple functions. Catalytic sites are always maintained within close proximity by having the catalytic domains reside on a single polypeptide chain.

The second group consists of the tightly bound enzyme complexes, which can be defined as complexes that have stoichiometric amounts of noncovalently associated enzymes. Examples of these complexes include the

well studied pyruvate dehydrogenase complex , the glycine cleavage system, and tryptophan synthetase. This organization requires assembly of the complexes and the ability to associate in a functional manner.

The third, and most controversial group, consists of enzymes that associate using weak noncovalent interactions. This third group of loosely bound enzyme complexes is often difficult to isolate intact. While the first two patterns can be distinguished by having a distinct physical difference, i.e., one organization is based on a single polypeptide with multiple catalytic domains and the other involves multiple polypeptides associations, the second and the third groups are arbitrarily differentiated by the ease of isolation in intact form. The differences between *in vitro* stabilities may not reflect the true intrinsic *in vivo* stability of organization.

What determines the strength of a protein-protein interaction or whether one is even favorable to form? In order for two proteins to associate, they first must have surfaces that are complementary to one another (Friedrich, 1984). Complementarity can be defined as the association of protein surfaces that share the same van der Waals envelope over a certain area.

The second requirement for specific association of two proteins is the proper positioning of the polar groups. For an interaction to occur any charged side chain that resides within the complementary surface area will be required to form a salt bridge with an opposite charged group on the adjacent protein.

The net contribution of the formation of a salt bridge on the free energy of binding, -4 to -13 kJ/mole, is small and would not seem to be a prerequisite for protein-protein interaction. The requirement for salt bridges arises due to the fact that without their formation a positive charge would have to transfer from the surface to a hydrophobic environment. This transfer would be very energetically unfavorable, +40 kJ/mole for each charge that is not canceled by

formation of a salt bridge (Friedrich, 1984). In the same vein the number of hydrogen bonds formed between the complementary surfaces should not be less than the number that the interacting surfaces could form by themselves with water.

The Deoxynucleoside Triphosphate Synthetase Complex

For the past twenty years a body of evidence has been collected by several research groups, including our own, which support the idea that the bacteriophage T4 employs a weakly associated multienzyme complex to carry out dNTP synthesis. This organization of deoxyribonucleotide biosynthesis enzymes is postulated to increase the local concentration of dNTPs at sites of DNA replication.

One of the first indications of the existence of the dNTP synthetase complex in bacteriophage T4 was the recognition that the thymidylate synthase and dCMP hydroxymethylase intracellular activity was delayed in the infected cell relative to synthesisi of the respective proteins (Tomich et al., 1974). The estimated reaction flux at early time points *in vivo* for both enzymes was found to be low even though the active enzymes could be isolated at these early time points. The delay was attributed to the time needed for assembly of the two enzymes into a multienzyme complex.

Other early evidence involved the study of permeabilized T4-infected bacteria. T4 containing defects in their ability to synthesize hm-dCTP were allowed to infect permeablized cells with exogenous hm-dCTP present. The surprising finding was that the addition of hm-dCTP would not bypass the defect (Wovcha et al., 1973, and North et al., 1976). Mutations in either the T4 phage genes encoding either dCMP hydroxymethylase or dNMP kinase could not be bypassed by the addition of exogenous hm-dCTP. This led to the

proposal that a link, physical or kinetic, existed between deoxyribonucleotide metabolism and DNA replication. The further study of permeabilized T4-infected E. coli demonstrated that the incorporation of either exogenous deoxyribonucleoside monophosphates or ribonucleoside diphosphates into T4 DNA was threefold higher than the incorporation of the deoxyribonucleoside triphosphate (Reddy and Mathews, 1978).

More direct evidence for the complex was obtained by looking at the kinetic linkage of aggregated dNTP-biosynthesis enzymes. Experiments demonstrated that it was possible to show kinetic coupling by the criterion of reduced accumulation of intermediates, for the three enzymes thymidylate synthase, dNMP kinase, and NDP kinase (Reddy and Mathews, 1978). An interesting observation made by Mathews was that the coupling requires functional dCMP hydroxymethylase, even though the dCMP hydroxymethylase is not part of the reaction sequence.

The enzyme dCMP hydroxymethylase was further shown to be required for the functional complex by mutation analysis. By examining the kinetic coupling of bacteriophages which contained C-terminal truncations in their dCMP hydroxymethylase enzymes it was shown that dCMP hydroxymethylase mutants which were less than half the size of the full length protein were unable to kinetically couple the three step pathway dCTP $\rightarrow$ dCMP $\rightarrow$ dUMP $\rightarrow$ dTMP. It was also shown in the same studies that a nearly full length deletion, while enzymatically inactive, could still function in the assembly of the complex by the criterion of kinetic coupling (Thylén and Mathews, 1989).

A number of attempt have been made at purification of the dNTP synthetase complex. Early work indicated that the interactions between the enzymes were

weak and easily dissociated. The first attempts at purification of the dNTP complex were attempted by Chiu et al. (1982) and Allen et al. (1983), and showed only low level enrichment of the dNTP enzymes. These first attempts did not confirm the physical linkage of the enzymes.

Through the knowledge gained from the earlier studies, Moen et al. (1988) were able to isolate a highly enriched preparation of the multienzyme complex. The complex was found to dissociate when in the presence of high concentrations of NaCl or $MgCl_2$. The overall recovery of the coupled activity was low, either reflecting loss during the purification or a reflection of the *in vivo* proportions of complexed verus free enzymes. Experiments with T4 mutants showed that the deoxycytidylate deaminase, both subunits of ribonucleotide reductase, and the gene product encoded by *regA* were required to retain the physical integrity of the dNTP complex. Finally no DNA polymerase or other replication proteins were isolated in this preparation. The essential role of the RegA protein was an unexpected result of the study.

Translational Regulation of Deoxyribonucleotide Metabolism Enzymes by RegA:

While the bacteriophage T4 dCMP hydroxymethylase and thymidylate synthase enzymes do share homology in structure and function, the regulation of their synthesis is quite different. The dCMP hydroxymethylase messenger RNA, but not thymidylate synthase messenger RNA, belongs to a class of early mRNAs that is regulated by the T4 gene product *regA*.

Since the early 1960s it had been recognized that during a T-even bacteriophage infection the synthesis of many early proteins ceased at 12 minutes in a wild-type infection (Dirksen et al., 1960). However, it was

observed that the early mRNA species were still present later in the infection (Hall et al., 1964). The 12-minutes shutoff could be removed by blocking DNA synthesis, and in a DNA$^-$ mutation the early gene products were now synthesized until 20 minutes, when they were again shutoff. The 12-minute shutoff was termed S1, first shutoff, while the 20 minute shutoff was named S2.

A mutation in bacteriophage T4 was later identified that allowed synthesis of early gene products past the S2 shutoff (Wiberg et al., 1973). This mutation was within a new gene, *regA*, which was determined to be a translational regulator of a number of early T4 genes. The T4 *regA* gene was determined to reside within a cluster of essential genes that encode DNA replication enzymes. Defects in the *regA* gene, however, cause no adverse effects on phage DNA replication.

The *regA* protein inhibits the translation of the regulated mRNAs at the level of initiation, by being able to compete with ribosomes for binding to the translational initiation region of the target mRNAs (Winter et al., 1987). The *regA* protein is also subject to autogenous regulation, similar to two other bacteriophage T4 enzymes, gp32, a single stranded binding protein, and gp43, the DNA polymerase (Andrake et al., 1988).

The regulatory effect that RegA protein has on other T4 gene products appears to be quite complex (Table I-2). While a number of early enzymes are believed to be down regulated by the *regA* gene product, a number of early enzymes appear to be unaffected by the presence of the RegA protein and a minority of enzymes may be stimulated in their synthesis by the presence of the transcriptional regulator. This stimulation of certain early enzymes is believed to be caused in part by the decrease in competition for ribosome inititiation. It has also been suggested that the RegA protein may increase gene product yields by direct interaction with the messenger RNA (Miller et al., 1987).

Table I-2. Effect of the Transcriptional Regulator RegA on Production of T4 Early Proteins[1]

Protein	T4 gene	Effect[2]
Deoxyribonucleotide Biosynthetic Enzymes		
dCMP hydroxymethylase	42	-
Deoxyribonucleotide kinase	1	-
dCTPase/dUTPase	56	-
dCMP deaminase	*cd*	-
Thioredoxin	*nrdC*	-
Ribonucleotide reductase, large subunit	*nrdA*	0
Ribonucleotide reductase, small subunit	*nrdB*	0
Thymidylate synthase	*td*	+
T4 DNA Replication Proteins		
DNA topoisomerase subunit	39	-
DNA topoisomerase subunit	52	-
DNA polymerase accessory	44	-
DNA polymerase accessory	45	-
Single stranded binding protein	32	0
DNA polymerase	43	0 to +
DNA helicase	41	+
DNA ligase	30	+
Other Early Proteins		
RegA (autogenous control)	*regA*	-
α-glucosyltransferase	*α-gt*	-
β-glucosyltransferase	*β-gt*	-
DNA-adenine methylase	dam	-
rIIA	rIIA	-
rIIB	rIIB	-
Lysozyme	*e*	-

[1]The effect that RegA protein has on the early enzymes was determined by comparing the expression of the early enzymes in bacteriophage T4 containing *regA*$^-$ mutations versus wild-type infection. An increase in production of an enzyme when a *regA* mutation is present is assumed to represent repression of the enzyme in the wild-type infection.

[2] -, underproduced; +overproduced; 0, no effect. (Wiberg and Karam, 1983)

It has been estimated that between 10-15% of all the T4-induced early proteins are normally under translational regulation by the *regA* protein (Miller et al., 1987). While no additional T4 products are required for RegA-mediated translational repression there is evidence that the RegA protein may have multiple functions and may participate in protein-protein interactions during the T4 infection (Miller et al., 1987).

A number of seemingly unrelated properties of *regA*$^-$ mutants have been observed and may represent undescribed functions of the *regA* gene product. One of the first distinctions recognized between the wild type T4 infection and the *regA*$^-$ infection was that at increased temperatures, 43-45°C, *regA*$^-$ mutants had vastly decreased numbers of viable phage. Surprisingly the DNA synthetic rates of the *regA*$^-$ mutants were reduced only by 20% of the normal wild type rate (Wiberg et al., 1973). Other observed characteristics of *regA*$^-$ mutants included increased sensitivity to hydroxyurea, decreased proteolytic cleavage of head proteins and partial inhibition of host DNA breakdown. Work in our own laboratory using *regA*$^-$ mutants demonstrated that the *regA* gene product was necessary for assembly of kinetically coupled dNTP synthetase complex (Moen et al., 1988).

The biological role of the *regA* gene product is still unclear. Past proposals for the biological role include the RegA protein binding to sites on RNA primers for DNA replication and assisting in the formation of the DNA replisome (Campbell and Gold, 1982). This proposal suggested that the primary binding site for the RegA protein is to RNA primers on DNA. At early stages in the bacteriophage infection the RegA protein would be sequestered, allowing the deoxyribonucleotide enzymes to be expressed in sufficient quantities until DNA synthesis was under way.

It has also been suggested by Wiberg and Karam (1983) that the RegA protein may be a component of a multienzyme complex that couples phage DNA replication to late transcription and DNA packaging. A final proposal suggests that the role of the *regA* gene product is to fine-tune the levels of enzymes for optimum efficiency (Karam et al., 1982).

In other organized assemblies translational regulation is used to help coordinate the assembly of complexes. In *E. coli*, certain ribosomal proteins can act as feedback regulators. Excess unassembled ribosomal proteins can feedback repress the synthesis of new proteins (Nomura et al., 1977). This feedback replication while maintaining the proper ratios of the ribosomal proteins also minimizes the number of partially assembled ribosomes which could compete and interfere with translation in the bacteria.

With this in mind a question about the benefit of having the bacteriophage T4 enzymes of deoxyribonucleotide synthesis being arranged in a complex can be asked. It could be proposed that while intact and fully functional complexes would be beneficial to the replication of the phage, the benefit could be lost if incomplete and partial complexes were also present. By creating imbalanced pools partially assembled complexes would be likely detrimental and even mutational to the phage replication. Investigating the role of the RegA protein with the dNTP complex may lead to an understanding on how proper assembly of the complex is maintained and incomplete assemblies are kept to a low level.

Present Work

If the dNTP synthetase complex can kinetically channel intermediates it must have a structural arrangement of the component enzymes. A difficult problem with studying the interactions of a complex that is supposed to contain a large collection of different components is differentiating a direct interaction of two components of the complex versus the indirect interactions, or "piggybacking". The goal of my research was to develop techniques to identify enzymes that are directly interacting within the dNTP synthetase complex. Once an initial identification of a possible direct interaction had been seen, the goal was to then characterize the interaction and understand it.

Specifically the enzyme dCMP hydroxymethylase was chosen as a starting point for the identification of direct interactions. dCMP hydroxymethylase was well documented to be a critical component of the dNTP synthetase complex and had been previously cloned and antibodies had been generated.

The use of anti-idiotypic antibodies was an attempt to develop a new method to look at enzyme-enzyme associations. While anti-idiotypic antibodies were well documented in the field of receptor research little published data existed on the efficacy in studying enzyme-enzyme interactions.

A second project with a related theme was involved with the translational repressor RegA. As shown previously the presence of RegA is critical for dNTP synthetase complex formation. Is the role of RegA in the formation of the complex an indirect one, or is RegA actively participating in the formation of the dNTP synthetase complex. To distinguish between the two possible explanations I tried to identify RegA protein-protein interactions using two

techniques which in the past have been used successfully in our lab. The first technique is enzyme affinity chromatography involving RegA bound to an Affi-Gel column and the second method is the generation of anti-idiotypic antibodies to RegA protein.

CHAPTER 2

Interactions Between T4 Phage-Coded Deoxycytidylate Hydroxymethylase and Thymidylate Synthase as Revealed with an Anti-idiotypic Antibody

J. Patrick Young, and Christopher K. Mathews

Summary

Anti-idiotypic antibodies were used to mimic the binding surface of the T4 bacteriophage deoxycytidylate hydroxymethylase enzyme, providing an immunological probe for protein-protein interactions involving this enzyme. Polyclonal dCMP hydroxymethylase antibodies were affinity purified and used to generate anti-idiotypic antibodies. The anti-idiotypic serum immunoprecipitated two native viral proteins, deoxycytidylate hydroxymethylase (EC 2.1.2.8) and thymidylate synthase (EC 2.1.1.45), from a sonicated detergent-treated extract of T4-infected *E. coli*. The anti-anti-dCMP hydroxymethylase antibody was found to be specific in binding to the T4 dTMP synthase, with no detectable affinity for the host dTMP synthase. Previous work in our laboratory has demonstrated the viral dCMP hydroxymethylase and dTMP synthase to be associated in a deoxyribonucleotide synthetase enzyme complex. Our current approach using anti-idiotypic antibodies as probes for protein-protein interactions, and complementary studies involving dCMP hydroxymethylase enzyme affinity columns, indicate a direct association between bacteriophage T4 dCMP hydroxymethylase and dTMP synthase.

Introduction

Bacteriophage T4 is a large DNA virus, which scavenges deoxyribonucleotides released by breaking down the host's DNA, while also synthesizing its DNA precursors by de novo pathways using virus-encoded enzymes. For its own protection, the T4 modifies deoxycytidine monophosphate to 5-hydroxymethyl deoxycytidine monophosphate using an enzyme, deoxycytidylate hydroxymethylase. The hm-dCMP[1] incorporated into viral DNA confers resistance to the DNA restriction system of the virus itself (Warner and Snustad, 1983).

The T4 bacteriophage genome encodes a variety of enzymes of nucleotide biosynthesis, including dCMP hydroxymethylase and dTMP synthase. The two enzymes carry out similar reactions on pyrimidine deoxyribonucleoside monophosphates.

The T4 viral enzymes involved in deoxyribonucleotide synthesis have been implicated to reside in a multienzyme complex (Moen et al., 1988; Reddy et al.,1977). As shown in our laboratory, a dCMP hydroxymethylase protein affinity column specifically binds a variety of T4 proteins, including dTMP synthase (Wheeler et al., 1992). Each enzyme of the complex should have specific protein-protein interactions with its nearest neighbors in the complex. Figure II-1 shows the pathways of dNTP biosynthesis in T4 infection, with an indication of the subunit structure of each enzyme. This diagram may provide a framework for predicting interactions among enzymes catalyzing sequential reactions.

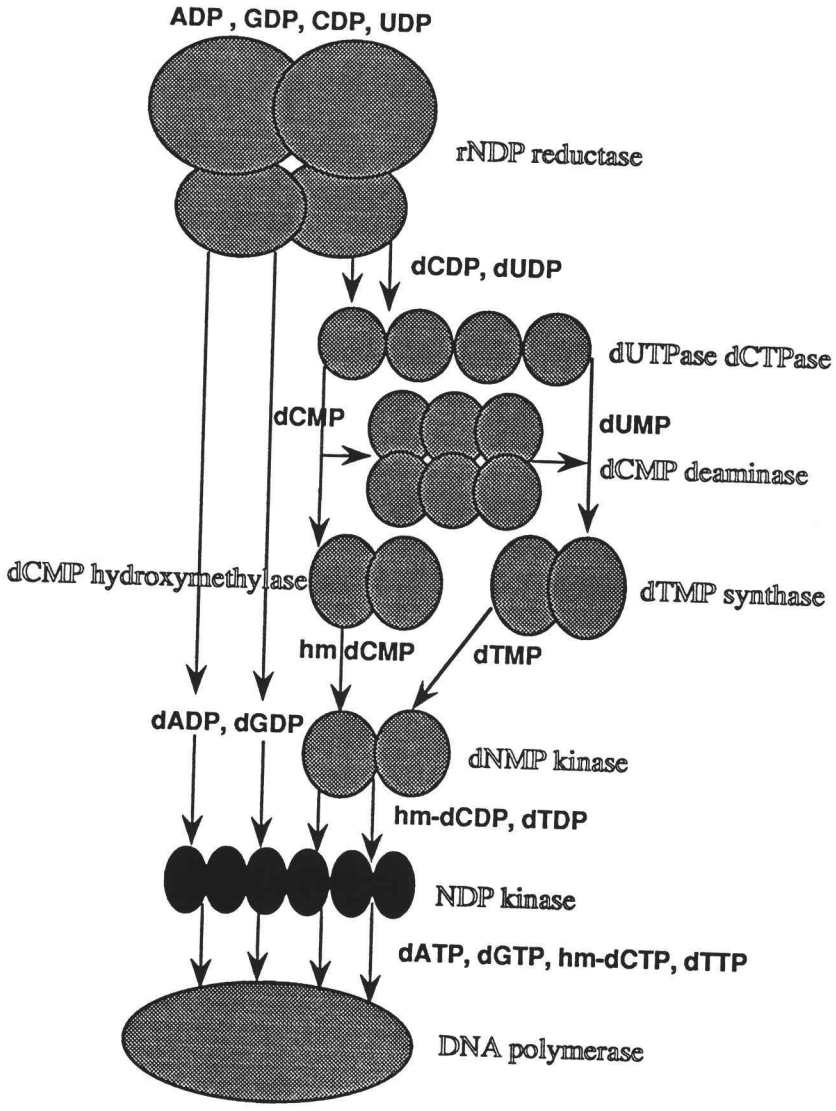


Figure. II-1. The Enzymology of Synthesis of Deoxyribonucleotides for T4 DNA. The pyrimidines, dUMP and dCMP, are modified by the addition of a methyl or hydroxymethyl group, respectively, at the 5-position. The number of subunits is represented for each individual enzyme. The virus-coded enzymes are shaded gray. Nucleoside diphosphate kinase, NDP kinase, is encoded by the host.

The work of Chiu and Greenberg demonstrated the ability of T4 to maintain a 2 to 1 ratio of dTMP to hm-dCMP synthesis flux in vivo over a wide variety of environmental conditions (Chiu et al., 1977). The synthesis ratio was determined to match closely the pyrimidine composition found in T4 DNA.

The current understanding of in vitro feedback control of individual enzymes does not fully explain this balance of modified deoxypyrimidine synthesis.

A problem with the investigation of specific protein-protein interactions in a complex is the difficulty in distinguishing between a direct interaction and secondary binding, or "piggybacking". The generation and use of anti-idiotypic antibodies is an attempt to develop a tool to identify direct enzyme-enzyme interactions. The generation of anti-idiotypic antibodies also may help to identify intramolecular subunit interactions, since most or all of the enzymes involved are oligomeric, as shown in (Figure II-1).

Anti-idiotypic antibodies have been shown to mimic the binding specificity of the original antigen (Figure II-2). Receptors for morphine, insulin, beta-adrenergic agonists, acetylcholine, and renal mineralocorticoid have all been successfully studied by using anti-idiotypic antibodies (Schulz and Gransch, 1984; Schulz and Gransch, 1985; Gransch et al., 1988; Lombes et al., 1990; for review, see Venter et al., 1984). Anti-idiotypic antibodies have also been used to study structural similarities among enzyme pterin binding sites (Jennings and Cotton, 1990) and an intracellular membrane protein that recognizes a mammalian endoplasmic reticulum retention signal (Vaux et al., 1990).

Somerville et al. have recently studied protein-protein associations involving the *E. coli* trp repressor by using anti-idiotypic antibodies as probes on immunoblots (Somerville et al., 1990). The technique seemed promising as a tool for exploring T4 bacteriophage protein-protein interactions. We have modified their methods by using the anti-idiotypic antibodies to immunoprecipitate proteins from sonicated non-denaturing detergent-treated extracts of T4-infected cells.

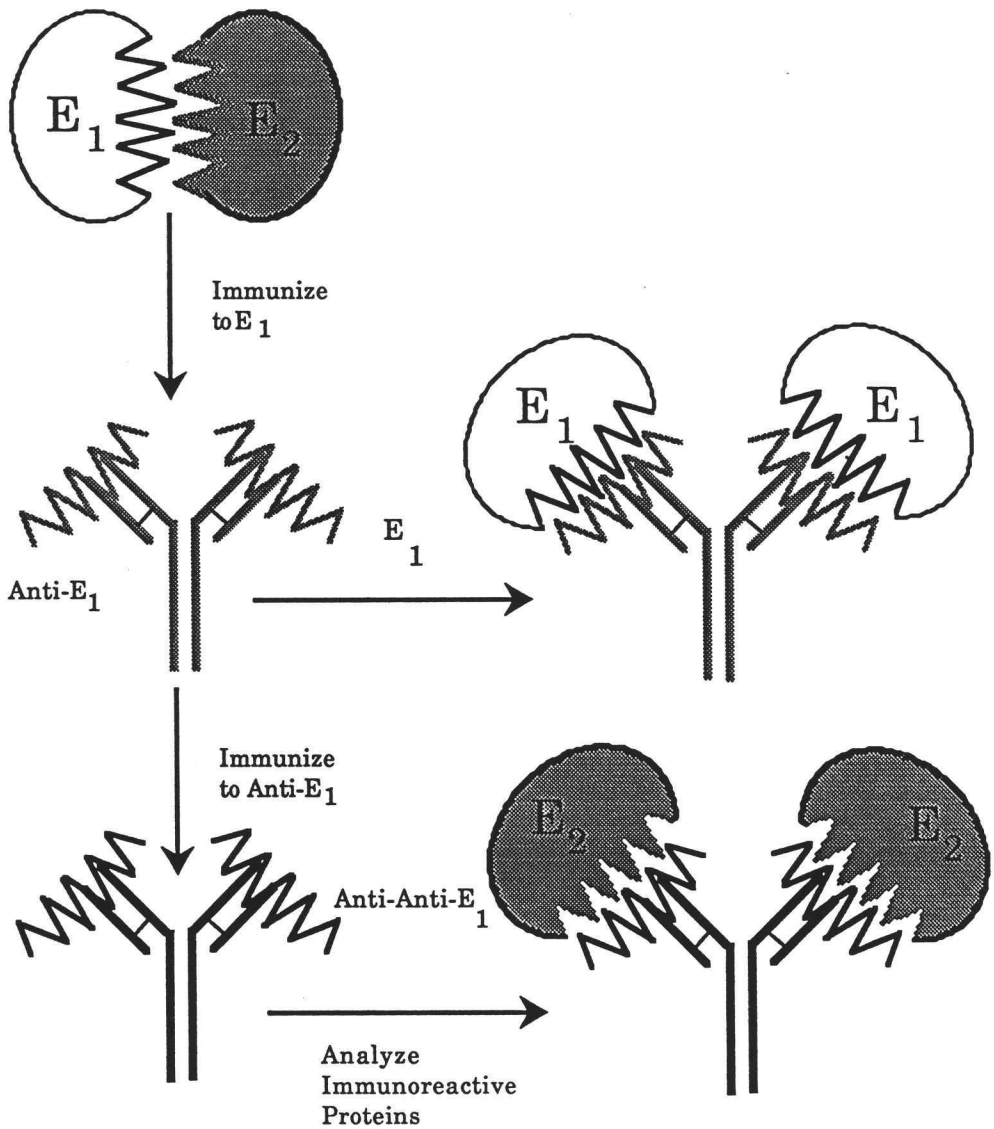


Figure II-2. Use of Anti-idiotypic Antibodies to Identify Interaction Between Two Enzymes (E1 and E2). A polyclonal serum contains a population of antibodies, which recognize a variety of epitopes. This population of antibodies can be used to generate anti-idiotypic antibodies. Certain anti-idiotypic antibodies may reflect the interaction site for protein-protein association.

Materials and Methods

Materials:

The MPL+TDM adjuvant used in generating the antibody response was purchased from RIBI ImmunoChem Research Inc. Protein A-Sepharose was purchased from Pharmacia. Affi-Gel 15 was purchased from Bio-Rad Laboratories. NitroScreen West immunoblot membrane was purchased from NEN Research, Dupont. Purified *E. coli* dTMP synthase was a gift from J. Ernest Villafranca, Agouron Pharmaceuticals Inc.

Protein Purification:

dCMP hydroxymethylase was purified and rabbit polyclonal antibodies were generated to denatured dCMP hydroxymethylase monomers and to native dCMP hydroxymethylase as previously described (Wang, 1989). dCMP hydroxymethylase antibodies were purified by 25-50% ammonium sulfate precipitation, followed by affinity chromatography on a dCMP hydroxymethylase column, and then Protein A purification. T4 thymidylate synthase was purified from pKTd2/MB901 as described in previous papers (Belfort et al., 1983; Purohit and Mathews, 1984)

Antibody Affinity Purification:

For the affinity column dCMP hydroxymethylase was purified from 50 grams (wet weight) of *E. coli* K38 carrying the plasmid pT7-42 (Lamm, et al. 1988). A simplified three-step procedure was used to purify dCMP hydroxymethylase for the affinity column. The steps involved streptomycin sulfate and ammonium sulfate precipitations, a DEAE Sepharose column, and an FPLC gel exclusion column (Superose 6, Pharmacia). The purified dCMP hydroxymethylase, 7.2

mg, was incubated with 2 ml of Affi-Gel 15 and the product washed 3 times with Milli Q water and 3 times with 0.1 M MOPS, 250 mM KCl, pH 7.5. After an overnight incubation at 4°C, 200 µl of 1 M ethanolamine-HCl, pH 8.0, was added, and allowed to mix for two hours and react with the still active groups on the Affi-Gel. The Affi-Gel 15 was spun down after the two-hour incubation, and the supernatant was measured for unbound protein. This was found to represent 0.8 mg of unreacted enzyme. The gel was placed in a 3-ml syringe and washed with 4 volumes of 1M glycine-HCl, pH 2.6. This wash contained a substantial amount of unbound dCMP hydroxymethylase, 18% of the total incubated.

The ammonium sulfate-precipitated dCMP hydroxymethylase polyclonal antiserum was resuspended in 0.3 volumes of PBS and dialyzed versus three changes of PBS for 10 hours. The antiserum was then applied overnight at a flow rate of 1 ml per minute at 4°C to the dCMP hydroxymethylase-Affi-Gel. The column bed was first washed with a PBS solution. A second wash involving a solution of 0.1 M boric acid-0.025 M Na borate-1 M NaCl-0.1% Tween 20 (v/v), pH 8.3, was applied at a flow rate of 1 ml per min. The final elution to remove the bound antibody was carried out with 1 M glycine-HCl, pH 2.8, which was collected in a neutralizing solution of 1 M Tris base, pH 9.6. A second affinity purification was carried out on a second sample. On the second purification the column was pre-washed with all three buffers before addition of the antibody.

The second sample was also bound to a Protein A column, which was washed and the sample eluted off with a 0.1 M glycine-HCl, pH 2.5, buffer, followed by neutralization by the addition of 1/20 (v/v) 1.0 M Tris-HCl, pH 9.6, solution.

Analysis of Purified Antibody:

To determine if the affinity-purified dCMP hydroxymethylase antibody was

still able to bind antigen, a western blot of dCMP hydroxymethylase was probed with the affinity-purified antibody. The immunoblot displayed a band corresponding to the molecular weight of dCMP hydroxymethylase (Figure II-3). A corresponding immunoblot developed with crude antiserum showed far higher background staining (data not shown).

In addition samples of the affinity-purified dCMP hydroxymethylase antibody were run on a polyacrylamide gel, which showed that the antibody had been purified but a considerable amount of serum albumin protein was still present in sample A. Sample A was purified further with a Protein A column to remove albumin.

Generation of Anti-idiotypic Antibodies:

One milligram of purified dCMP hydroxymethylase antibody was injected into each of two New Zealand White rabbits along with a synthetic adjuvant, MPL+TDM emulsion. After 4 weeks the rabbits were individually boosted with an additional 1.0 mg of the purified dCMP hydroxymethylase antibody. One week after the boost a test bleed was taken and analyzed for signs of antibody generation. After positive indications of antibody production, a major bleed, 40 mls, was carried out on each of the two rabbits the following week. Before the injection of dCMP hydroxymethylase antibody the two rabbits were bled, 10 ml each, for control pre-immune rabbit serum.

Immunoprecipitation with Anti-(dCMP Hydroxymethylase Antibody) Serum:

To identify the antibodies generated in the rabbits, immunoprecipitations on [^{35}S]methionine-labeled T4-infected and uninfected *E. coli* samples were carried out. Pre-immune serum was used as a control to confirm that the proteins precipitated were due to antibodies generated by the injection of dCMP hydroxymethylase antibody.

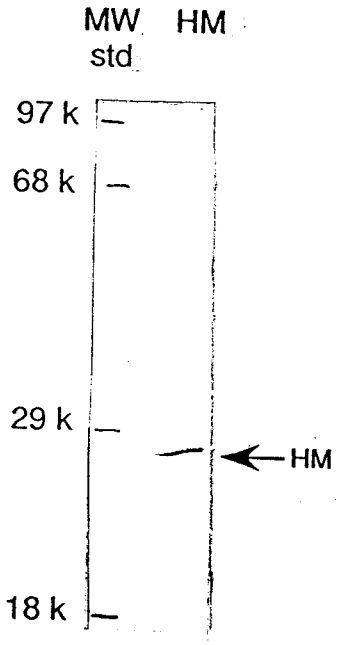


Figure. II-3. Analysis of the Affinity-purified dCMP Hydroxymethylase Antibody. An immunoblot was carried out with 5 µg protein from an unfractionated extract of pT7-42/K38 which had been induced to overexpress dCMP hydroxymethylase. A 12% polyacrylamide gel was run and the proteins were transferred to NitroScreen. The immunoblot was probed with affinity purified hydroxymethylase antibody, 0.5 µg (total protein) at a concentration of 100 ng/ml.

T4 and *E. coli* proteins were labeled by incorporation of [^{35}S]methionine in a growing culture. The T4-infected culture was labeled from 3 to 8 minutes post infection. The *E. coli* samples were labeled for 20 minutes. The samples were sonicated and unless otherwise stated treated with DNase I 10 µg/ml and micrococcal nuclease 3 µg/ml as described previously (Wheeler et al., 1992). Immunoprecipitations were carried out in NP-40 buffer: 150 mM NaCl, 1.0% NP-40, 50 mM Tris-HCl, pH 8.0. The sera and labeled extracts were incubated at 4°C, 100 µl of Protein A beads were added and incubation continued for two hours. The Protein A beads were washed three times by resuspension in 1 ml of NP-40 buffer, followed by centrifugation. Each one-ml aliquot of NP-40 was allowed to sit for 10 minutes between centrifugations. 20 µl of 4x loading buffer--40% glycerol-4% SDS-4% 2-mercaptoethanol-0.5 M Tris-HCl, pH 6.8--was added and the samples were heated at 80°C for 15 minutes. The samples were run on polyacrylamide gels, and treated with 1 M sodium salicylate, dried and placed over film.

The immunoprecipitations using crude serum showed wide variation in background levels. Overnight incubations of sera with labelled extracts precipitated a greater amount of antigen but also increased the number and intensity of background bands. Changes in intensity and number of background bands were also seen with different T4 infected extracts. Immunoprecipitations with PBS present (Figure II-6) appeared to decrease the background signal.

Separation of Antibodies in the Anti-idiotypic Serum:

A 100-µl aliquot of serum A was placed on a dCMP hydroxymethylase affinity column with 5 ml of PBS. The PBS serum solution was circulated over the column for 3 hrs. After 3 hrs the PBS solution was collected and 5 ml of additional PBS was added to the column and washed through. This PBS

fraction was concentrated down to 1 ml; it contained the antibodies that had no affinity for dCMP hydroxymethylase. To remove antibodies that did have an affinity for the bound dCMP hydroxymethylase protein, 5 ml of 100 mM glycine, pH 2.5, was added to the column and circulated for 1 hr. The acidic solution was then collected, and the column was washed with an additional 5 ml of the glycine buffer. This solution was neutralized with 1/20 vol of 1 M potassium phosphate, pH 8.0. The second wash was then concentrated to 700 µl. A 1/10th volume of 10X NP40 buffer and also [^{35}S]methionine-labeled T4 extracts, 300 µl, were then added to 100 µl serum, to the non-dCMP hydroxymethylase-bound fraction, and to the dCMP hydroxymethylase-bound fraction. An immunoprecipitation was then carried out on the three samples.

Results

Generation of isologous anti-idiotypic antibodies was attempted with the purified rabbit anti-dCMP hydroxymethylase antibody. The cross-immunization between animals of the same species carried the benefit of decreasing the number of antibodies generated to the constant regions of the hydroxymethylase antibody.

The reasons for use of affinity chromatographic purification were 1) the likelihood that the anti-dCMP hydroxymethylase antibody is very non-immunogenic, creating a need to inject the maximum amount of anti-dCMP hydroxymethylase-specific antibody, 2) the likelihood that dCMP hydroxymethylase-specific antibody comprised less than 10% of the total concentration of antibody found in the serum (Harlow and Lane1988), and 3) the desire to inject antibodies that bound only to the surface features of native

dCMP hydroxymethylase. Presumably, these antibodies would be enriched among proteins binding to a dCMP hydroxymethylase column. The latter property is sought to maximize the chance of generating antibodies directed against domains involved in protein-protein interactions.

Immunoprecipitation Using Anti-idiotypic dCMP Hydroxymethylase Serum:

NP-40 buffer was selected for the immunoprecipitations to allow separation of proteins from complexes with minimal disruption of the native structure of the proteins. It was also assumed that nonspecific cross-reactive epitopes would be minimized when the *E. coli* and T4 proteins were kept in the native conformation.

Under these conditions serum A was found to immunoprecipitate specifically two viral proteins (Figure II-4). The proteins had apparent molecular weights of 28 kDa and 32 kDa, respectively. Also six *E. coli* proteins were immunoprecipitated by serum A. Both the affinity-purified dCMP hydroxymethylase antibody and serum B immunoprecipitated a single 28-kDa viral protein. Control immunoprecipitations using pre-injected rabbit serum and [^{35}S]-labelled *E. coli* proteins were also carried out and did not reveal immunoprecipitation of either protein. However, a protein of Mr about 34-kDa immunoprecipitated in both the pre-immunized and immunized sera. Immunoprecipitations that were carried out with labeled extracts preincubated with deoxyribonuclease showed much lower amounts of the 34-kDa protein. We have tentatively identified this protein as gp32, the T4 single-strand DNA-binding protein. However because it is recognized by the preimmune serum, and its precipitation appears to be dependent on the presence of DNA, the association seems not to be specific and we have not pursued it further.

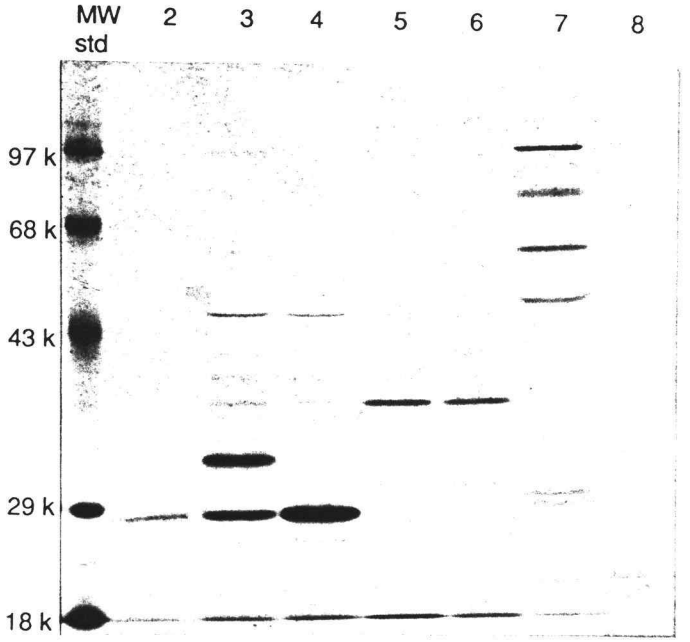


Figure II-4. Immunoprecipitation of ^{35}S-Labeled T4 and *E. coli* Proteins. Lanes 1 through 5 depict immunoprecipitations of a ^{35}S-labeled T4-infected *E. coli* extract. Lanes 6 and 7 are immunoprecipitations of ^{35}S-labeled *E. coli* extract. The following antibodies were used: Lane 1, affinity-purified dCMP hydroxymethylase antibody; Lane 2, anti-idiotypic-dCMP hydroxymethylase serum A; Lane 3, anti-idiotypic-dCMP hydroxymethylase serum B; Lane 4, pre-immune serum A; Lane 5, pre-immune serum B; Lane 6, anti-idiotypic dCMP hydroxymethylase serum A; Lane 7, anti-idiotypic dCMP hydroxymethylase serum B.

Identification of Immunoprecipitated Proteins as dCMP Hydroxymethylase and dTMP Synthase:

Serum A was determined to immunoprecipitate dCMP hydroxymethylase and T4 dTMP synthase. The identification of dTMP synthase was confirmed by the ability of excess purified T4 dTMP synthase to inhibit, by dilution, the immunoprecipitation of the 32-kDa band (Figure II-5). The addition of an excess amount of nonradioactive dCMP hydroxymethylase in comparable fashion was also specific for extinguishing the 28-kDa band. Purified T4 dTMP synthase and dCMP hydroxymethylase were also found to co-migrate with the 32-kDa and 28-kDa bands, respectively, under similar conditions of electrophoresis.

The labelled extracts in this experiment were not treated with deoxyribonuclease. The appearance of the 34-kDa band was not affected by either the addition of excess nonradioactive dCMP hydroxymethylase or T4 dTMP synthase.

The Generated dTMP Synthase Antibody is not a dCMP Hydroxymethylase Antibody:

T4 dTMP synthase and dCMP hydroxymethylase share some sequence homology (Lamm et al., 1988). Therefore, we had to consider the possibility that recognition of both proteins by one antiserum represented immunological cross-reactivity, and that both proteins were recognizing the same antibodies. This possibility was conclusively ruled out by fractionating the antibodies on a dCMP hydroxymethylase affinity column. As seen in Figure II-6, a dCMP hydroxymethylase-specific antibody is retained by the dCMP hydroxymethylase column, while the dTMP synthase antibody is eluted. The dTMP synthase-specific antibody has no affinity for bound dCMP hydroxymethylase and therefore is not a cross-reactive dCMP hydroxymethylase antibody. In other words, dTMP synthase and dCMP hydroxymethylase are recognizing distinct antibodies in anti-anti-hydroxymethylase serum A.

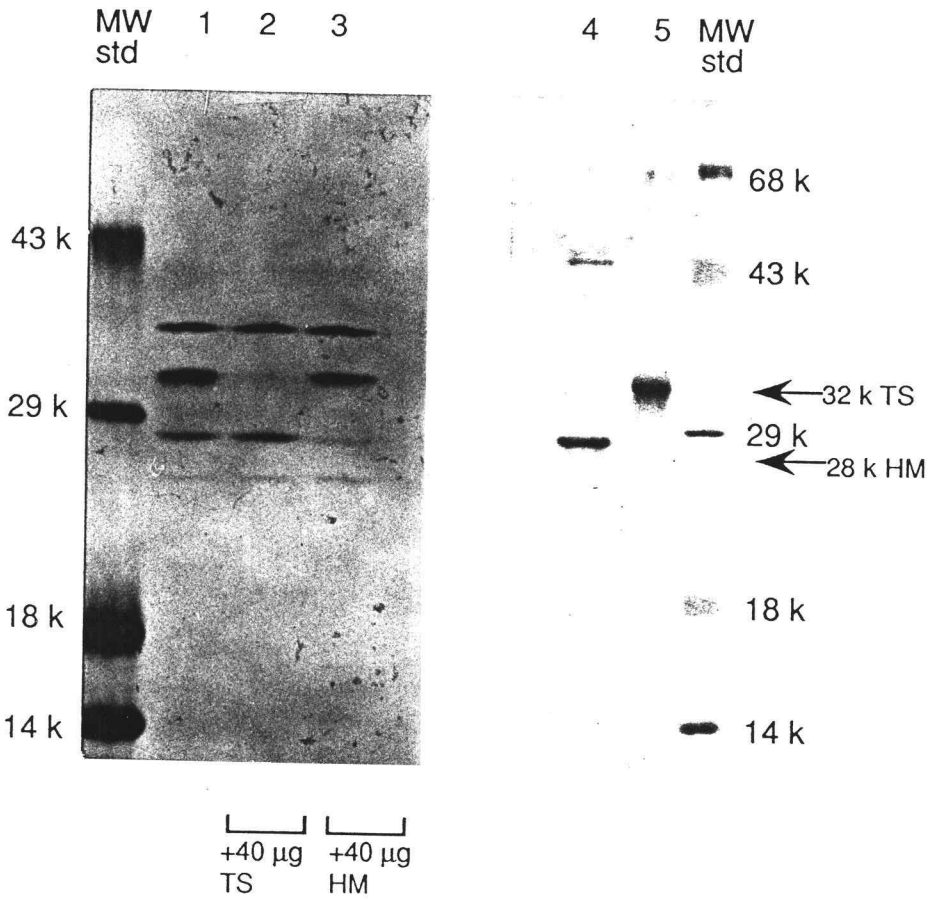


Figure II-5. Identification of 32-kDa Band as T4 dTMP Synthase. Lanes 1, 2, and 3 depict immunoprecipitations of a ^{35}S-labeled T4-infected *E. coli* extract that was not deoxyribonuclease treated. Lane 1 represents an immunoprecipitation using serum A. Lane 2 represents an immuno-precipitation using serum A in the presence of 40 µg unlabeled T4 dTMP synthase. Lane 3 represents an immunoprecipitation using serum A in the presence of 40 µg dCMP hydroxymethylase. Lanes 4 and 5 are Coomassie blue-stained dCMP hydroxymethylase and T4 dTMP synthase, respectively. The outer (unnumbered) lanes depict molecular weight markers.

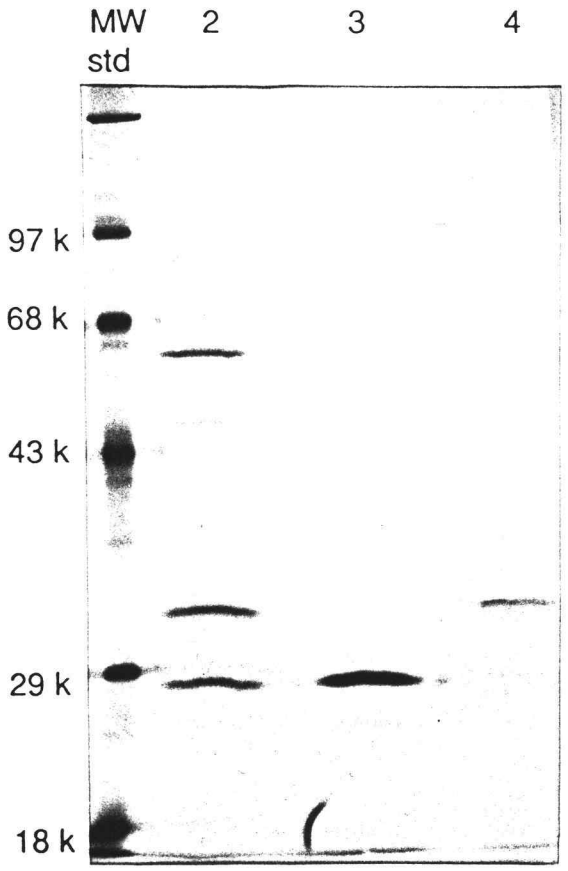


Figure II-6. Distinct Anti-dTMP Synthase and Anti-dCMP Hydroxymethylase Antibodies in the Anti-idiotypic Serum. Serum A was bound to a dCMP hydroxymethylase-Affi-Gel column and eluted off. Lanes 1, 2, and 3 depict immunoprecipitations of an ^{35}S-labelled T4-infected *E. coli* extract. Lane 1, immunoprecipitation with crude serum A. Lane 2, immunoprecipitation with the serum A fraction that had affinity for a dCMP hydroxymethylase-Affi-Gel column. Lane 3, immunoprecipitation with the serum A fraction that had passed through a dCMP hydroxymethylase-Affi-Gel column.

The dTMP Synthase Antibody is Specific for the Bacteriophage T4 Protein:

If the dTMP synthase antibody is mimicking part of the dCMP hydroxymethylase enzyme surface that participates in a protein-protein interaction specific for the phage-encoded enzymes, one would expect the antibody to be also phage-specific. An immunoprecipitation was carried out where the antibody's ability to bind ^{35}S-labelled phage dTMP synthase was challenged with purified T4 or *E. coli* dTMP synthase (Figure II-7). As seen in previous immunoprecipitations the unlabeled phage-coded dTMP synthase could dilute out the 35S-labelled phage dTMP synthase, while the *E. coli* dTMP synthase did not inhibit the immunoprecipitation of the T4 enzyme or any of the *E. coli* proteins immunoprecipitated by serum A. The dTMP synthase antibody binds the phage enzyme and not that of the host.

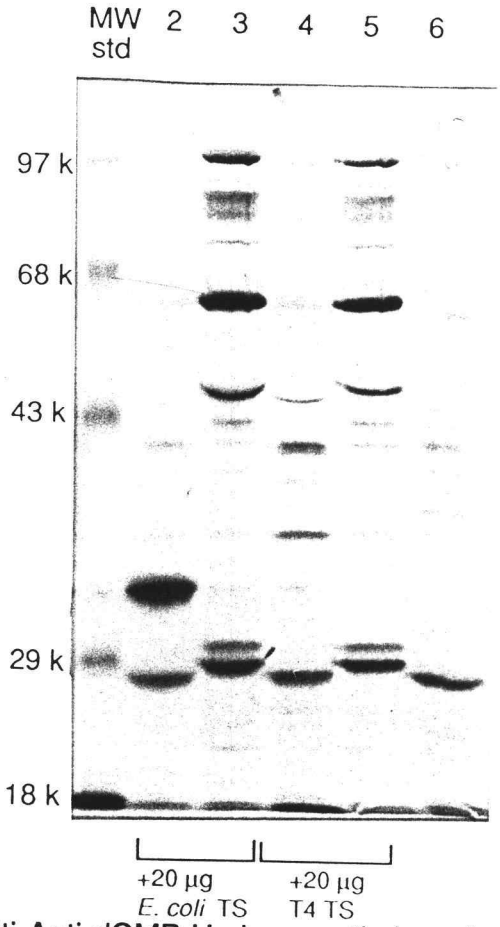


Figure II-7. The Anti-Anti-dCMP Hydroxymethylase Antibody is Specific for Phage-coded dTMP Synthase. Lane 1, T4 proteins precipitated by overnight incubation with anti-anti-hydroxymethylase in the presence of 20 µg of purified *E. coli* dTMP synthase, showing that the bacterial enzyme does not interfere with the binding between the phage enzyme and the anti-idiotypic serum; lane 2, *E. coli* proteins precipitated by anti-anti-HM in the presence of 20 µg of *E. coli* dTMP synthase; lane 3, T4 proteins precipitated by anti-anti-HM in the presence of 20 µg of purified T4 dTMP synthase, confirming that the labeled 32-kDa band represents T4 dTMP synthase; lane 4, *E. coli* proteins precipitated by anti-anti-HM in the presence of 20 µg of T4 dTMP synthase; lane 5, proteins precipitated by polyclonal antiserum against T4 dCMP hydroxymethylase, confirming that the labeled 28-kDa protein in lanes 1 and 3 is T4 dCMP hydroxymethylase.

Discussion

dCMP hydroxymethylase has been implicated to reside in a deoxyribonucleotide synthesis complex, and has been previously recognized to interact with other viral proteins when bound to affinity columns. Recent work has shown that dTMP synthase is retained on a dCMP hydroxymethylase protein affinity column along with a number of other bacteriophage T4 enzymes.

The structure of entire protein-antibody complexes has been established for several proteins (Davies et al., 1988). The surface areas were determined to be large, 500-700 Å2. The interaction sites were found to exclude solvent and were tightly packed. For lysozyme, at a resolution of 2.8 Å, no distortion of either the antibody or protein was recognized (Amit et al., 1986).

The ability of an antibody to cover a large surface area of a protein in a mirror-like image of the protein's surface suggests that anti-idiotypic antibodies to such an antibody may be able to mimic a portion of the protein's surface. However, recent work involving the determination of the three-dimensional structure of an idiotypic-anti-idiotypic complex has shown that not all such complexes indeed contain idiotypic mimicry (Bentley et al., 1990). A number of anti-idiotypic antisera may have to be generated before one is complementary in structure.

The use of cloned dCMP hydroxymethylase for generation of antibodies was advantageous in that antibodies generated later in the project could not possibly have been caused by trace amounts of T4 protein contaminants contained in the original purified dCMP hydroxymethylase protein. dCMP hydroxymethylase is the only T4 protein present in the original extract.

Serum B did not show a dTMP synthase-binding antibody, but did show a

large production of dCMP hydroxymethylase antibody. A possible explanation for the dCMP hydroxymethylase antibody seen in both serum samples is that anti-idiotypic dCMP hydroxymethylase antibodies were produced that recognized the dimer binding site of dCMP hydroxymethylase. However, we can not rule out the possibility that the hydroxymethylase antibodies generated were produced by either dCMP hydroxymethylase enzyme contamination from the affinity column or that the dCMP hydroxymethylase antibody found in the final bleeds was from the original antibody.

An anti-idiotypic antibody that recognizes only the phage-encoded dTMP synthase is indicative of direct interaction between the T4 dCMP hydroxymethylase and phage dTMP synthase. Studying the interaction directly by the use of dCMP hydroxymethylase enzyme affinity columns had shown such an interaction, but did not answer the question whether the dCMP hydroxymethylase was bound directly to the phage dTMP synthase or just associated with a complex that contained dTMP synthase. The function of a direct association of the two enzymes could be control of synthesis of modified deoxypyrimidines. Binding of T4 phage dTMP synthase with dCMP hydroxymethylase may contribute toward the maintenance of the phage dTMP/hm-dCMP ratio of intracellular synthetic rates. The determination that dTMP synthase interacts with dCMP hydroxymethylase is the first evidence that the arrangement of enzymes in the T4 deoxyribonucleotide synthetase enzyme complex is organized with functionally similar enzymes juxtaposed to one another. Extension of anti-idiotypic antibodies as tools for probing protein-protein interactions may be applicable to further analysis of this complex and in a variety of other biochemical systems that involve protein-protein interactions.

Acknowledgements

We thank Linda Wheeler for purification of the bacteriophage T4 thymidylate synthase, Nancy Roseman for help in generation of antibodies, and Ralph Davis for his ideas and suggestions.

Footnotes

1 Abbreviations used include: TS, thymidylate synthase; HM, deoxycytidylate hydroxymethylase; hm-dCMP, 5-hydroxymethyl-deoxycytidine 5'-monophosphate; dNTP, deoxyribonucleoside 5'-triphosphate; Tris, tris(hydroxymethyl)aminomethane; PBS, phosphate-buffered saline, 137 mM NaCl, 3 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4.

CHAPTER 3

Protein-Protein Interactions of Purified T4 Thymidylate Synthase and dCMP Hydroxymethylase in vitro

Introduction

Previous studies in our laboratory have supported the idea that two homologous bacteriophage T4 enzymes, thymidylate synthase, and dCMP hydroxymethylase, directly interact within the deoxyribonucleoside synthetase complex. The generation of anti-idiotypic antibodies to dCMP hydroxymethylase were found to immunoprecipitate the phage thymidylate synthase specifically. In addition experiments using an immobilized dCMP hydroxymethylase affinity column demonstrated that the T4 thymidylate synthase was one of roughly a dozen enzymes that specifically bound to an immobilized dCMP hydroxymethylase enzyme (Wheeler et al., 1992).

The unique feature present on the T4 thymidylate synthase that interacts with the anti-idiotypic antibody should also correspond to the thymidylate synthase domain that forms the junction with the dCMP hydroxymethylase enzyme. The generation of thymidylate synthase C-terminal truncations were carried out in an attempt to map the interaction site of the anti-idiotypic antibody with the enzyme.

Reconstitution of the protein-protein interactions that occur between the enzymes involved in the complex should also prove to be a powerful tool in understanding the nature of the association and its significance to the synthesis of deoxyribonucleotides. To characterize the interaction of the dCMP hydroxymethylase with thymidylate synthase, purification of the two enzymes to homogeneity was carried out, and a number of attempts were made to study their binding in an isolated system.

Materials and Methods

Materials:

The cation exchanger, S-Sepharose, was purchased from Pharmacia. The *E. coli* strain AR120 and the overexpression plasmids pKTd2 and pKTdΔI were gifts from Marlene Belfort. The S30 cell free transcription/translation system was purchased from Promega Biotec. LB medium was composed of 10 g Bacto-tryptone, 5 g yeast extract, 10 g NaCl 1 ml 1N NaOH per liter.

Mapping the Anti-idiotypic Binding Domain of T4 Thymidylate Synthase:

For the initial attempt at mapping the binding domain of the thymidylate synthase ExoIII deletions of the 3' end of the pkTdΔI plasmid were carried out as described by Gross and Rohrmann, 1990. The plasmid had been previously been linearized with HpaI and BstXI, restriction enzymes that cut adjacent to the 3' end of the thymidylate synthase gene. For the generation of deletions 20 μg of the linearized pkTdΔI plasmid was digested at 37°C with 1,258 units of ExoIII in at total volume of 60 μl. Aliquots were removed at 30 second intervals and treated with S1 nuclease for 30 minutes at 4°C. The deleted plasmids were ammonium acetate precipitated and brought up in T4 DNA Pol. buffer. The ends of the deletions were polished with T4 DNA polymerase and the samples were analyzed on a 1% agarose gel (Figure III-1). Digestions corresponding to deletions within the 3' end of the thymidylate synthase gene were recircularized by T4 ligase and transformed into the AR120 strain using a $RbCl_2$ method, described in Appendix D.

Transformed colonies were isolated and minipreps were carried out as, described in Appendix E, on the individual colonies to confirm the size of the deletions (Figure III-2). Inductions of the 3' deletions along with the full length pKTdΔI plasmid were carried out by the addition of nalidixic acid, 40 μg/ml, in the presence of 100 μg/ml ampicillin and 12 μg/ml tetracycline. Polyacrylamide

gel analysis for expression of the induced deletions was carried out (Figure III-3).

In Vitro Transcription/Translation of Thymidylate Synthase:

The expression of thymidylate synthase was also carried out by using a cell free *E. coli* S30 system. Using the S30 *in vitro* system expression of full length thymidylate synthase was also found to generate a series of soluble truncated products. Expression of a pKTdΔI plasmid containing a EcoRV generated 3' deletion demonstrated that the truncated forms were C-terminal deletions, data not shown. Using the standard incorporation reaction protocol the expression of ^{35}S labeled thymidylate synthase was carried out for 2 hours at 37°C. Polyacrylamide gel analysis of the labeled thymidylate synthase with the truncated products was carried out alongside an immunoprecipitation of the products using the anti-idiotypic antibody that recognizes T4 thymidylate synthase (Figure III-4).

Affinity of Thymidylate Synthase With Immobilized dCMP Hydroxymethylase:

An initial attempt was made to look at the interaction of the purified thymidylate synthase with immobilized dCMP hydroxymethylase. For the experiment, 200 µg of T4 thymidylate synthase, which had been previously purified by Linda Wheeler, was dialyzed into 50 mM Tris-acetate, pH 8.0, 25 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol. The thymidylate synthase was recirculated through a 5-mg dCMP hydroxymethylase Affi-gel 15 column at a flow rate of 1 ml/min for 1 hour. The buffer was collected and three increasing NaCl washes, 200 mM, 600 mM, and 2 M, were then recirculated over the column at 1 ml/min for 1 hour each. The individual salt washes were collected and the amount of thymidylate synthase present was determined by Bradford protein analysis (Figure III-5).

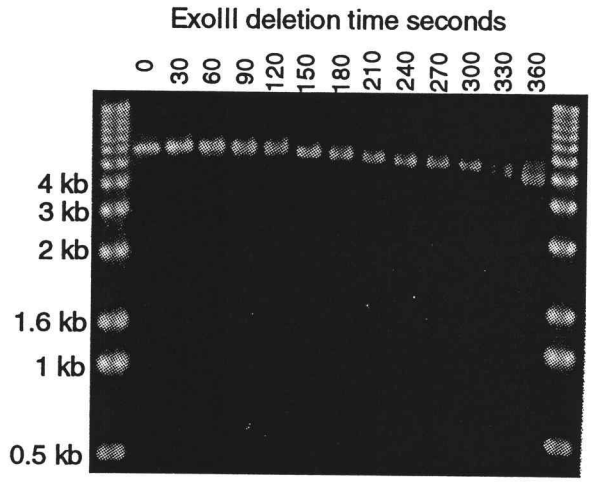


Figure III-1. Analysis of ExoIII 3' Deletions of T4 thymidylate Synthase Gene. The pKTdΔI plasmid was linearized and digested with ExoIII nuclease. Aliquots were removed from the reaction every 30 seconds. The deletions were analyzed on a 1% agarose gel.

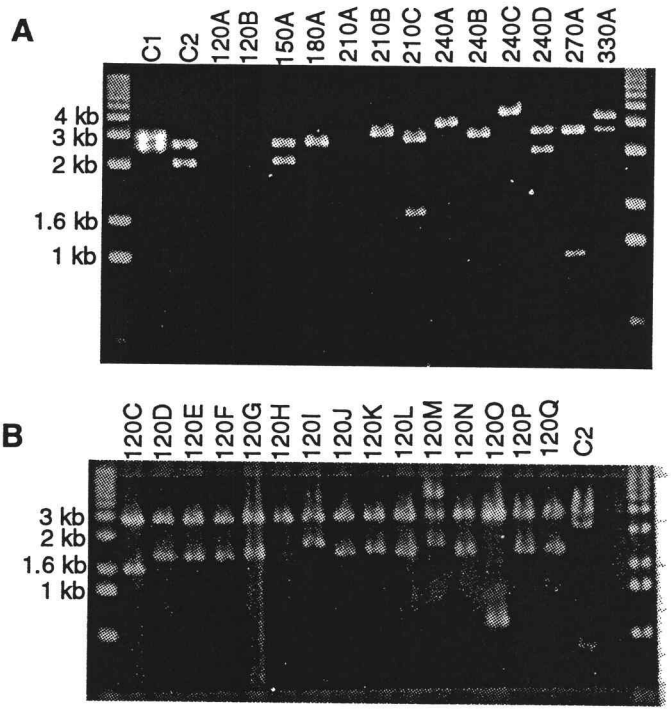


Figure III-2. Analysis of Transformed pKTdΔI 3' Deletions into a AR120 Strain. AR120 colonies were selected from the $RbCl_2$ transformations of ligated ExoIII digested pKTdΔI plasmid and grown up individually in 3 ml LB media. The plasmids were purified and cut with the restriction enzymes, BamH1 and EcoR1. The digested plasmids were analysed on a 1% agarose gel. The top gel, (A) represents the recovered plasmids from a variety of time points. Lane C1 corresponds to the original pKTdΔI plasmid cut with BamH1 and EcoR1. Lane C2 is the original linearized pKTdΔI also cut with BamH1 and EcoR1 restriction enzymes. Gel (B) represents the various deletion lengths recovered from a single ExoIII deletion time point transformation, 120 seconds.

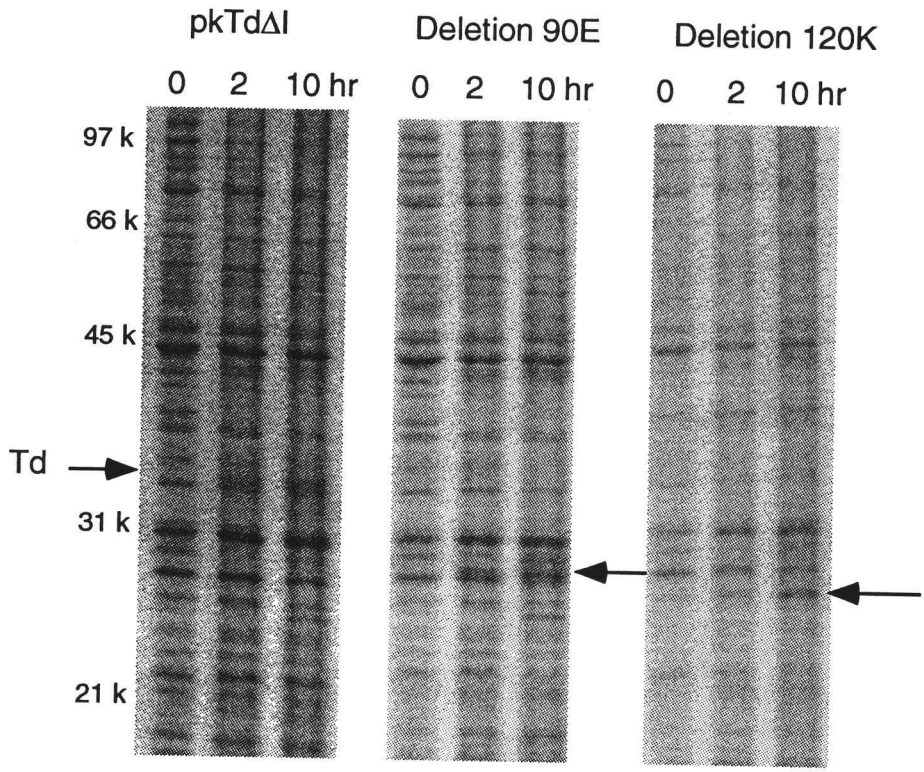


Figure III-3. Expression of C-terminal Truncated pKTdΔI/AR120 Clones. The original pKTdΔI/AR120 construct along with the ExoIII deletions from the 90, and 120 second time points were induced with nalidixic acid. The inductions were sampled at 0, 2, and 10 hours for each construct and analyzed on a polyacrylamide gel.

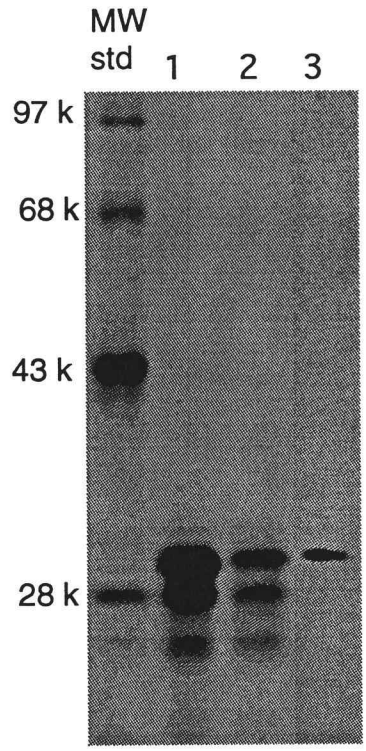


Figure III-4. Immunoprecipitation of Thymidylate Synthase Truncations. Lanes 1, 2, and 3 represent the ^{35}S labeled products of the *in vitro* S30 transcription/translation of the pkTdΔI plasmid. Lanes 1 and 2 represent varying amounts of the full length thymidylate synthase products and C-terminal truncation products. Lane 3 is an immunoprecipitation of the products using the dCMP hydroxymethylase anti-idiotypic antibody. Only the full length thymidylate synthase is immunoprecipitated by the anti-idiotypic antibody.

Overexpression of Intron and Intron-deleted T4 *td* Gene:

An initial series of test inductions were carried out to compare the overexpression of plasmid encoded bacteriophage thymidylate synthase with and without the 1-kbp intron present. The test inductions of 500 ml-LB cultures of AR120/pKTd2 (with intron), and AR120/pKTdΔI (no intron), were carried out at 37°C in the presence of 100 μg/ml ampicillin and 12 μg/ml tetracycline. The test inductions were carried out with nalidixic acid, 40 μg/ml final concentration between an A_{650} of 0.21 and 0.25. Aliquots were removed at 30 minute intervals from the cultures and assayed for thymidylate synthase activity by using the Wahba and Friedkin method (1961). Comparisons of the extent of thymidylate synthase activity induced and the occurrence of maximum activity were made between the cloned wild type and intron-deleted *td* gene (Figure III-6).

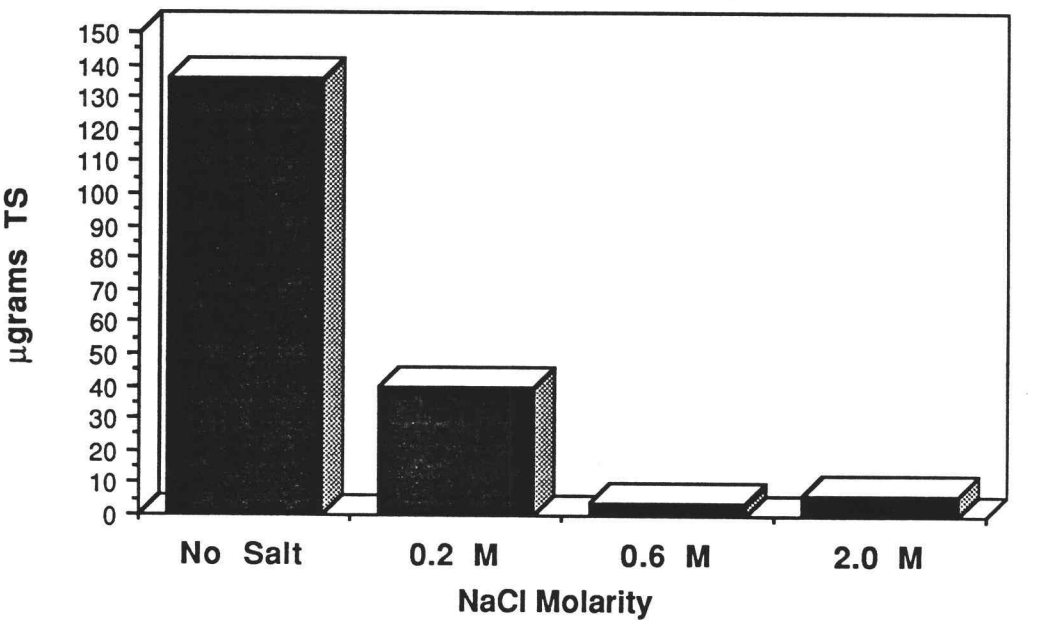


Figure III-5. Interaction of Purified T4 Thymidylate Synthase with a dCMP Hydroxymethylase Affi-Gel 15 Column. The no salt fraction, corresponds to the original buffer, 50 mM Tris-acetate, pH 8.0, 25 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol. For this initial experiment 200 µg of phage thymidylate synthase was applied to the dCMP hydroxymethylase column. The recovery of thymidylate synthase from the salt elutions was 96%.

Purification of Bacteriophage T4 Thymidylate Synthase:

For a large scale purification of bacteriophage T4 thymidylate synthase the intron-deleted overexpression system was selected. Eight 500-ml inductions of AR120/pKtdΔI were carried out in LB medium. The 500-ml cultures were grown in 2.5-liter flasks at 37°C in the presence of 100 μg/ml ampicillin and 12 μg/ml tetracycline. Each 500-ml culture was inoculated with 5 ml of a freshly saturated overnight culture. The overnight culture was started from a -80° C glycerol stock of the AR120/pKtdΔI line. At an A_{650} nalidixic acid, 40 μg/ml final concentration, was added to each flask and allowed to induce for 3 hours.

The purification protocol for the bacteriophage T4 thymidylate synthase involved a modification of the procedure first developed by Linda Wheeler. The frozen bacterial pellet was brought up in S-Sepharase buffer, 100 ml of 50 mM KCl, 10 mM ß-mercaptoethanol, 1mM EDTA, 40 μg/ml PMSF, and 50 mM Tris-HCl, pH 6.5. The sample was sonicated three times on ice with each sonication lasting two minutes. The cell debris was centrifuged at 10,000 g for 45 minutes.

A 30 to 60% ammonium sulfate cut was carried out at a pH of 7.4. The pellet was resuspended in the S-Sepharose buffer with the addition of fresh PMSF. The sample was dialyzed overnight in two changes of 4 L S-Sepharose buffer. The insoluble material was removed by a 10,000 rpm centrifugation in a SS34 rotor.

The supernatant was applied to a 100-ml S-Sepharose cation exchange column at a flow rate of 0.2 ml/min. The absorbance was monitored at 280 nm and a gradient elution was carried out at a flow rate of 0.5 ml/min after a baseline absorbance value was attained. The gradient was composed of an initial reservoir containing 250 ml of 50 mM Tris-HCl, pH 6.5, 50 mM KCl, 10 mM ß-mercaptoethanol and a second reservoir containing 250 ml of 50 mM Tris-HCl, pH 7.5, 350 mM KCl, 10 mM ß-mercaptoethanol. Fractions were collected and assayed for thymidylate synthase activity.

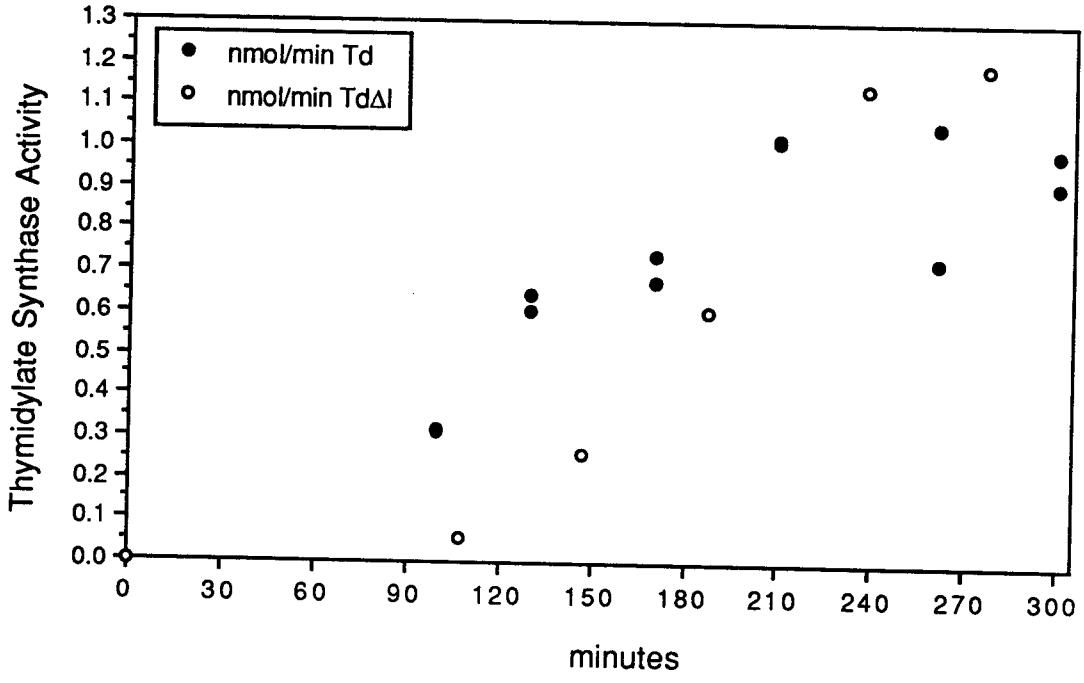


Figure III-6. Effect of the 1-kbp Intron on Overexpression of T4 Thymidylate Synthase. Two separate inductions of the AR120/pKTd2, filled circles, which contain an intron within the *td* gene were measured along with a single induction of a similar strain and plasmid which had the intron removed, AR120/pKtdΔI, open circles.

The cation exchange fractions which contained activity were precipitated by the addition of a 100% saturated solution of ammonium sulfate, pH 7.4, to a final concentration of 70%.

The precipitated protein was dissolved in 2 ml of the gel exclusion buffer, 50 mM Tris-HCl, pH 7.5, 250 mM KCl, 5 mM ß-mercaptoethanol. The sample was applied to a Superose 6 prep. grade FPLC column at a flow rate of 0.4 ml/min. Fractions were collected and assayed for thymidylate synthase activity (Figure III-7). Protein determinations for all the fractions and the previous steps in the purification were carried out using the Bradford protein assay. The purified thymidylate synthase was run out on a 12% polyacrylamide gel and Coomassie blue-stained (Figure III-8). Table III-1 summarizes the purification of the bacteriophage T4 thymidylate synthase.

Table III-1. Purification of Bacteriophage T4 Thymidylate Synthase

Step in Purification	mg Total Protein	Activity nmol/min	Specific Activity nmol/min / mg	% of Crude Activity	Fold Purification
Crude Supernatant	780	258,000	330	100%	1
Ammonium Sulfate cut (30-60%)	231	165,262	720	64%	2
Cation Exchange (S-Sepharose)	25	81,900	3,200	32%	10
FPLC, Gel Exclusion (Superose 6)	6	40,908	6,800	16%	21

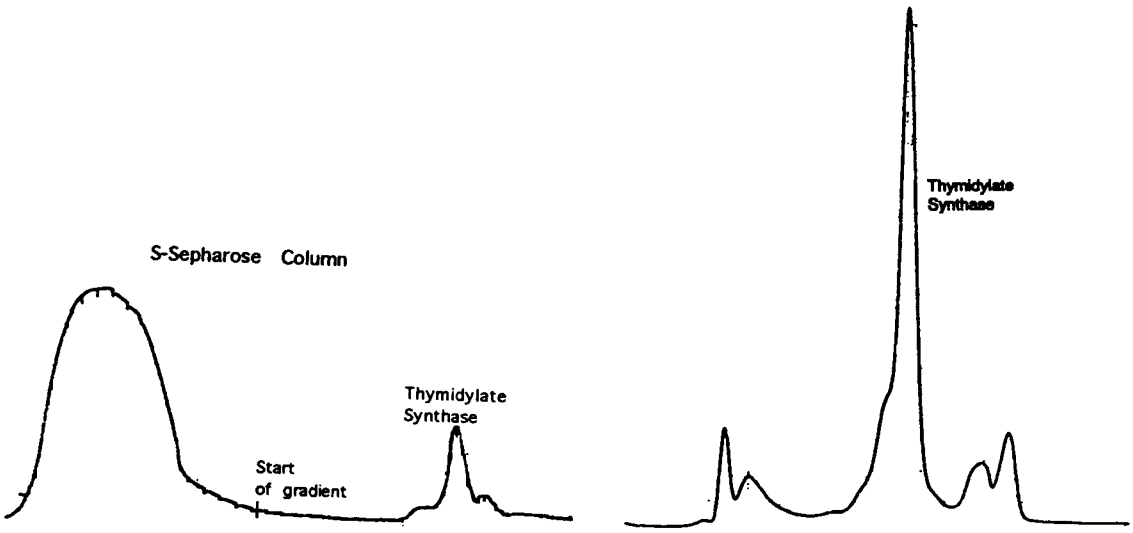


Figure III-7. Elution of Phage Thymidylate Synthase From a S-Sepharose Cation Exchange Column and an FPLC Gel Exclusion Column. The absorbance was monitored at 280 nm. The S-Sepharose column and Superose 6 gel exclusion were monitored at 280 nm with a fullscale reading equal to 2.0 OD units for the S-Sepharose column while the gel exclusion fullscale reading equaled 1.0 OD.

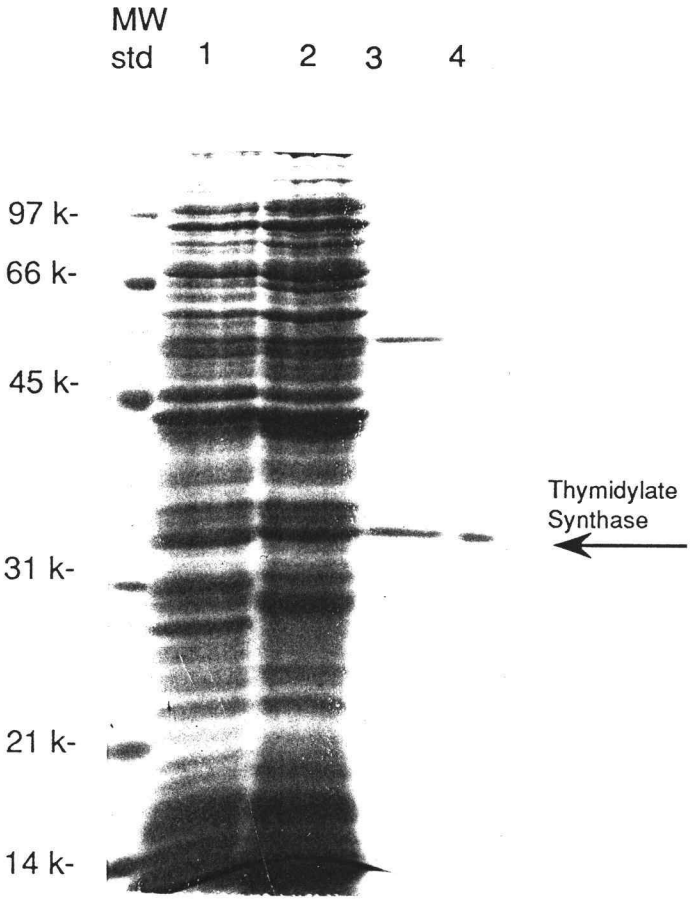


Figure III-8. Polyacrylamide Gel Analysis of Thymidylate Synthase Purification Steps. Lane 1 represents Coomassie blue-stained crude supernatant. Lane 2 represents the ammonium sulfate cut fraction. Lane 3 corresponds to the collected fractions from the S-Sepharose chromatography. Lane 4 is the purified T4 thymidylate synthase after passage through a FPLC gel-exclusion column.

Km and Turnover Determinations for T4 Thymidylate Synthase:

The determination of the K_m values for the two substrates of T4 thymidylate synthase, dUMP and 5,10 methylenetetrahydrofolate were carried out for purified enzyme (Figures III-9, and III-10). The reactions were carried out in a buffer volume of 1 ml containing 100 mM Tris-HCl, pH 7.4, 100 mM KCl, 100 mM ß-mercaptoethanol, 25 mM $MgCl_2$, 15 mM formaldehyde at 25°C. The reactions were followed at 338 nm for the production of dihydrofolate, ΔE_m = 6,600 L $mol^{-1}cm^{-1}$. The concentration range of dUMP varied from 218 µM to 3.6 µM while the 5, 10- 5,10 methylenetetrahydrofolate varied from 23 µM to 1.2 µM. The thymidylate synthase concentration was 11 nM for all measurements. The concentration of a dUMP standard was determined by measuring the absorbance at 260 nm at pH 2.0 E = 9,800 $mol^{-1}L^{-1}cm^{-1}$.

Purification of dCMP Hydroxymethylase by Low-Affinity Antibody Chromatography:

The generation of an anti-dCMP hydroxymethylase column was carried out by the initial purification of low affinity anti-dCMP hydroxymethylase polyclonal antibodies (Kellogg and Alberts, 1992). Figure III-11 diagrams the overall purification scheme. The initial chromatography involved passage of anti-dCMP hydroxymethylase rabbit serum over a 3-ml Affi-gel-10 dCMP hydroxymethylase column which had been previously used for antibody purification and protein-protein interaction studies. A total of 10 ml of serum was loaded onto the column at a flow rate of 1 ml/min at room temperature.

The column was washed with TBS, 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, until no protein was detected in the flow-through. Low affinity anti-dCMP hydroxymethylase antibodies were eluted with buffer containing, 1.4 M $MgCl_2$, 10% glycerol, and 50 mM HEPES, pH 7.6. The elution was carried out by applying 1-ml aliquots of the elution buffer directly onto the column bed.

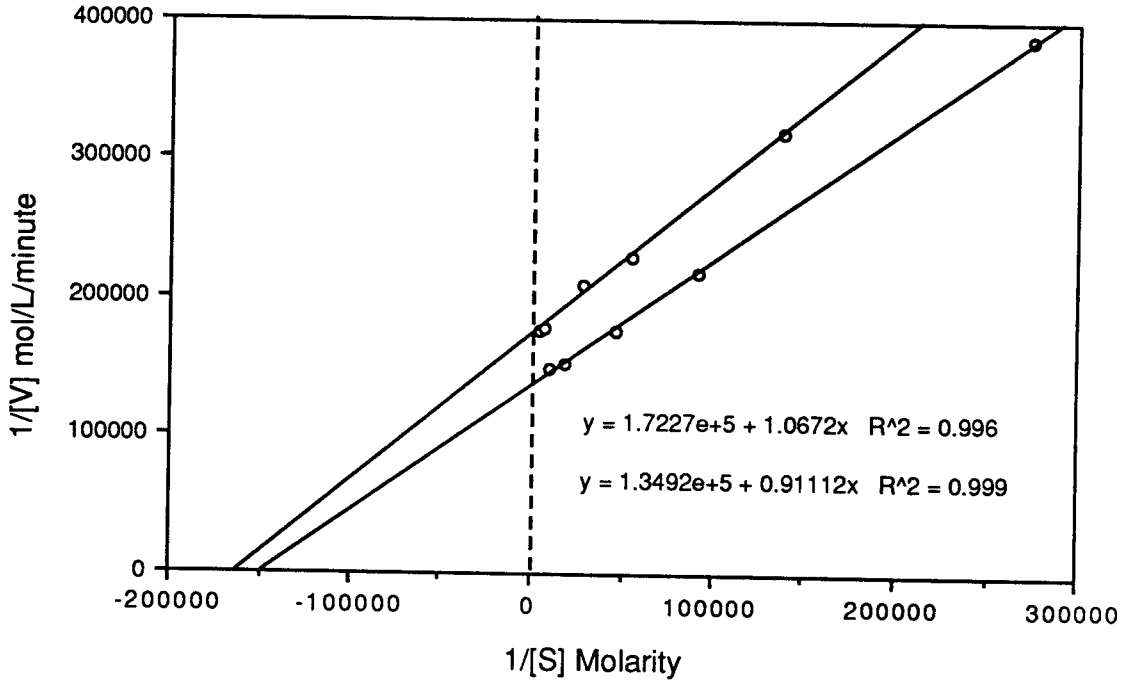


Figure III-9. The Determination of K_m of T4 Thymidylate Synthase for dUMP. Two independent measurements were carried out for the kinetic determinations. The second set of velocity measurements showed a marked decrease in the phage thymidylate synthase V_{max}, 681 to 533 and a slightly decreased K_m, 6.75 μM to 6.17 μM. The average K_m value was determined to be 6.5 μM. The average turnover value was equal to 607 moles dUMP/moles enzyme (dimer)/minute.

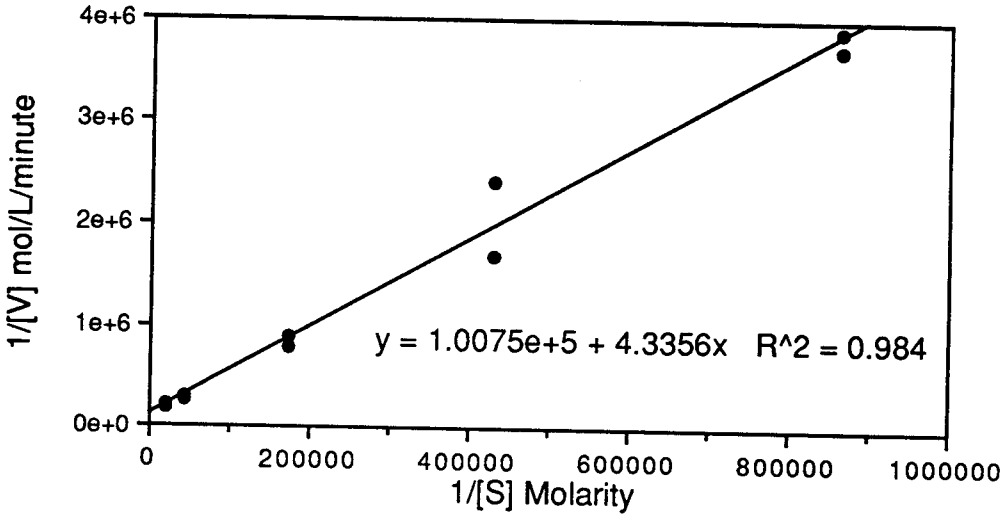


Figure III-10. Determination of the K_m Value of T4 Thymidylate Synthase for 5,10-Methylene Tetrahydrofolate. Duplicate measurements were carried out at each concentration of 5,10 methylene tetrahydrofolate A K_m value of 43 μM was determined for the cofactor. The turnover number was found to be 912 moles FH_2/mole enzyme (dimer)/minute. Using the turnover values for the dUMP and cofactor an average turnover was determined to be 760 ± 152 moles substrate/mole enzyme (dimer)/minute.

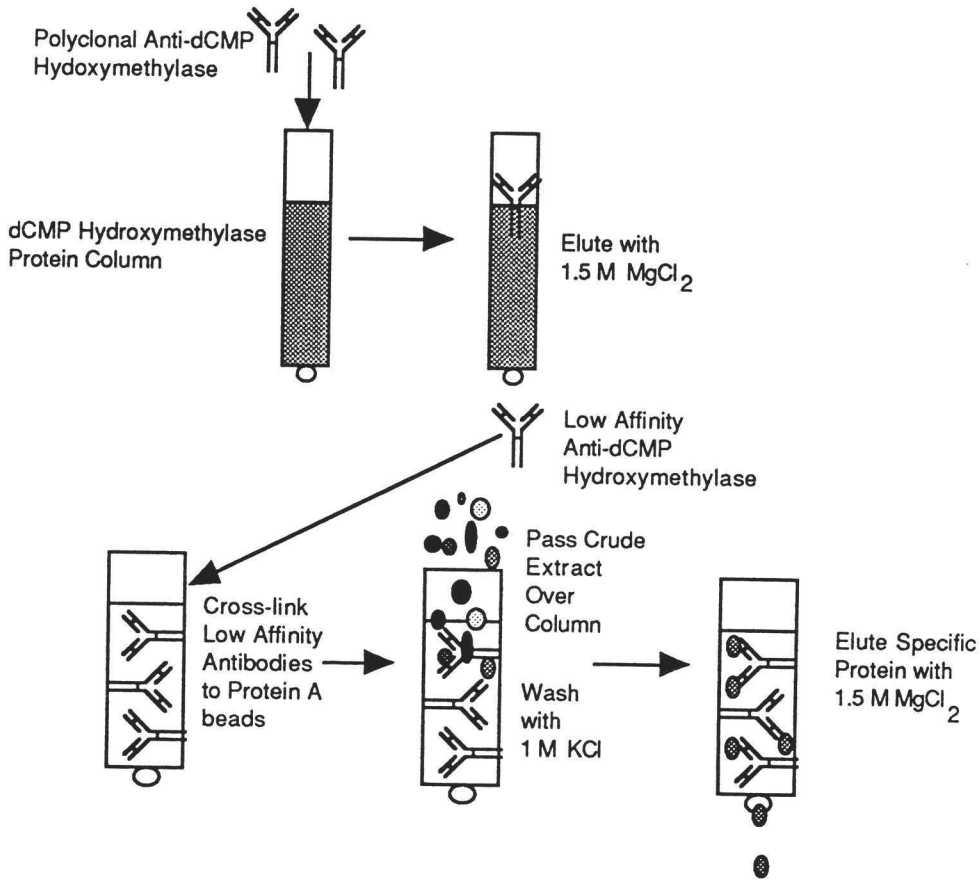


Figure III-11. Schematic for the Purification of dCMP Hydroxymethylase using a Low Affinity Antibody Column. The crude extract passaged over the low-affinity column was ^{35}S-labeled T4-infected *E.coli* lysate. The one step procedure is advantageous when working with labeled extracts by minimizing the amount of equipment that comes into contact with the radioactive fractions.

The elution fractions were assayed for antibody by Bradford protein determination. The high affinity dCMP hydroxymethylase antibodies were eluted with 100 mM glycine, pH 2.2. The eluted fractions were neutralized with a 1:20 addition of 1 M phosphate, pH 8.0.

The low-affinity dCMP hydroxymethylase antibodies were linked to protein A-Sepharose Cl-4B beads by using dimethylpimelimidate as described by Harlow and Lane, 1988. A high affinity dCMP hydroxymethylase protein A column was also made following similar procedures. For each dCMP hydroxymethylase column, 250 µl of protein A beads were incubated overnight at 4°C with the antibody solution in 5 ml PBS, 137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4. The protein A beads were then washed with 10 volumes of 0.2 M sodium borate, pH 9.0. The beads were resuspended in 5 ml of borate buffer and solid dimethylpimelimidate was added to a final concentration of 20 mM. The cross-linking reaction was carried out for 30 minutes at room temperature and quenched by washing the beads with 0.2 M mono-ethanolamine, pH 8.0.

A 500-ml ^{35}S methionine-labeled T4-infected *E.coli* extract was prepared as described before (Wheeler et al., 1992). The labeled extract was brought up in 6 ml of column buffer, 50 mM HEPES, pH 7.6, 50 mM KCl, 1.0 mM $MgCl_2$ and 1.0 mM $CaCl_2$. The extract was sonicated and treated with 10 µg/ml DNAse I, 3 µg/ml micrococcal nuclease, and a 1:500 addition of 20 mg/ml PMSF stock solution. The labeled extract was centrifuged for 30 minutes at 10,000 rpm in a SS34 rotor and the supernatant was collected. The supernatant was passed twice through a 1-ml protein A-Sepharose column. This step was carried out to remove proteins that might bind non-specifically to the protein A column.

The low affinity anti-dCMP hydroxymethylase protein A beads were washed

with the final elution buffer, 50 mM HEPES, pH 7.6, 1.5 $MgCl_2$, and 10 % glycerol, to remove any uncross-linked antibody. The high affinity anti-dCMP hydroxymethylase beads were washed with 100 mM glycine, pH 2.2. The beads were equilibrated in column buffer and placed in 5-ml tubes containing 3 ml of T4-infected extract. The slurries were mixed overnight at 4°C in the presence of protease inhibitor, PMSF.

The immunoaffinity beads were placed in small columns and the extracts collected. The immunoaffinity columns were washed 3 times with 5 ml aliquots of column buffer. To remove associated proteins from the dCMP hydroxymethylase enzyme, the columns were eluted with 0.3-ml aliquots of column buffer containing 1.0 M KCl. To elute the dCMP hydroxymethylase enzyme the low affinity immunoaffinity column was washed with 0.3-ml aliquots of 50 mM HEPES, pH 7.6, 1.5 $MgCl_2$, and 10% glycerol. The high affinity column was eluted with 100 mM glycine, pH 2.2. After the elutions a small sample of both gel matrices was counted for bound labeled protein. The high affinity immunoaffinity column retained a total 120,000 cpm while the low affinity column had 80,000 cpm attached. The collected fractions were counted for radiolabeled protein (Figure III-12). A 12% polyacrylamide gel was run with the 1.5 M $MgCl_2$ eluted fractions, Coomassie stained, and placed under film (Figure III-9).

The 1.5 M-eluted dCMP hydroxymethylase was concentrated in a Centricon-30 device and brought up in buffer to be used to study the protein-protein interaction 15 mM potassium glutamate, pH 7.6, 15 mM Mg acetate, 20 mM KCl. The protein concentration of the purified dCMP hydroxymethylase was determined by carrying out a Bradford assay. A total of 14.3 µg of dCMP hydroxymethylase containing 440,000 cpm, 30,600 cpm/µg was purified.

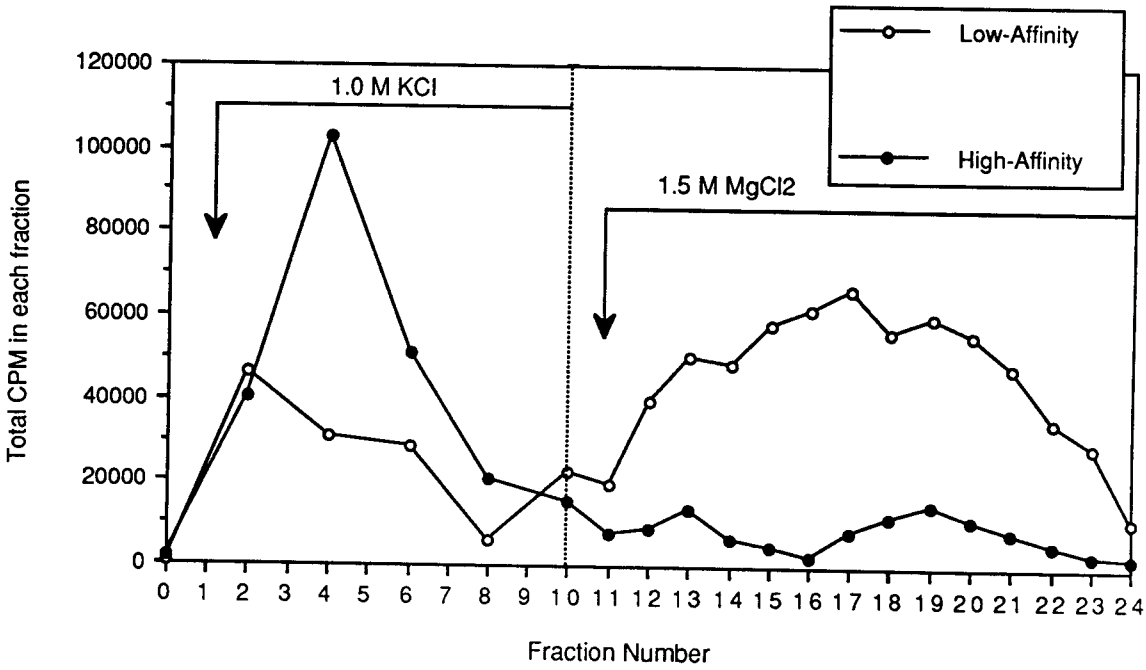


Figure III-12. Elution of ^{35}S-Labeled T4 Proteins From Low and High Affinity Anti-dCMP Hydroxymethylase Columns. The two columns were washed with 1.0 M KCl to remove contaminating proteins. The elution of dCMP hydroxymethylase was carried out by the passage of 1.5 M $MgCl_2$ over the columns.

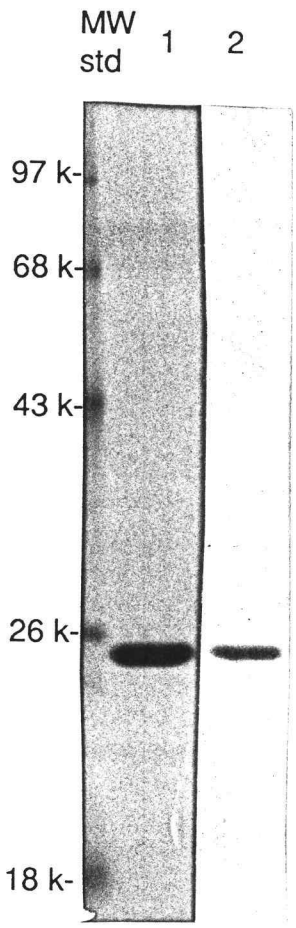


Figure III-13. Polyacrylamide Gel Electrophoresis of ^{35}S-Labeled dCMP Hydroxymethylase. Lane 1 corresponds to the dCMP hydroxymethylase enzyme eluted with 1.5 M $MgCl_2$ from the low affinity antibody column. Lane 2 represents a 24 hour film exposure of the eluted dCMP hydroxymethylase.

Results

To more fully understand the interaction between dCMP hydroxymethylase and T4 thymidylate synthase an attempt was made to map the anti-idiotypic binding domain to T4 thymidylate synthase. The generation of 3' deletions within the T4 thymidylate synthase gene was carried out. As seen in Figure III-1 the deletion of plasmid sequence using the ExoIII nuclease was quite controllable. However, the transformants resulting from these well defined deletions often gave quite random lengths for the plasmids within the AR120 strain, see Figure III-2, gel A. Several clones were shown to contain deletions of the correct size for epitope mapping, and were able to be induced as shown in Figure III-3. However, the cloned C-terminal deletions were all determined to be insoluble, data not shown.

The expression of soluble C-terminal deletions of T4 thymidylate synthase was possible using an *E. coli* S30 *in vitro* transcription/translation system. As shown in Figure III-4, an immunoprecipitation of a mixture of full length and truncated products showed that only the full length protein was recognized by the anti-idiotypic antibody.

An initial experiment to examine the interaction of the two purified enzymes was carried out involving the passage of T4 thymidylate synthase over an immobilized dCMP hydroxymethylase column. The protocol for this experiment used conditions that were similar to previous studies that involved the passage of crude T4 lysates over an immobilized dCMP hydroxymethylase column (Wheeler et al., 1992). In the previous study the immobilized dCMP hydroxymethylase column bound thymidylate synthase specifically at 0.2 M NaCl and eluted the enzyme at 0.6 M NaCl. As seen in figure III-5 the retention of purified thymidylate synthase on the dCMP hydroxymethylase column is low even at 0.2 M NaCl. The significance that 20% of the thymidylate synthase was

initially retained and eluted at 0.2 M NaCl is unclear with this type of affinity experiment. To try to examine the interaction in a more informative manner an attempt was made to study the two proteins using analytical affinity chromatography. Using an equilibrium binding method a member of our lab, Dr. Wayne Thresher, had previously shown that the dissociation constants for macromolecules could be determined by immobilization of enzymes on controlled-pore glass. The purification of the phage thymidylate synthase and ^{35}S-labeled dCMP hydroxymethylase was carried out to initiate such studies.

The bacteriophage T4 thymidylate synthase gene contains a 1017- base pair intron (Chu et al., 1984), which could affect the overexpression of the enzyme. The bacteriophage introns are related to the class I introns found in certain eukaryotic genes and have been shown to self-splice. However, additional bacteriophage gene products may exist within an infected *E. coli* that increase the rate of splicing. The phage thymidylate synthase has previously been cloned into several overexpression systems which either have the full length intron present or have the intron completely deleted (Belfort et al., 1985 and West et al., 1986). A comparison of the two systems was made to determine if the presence of the intron affected the degree of induction of the phage thymidylate synthase.

The test inductions as shown in Figure III-6 demonstrate that the presence of the intron did not greatly affect the expression of the thymidylate synthase enzyme. The intron-containing construct surprisingly showed a faster induction of activity than the intron deleted clone. However, the intron-deleted construct did have slightly higher maximum induction of thymidylate synthase activity. Previous studies by West et al. (1986) had demonstrated no difference in the time of expression for the two plasmids, and they made no comparisons on maximum activities. The differences that I observed were small and could be due to natural variation in the AR120 strain and not an intrinsic effect of the *td*

intron. The intron-deleted overexpression plasmid was selected for the large scale induction of thymidylate synthase.

The purification of thymidylate synthase generally yielded between 6 to 10 mg of purified enzyme. The use of cation exchange chromatography was very useful in the purification. The phage thymidylate synthase showed a strong affinity for the cation column and could be separated from the majority of *E. coli* proteins, see lane 3 Figure III-8.

A range of values have been reported in the past for the K_m value of T4 thymidylate synthase, with the the lowest value equaling 20 μM for enzyme purified from infected *E. coli* (Capco et al., 1973). The variation in apparent steady-state values may be simply due to differences in the assay conditions used. The phage thymidylate synthase has also been found to be sensitive to storage conditions and is easily denatured. In the past multiple purification steps were required for the purification of the enzyme from infected *E.coli* extracts. The intrinsic instability of the thymidylate synthase may also play a role in the wide variations in the steady-state values.

The determination of the K_m values from the overexpressed phage thymidylate synthase, as shown in Figures III-9 and III-10, were lower than previously observed. The average K_m value was determined to be 6.5 μM for dUMP and 43 μM for 5,10-methylene tetrahydrofolate. The measure of enzyme efficiency, $kcat/K_m$, for T4 thymidylate synthase was determined to be 1.6×10^6 $(s^{-1})(mol/L)^{-1}$ while dCMP hydroxymethylase has a $kcat/K_m$ that is only 7.6×10^3 $(s^{-1})(mol/L)^{-1}$(Pizer and Cohen, 1962).

The study of the interactions of the two enzymes by analytical affinity chromatography involves the measurement of the mobile macromolecule over a wide range of concentrations. To study the interaction at low dCMP hydroxymethylase concentrations the generation of ^{35}S labeled dCMP

hydroxymethylase was attempted. A one step purification of the labeled dCMP hydroxymethylase enzyme was attempted by using a novel chromatography method described by Kellogg and Alberts (1992), which involves purification of enzymes by generation of a low-affinity antibody column which elutes purified protein under non-denaturing conditions. As shown in Figure III-12, the elution of radioactive protein was seen when a low-affinity antibody column was washed with 1.5 M $MgCl_2$. A column composed of high-affinity anti-dCMP antibodies was constructed as a comparison control. Surprisingly the high affinity antibody column did not elute the dCMP hydroxymethylase enzyme even at pH 2.2. Figure III-13 demonstrates that the eluted dCMP hydroxymethylase was highly purified.

For an initial attempt to use the two proteins in an analytical affinity experiment 6 mg of the purified thymidylate synthase was coupled to controlled-pore silica beads as described previously (Thresher and Swaisgood, 1990). After coupling, the immobilized thymidylate synthase was determined to be highly active. However, the immobilized enzyme was determined to be unstable and was found to lose a majority of activity during a set of control experiments.

Discussion

The generation of 3' deletions within the plasmid encoded T4 thymidylate synthase gene failed to map the epitope binding domain of the dCMP anti-idiotypic antibody. The overexpression of truncated thymidylate synthase within a AR120 *E. coli* strain generated only insoluble protein. However, the *in vitro* generation of soluble thymidylate synthase truncations was successful. The inablility of the truncations to be immunoprecipitated by the anti-idiotypic antibody demonstrates that the C-terminal region is required for binding. These results indicate that either the anti-idiotypic antibody binds to the C-terminal end of the thymidylate synthase, or that the C-terminal section is required for the

proper folding of the protein-protein interaction domain which resides elsewhere on the thymidylate synthase.

An initial study demonstrated that purified thymidylate synthase appears to have lower affinity for immobilized dCMP hydroxymethylase than thymidylate synthase in a crude extract. While the discrepancy seen in the two experiments may be caused by the different buffers used in the affinity experiments, other possible explanations include the possibility that molecular crowding in the lysate experiments increases the affinity of thymidylate synthase for dCMP hydroxymethylase, or that the interaction between thymidylate synthase and dCMP hydroxymethylase is stabilized by other proteins that interact with the immobilized dCMP hydroxymethylase.

The comparison of the overexpression of intron deleted thymidylate synthase with the wild type gene demonstrated no time induction lag with the intron-containing gene and in fact the induction of the intron containing plasmid was faster than the intron-deleted plasmid. A slightly higher overall induction of activity was seen with the intron-deleted gene.

Finally, we extend the demonstrated versatility of the low-affinity antibody method for purification of enzymes from T4-infected extracts.

CHAPTER 4

Investigation of Protein-Protein Interactions Involving RegA Using Anti-idiotypic Antibodies and Protein Affinity Columns

Introduction

Soon after the infection of *E. coli* by bacteriophage T4 rapid transcription of the phage genome occurs within the host cell. During the 25-minute T4 reproductive cycle both transcriptional and translational regulation occurs within the infected *E. coli*. The *regA* gene product is a small protein of 15kDa which is known to act as a translational regulator of at least 12 T4 early and middle T4 genes (Webster et al., 1989). The levels of RegA protein in the infected cell are also translationally autoregulated, and the protein is present at low levels, 1.5 to 7.5 μM (Miller et al, 1985).

The RegA protein represses translation through occlusion of a portion of the mRNA sequence recognized by the ribosome during translation initiation (Gold, 1988). It has been demonstrated in a plasmid-encoded *regA* system that the *regA*-mediated translational repression requires no other T4 products (Miller et al., 1987). RegA-sensitive mRNAs have neither a common secondary structure nor sequence homology at the binding site. Many of the mRNAs which are targets do contain a uridine-rich area at the translation initiation site. However, the position and amount of uridine varies greatly among transcripts.

Work from this laboratory and others has demonstrated that bacteriophage T4 employs a multienzyme complex to synthesize deoxyribonucleotides. Experiments with T4 mutants demonstrated that the *regA* gene product is necessary to retain kinetic and physical integrity of this complex (Moen et al., 1988).

Why is the *regA* gene product necessary for the formation of the dNTP complex? We have proposed that either the *regA* gene product interacts directly with the complex as an integral member of the complex or interacts through protein-protein interactions during the assembly.

A second possible explanation is that the *regA* gene product does not directly interact with the dNTP complex but instead is involved in coordinating the stoichometric levels of the individual enzymes of the complex at the proper concentrations to allow proper association. To determine which of the two possible proposals is correct we have set out in this study to determine whether the RegA protein is interacting with any other T4 or *E. coli* proteins.

Two methods which have been used successfully in the past in our lab to identify protein-protein interactions were carried out to identify any possible associations of dNTP biosynthesis enzymes with the RegA protein. The first method involved immobilization of RegA protein onto an agarose matrix and using it for affinity chromatography of lysates of [^{35}S] methionine-labeled T4-infected *E. coli* extracts. A second complementary method to identify protein-protein interactions involved the attempt to generate anti-idiotypic antibodies to the RegA protein. The anti-idiotypic antibodies would serve as immunological mimics of the RegA protein binding domains, and allow the identification of protein-protein interactions.

Materials and Methods

Materials:

The MPL+TDM adjuvant used in generating the antibody response in rabbits was purchased from RIBI ImmunoChem Research Inc. Freund's

complete adjuvant was purchased from Sigma. Protein A-Sepharose was purchased from Pharmacia. Affi-Gel 15 was purchased from Bio-Rad Laboratories. NitroScreen West immunoblot membrane was purchased from NEN Research, Dupont. RegA protein used for these experiments was a generous gift from Drs. William Konigsberg and Eleanor Spicer. For the control bovine serum albumin (BSA), affinity column, 1.2 mg fraction V-protease free, was purchased from Sigma.

Preparation of a RegA Affinity Column:

RegA, 1.93 mg, was dialyzed overnight in a coupling buffer consisting of 300 mM NaCl, 100 mM MOPS, pH 7.5. The RegA protein was then concentrated to 1200 µl after dialysis. A 400-µl slurry of Affi-Gel 15 was thawed and washed at 4°C with 5 ml water followed by 5 ml coupling buffer. The gel slurry was incubated with the RegA protein for 4 hours at 4°C on a rotating platform. The Affi-Gel reactive group was quenched with 25 µl of 1 M monoethanolamine, pH 8.0. The amount of uncoupled protein was determined by a Bradford protein assay. The coupling efficiency was determined to be 66%, yielding a RegA protein affinity column with 1.27 mg of bound protein.

Generation of Rabbit Anti-RegA Polyclonal Antibodies:

For the generation of the initial anti-RegA antibody, two female New Zealand White rabbits were each injected at an intramuscular site with 800 µg of native RegA protein. The RegA protein had been previously dialyzed into PBS, concentrated to 1 mg/ml, and vortexed with an equal volume of MPL+TDM synthetic adjuvant. After 4 weeks the rabbits were boosted with an additional 800 µg RegA. Two weeks following the booster injections cardiac

punctures were carried out and 30 ml of blood was collected from each rabbit. A second bleed was carried out two weeks after the initial bleed. No additional boosting with RegA protein was carried out between the two collections.

An immunoblot was carried out to determine whether anti-RegA antibodies had been generated (Figure IV-1). As determined by the immuno-blot, both rabbits had generated anti-RegA antibodies, with rabbit B apparently giving a larger response to the RegA injections. The crude sera showed high backgrounds when incubated with T4-infected *E. coli* extracts.

Purification of Anti-RegA Antibody:

The first step in purification of the anti-RegA antibodies was passage of the crude sera over a protein A-Sepharose Cl-4B column (Harlow and Lane, 1988). Before the protein A purification, 4.5 ml of serum was thawed and centrifuged 15,000 g for 15 minutes and then a 1:10 volume of 1 M Tris, pH 8.0 was added. The serum was passed over a 2-ml protein A-Sepharose Cl-4B column. The column was washed with 10 volumes of 10 mM Tris, pH 8.0. The rabbit IgG was eluted with 100 mM glycine, pH 3.0, and ten 1.5-ml fractions were collected. The glycine buffer was neutralized by the addition of 100 µl of 1 M Tris, pH 8.0. Bradford protein assays were carried out to identify the fractions containing IgG.

The protein A column was found to be quite stable, and the purification of 30 ml serum from rabbit B was carried out by repeated passage of serum samples over the protein A column. After purification of rabbit B serum the column was washed with 10 column volumes each of 2 M urea, 1 M LiCl, and finally with 100 mM glycine, pH 2.5. Purification of rabbit A serum was then carried out as described previously. The typical yield of IgG was between 1-3 % of the total protein in the crude serum.

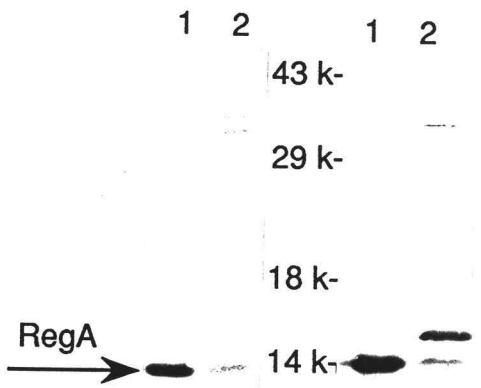


Figure IV-1. The Generation of Anti-RegA Antibodies. An immunoblot was carried out to determine whether anti-RegA antibodies were generated in the two rabbits. A 15% polyacrylamide gel was run and the proteins were transferred to NitroScreen. Lanes 1 and 3 were loaded with 0.5 μg of purified RegA protein. Lanes 2 and 4 were loaded with 20 μg of crude bacteriophage T4-infected *E. coli* extract. The immunoblot was divided in half and lanes 1 and 2 were incubated with serum A, diluted 1:200, while lanes 3 and 4 were incubated with serum B diluted 1:200. Serum B showed the stronger signal to RegA and was also cross-reactive to an unidentified 16-kDa protein.

The second step in purification of the RegA antibodies involved passage over a RegA protein affinity column. The affinity purification was carried out to separate the anti-RegA antibodies from the non-specific IgG protein. A RegA protein Affi-Gel 15 column which contained 1 mg of RegA protein was washed with 5 column volumes of PBS, 137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4. For each affinity purification run, 2.3 mg of rabbit IgG was passaged three times through the column at a flow rate of 0.1 ml/min. The column was washed with 20 bed volumes of 4°C PBS. The RegA antibodies were eluted with a 100-mM glycine wash, pH 2.2. The eluted RegA antibodies were collected into 1-ml fractions and neutralized with 1 M phosphate pH 8.0. For the affinity purification 7% of the total IgG from rabbit B bound to the RegA protein column (Figure IV-2). For each 1 ml of serum from rabbit B roughly 100 µg of purified anti-regA antibody was recovered. Purification of rabbit A anti-RegA antibody consistently produced lower yields of the purified antibody, approximately 3% of the total IgG.

Papain Digestion of Anti-RegA IgG to Fab Fragments:

For the digestion of anti-RegA to Fab fragments 1:20 volume of 1 M cysteine, and EDTA, 1 mM final concentration, were added to the purified anti-RegA antibodies. Both protein A-purified RegA antibody and protein A-purified plus affinity-purified RegA antibodies were used as substrates to generate Fab fragments. The antibody had been previously dialyzed into 100 mM Na acetate, pH 5.5, and concentrated to 5 mg/ml. For 1 mg of IgG, 10 µg of papain was added and incubated at 37°C for 2 hours (Figure IV-3). To stop the digestion a 1:10 addition of 750 mM iodoacetamide was added.

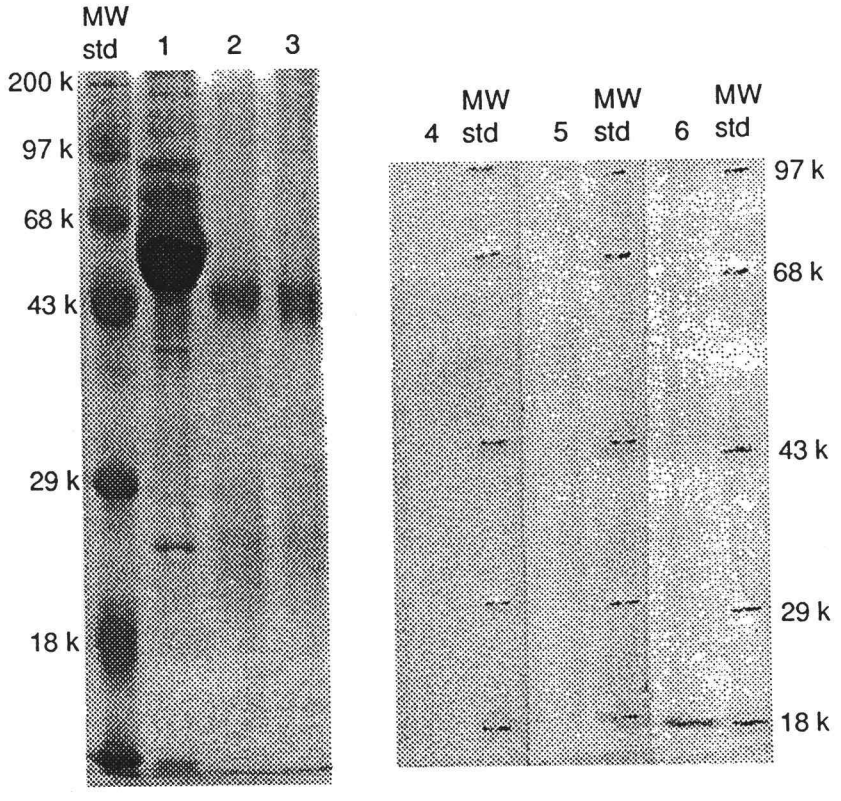


Figure IV-2. Anti-RegA Rabbit Antibody Affinity Purification.
A 13% polyacrylamide gel was run and Coomassie blue-stained containing the three collected fractions from the anti-RegA antibody purification. Lane 1 was loaded with 10 μg of the protein-A column flow through. Lane 2 was loaded with 3 μg of the RegA column flow through. Lane 3 contains 3 μg of protein that was eluted from the RegA affinity column. An immunoblot was also carried out with 30 μg of T4-infected *E. coli* lysate in each of three blots. Each immunoblot was incubated with an equivalent amount of protein, 0.5 μg (total protein) at a concentration of 100 ng/ml. Lane 4 was incubated with the protein-A column flow through. Lane 5 was incubated with the RegA column flow through, while lane 6 was developed with the protein that bound to the RegA affinity column. All three immunoblots were developed with equivalent amounts of alkaline phosphatase secondary antibody.

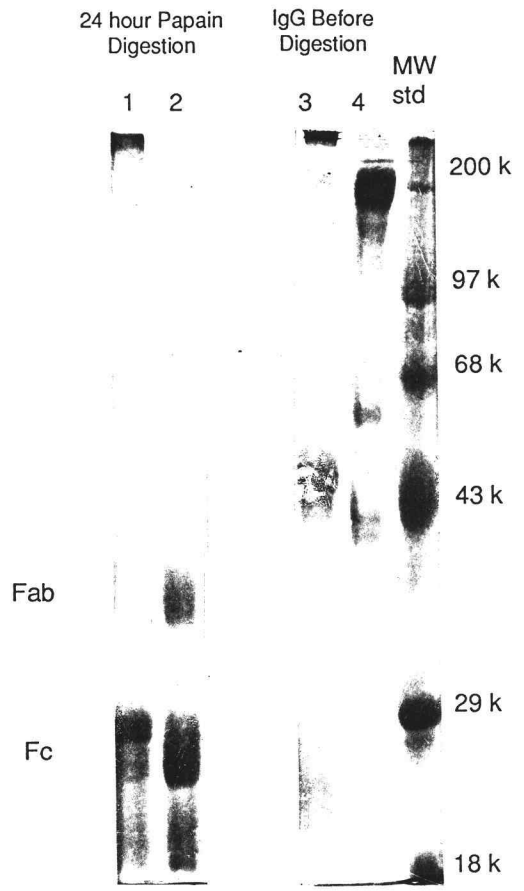


Figure IV-3. Generation of Anti-RegA Fab Fragments by Papain Digestion. A 24-hour digestion of anti-RegA rabbit antibody was carried out and a 10% polyacrylamide gel was run. Lane 1 contained anti-RegA IgG that had been digested for 24 hours with papain and was loaded onto the gel in sample buffer containing ß-mercaptoethanol. Lane 2 is a similar sample but loaded without ß-mercaptoethanol. Lane 3 and Lane 4 were loaded with anti-RegA IgG that was not treated with the protease.

Separation of Fab Fragments from Fc Regions:

After digestion of the RegA antibodies the fragments were mixed with 2 volumes of PBS and concentrated in a Centricon 30 device. The fragments were purified by a final passage over a RegA protein affinity column as described before for the purification of the intact antibody. When protein A purified plus affinity purified regA antibodies were used in the papain digestion the yield of affinity purified Fab approached 20% of the initial RegA antibody concentration (Figure IV-4). When protein A-purified RegA antibody was digested and purified the yield was less than 1% of the initial antibody protein concentration.

Generation of Mouse Anti-idiotypic Antibodies:

For the generation of anti-idiotypic antibodies Swiss-Webster mice were injected intraperitoneally with 50 µg of affinity-purified RegA Fab (Harlow and Lane, 1988). The Fab fragments were mixed prior to the injection with 9 volumes of complete Freund's adjuvant. The total volume of the injected material was 200 µl. Each mouse received Fab fragments from only one Fab purification. Fab fragments originating from different rabbits were not mixed during the purification nor during the injections. For each anti-RegA Fab isolate from an individual rabbit, two mice were injected with identical purified Fab. After 28 days the first booster injection was administered by using the same protocol as the primary injection. Additional boosts were then carried out on a weekly basis.

After three booster injections all mice showed signs of peritoneal swelling but the development of ascites fluid did not occur. Tail bleeds were carried out on the mice as an alternative to collection of ascites fluid. An additional mouse which did not receive any injections was also bled. Finally as a second control a mouse was injected and boosted with Freund's complete adjuvant without Fab fragments.

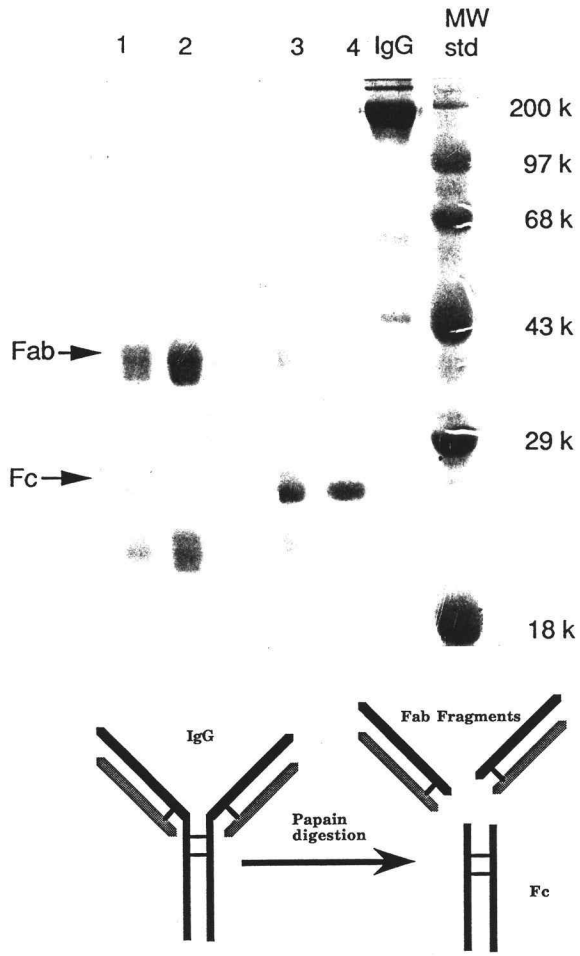


Figure IV-4. Affinity Purification of RegA Fab Fragments. A 12% polyacrylamide gel was loaded using sample buffer that did not contain ß-mercaptoethanol. Lane 1 was loaded with affinity purified Anti-RegA Fab fragment which was generated from protein A-purified rabbit A IgG. Lane 2 was loaded with affinity purified Anti-RegA Fab fragment which was generated from both a protein A column and a RegA affinity column purification of rabbit B IgG. Lane 3 represents the flow through of the affinity purification of the anti-RegA Fab from protein A-purified rabbit A IgG. Lane 4 is the flow through of the affinity purification of the anti-RegA Fab from the protein A column and a RegA affinity column purification of rabbit B IgG. The last lane contains purified anti-RegA IgG from rabbit B that was not treated with papain.

Testing for Anti-idiotypic Antibodies:

To test for the generation of mouse antibodies to the rabbit Fab fragments a preliminary slot blot was carried out after the first boost. Anti-RegA Fab fragments from each rabbit were bound to nitrocellulose along with a non-specific rabbit Fab as a control. The full length anti-RegA IgG from rabbit A and rabbit B were also blotted. Finally a crude extract of T4-infected *E. coli* was bound to the nitrocellulose. The slot blots were incubated with the sera of the anti-RegA Fab challenged mice. The secondary anti-mouse alkaline-phosphatase antibody was then added to the blots and also placed in a control blot that had not been incubated with a primary antibody (Table IV-1).

A western immunoblot was also carried out on the non-specific rabbit Fab and the anti-RegA Fab fragments (Figure IV-5). All the anti-Fab mice sera were able to stain a band corresponding to the rabbit Fab fragments. Non-specific rabbit Fab fragment was also developed by the anti-Fab mice sera, demonstrating that at least part of the immunogenic response generated in the mice was to a conserved area of the Fab fragments. Mouse serum injected only with Freund's complete adjuvant did not develop a band on the immunoblots.

After one booster injection anti-rabbit Fab sera failed to develop a positive signal against T4 proteins. Two more additional boosts were carried out and immunoprecipitations were then carried out.

Table IV-1. Generation of Mouse Antibodies to Rabbit Fab Fragments.

Mouse Serum	injected antigen	anti-RegA Fab A	anti-RegA Fab B	anti-RegA IgG A	anti-RegA IgG B	T4 Proteins	Control Rabbit Fab
A1	Rabbit Fab A	+	+	+++	+++	-	++
B1	Rabbit Fab B	++	++	++++	++++	-	++
Control, anti- mouse 2°		-	-	-	-	-	-

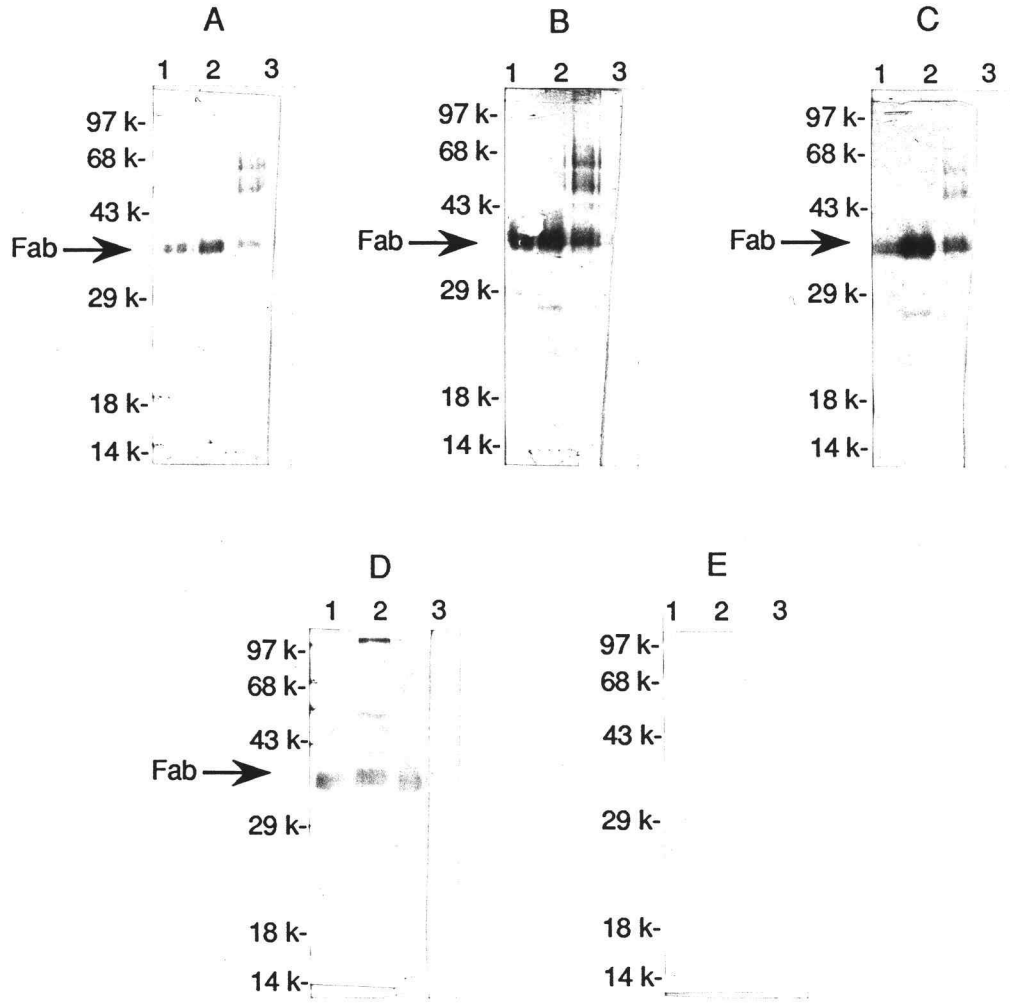


Figure IV-5. The Generation of Anti-Fab Antibodies. An immunoblot was carried out to confirm that anti-Fab antibodies were generated in the mice. A 12% polyacrylamide gel was run and the proteins were transferred to NitroScreen. Lanes 1 and 2 of each immunoblot was loaded with 0.2 µg of affinity purified RegA Fab fragments. Lane 3 for each immunoblot was loaded with 0.2 µg of non-specific rabbit Fab fragment. The Fab fragments were loaded onto the gel in sample buffer that did not contain ß-mercaptoethanol. Immunoblot A, B, C, and D were developed with 1:1000 dilution of A1, A2, B1, B2 anti-rabbit Fab mouse sera, respectively. Immunoblot E was developed with 1:1000 dilution of Freund's injected mouse serum.

Immunoprecipitation with Mouse Anti-anti-RegA:

T4 and *E. coli* proteins were labeled by incorporation of [^{35}S]methionine in a growing culture. The T4-infected culture was labeled from 3 to 8 minutes post infection. The *E. coli* samples were labeled for 20 minutes. The samples were sonicated and unless otherwise stated treated with DNase I 10 µg/ml and micrococcal nuclease 3 µg/ml as described previously (Wheeler et al., 1992). Immunoprecipitations were carried out in either a "mild" NP-40 buffer: 150 mM NaCl, 1.0% NP-40, 50 mM Tris-HCl, pH 8.0 (Figure IV-6 and IV-7) or a more stringent RIPA buffer: 150 mM NaCl, 1.0% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris pH 8.0 (Figure IV-8). The sera and labeled extracts were incubated at 4°C, 100 µl of Protein A beads were added and incubation continued for two hours. The Protein A beads were washed three times by resuspension in either 1 ml of NP-40 or RIPA buffer, followed by centrifugation. Each one-ml aliquot of buffer was allowed to sit for 10 minutes between centrifugations. 20 µl of 4x loading buffer--40% glycerol-4% SDS-4% 2-mercaptoethanol-0.5 M Tris-HCl, pH 6.8--was added and the samples were heated at 80°C for 15 minutes. The samples were run on polyacrylamide gels, and treated with 1 M sodium salicylate, dried and placed over film.

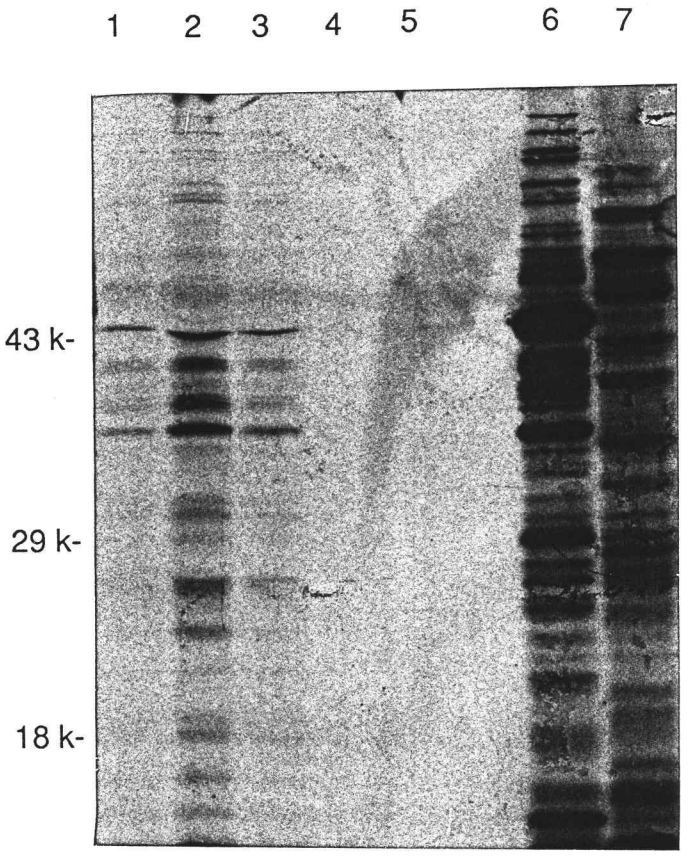


Figure IV-6. Immunoprecipitations in NP-40 Buffer of ^{35}S-Labeled T4 and *E. coli* Proteins. Lanes 1 through 3 depict immunoprecipitations of a ^{35}S-labeled T4-infected *E. coli* extract. Lanes 4 and 5 are immunoprecipitations of ^{35}S-labeled *E. coli* extract. The following antibodies were used: Lane 1, pre-immune mouse antibody; Lane 2, anti-rabbit Fab (B1) mouse serum; Lane 3, anti-rabbit Fab (A1) mouse serum; Lane 4, anti-rabbit Fab (B1) mouse serum; Lane 5, anti-rabbit Fab (A1) mouse serum; Lane 6, ^{35}S-labeled T4-infected *E. coli* extract ; Lane 7, ^{35}S-labeled *E. coli* extract.

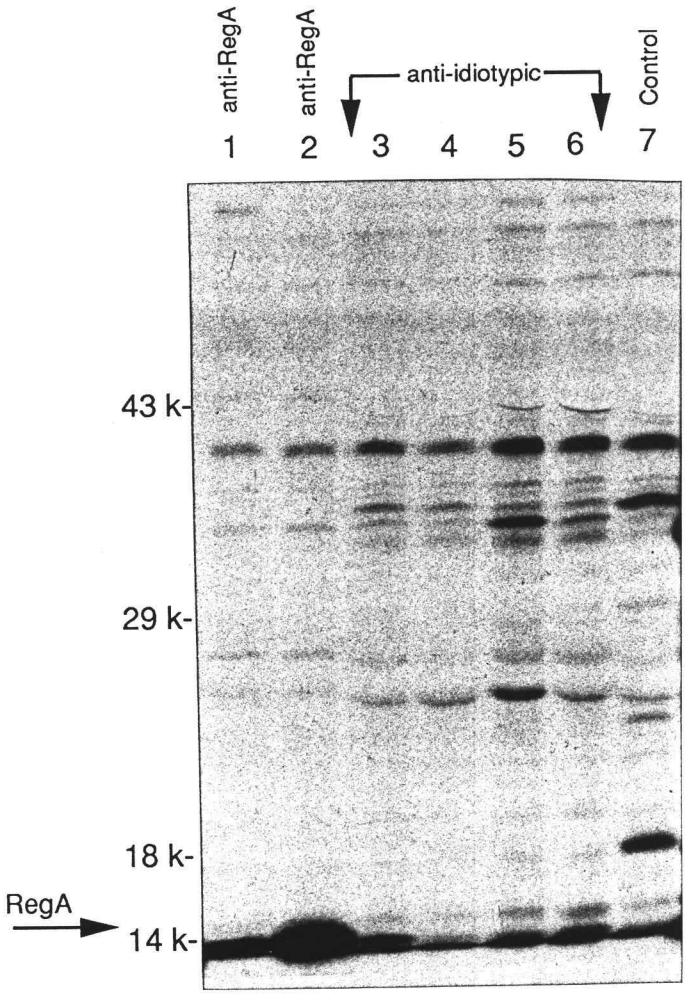


Figure IV-7. Immunoprecipitation in NP-40 Buffer with Anti-RegA Sera, Anti-rabbit Fab Sera, and Freund's Challenged Mouse Serum. Lanes 1 through 7 depict immunoprecipitations of a ^{35}S-labeled T4-infected *E. coli* extract. The following antibodies were used: Lane 1, anti-RegA (A) affinity purified rabbit IgG; Lane 2, anti-RegA (B) affinity purified rabbit IgG; Lane 3, anti-rabbit Fab (A1) mouse serum; Lane 4, anti-rabbit Fab (A2) mouse serum; Lane 5, anti-rabbit Fab (B1) mouse serum; Lane 6, anti-rabbit Fab (B2) mouse serum; Lane 5, anti-rabbit Fab (B1) mouse serum; Lane 6, Freund's injected mouse serum.

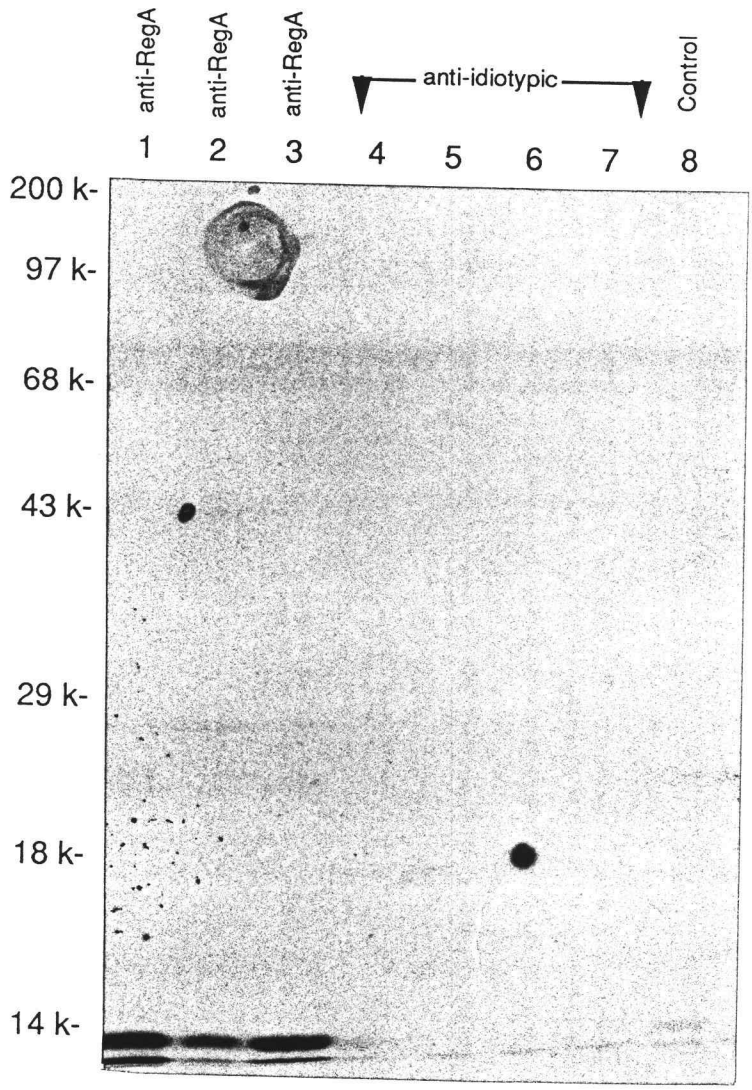


Figure IV-8. Immunoprecipitation of ^{35}S-Labeled T4 Proteins in RIPA Buffer. Lanes 1 through 8 depict immunoprecipitations of a ^{35}S-labeled T4-infected *E. coli* extract carried out under identical conditions as done in Figure IV-6, except that the buffer used was RIPA instead of NP-40.. The following antibodies were used: Lane 1, anti-RegA polyclonal serum from Eleanor K. Spicer, Yale University; Lane 2, anti-RegA (A) affinity purified rabbit IgG; Lane 3, anti-RegA (B) affinity purified rabbit IgG; Lane 4, anti-rabbit Fab (A1) mouse serum; Lane 5, anti-rabbit Fab (A2) mouse serum; Lane 6, anti-rabbit Fab (B1) mouse serum; Lane 6, anti-rabbit Fab (B2) mouse serum; Lane 7, anti-rabbit Fab (B1) mouse serum; Lane 8, Freund's injected mouse serum.

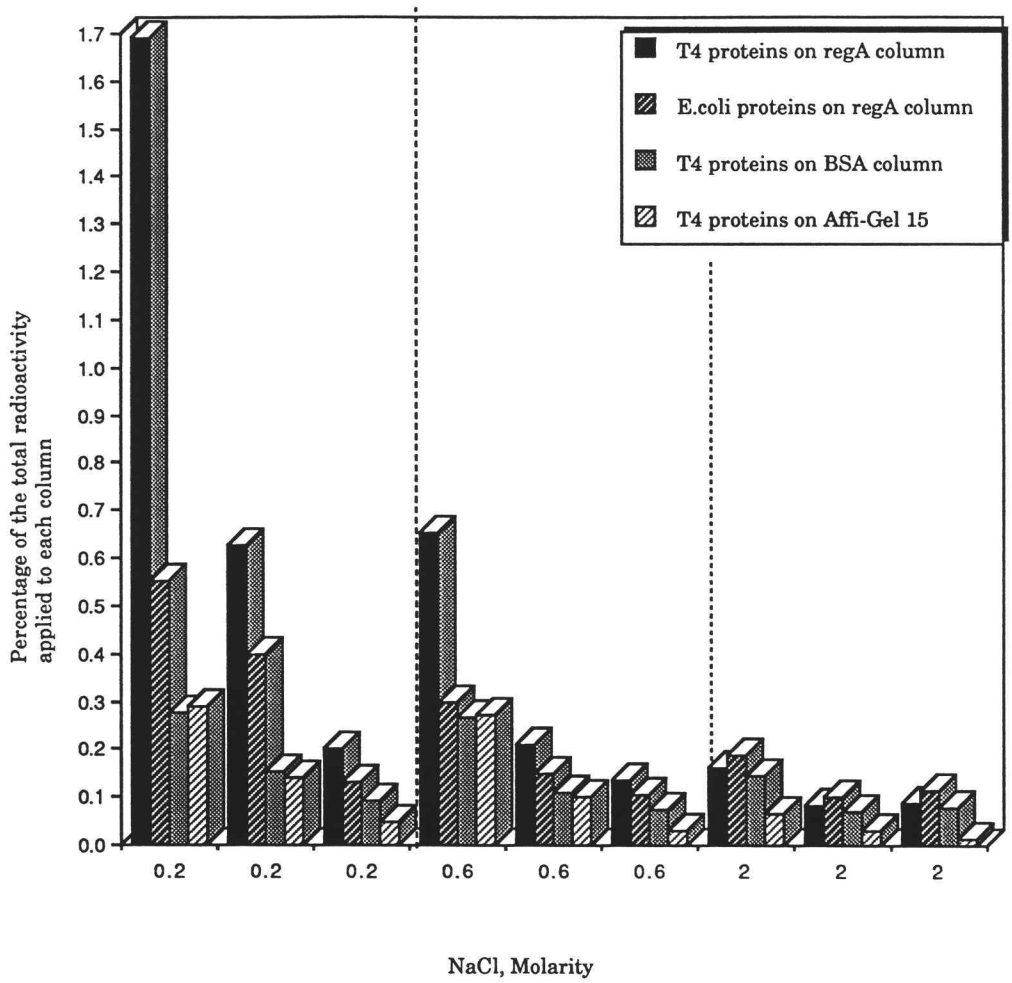
Specific Associations of *E. coli* and T4 proteins with Immobilized RegA:

To examine interactions of bound RegA protein with *E. coli* and T4 proteins labeled [35] S-methionine extracts of *E. coli* and T4 infected *E. coli* were made as described previously. The T4-infected culture was labeled from 3 to 8 minutes post infection, and the *E. coli* samples were labeled for 20 minutes. Before being passed through the RegA affinity column, the labeled extracts were centrifuged at 15,000 g for 5 minutes. A total of $3x10^6$ cpm of labeled extract was mixed with the RegA Affi-Gel matrix in 1 ml of column buffer,1 mM EDTA, 1 mM ß-mercaptoethanol, 5 mM $MgCl_2$, 10 % (W/V) glycerol, 25 mM NaCl, 20 mM Tris-HCl, pH 8.0, overnight at 4°C. The RegA matrix was placed in a small column and washed with increasing salt concentrations, 25 mM, 200 mM, 600 mM, 2 M NaCl. For each salt concentration three sequential washes of 2 ml were carried out with each 2 ml volume allowed to recirculate for 15 minutes before being drained off and counted for radioactivity. The column was further washed with 2 M NaCl until no additional cpm were seen in the wash. The column was re-equilibrated with 25 mM NaCl column buffer and the affinity experiment was repeated using labeled *E. coli* extract

The fractions from the affinity experiment were placed in scintillation cocktail and counted for ^{35}S methionine (Figure IV-9). The fractions were also concentrated and applied to a 10% polyacrylamide gel. Entire fractions of each of the three high salt washes, 200 mM, 600 mM, 2 M NaCl, were loaded on the gel. For the initial low salt wash, 25 mM NaCl, only 1 %, 25 µl, of the fraction was loaded on the gel for both the T4 and *E. coli* extracts (Figure IV-10).

As controls to identify possible non-specific interactions the above procedure was repeated by passing labeled T4 extract over a blocked ethanolamine Affi-Gel 15 column and a BSA coupled Affi-Gel 15 column (Figure IV-11). The BSA Affi-Gel column contained 1.2 mg of coupled BSA to the matrix.

Figure IV-9. Percentage Total Radioactivity of ^{35}S-Labeled T4 and *E. coli* Proteins Eluted from Affinity Columns. Aliquots were removed from the three sequential washes at a given salt elution concentration. The aliquots were placed in scintillation cocktail and the radioactivity determined. The percent of total applied radioactivity was calculated for the three highest salt washes 200 mM, 600 mM, 2 M NaCl, for each of the four affinity experiments; ^{35}S-labeled T4-proteins bound to a RegA column; ^{35}S-labeled *E. coli*-proteins bound to a RegA column; ^{35}S-labeled T4-proteins to a BSA column; ^{35}S-labeled T4-proteins to an ethanolamine-blocked Affi-Gel 15 column.


T4 proteins on regA column
E.coli proteins on regA column
T4 proteins on BSA column
T4 proteins on Affi-Gel 15
Percentage of the total radioactivity applied to each column
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
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2
2
2
NaCl, Molarity

Figure IV-9.

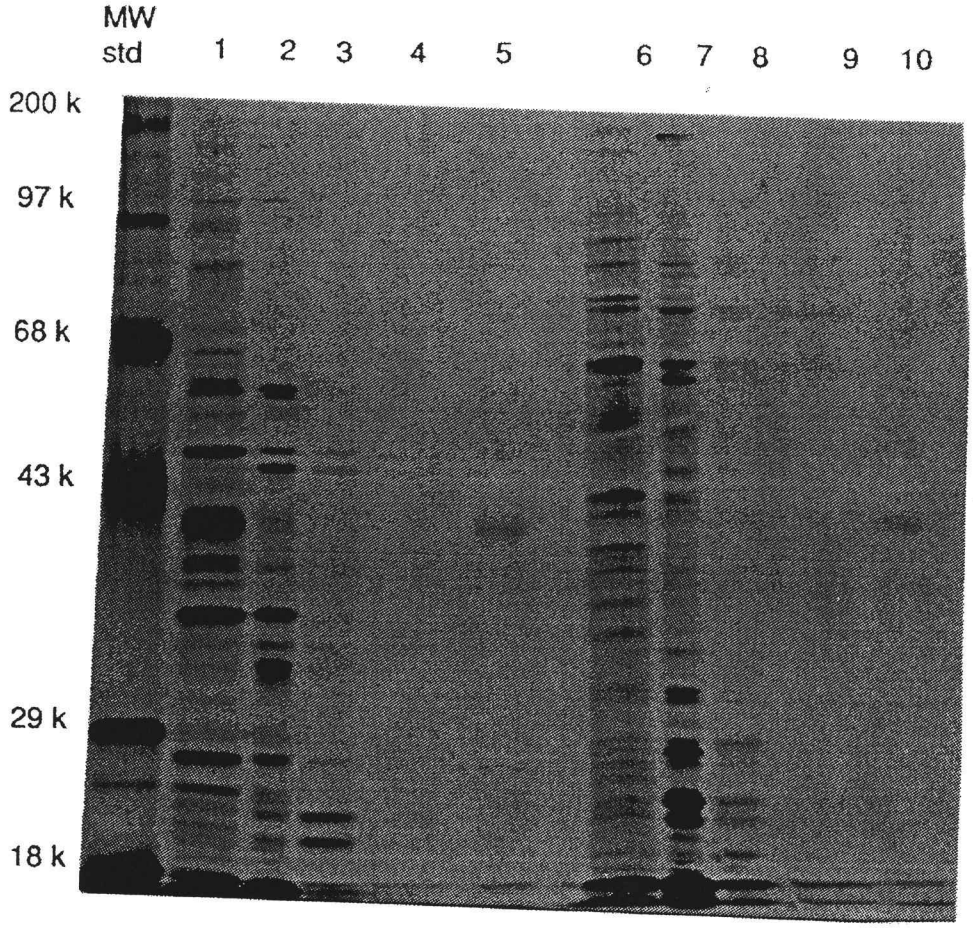


Figure IV-10. Analysis of T4 and *E. coli* Proteins Bound to a RegA Affinity Matrix. Lanes 1-4 depict ^{35}S-labeled T4-proteins that were eluted from a RegA Affi-Gel 15 column at increasing salt concentrations 0.025, 0.2, 0.6 and 2 M NaCl, respectively, as described in the Methods section. Lane 1 represents only 1% of the total eluted protein from the first 0.025 M wash. Lanes 2, 3, and 4 represent the entire fractions for each salt concentration. Following the final salt wash 20 µl of the RegA affinity gel matrix was removed and boiled in $NaDodSO_4$ sample buffer and loaded as lane 5. Lanes 6-9 depict ^{35}S-labeled *E. coli* proteins that were eluted from the RegA affinity column using identical NaCl concentrations as in lanes 1-4. Lane 10 depicts 20 µl of the affinity matrix after the final salt wash of the *E. coli* proteins. The 10% polyacrylamide gel was treated with 1 M sodium salicylate and placed on film at -70°C for 8 days.

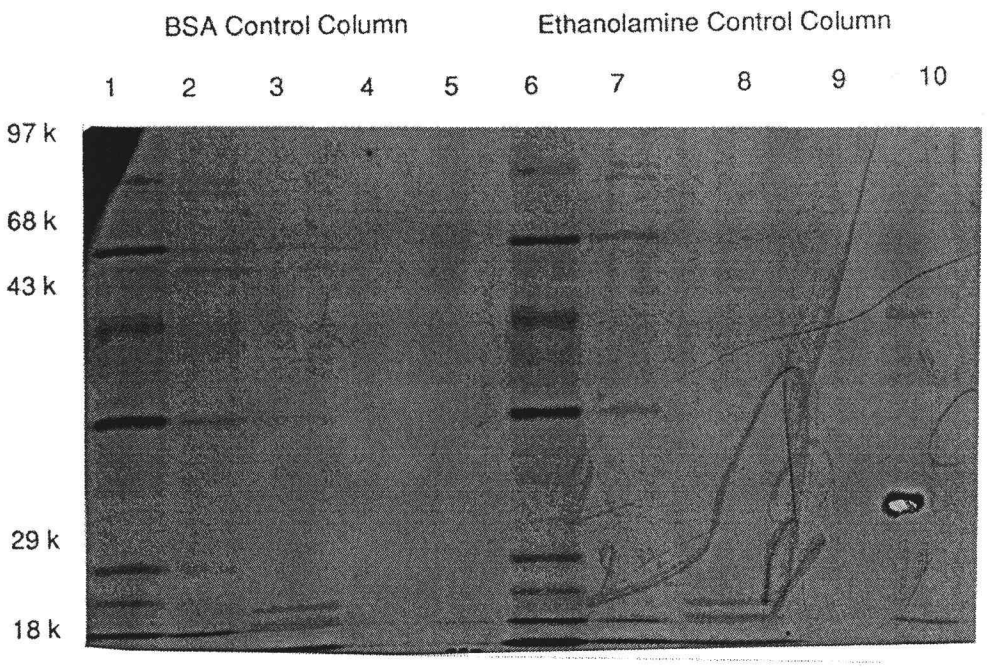


Figure IV-11. Analysis of T4 Proteins Bound to Control BSA Affi-Gel and Ethanolamine Affi-Gel Columns. The two control columns were incubated with an equivalent amount of ^{35}S-labeled T4-infected *E. coli* extract as passed over the RegA affinity column. Lanes 1-4 depict ^{35}S-labeled T4-proteins that were eluted from a BSA-Affi-Gel 15 column at increasing NaCl concentration; lane 1, 0.025 M; lane 2, 0.20 M; lane 3, 0.6 M; lane 4, 2 M NaCl. The boiled BSA column matrix, lane 5, contains no detectable protein. Lanes 6-10 depict T4 proteins eluted from an ethanolamine-blocked Affi-Gel 15 column at increasing NaCl concentrations as in lanes 1-4. The boiled ethanolamine Affi-Gel 15 column also had no detectable T4 proteins, lane 11.

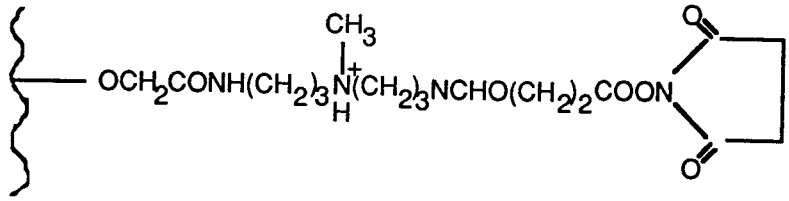
Results

RegA protein was coupled to an affinity column matrix to answer the question, does RegA interact with other bacteriophage T4 or host proteins. The column used in the affinity experiment was Affi-Gel 15, which is an N-hydroxysuccinimide ester of a derivatized crosslinked agarose matrix (Figure IV-12). Affi-Gel 15 is distinguished from other affinity supports by containing a positive charge in its 15-atom spacer arm.

For high coupling efficiency using Affi-Gel 15 the manufacturer recommends that the matrix and protein be incubated in a buffer that is above the pI of the protein. Under these conditions the protein of interest carries a net negative charge during the coupling reaction and is not repelled from the positive matrix.

A second type of agarose affinity matrix, Affi-Gel 10, contains a neutral charged spacer, and is recommended to be coupled in a buffer at or below the pI of the protein. For the above reasons the Affi-Gel 15 is generally used with acidic proteins while Affi-Gel 10 is used with neutral to basic proteins.

However, when looking at weak protein-protein interactions the most important factor in carrying out affinity chromatography is not the coupling efficiency of the bound protein, but the ability of the bound protein to interact with and bind soluble proteins in a manner that resembles the interaction found *in vivo*. If the protein-protein interactions being studied are dependent on ionic interactions it is possible that a majority of the proteins binding to the affinity column may carry a net charge that is opposite to that of the bound protein, i.e., a bound acidic protein will bind mostly basic proteins, and a basic protein will tend to bind negatively charged acidic proteins. An affinity column matrix that is opposite in charge to the bound protein allows efficient coupling but may interfere with protein-protein interactions due to the fact that the matrix would have the same charge as the soluble protein which have weak affinities to the bound protein.



Affi-Gel 15

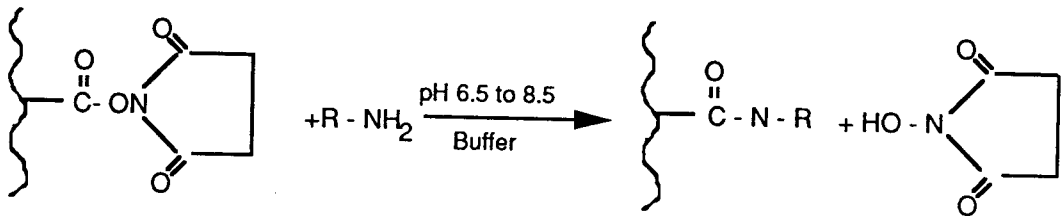


Figure IV-12. Structure and Coupling Reaction of Affi-Gel 15.

For the aforementioned reasons the affinity gel selected to bind RegA, a positive protein with a pI of 9.8, was Affi-Gel 15, which also carries a positive charge. To attenuate the charge-charge repulsion during the coupling reaction a high ionic strength buffer was used containing 0.3 M NaCl. Under these conditions the RegA protein was able to be coupled to the positive matrix at a yield of 66%.

The Affi-gel 15 RegA column retained a number of T4 and *E. coli* proteins that eluted at low NaCl concentrations as seen in Figure IV-10. The amount of labeled T4 proteins retained on the RegA column was significantly higher than found with the ethanolamine-blocked column matrix or a control BSA Affi-gel 15 column as seen in Figure IV-9. The majority of the proteins retained on the RegA column showed weak interaction and were eluted at low NaCl concentration of 200 mM. Two prominent T4 proteins were found to be eluted at a NaCl concentration of 600 mM, while 4 *E. coli* proteins were found to be eluted at the higher salt wash of 600 mM. However the two prominent T4 proteins that were eluted at 600 mM were also eluted by the control columns. A majority of the bacteriophage T4 early proteins have been identified and their apparent molecular weight migrations are known. Table IV-2 represents the tentative identification of the T4 proteins bound to the RegA column based upon their migration values through polyacrylamide gels. Several of the early T4 proteins that were eluted could not be identified because they did not have distinctive molecular weights that allowed unambiguous identification.

The preliminary identifications of the T4 proteins suggest that they are involved in DNA replication. While the most prominent bands corresponded to the migration values of the DNA polymerase accessory proteins only a weak band was eluted at 200 μM NaCl for DNA polymerase.

Table IV-2 Tentative Identification of Bacteriophage T4 Early Proteins that Specifically Associate with an Affi-gel 15 RegA Column

Early T4 Proteins Eluted at 0.2 M NaCl	
Mol. Wt. Observed	Tentative Identification by apparent MW
58,000	gp39 DNA topoisomerase, subunit large
57,500	gp41 primase subunit,
47,000	gp52 DNA topoisomerase, subunit small
46,500	?
36,000	gp44, DNA polymerase accessory protein
34,000	gp32, Single stranded DNA-binding protein
33,500	?
33,000	*pseT* or *td*, Thymidylate synthase
28,000	gp45, DNA polymerase accessory protein or gp42 dCMP hydroxymethylase

The generation and purification of anti-RegA rabbit antibody was successful but the yield was low. Due to the small supply of RegA protein further booster injections of the rabbits was not possible. Due to the small yield of affinity purified antibody an attempt was made to generate anti-idiotypic antibodies in mice. This cross immunization of rabbit Fabs into mice has been a successful method to generate anti-idiotypic antibodies in the past and was a recommended method in a review for the generation of anti-idiotypic antibodies (Catty, D. and Raykundalia, 1988).

The injection of the Fab anti-RegA developed a response. However, non-specific rabbit Fab was also recognized, demonstrating that at least part of the response was to a structural region of the Fab fragment and not the protein-protein interaction site.

After the first series of booster injections a strong response was seen generated in the mice to both the anti-RegA Fab fragments and to the anti-RegA IgG. However, no response was seen in the first test bleeds to T4-infected *E. coli* lysates. After additional boosts immunoprecipitations were carried out with the mouse serum samples.

As shown in Figure IV-6, the initial immunoprecipitations using an anti-idiotypic serum sample brought down a number of T4 proteins but no *E. coli* proteins. A control using pre-immune mouse serum did not immunoprecipitate the T4 proteins. Further experiments showed that the immunoprecipitation of T4 proteins was dependent on the type of buffer used during the incubations of sera with the labeled extracts. A second finding was that the control mouse serum which was injected with only Freund's complete adjuvant also brought down a large number of T4 proteins, Figure IV-7.

Specifically, imunoprecipitations using NP-40 buffer brought down a number of T4 proteins both in the serum samples collected from anti-RegA Fab

injected mice and in the Freund's injected control mouse. However, immunoprecipitations carried out in the more stringent RIPA buffer did not bring down the T4 proteins when using serum from either anti-RegA injected mice or the Freund's control, Figure IV-9.

From the following immunoprecipitations it was concluded that the T4 proteins that were immunoprecipitated were due to the repeated injections of Freund's complete adjuvant and not due to the generation of anti-idiotypic antibodies mimicking the RegA binding domains.

Freund's complete adjuvant contains a suspension of heat killed *Mycobacterium butricum* or *Mycobacterium tuberculosis*. The repeated injections of the denatured bacterial proteins to induce ascites fluid may be the cause of the high backgrounds seen in the immunoprecipitations using NP-40 buffer.

Discussion

The RegA protein is believed to be a global regulator of bacteriophage T4 early genes. The biological significance of this global regulation is not well understood. The RegA protein has been cloned and overexpressed but little is known about the functional domains of the protein. Although the RegA protein is nonessential during normal laboratory growth, it has been shown to be highly conserved in most phages studied (Miller and Jozwik, 1990).

The attempt to generate RegA anti-idiotypic antibodies was not successful in this study. The attempt to generate ascites fluid using repeated injections of Freund's complete adjuvant created extremely high backgrounds in the immunoprecipitations that were carried out under mild conditions. No T4 proteins were immunoprecipitated with the supposed anti-idiotypic sera when

a more stringent buffer, RIPA, was used in the immunoprecipitations. This leads me to conclude that either no anti-idiotypic antibodies were generated that recognized other T4 proteins, or that the anti-idiotypic antibodies associated weakly with the T4 proteins and were masked by the high background signals when NP-40 buffer was used.

In this study we attempted to determine whether the RegA protein is associating directly with the deoxyribonucleotide biosynthesis enzymes. By the generation of a RegA affinity column it was determined that certain T4 proteins do appear to interact with the RegA protein. This is the first direct evidence that the RegA protein may also be involved in protein-protein interaction within the T4-infected *E. coli.* Most of the associations were disrupted at a relatively low salt concentration of 200 mM NaCl. Two T4 proteins did show higher affinity and were eluted at 600 mM NaCl.

From past work the molecular weight migration on polyacrylamide gels of most of the early T4 proteins is known. The identification of the eluted proteins cannot be conclusively made by just the molecular weight migrations due to many early T4 proteins having equivalent values. However, from the tentative identifications a pattern emerges with the eluted proteins likely being proteins involved in T4 DNA replication. Enzymes involved in T4 deoxyribonucleotide biosynthesis may also be binding to the RegA column, but this can only be conclusively demonstrated by additional two-dimensional electrophoresis of the eluted proteins.

A past proposal for the biological role of the RegA protein suggested that it assisted in the formation of the DNA replisome (Campbell and Gold, 1982). This initial investigation for protein-protein interactions involving the *regA* gene product gives preliminary support to Campbell and Gold's theory that the

RegA protein is a "nonessential" component of the replication complex. Further study of the protein-protein interactions of RegA may uncover the significance that RegA has on the biology of T4. The RegA protein may still prove to be a bridge between bacteriophage T4 DNA replication and dNTP biosynthesis.

Summary and Future Directions

The research described in this thesis demonstrates that the generation of anti-idiotypic antibodies can provide information on enzyme protein-protein interactions. The identification of a dCMP hydroxymethylase anti-idiotypic antibody that recognizes the bacteriophage thymidylate synthase implicates a direct interaction between the two enzymes. The determination that the anti-idiotypic antibody is specific for the phage encoded thymidylate synthase, strengthens the argument that the antibody is demonstrating a direct interaction between the two enzymes.

Previous studies using affinity chromatography had identified the T4 thymidylate synthase as being one of approximately a dozen proteins that interacted with the immobilized dCMP hydroxymethylase. However, the affinity experiments did not resolve the question of which proteins were directly interacting with the immobilized enzyme or which were binding in a "piggyback", or indirect, fashion.

What biochemical function would be served by having the T4 thymidylate synthase and dCMP hydroxymethylase interact? As mentioned in the introduction there is precedent for a thymidylate synthase active site to be localized near a second catalytic site. In the case of parasitic organisms the thymidylate synthases are known to be bifunctional enzymes containing a dihydrofolate reductase activity within the same gene. The carboxy-terminal side of the bifunctional polypeptide contains a thymidylate synthase sequence that is very homologous to other thymidylate synthases which do not show multiple activities.

The purpose for the bifunctional nature of the parasitic enzyme has not been clearly demonstrated. A possible advantage for having the dihydrofolate reductase linked to the thymidylate synthase is the protection of the labile cofactor.

A possible role for the interaction of thymidylate synthase and dCMP hydroxymethylase in bacteriophage T4 would not be the protection of a substrate but more likely the regulation of the end products, dTTP and hm-dCMP.

From an organism's point of view evolution has not selected for the excellence of a single enzyme but rather the controlled output of the entire pathway which the enzyme is involved (Friedrich, 1984). While the interaction of the thymidylate synthase with the dCMP hydroxymethylase may not improve the ability of the two enzymes to catalyze their respective reactions the association may allow better coordination of their deoxypyrimidine products.

My research demonstrates that the anti-idiotypic antibody recognizes a unique feature on the T4 thymidylate synthase which the host enzyme does not possess. Mapping the epitope binding domain of the anti-idiotypic antibody could in theory identify the protein-protein binding domain on the bacteriophage thymidylate synthase and within the last year I have pursued the mapping of the epitope binding domain between the anti-idiotypic antibody and thymidylate synthase. A variety of techniques were attempted to map the epitope site including limited proteolysis of the thymidylate synthase and generation of C-terminal deletions of the cloned *td* gene. Both techniques failed to map the epitope site. A major hurdle in the attempts to map the epitope site is that the antibody only recognizes the

native T4 thymidylate synthase and not the denatured protein.

Recent efforts to map the epitope site have involved generation of C-terminally truncated thymidylate synthases using an in vitro transcription-translation system. With this technique I have determined that even a small deletion into the C-terminal region prevents recognition of thymidylate synthase by the anti-idiotypic antibody. This result implies that either the thymidylate synthase C-terminal region contains the epitope site or that the C-terminal region is needed for the overall correct conformation of the enzyme. It may prove that the intact dimer of T4 thymidylate synthase is required for the presence of the dCMP hydroxymethylase binding domain. Therefore, mapping of the epitope by cleavage of the thymidylate synthase protein or by deletion of sequence within the open reading frame will be ineffective.

Characterizing the interaction of the thymidylate synthase with dCMP hydroxymethylase through *in vitro* reconstitution of the two enzymes should be carried out. I have carried out purifications to homogeneity of both the thymidylate synthase and the dCMP hydroxymethylase for future studies. An interesting and worthwhile future study would be the determination of the effect upon the interaction of the two enzymes in the presence of substrates, deoxyribonucleoside monophosphates or methylene tetrahydrofolate.

A currently unexplained observation is the apparent high affinity that the dCMP hydroxymethylase anti-idiotypic antibody has for T4 thymidylate synthase while the initial studies using the two enzymes suggest a weak protein-protein interaction. This paradox suggests that the anti-idiotypic antibodies' binding domain does not truly mimic the dCMP hydroxymethylase surface and is instead an analog surface which has

higher affinity than the original binding domain.

The generation of the polyclonal anti-idiotypic serum probably contained a number of different anti-idiotypic antibodies which had a wide spectrum of affinities that were analogous to the dCMP hydroxymethylase protein-protein binding domain. The anti-idiotypic antibodies with high affinities for thymidylate synthase were able to maintain their interaction during the immunoprecipitations while weaker affinity analogs failed to immunoprecipitate labeled proteins. If this proposal is correct it points to a possible problem with the use of anti-idiotypic antibodies, the anti-idiotypic antibodies may have affinities that do not reflect the true strength of the actual interaction between the two macromolecules.

Examination for protein-protein interactions involving the transcriptional regulator, RegA, demonstrated that the immobilized translational repressor had specific affinities for *E. coli* and other T4 proteins. An attempt was made to identify the T4 proteins that were retained on the affinity column. The migration values on polyacrylamide gels of known T4 proteins were used in an attempt to identify the interacting proteins. However, this initial identification was not conclusive and leaves in doubt the identification of a majority of the proteins.

Future research involving the use of 2D electrophoresis of the eluted T4 extracts would validate the initial conclusion that RegA is associating with replication proteins at low to moderate ionic strengths. In addition, the identification of the *E. coli* proteins that were retained on the affinity column would also be useful in understanding the role that RegA has within the T4 infected *E. coli* . Finally a comparison of the elution patterns attained with RegA bound to both a positive agarose matrix and a neutral affinity column would be an appropriate future control to confirm specific interactions of the

RegA protein.

The attempt to generate anti-idiotypic antibodies to RegA by the injection of rabbit Fab fragments into mice proved problematic. While unsuccessful, it did demonstrate the limits and possible pitfalls with generating anti-idiotypic antibodies. The repeated use of Freund's complete adjuvant created high non-specific backgrounds in the immunoprecipitations using our standard incubation buffer.

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Appendices

APPENDIX A.

Association of the Large Subunit of Ribonucleotide Reductase with HeLa Cell Mitochondria

Another project on which I have also spent a considerable amount of time, examined the association of ribonucleotide reductase with HeLa mitochondria. This project is a continuation of the work initiated by Janet Leeds. I have concentrated my efforts on attempting to determine if the low level of ribonucleotide reductase activity associated with mitochondria is biologically significant or if it is due to contamination from the cytoplasm.

Introduction

Past work by Janet Leeds involved the isolation of intact mitochondria and assaying the extracts for deoxynucleotide metabolizing enzymes. Ribonucleotide reductase assays which were carried out by Dr. Mary Slabaugh demonstrated that 3% of the whole-cell activity was associated with the mitochondria (Leeds, 1987). More interesting was the observation that 1.0 mM dATP, while inhibiting 96% of the whole-cell activity, stimulated the mitochondrial associated activity two-fold. I initiated a project to determine if the observed differences in the regulation of activity were due to a unique form of ribonucleotide reductase being present in the mitochondria.

The initial experiment involved isolating mitochondria from rapidly dividing HeLa cells and carrying out immunoblots on cytoplasmic and mitochondrial fractions. The antibody selected to probe the immunoblots was a monoclonal antibody, AD203, which is specific for the mammalian large subunit (Engström, 1982). The large subunit, R1, contains nucleotide-binding sites that regulate

enzyme activity. The AD203 monoclonal antibody was selected because it had very low cross-reactivity with other proteins. It was expected that a unique mitochondrial large subunit would either be non-cross reactive with the antibody or would be shown to have a molecular weight that was different from the cytoplasmic form.

Material:

The AD203 monoclonal antibody was purchased from InRo Biomedtek. The glyceraldehyde-3-phosphate dehydrogenase polyclonal antibody was a gift from Dr. Ann Brodie. NitroScreen West immunoblot membrane was purchased from NEN Research, Dupont. Trypsin-EDTA, 0.05% trypsin, 0.53 mM EDTA, was purchased from Gibco.

Mitochondrial Isolation Procedure:

HeLa cells were removed from the cell culture plates by either trypsin digestion or by scrapping with a rubber policeman. The mitochondria were isolated using the protocol described in appendix B. For the comparison of trypsin sensitivity a HeLa 1 ml cell homogenate was incubated at 37°C in the presence of 100 µl trypsin-EDTA, 0.05% trypsin, 0.53 mM EDTA, for 15 minutes. The proteolysis was stopped with the 1:50 addition of 20 mg/ml PMSF. The mitochondria were then purified as described in appendix B. Additional purification of the mitochondrial extracts were carried out by sucrose gradients as described in appendix C. The HeLa fractions were also fixed and examined under a transmission electron microscope to determine purity.

A polyclonal antibody to glyceraldehyde-3-phosphate dehydrogenase was used as an example of non-specific association of an enzyme with membranes. The mammalian glyceraldehyde-3-phosphate dehydrogenase is a known

cytoplasmic enzyme which has been found to adhere non-specifically to plasma membrane and organelles (Ryzlak and Pietruszko, 1988). The purpose of this control was to determine if the large subunit had a similar association as the non-specific glyceraldehyde-3-phosphate dehydrogenase.

Results

Initial immunoblots using the anti-R1 antibody clearly demonstrated a difference in the molecular weights of the cytoplasmic and mitochondrial R1 protein, see lanes 1 and 4, Figure 1. However, additional work determined that the difference in the molecular weight pattern was not due to a distinct mitochondrial form of ribonucleotide reductase large subunit. Instead the difference was determined to be an artifact from the trypsin isolation procedure used to detach the HeLa cell monolayer from the cell culture plates.

When HeLa cells were isolated by mechanical scraping the R1 immunoblots were identical in molecular weight, see lanes 3 and 4, Figure 1. The trypsin sensitivity of R1 proved fortuitous in that it demonstrated a distinct difference between the R1 subunit found in the cytoplasm and that which was present in the crude mitochondrial fraction.

The trypsin treatment of a homogenized cell extract confirmed that the mitochondrial R1 was sequestered away from the protease, see lanes 2 and 5, Figure 1. Finally by probing with the polyclonal antibody to glyceraldehyde-3-phosphate dehydrogenase it was determined that both the cytoplasmic and crude mitochondrial fractions contained glyceraldehyde-3-phosphate dehydrogenase enzyme.

To compare the localization of the R1 subunit and the non-specific binding of glyceraldehyde-3-phosphate dehydrogenase an additional sucrose gradient purification step was carried out on a mitochondrial fraction. As seen in Figure 2, the R1 subunit co-migrated with the intact mitochondria, while the

glyceraldehyde-3-phosphate dehydrogenase enzyme was distributed throughout the gradient.

From repeated mitochondrial purifications and immunoblots it was determined that a mitochondrial protein extract contains approximately as much R1 subunit as does an equal fraction of cytoplasmic extract. In other words, the R1 immunoblot for the cytoplasmic and mitochondrial fractions are of roughly equal intensity when an equivalent amount of total protein is loaded onto each lane of the gel. However, the total amount of purified mitochondrial protein is much lower than the amount of total protein found in the whole cytoplasmic fraction, roughly 5%. Also the amount of total mitochondrial protein present after purification varies greatly with each HeLa cell homogenization.

Examination of the mitochondrial fractions with a transmission electron microscope, TEM, showed that the mitochondrial extract appeared to be fairly pure and fragments of cell membrane, nuclear membrane or other organelles were not in abundance. The use of TEM showed that the mitochondria were swollen and many appeared to have lost their outer membranes. Inspection of the homogenized HeLa cell pellet showed a large proportion of organelles entangled within the lysed cell by cytoskeletal material and thereby preventing the release of many of the mitochondria. This observation may explain the low yield of mitochondria obtained from the HeLa cells.

Discussion

The occurrence of enzymes in multiple molecular forms, isozymes, is a common motif found within in cells of higher organisms. It has recently been demonstrated that *Saccharomyces cervisiae* encodes two alternate large subunits of ribonucleotide reductase, *RNR1* and *RNR3*. Under normal growth conditions the gene product of *RNR1* is used to form the native enzyme with

the small subunit. However, after the occurence of DNA damage the *RNR3* is expressed (Yagle and McEntee, 1990). It would seem likely that other eukaryotic cells could contain multiple molecular forms of the ribonucleotide reductase enzymes.

Past work had identified low level ribonucleotide reductase activity localized with HeLa mitochondria. The use of immunoblots demonstrated that the mitochondrial extract contained a ribonucleotide reductase large subunit that migrated at the same molecular weight as found in the cytoplasm, indicating that the mitochondrial R1 is not a unique form. However, the protection of the mitochondrial R1 from proteolysis may indicate internalization of the enzyme within the mitochondria.

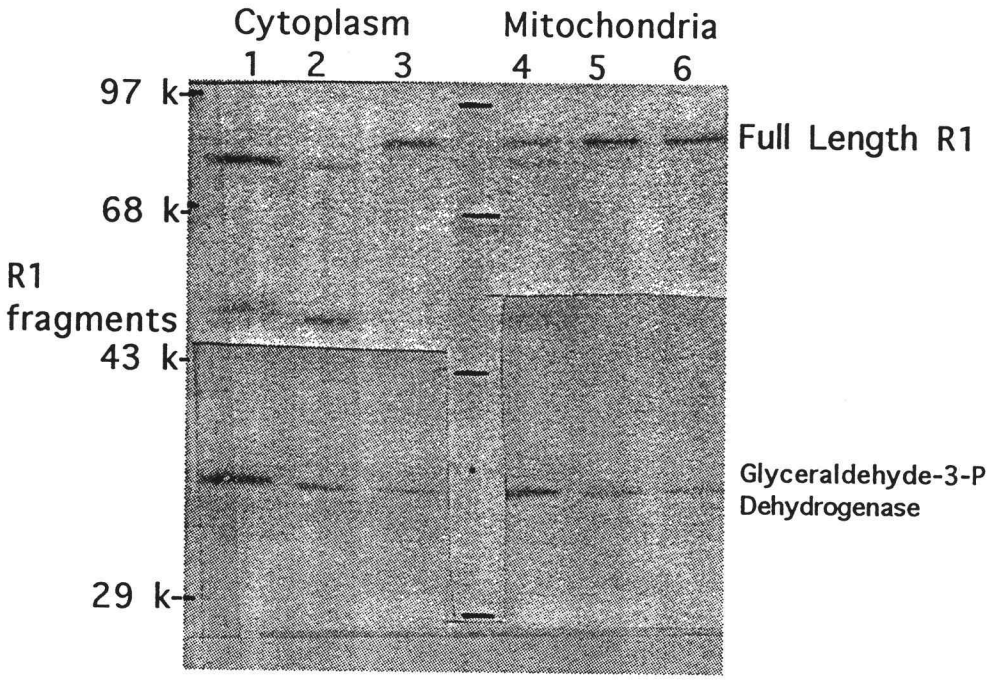

Figure A-1. Immunoblot Analysis of Trypsin Treated HeLa Fractions. Lanes 1 and 4 correspond to fractionations of HeLa cells which were isolated by trypsin treatment of the cell plates. Lanes 2,3,5, and 6 represent fractionations of cells that were collected by mechanical scraping of the plates. After homogenization, a cell lysate was incubated with trypsin for 15 minutes at 37°C and the proteolysis stopped with protease inhibitor, PMSF. The mitochondria were then purified from the treated homogenate. Lanes 2 and 5 represent the trypsin treated fractions. Lanes 5 and 6 are untreated control fractionations. The immunoblot was also probed with a glyceraldehyde-3-phosphate dehydrogenase antibody as a control.

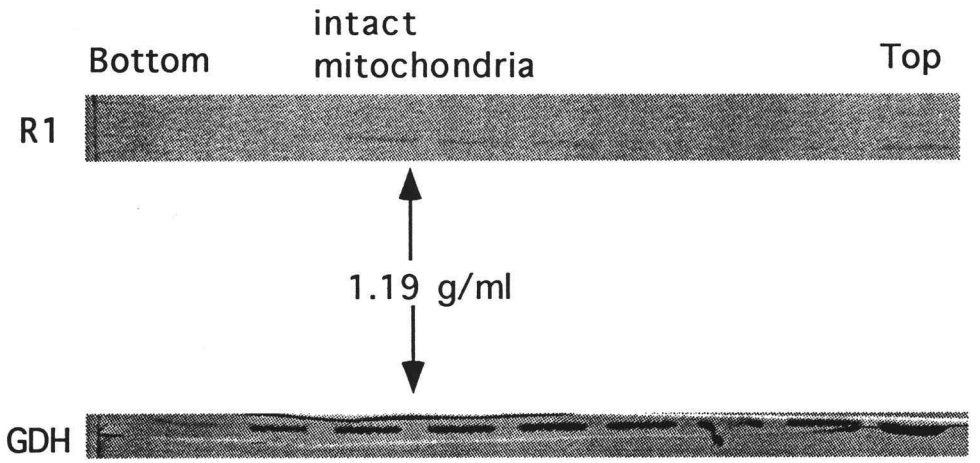

Figure A-2. Immunoblot Analysis of Sucrose Gradient Purification of HeLa Mitochondrial Extracts. A sucrose gradient purification of crude HeLa mitochondria was carried out as described in appendix C. The density of the fractions was determined by the use of a refractometer. The position of intact mitochondria was also confirmed by measuring the collected fractions for citrate synthase activity. The top western represents the anti-R1 immunoblot of the sucrose fractions, while the bottom immunoblot was probed with anti-glyceraldehyde-3-phosphate dehydrogenase. The large subunit of the ribonucleotide reductase was found to be located at the top of the gradient and also present with the intact mitochondria. The anti-GDH was distributed throughout the gradient and not localized with the mitochondria.

Ribonucleotide Reductase Localization References

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Ryzlak, M.T. and Pietruszko, R. (1988) Heterogeneity of glyceraldehyde-3-phosphate dehydrogenase form human brain *Biochimica et Biophysica Acta* 954: 309-324.

Yagle, K., and McEntee, K. (1990) The DNA Damage-Inducible Gene DIN1 of *Saccharomyces cervisiae* Encodes a Regulatory Subunit of Ribonucleotide Reductase and Is Identical to RNR3. *Mol. Cell. Biol.* 5553-5557.

APPENDIX B.

Purification of Mitochondria From Cell Culture.

1. HeLa cells are typically grown in MEM media supplemented with glutamine, penicillin/streptomycin, and 5% bovine calf serum.
2. Grow HeLa cells to 75% confluency, trypsinize cells off the cell culture plates and plate out in fresh media at 60% confluency. The 24 hour pre-trypsination helps weaken the cell membrane for later cell rupture.
3. Aspirate off the medium and and wash plates with Ca^{2+} Mg^{2+} free PBS. Scrape cells off the plates with a sterilized rubber policeman. To prevent the cells from drying out have approximately 2 ml of PBS on each plate when scrapping the cells.
4. Rinse cells off the plates with PBS and place solution in 30 ml corex tubes.
5. Perform all further manipulations on ice at 4°C; use MilliQue water for all solutions.
6. Spin cells down in Sorvall SS34 rotor, 2500 rpm, 3 minutes. Resuspend with three pellet volumes of Ca^{2+} Mg^{2+}-free PBS. Freeze HeLa cells in -70°C freezer overnight or longer. An alternative method is to freeze the cells in liquid nitrogen and precede directly to the next step.
7. Allow the cell suspension to melt and again spin cells down in Sorvall SS34 rotor, 2500 rpm, 3 minutes. Remove the cloudy supernatant and resuspend the cell pellet in 1 cell volume of, 0.25 M sucrose, 1mM EGTA, 0.5% BSA, 10 mM Hepes-NaOH, pH 7.4.
8. Place the cell suspension in a glass/teflon homogenizer and disrupt the

cell membrane with 8 strokes of the piston. Confirm cell disruption by examining a 1/50 dilution of the extract in a cytotometer. Compare the difference between an untreated fraction versus the homogenized extract.

9. Spin the homogenized extract in 1.5 ml microfuge tubes at 500 g to pellet the cell membrane. Place the supernatant in 1.5-ml microfuge tubes and spin the crude mitochondria a second time at 500 g.

10. Pellet the mitochondria by spinning at 16,000 g, save the supernatant as cytoplasmic fraction.

APPENDIX C.

Sucrose Gradient Purification of Mitochondria

1. Prepare the sucrose gradients the night before the actual run. Stack 2.0, 1.6, 1.3, 1.0 M sucrose , 1mM EDTA, 0.1% BSA, 10 mM Hepes-NaOH, on top of each other in SW41 tubes. After pipetting a fraction into a tube, freeze with liquid nitrogen and then apply the next lower sucrose solution. Allow the tubes to liquify at 4°C overnight. A continual gradient will be formed after 12 hours.
2. Place the crude mitochondria on the gradients and run in an ultracentrifuge SW 41 rotor at 80,000 g for 2 hrs at 5°C.
3. Collect 500 µl fractions into 1.5 ml microfuge tubes, by punching a hole in the bottom of the centrifuge tubes or by pippeting off the top.
4. Measure the density of the fractions using a refractometer. Intact mitochondria should sediment at 1.19 g/ml.
5. Dilute the samples with 2 volumes of 1 mM EDTA, 10 mM Hepes, pH 7.4. Centrifuge at 16,000 g, 20 minutes. Resuspend pellets and repeat centrifugation to remove BSA containing gradient solution.
6. Assay fractions for mitochondrial activity, such as citrate synthase. Store samples at -20°C.

APPENDIX D.

$RbCl_2$ Method for Competent Cell Making

$RbCl_2$ Buffer
- Millique water
- pH to 5.7 with 1N HCl
- Filter sterilize

100 mM $RbCl_2$
45 mM $MnCl_2$
10 mM $CaCl_2$
35 mM CH_2COOK
15% (W/V) Sucrose

SOB & SOC Media:
To 950 ml of Millique water, 20 g bacto-tryptone, 5 g bacto-yeast extract, 0.5 g NaCl, 0.18 g KCl, 1 ml 1 N NaOH. Autoclave 20 min. Just before use add 10 ml 1 M $MgCl_2$ and 20 ml 1 M $MgSO_4$. For SOC media same as SOB medium except add 20 ml 1 M glucose per liter, 20 mM final conc.
All glassware, SOB & SOC media flasks, incubator test tube, incubator flask, corex tubes, should be washed 3x with millique water and autoclaved containing millique water.

1. Start cells from -80° C stock. Very important to streak out on a plate the night before.
2. Place 4-5 regular shaped colonies into 2 ml SOB media, vortex. Shake at 300 rpm 37°C until A_{600} approx. 0.8. Dilute the culture into a second flask so that the A_{600} does not exceed 0.06. You will want 5 ml for every transformation.
3. Measure A_{600} every 20 minutes. If the A_{600} is not doubling every 20 minutes your transformation efficiency will be lower than optimum.
4. Grow cells in SOB media to A_{600} of 0.3, ~ $5x10^7$ cells/ml. Place in clean corex tubes on ice 5 minutes.
5. Spin cells down 3,000 rpm SS34 for 3 minutes. Pour off media and aspirate off as much media as possible.
6. For each 5 ml of cells gently resuspend with 1 ml ice cold $RbCl_2$ buffer in the corex tubes.
7. Spin cells down 3,000 rpm SS34 for 3 minutes. Pour off buffer, add 200 µl of ice cold $RbCl_2$ buffer per transformation, resuspend gently.
8. Place 200 µl of bacteria in each polyprop. tube. Add DNA to tubes and as a

control add 1 ng of supercoiled plasmid DNA to one tube. Gently rock tubes on ice for 1 hr.

9. Heat shock for 90 seconds at 42°C, do not shake, cool on ice 2 min.

10. Add 1 ml SOC media, no antibiotic, 37°C for 45 minutes. Slow speed on shaker, 225 rpm. The polyprop. tubes should be standing vertically.

11. Spread out on plates, 100 µl, 50 µl, 1 µl for each. For 1 µl dilute with 100 µl media before spreading.

APPENDIX E

Mini-Prep Method

1. Grow a 3 ml bacterial culture to an A_{600} of 0.8 and add chloroamphenicol to a final concentration of 170 µg/ml, stock solution is in ETOH 34 mg/ml. Grow for 12 more hours

2. Transfer 1.5 ml of the bacterial culture into 1.5 ml eppendorf tubes

3. Centrifuge for 1 minute and aspirate off the media supernatant. If your plasmid is expressed at a low copy number add the remaining 1.5 ml to the eppendorf tubes and repeat steps 2 and 3.

4. Add 300 µl STET to each tube. Make the STET buffer up fresh

STET		100 ml
Sucrose	8%	8 g
Triton X	0.5%	0.5 ml
EDTA pH 8.0	50 mM	10 ml of 0.5 M EDTA
Tris pH 8.0	10 mM	1 ml of 1 M Tris

5. Prepare lysozyme cocktail: (Recipe shown is for 24 samples) 10 mg lysozyme,1 ml TE (Mix by vortexing)

6. Add 25 µl lysozyme cocktail to each eppendorf tube.

7. Resuspend pellets by repeated pipetting or vortexing.

8. Place samples in a rapidly boiling water bath for 40 seconds

9. Centrifuge for 2 minutes. Pull the white gel like "goober" pellet out with a toothpick. For a successful miniprep the pellet should be approximately 20-50% of the liquid volume. A very small chalk like pellet that does not stick to the toothpick is a bad omen. Also a pellet that is larger than the supernatant is also a bad sign.

10. Add 300 µl isopropanol to each tube and vortex.

11. Centrifuge for 10 minutes pour off the supernatant and spin the tubes an additional 5 minutes. The second spin without the supernatant brings the DNA to the bottom of the tube and off the sides of the eppendorf tubes.

12. Carefully aspirate off the rest of the liquid and add 166 µl TE buffer or water to each tube. Resuspension is much easier if allowed to soak a half an hour to an hour. This is a good place to stop by storing the tubes at 4° C or -20° C. If the plasmid DNA is to be used for sequencing, it should be stored at 4° C only, and used within a week. When using high copy number plasmids, the minipreps may be used at this point, to further clean up do the following ETOH precipitation.

13. Resuspend pellet with a yellow pipet tip or by vortexing.

14. Add 83 µl 7.5 M ammonium acetate and (An optional step to help clean up minipreps is to centrifuge for 2 minutes and place the supernatants into new eppendorfs).

15. Add 500 µl 100% ETOH. Place in the -80° C for 15 minutes or longer.

16. Centrifuge for 10 minutes pour off supernatant and spin an additional 5 minutes.

17. Aspirate off any additional supernatant and pipet 1 ml 70% ETOH from the -20° C freezer into each sample being careful to not disturb the DNA pellet.

18. Aspirate off ETOH, centrifuge for 30 seconds and remove final ETOH. Allow to air dry, approx 10 minutes.

19 Bring pellets up in TE buffer or in water, 20-100 µl depending on DNA pellet size.

20. (optional) Determine DNA versus protein concentration using Warburg spectrophotometric program. 1µg/ul is a common and successful result.