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PROGRESS REPORT

Summary Progress Report

This project focuses on the DNA polymerase and accessory proteins of phage T7 for use in DNA sequence analysis. T7 DNA polymerase (gene 5 protein) interacts with accessory proteins for the acquisition of properties such as processivity that are necessary for DNA replication. One goal is to understand these interactions in order to modify the proteins to increase their usefulness with DNA sequence analysis. Using a genetically modified gene 5 protein lacking 3' to 5' exonuclease activity we have found that in the presence of manganese there is no discrimination against dideoxynucleotides, a property that enables novel approaches to DNA sequencing using automated technology. Pyrophosphorolysis can create problems in DNA sequence determination, a problem that can be eliminated by the addition of pyrophosphatase. Crystals of the gene 5 protein/thioredoxin complex have now been obtained and X-ray diffraction analysis will be undertaken once their quality has been improved. Amino acid changes in gene 5 protein have been identified that alter its interaction with thioredoxin. Characterization of these proteins should help determine how thioredoxin confers processivity on polymerization. We have characterized the T7 DNA binding protein, the gene 2.5 protein, and shown that it interacts with gene 5 protein and gene 4 protein. The gene 2.5 protein mediates homologous base pairing and strand uptake. Gene 5.5 protein interacts with *E. coli* H1 protein and affects gene expression. Biochemical and genetic studies on the T7 56-kDa gene 4 protein, the helicase, are focused on its physical interaction with T7 DNA polymerase and the mechanism by which the hydrolysis of nucleoside triphosphates fuels its unidirectional translocation on DNA.

Previous Work Accomplished and Background Information

The major overall goal of this project is to exploit the unique features of the bacteriophage T7 DNA replication system for DNA sequence analysis using the dideoxynucleotide chain termination method. In some instances it has been possible to make use of normal properties of the replication proteins while in others it has or will be necessary to eliminate or alter properties that are undesirable for DNA sequence analysis. For example, processivity of polymerization of nucleotides is a desirable property conferred by the processivity factor, *E. coli* thioredoxin.[Tabor, S., and Richardson, C. C. (1987) *J. Biol. Chem.* **262**, 15330-15333]. However, the 3' to 5' proofreading exonuclease of T7 DNA polymerase is so efficient that it removes dideoxynucleotides and thus makes the native enzyme unsuitable for DNA sequence analysis. A major advance in this endeavor, accomplished during previous project periods, was the acquisition of a T7 DNA polymerase having greatly reduced levels of 3' to 5' exonuclease activity. Initially, the exonuclease activity was reduced to 0.1 to 1.0% of that of wild-type T7 DNA polymerase by a chemical modification of the gene 5 protein [Tabor, S., and Richardson, C. C. (1987) *J. Biol. Chem.* **262**, 15330-15333]. Subsequently, based on the properties of the chemically modified enzyme, we obtained a genetically modified T7 DNA polymerase lacking exonuclease activity [Tabor, S., and Richardson, C. C. (1989) *J. Biol. Chem.*264, 6447].

In some instances modifications in the reaction conditions themselves alters the characteristics of the reaction dramatically. Two such examples include effects on discrimination against dideoxynucleotides and the hydrolysis of dideoxynucleotide terminated chains by pyrophosphorolysis. Most nucleotide analogs are incorporated less efficiently than unmodified

nucleotides, the extent of discrimination depending on both the analog and the DNA polymerase. T7 DNA polymerase discriminates against a ddNTP several-fold with the relative rates of ddNMP and dNMP incorporation being dependent on neighboring DNA sequences. This differential incorporation leads to variation in band intensities in autoradiographs of DNA sequencing gels. We have shown [Tabor, S., and Richardson, C. C. (1989) *Proc. Natl. Acad. Sci., USA* **86**, 4076] that incorporation of dideoxynucleotides (ddNMPs) by T7 DNA polymerase and *E. coli* DNA polymerase I is more efficient when Mn^{2+} rather than Mg^{2+} is used for catalysis. With T7 DNA polymerase and Mn^{2+}, ddNMPs and dNMPs being incorporated at virtually the same rate. The lack of discrimination against ddNMPs using T7 DNA polymerase and Mn^{2+} results in uniform terminations of DNA sequencing reactions, with the intensity of adjacent bands on polyacrylamide gels varying by less than 10%.

We were surprised to find that specific fragments generated by T7 DNA polymerase in DNA sequencing reactions disappear with time of incubation despite the absence of exonuclease activity. During the past project period we found that pyrophosphorolysis by bacteriophage T7 DNA polymerase leads to the degradation of specific dideoxynucleotide-terminated fragments on DNA sequencing gels [Tabor, S., and Richardson, C. C. (1990) J. Biol. Chem. 265. 8322-8328]. This reaction can be prevented by pyrophosphatase. The use of pyrophosphatase, a genetically modified T7 DNA polymerase that lacks exonuclease activity, and Mn^{2+} rather than Mg^{2+} to eliminate discrimination between dideoxynucleotides and deoxynucleotides generates bands of uniform intensity on a DNA sequencing gel. Uniform band intensities simplify the analysis of a DNA sequence, particularly with automated procedures.

Crystallization of T7 DNA Polymerase

One goal of this project is to obtain the three dimensional structure of T7 DNA polymerase. During the past two years we have collaborated with Dr. William E. Royer of the University of Massachusetts Medical School in attempts to crystallize T7 DNA polymerase, the T7 DNA polymerase/thioredoxin complex, and the T7 DNA polymerase/thioredoxin complex in the presence of a DNA primer/template. During the current project period we have expanded this phase of the project by establishing a collaboration with Dr. Alex Rich at the Massachusetts Institute of Technology. Dr. Royer has provided Dr. Rich with gene 5 protein and thioredoxin and discussed his previous attempts at crystallization with him. Within the past six months Dr. Chul-Hee Kang, a senior research fellow in Dr. Rich's laboratory has obtained crystals of the gene 5 protein/thioredoxin complex. Present efforts are directed toward obtaining reproducible crystals of higher quality prior to initiating X-ray diffraction studies.

We are most anxious to obtain co-crystals of gene 5 protein/thioredoxin bound to a primer-template and deoxynucleoside triphosphate. As discussed below, we have recently shown that, under conditions of dideoxynucleotide chain termination, gene 5 protein/thioredoxin forms an extremely stable complex with a primer template at specific sequences within the template in a deoxynucleoside triphosphate dependent reaction. By using genetically modified T7 DNA polymerase lacking exonuclease activity the resulting primer-template bound to the enzyme remains intact, as does the deoxynucleoside triphosphate. Dr. A. Nussbaum has completed the synthesis of sufficient amounts of one such defined primer/template for the co-crystallization studies. For these latter studies we have provided Dr. Rich with large amounts of the genetically modified T7 DNA polymerase lacking exonuclease activity.

Interaction of Mutant Gene 5 Proteins with Thioredoxin (5)

The gene 5 protein of phage T7 is a DNA polymerase of low processivity, dissociating from the primer-template after catalyzing the incorporation of 1-50 nucleotides. Upon infection, phage T7 annexes a host protein, thioredoxin, as a processivity factor for the gene 5 protein. E. coli thioredoxin binds tightly (K_m = 5 nM) to T7 DNA polymerase in a 1:1 stochiometry and bestows processivity on the polymerization reaction by increasing the affinity of the gene 5 protein for a primer-template. Thioredoxin, the product of the *trxA* gene of *E. coli*, is a 12-kDa, heat-stable protein. It contains two reversibly oxidizable cysteine residues and functions as a general protein disulfide oxidoreductase within the cell. Oxidized thioredoxin does not interact with T7 DNA polymerase. One surface of the thioredoxin molecule is especially flat and hydrophobic, and it has been proposed that this surface of thioredoxin may provide a binding site for interactions between thioredoxin and other proteins.

In order to dissect the gene 5 protein-thioredoxin interactions we examined nine mutant thioredoxins, representing amino acid residue substitutions at five different positions, for their ability to interact with wild-type gene 5 protein both *in vivo* and *in vitro*. In an *in vitro* reconstitution assay with gene 5 protein, mutant thioredoxins with alterations at either one or both active-site cysteine residues could restore nearly full polymerase activity. Another thioredoxin mutant (gly-74 to asp-74) can restore nearly full polymerase activity, but only at a concentration that is several hundred fold higher than wild-type. A third thioredoxin mutant (gly-92 to asp-92) does not appear to bind gene 5 protein at all, even at extremely high concentrations. On the basis of the biochemical analysis, we have used the thioredoxin mutant (gly-74 to asp-74) to select for T7 revertants, and we have characterized the nature of the revertant mutations in the T7 phage. The gly-74 to asp-74 mutation in the thioredoxin gene is suppressed by a replacement of glu-319 in the gene 5 protein to either a valine or lysine residue.

The crystal structure of the large Klenow fragment of *E. coli* DNA polymerase I reveals a striking groove or crevice whose size and shape are compatible with its being the binding site for double-stranded DNA. The amino acid sequences of the large Klenow fragment of *E. coli* DNA polymerase I and T7 gene 5 protein show extensive homologies, especially in this DNA binding domain. On the basis of tertiary structure inference, we suggest that the glu-319 residue of gene 5 protein is located at the edge of the DNA-binding groove within the polymerization domain. We speculate that thioredoxin binds gene 5 protein at the edge of this crevice such that the two proteins together lock or clamp the duplex DNA into position.

Other mutations within gene 5, Ala-45 to thr, val-3 to ile, and val-32 to ala, can suppress the same asp-74 thioredoxin mutation. By tertiary structure inference to the large fragment of *E. coli* DNA polymerase I, we propose that all three of these revertant mutations are located within the 3' to 5' exonucleolytic domain of gene 5 protein. Because of the nature of the amino acid substitutions and the fact that all three revertant mutations are located at different positions, we suggest that these suppressor mutations do not represent contact points between thioredoxin and gene 5 protein. We are unable to explain how mutations in the 3' to 5' exonucleolytic domain of gene 5 can suppress the asp-74 thioredoxin mutant; however, it is tempting to speculate that perhaps an alteration of 3' to 5' exonucleolytic activity may increase the processivity of polymerization.

Current studies are directed toward purifying the mutant gene 5 proteins and examining their properties. For example, initial studies will measure the affinity of each of the mutant gene 5 proteins for wild-type and the asp-74 thioredoxin. Concurrent with these analyses we will determine the processivity of the polymerization reaction. In view of the location of some of the gene 5 protein alterations within the exonuclease domain, we are most interested in examining the specificity and the level of exonuclease activity in each of the mutant gene 5 proteins both in the presence and absence of each of the thioredoxins. The affinity of each of the combinations of gene

5 protein and thioredoxin for a primer-template will be carried out as was done previously for wild-type gene 5 protein and wild-type thioredoxin.

The Gene 5 Protein/Thioredoxin-Primer//Template:ate Complex

We have previously shown that the increase in processivity conferred by thioredoxin derives from its ability to stabilize gene 5 protein-primed DNA complexes [Huber, H. E., Tabor, S., and Richardson, C. C. (1987) *J. Biol. Chem.* **262**, 16224-16232] Gene 5 protein forms a short-lived complex with the primer-template in the absence of Mg^{2+} and nucleotides. Thioredoxin increases the half-life of the primer-template-polymerase complex from less than a sec to 5 min. Thioredoxin also reduces the equilibrium dissociation constant, K_d, of a gene 5 protein-poly(dA)·oligo(dT) complex 20 to 80-fold. During the current project period we have found that the gene 5 protein/thioredoxin complex forms an extremely stable complex at a dideoxyribonucleotide terminated primer/template provided that the next potential nucleoside 5'-triphosphate to be polymerized is also present. In addition, the sequence of the template at the primer/template junction has a marked effect on the observed dissociation constant. We are currently making use of the marked stability of the complex to examine by exonuclease and endonuclease digestion the region of the primer and template that are contacted by the enzyme. For example, the size of the region of the primer strand covered by the enzyme complex can be probed using T7 exonuclease 6 and lambda exonuclease, both of which hydrolyze from the 5'-terminus. On the other hand, the use of *E. coli* exonuclease III has allowed us to examine the size of the template strand protected by T7 DNA polymerase. We are currently obtaining footprints and Kd values for gene 5 protein in the presence and absence of thioredoxin. Furthermore, it is important to compare the footprint and stability with both native gene 5 protein and Δ28 gene 5 protein lacking exonuclease activity. Somewhat surprisingly, we find that the gene 5 protein alone is relatively stable at these sites. Finally, as mentioned above, we using these conditions and sequence specific primer/templates to prepare gene 5 protein/thioredoxin-primer/template-dNTP complex for crystallization.

Gene 2.5 Protein of Bacteriophage T7 (2,3,4,6)

Single-stranded DNA binding proteins that stimulate DNA polymerases are thought to act non-enzymatically, coating the DNA and removing secondary structures. The product of gene 2.5 has been implicated in T7 DNA replication, recombination, and repair. It was originally purified based upon its strong, specific affinity for single-stranded DNA, and its ability to stimulate DNA synthesis by T7 DNA polymerase. Gene 2.5 protein has been thought to be analogous in function to the *E. coli* single-stranded DNA binding (SSB) protein and the phage T4 gene 32 protein; both of these proteins have a strong, specific affinity for single-stranded DNA, and can stimulate DNA synthesis. In addition, like T7 gene 2.5 protein, both *E. coli* SSB protein and the T4 gene 32 protein have been shown by genetic and biochemical studies to be required for DNA replication, recombination, and repair .

Purification and physical properties - We have purified the gene 2.5 protein of bacteriophage T7 to homogeneity from cells over-expressing its gene. Native gene 2.5 protein consists of a dimer of two identical subunits of molecular weight 25,562. Gene 2.5 protein binds specifically to single-stranded DNA with a stoichiometry of ~7 nucleotides bound per monomer of gene 2.5 protein; binding appears to be noncooperative. Electron microscopic analysis shows that gene 2.5 protein is able to disrupt the secondary structure of single-stranded DNA. The single-stranded DNA is extended into a chain of gene 2.5 protein dimers bound along the DNA. In fluorescence quenching and nitrocellulose filter binding assays, the binding constants of gene 2.5 protein to single-stranded DNA are $1.2 \times 10^6\ M^{-1}$ and $3.8 \times 10^6\ M^{-1}$, respectively. *Escherichia coli* single-stranded DNA binding protein and phage T4 gene 32 protein bind to single-stranded

DNA more tightly by a factor of 25. Fluorescence spectroscopy suggests that tyrosine residue(s), but not tryptophan residues, on gene 2.5 protein interact with single-stranded

Interaction with other proteins - We have shown that bacteriophage T7 gene 2.5 protein interact with T7 DNA polymerase (T7 gene 5 protein/thioredoxin) by affinity chromatography and fluorescence emission anisotropy. T7 DNA polymerase binds specifically to a resin coupled to gene 2.5 protein, and elutes at an ionic strength of 250 mM NaCl. Thioredoxin does not bind to gene 2.5 protein. Steady state fluorescence emission anisotropy gives a dissociation constant of 1.1 ± 0.2 μM for the complex of gene 2.5 protein and T7 DNA polymerase, with a ratio of gene 2.5 protein to T7 DNA polymerase in the complex of one to one. Nanosecond emission anisotropic analysis suggests that the complex contains one monomer each of gene 2.5 protein, gene 5 protein, and thioredoxin. In addition, we have compared the ability of T7 gene 2.5 protein to stimulate the activity and processivity of T7 DNA polymerase with the ability of three other single-stranded DNA binding proteins: *E. coli* single-stranded DNA binding protein, T4 gene 32 protein, and *E. coli* recA protein. All except *E. coli* recA protein stimulate T7 DNA polymerase; *E. coli* recA protein inhibits these activities.

Gene 2.5 protein facilitates homologous base pairing - We have recently demonstrated that gene 2.5 protein facilitates the renaturation of single-stranded DNA. The rate of renaturation is increased over 1000-fold and is much more efficient than that carried out by *E. coli* recA protein, *E. coli* SSB, or T4 gene 32 protein. In contrast to the reaction promoted by T4 gene 32 protein the kinetics of renaturation are first order. Although the renaturation reaction catalyzed by recA protein is also first order, the gene 2.5 protein reaction differs in that it does not require ATP. In this regard it is similar to the previously characterized B protein of phage lambda and the sep protein of yeast. The reaction does not require Mg^{2+}; however, in the absence of Mg^{2+}, a higher NaCl concentration is required. Interestingly, in the electron microscope, the complexes formed in the absence of Mg^{2+} and NaCl, conditions under which gene 2.5 protein does not stimulate renaturation, the complexes are spread out with little evidence of protein-protein interactions beyond the nearest neighbors. On the other hand, in the presence of Mg^{2+} ions, conditions under which gene 2.5 protein does catalyze renaturation of single-stranded DNA, the single-stranded DNA appears highly compact with some indication of intrastrand interactions. Such interactions could be important for the catalysis of homologous pairing. If a single-stranded region is exposed on a duplex DNA molecule for exonuclease action, then the gene 2.5 protein will promote homologous base pairing of a complementary single-stranded DNA fragment eventually resulting in strand uptake and displacement of the duplex in the accepting DNA molecule. We are currently investigating the mechanism by which the binding of gene 2.5 protein to single-stranded DNA facilitates its ability to renature to a homologous single-stranded DNA molecule.

Gene 4 Protein of Bacteriophage T7 (2, 8)

T7 DNA replication requires helicase and primase activities, both activities being provided by the gene 4 proteins encoded by the phage. Preparations of gene 4 protein purified from phage-infected cells contain two mol. wt . species: a 56-kDa and a 63 kDa protein . The nucleotide sequence of T7 DNA contains an AUG initiation site with a strong ribosome binding site for protein synthesis within gene 4. The two proteins thus have the same amino acid sequence except for the additional 63 amino acid residues (7 kDa) at the amino terminus of the 63-kDa protein. During past project periods we have developed expression systems that enable the isolation of the 56-kDa and 63-kDa gene 4 proteins, each free of contamination by the other. The 56-kDa protein binds to- and translocates 5' to 3' on single-stranded DNA and has helicase activity; both reactions are dependent on the hydrolysis of a NTP. The 63-kDa protein, in addition to having helicase activity, also catalyzes the template-directed synthesis of oligoribonucleotides

Characterization of the 56-kDa gene 4 protein - Kinetic studies on the 56-kDa protein indicate that the it has two binding sites for dTTP; an allosteric site occupied at 1 mM dTTP, and a hydrolytic site with a higher K_m for dTTP. From a computer search, we located two regions (residues 65-109 and 305-366) that resemble the consensus sequence for a NTP-binding site. I will refer to these potential nucleotide binding sites as NBS-1 and NBS-2, respectively. In view of the proximity of NBS-1 to the N-terminal primase domain found on the 63-kDa protein we postulate that ribonucleotides such as ATP and CTP occupy this site for condensation to initiate primer synthesis. NBS-2 would be occupied by dTTP, promoting binding to single-stranded DNA, and then undergoing hydrolysis to support translocation.

During the current project period we have used site-directed mutagenesis to examine the NBS-2 site. In these studies we have altered two adjacent codons within the region encoding the abridged "A" motif present in several helicase. A conserved gly-254 and lys-255 was changed by random mutagenesis to obtain a set of 56-kDa proteins with 12 different amino acid combinations in these two positions. The changes in the altered genes were identified by DNA sequence analysis. Several of these altered 56-kDa gene 4 proteins not only can not support T7 growth but are actually inhibitory to T7 DNA replication carried out by wild type gene 4 protein. We are now purifying the altered gene 4 proteins in order to characterize them biochemically. Our preliminary evidence suggests that alterations in NBS-2 reduce greatly the DNA dependent hydrolysis of dTTP.

Characterization of the primase activity of the 63-kDa gene 4 protein - In order to study 63-kDa gene 4 protein free of 56-kDa gene 4 protein, mutations were introduced into the internal ribosome-binding site responsible for the translation of the 56-kDa protein. The 63-kDa gene 4 protein was purified 16,000-fold from *E. coli* cells harboring an expression vector containing the mutated gene 4. Purified 63-kDa gene 4 protein has primase, helicase, and single-stranded DNA-dependent dTTPase activities. The constraints of primase recognition sequences, nucleotide substrate requirements, and the effects of additional proteins on oligoribonucleotide synthesis by the 63-kDa gene 4 protein have been examined using templates of defined sequence. A three base sequence, 3'-CTG-5', is necessary and sufficient to support the synthesis of pppAC dimers. dTTP hydrolysis is essential for oligoribonucleotide synthesis. Addition of a 7-fold molar excess of 56-kDa gene 4 protein to 63-kDa protein increases the number of oligoribonucleotides synthesized by 63-kDa protein 100-fold. The increase in oligonucleotides results predominantly from an increase in the synthesis of tetramers, with relatively little change in the synthesis of dimers and trimers. The presence of 56-kDa protein also causes 63-kDa protein to synthesize "pseudo-templated" pppACCCC pentamers at the recognition sequence 3'-CTGGG-5'. T7 gene 2.5 protein, a single-stranded DNA binding protein, increases the total number of oligoribonucleotides synthesized by 63-kDa gene 4 protein on single-stranded M13 DNA, but has no effect on the ratio of dimers to trimers and tetramers.

The Gene 5.5 Protein of Phage T7 (7)

Gene 5.5 of T7 lies adjacent to the DNA polymerase gene and encodes a polypeptide with a molecular weight of about 11 kDa. Mutations in gene 5.5 lead to defective phages that have reduced plaque size and burst yield. As our attempt to study the functions of gene 5.5 during T7 growth, we cloned and over-expressed gene 5.5 using the T7 RNA polymerase expression system. In the process of purifying gene 5.5 protein, we found, surprisingly, that gene 5.5 protein purified as a complex with the *E. coli* nucleoid protein H1. Bacterial DNA is associated with several histone-like proteins. Among them, protein H1 (also called H-NS) is the second most abundant of the bacterial nucleoid and was original identified as a 15-16 kDa nucleoid-associated protein. This protein consists of 135-137 amino acids and is overall neutral but contains many basic and acidic domains. Protein H1 is highly conserved among different bacterial species. Purified protein H1 binds tightly to double-stranded DNA and increases its thermal stability. Upon binding DNA *in vitro*, protein H1 undergoes conformational change. The protein does not wrap

DNA *in vitro* but does seem to compact DNA significantly. In addition, protein H1 can prevent the formation of open complex by *E. coli* RNA polymerase for transcription *in vitro* . Protein H1 is important in DNA supercoiling and global gene regulation *in vivo*. Mutations in the protein H1-encoding gene are highly pleiotropic, *i.e.*, they affect various genes scattered around the genome. Phenotypes of mutants includes derepression of gene expression (*ozmZ, drdX, bglY*), increased site-specific recombination frequency (*pilG*), and higher frequency of spontaneous deletions (*bglY*). Some *osmZ* mutants also influence the superhelicity of reporter plasmids. These observations support that protein H1 plays a pivotal role in the global regulation of the bacterial genome, a hypothesis that is consistent with the fact that protein H1 is associated tightly with the nucleoid.

In order to facilitate the purification of gene 5.5 protein, a fusion protein was created between the *E. coli* maltose binding protein and gene 5.5 protein. This fusion also binds to protein H1 even though the N-terminus of gene 5.5 protein was covalently linked to the C-terminus of the maltose binding protein. Expression of this fusion protein or gene 5.5 protein alone is able to relieve the repression of the *E.coli proU* promoter by protein H1. Furthermore, the purified fusion protein is able to suppress the inhibitory effect of protein H1 on the *proU* promoter during *in vitro* transcription by the *E. coli* RNA polymerase. The protein H1-gene 5.5 protein complex remains bound to DNA but protein H1 can no longer repress transcription. In addition, protein H1 can also inhibit the *in vitro* transcription of the T7 genome by the T7 RNA polymerase. Such inhibition can be completely reversed by gene 5.5 protein.

Novel Strategies for DNA Sequence Analysis Using Modified T7 DNA Polymerase (1)

We have developed several applications that exploit the uniform band intensities obtained with T7 DNA polymerase lacking exonuclease activity, pyrophosphatase, and Mn^{2+} ions. The advantages of uniform band intensities are most evident with automated DNA sequencing procedures. We have developed a sequencing procedure that requires only a single fluorescent primer, a single reaction containing the four unmodified ddNTPs, and a single lane to separate the fragments. For example, one can use a 2-fold difference in ratios between each ddNTP, for a total range of 8-fold. A second reaction can be carried out containing different ratios for the purpose of error-checking. We have also used the ability to generate bands of equal intensity to detect heterozygotic sequences in genomic DNA. Heterozygotic sequences are readily detected because their bands have half the intensity of homozygotic sequences.

Publications.

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