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CHARACTERIZATION AND MODIFICATION OF PHAGE T7 DNA POLYMERASE FOR USE IN DNA SEQUENCING

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ABSTRACT

This project has focused on the DNA polymerase of phage T7 for use in DNA sequencing. A complex of T7 DNA polymerase and *E. coli* thioredoxin form a highly processive DNA polymerase. The exonuclease activity of the enzyme can be reduced by chemical or genetic modifications resulting in an enzyme that has several properties useful in sequencing including high processivity and lack of discrimination against dideoxynucleotides. Manganese ion eliminates all discrimination against ddNTPs allowing sequence determination based on band intensity. A single tyrosine residue in the active site of T7 DNA polymerase is responsible for the efficient incorporation of ddNMPs. Replacement of the phenylalanine at this position in Klenow or Taq DNA polymerase with tyrosine eliminates discrimination against ddNTPs, a property that has advantages for cycle sequencing. Pyrophosphorolysis catalyzed by a polymerase result s in the hydrolysis of specific fragments in DNA sequencing reactions, a problem that is eliminated by the addition of pyrophosphatase. The thioredoxin domain of gene 5 protein has been identified and transferred to Klenow DNA polymerase to make it processive. We have crystallized a complex of T7 DNA polymerase/thioredoxin bound to a primer-template in the presence of a dNTP.

Detailed Report on Work Accomplished

Chemical and genetic modification of the exonuclease domain of the gene 5 protein

Native T7 DNA polymerase having high levels of exonuclease can be converted to a form having low levels of exonuclease activity ("modified" T7 DNA polymerase). In the presence of the transition metal iron, the reducing agent dithiothreitol, and oxygen, the exonuclease activity of gene 5 protein can be reduced 4,000-fold while retaining greater than 70% of the polymerase activity. For optimal conditions ferrous ions are required in a one to one stoichiometry to gene 5 protein molecules. Both single-stranded and double-stranded DNA exonuclease activities are inactivated at the same rate and the presence of thioredoxin has no effect. Our results support a model in which ferrous iron, bound at the metal binding site in close proximity to the exonuclease site, reacts with oxygen to produce reactive oxygen species which in turn react with residues that constitute the active site

We have used *in vitro* mutagenesis to isolate T7 DNA polymerases that have reduced levels of exonuclease activity . A variety of chemical reagents specific for different classes of residues were reacted with native T7 DNA polymerase and the population of molecules were screened for the presence of molecules capable of polymerizing nucleotides through a region of strong secondary structure. These studies, followed by *in vitro* mutagenesis, led to the identification of histidine-123 as important to exonuclease activity. Subsequent *in vitro* mutagenesis of the region surrounding His-123 provided a collection of gene 5 proteins having alterations ranging from single amino acid changes up to a 28 amino acid deletion. All of these T7 DNA polymerases have reduced exonuclease activity (from 30% to less than 1×10^{-6} % of native T7 DNA polymerase) but either normal or elevated DNA polymerase activity.

Characterization of the Gene 5 protein-thioredoxin complex

The T7 gene 5 protein is a DNA polymerase of low processivity, dissociating from the primer-template after catalyzing the incorporation of 1-16 nucleotides. Upon infection, T7 annexes a host protein, thioredoxin, as a processivity factor. *E. coli* thioredoxin binds tightly to T7 gene 5 protein 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 protein disulfide oxido-reductase within the cell. We have used fluorescence emission anisotropy to examine the interaction between gene 5 protein and thioredoxin in solution. In these studies thioredoxin was coupled to anthranylate, a fluor that reacts specifically with lysine residues. The concentration of gene 5 protein required to give a maximum increase in emission anisotrophy when added to a fixed amount of the labeled thioredoxin demonstrates that the two proteins are present in a 1 to 1 stoichiometry. Furthermore, a dissociation constant of 4.0×10^{-8} M was calculated for the two proteins in the absence of DNA. Interestingly, in the presence of DNA the affinity of the two proteins for each other increases 4-fold.

Interaction of mutant thioredoxins with the T7 gene 5 protein

One approach to explore the interaction of thioredoxin with gene 5 protein is to use mutationally altered proteins. We have identified nine mutant thioredoxins that are altered in their interactions with gene 5 protein. The dissociation constant of these mutant thioredoxins for gene 5 protein is increased between 5 and several hundred fold compared to wild-type thioredoxin and in one case no detectable binding is observed. The maximum polymerase activity of the reconstituted gene 5 protein-thioredoxin complex, however, is at least 80% of the wild-type level. During the past

project period we have constructed an *E. coli* strain, *E. coli* JH20 (F^-*pcnB*$^+\Delta$*trxA*), that enables the construction of any *E. coli* thioredoxin mutant strain by transformation with a plasmid which harbors the appropriate mutant thioredoxin allele. The F^- genotype is required since T7 productively infects only female cells. The *pcnB*$^-$ mutation reduces the plasmid copy number to a single copy per cell since a high copy number will lead to a large intracellular concentration of the mutant thioredoxin that may overcome partial functional defects. Deletion of the thioredoxin gene removes the possibility of generating a wild-type thioredoxin gene between the plasmid and the bacterial chromosome. *E. coli* cells containing a mutant thioredoxin, *trx*A7, that has both active site cysteine residues replaced with serine residues supports T7 growth (e.o.p. = 0.4) thus confirming that the oxido-reductase function of thioredoxin is not necessary for its ability to function as a processivity factor. A second thioredoxin mutant, *trxA13* (Gly-92 to Asp), does not support T7 growth (e.o.p. = $<1 \times 10^{-9}$). A third mutant, *trxA11* (Gly-74 to Asp) also does not support T7 growth but T7 plaques appear at a frequency of 5×10^{-4}, suggesting that these phage contain suppressor mutations that enable them to grow on this thioredoxin mutant·

Identification of Gene 5 proteins altered in their interaction with thioredoxin

Based on the growth of T7 phage on various thioredoxin mutants we have used a genetic method to investigate the interaction between T7 gene 5 protein and *E. coli* thioredoxin. The strategy is to use thioredoxin mutants that are unable to support the growth of wild-type T7 to select for T7 revertant phage that suppress the defect in thioredoxin. As mentioned above, the thioredoxin mutant *trxA11* (Gly-74 replaced by Asp) does not support the growth of wild-type T7, but suppresser mutations arise in the phage at a frequency of 1 in 2,000 that enable the phage to grow on this mutant thioredoxin strain. We have characterized genetically six of these phage suppresser mutations. All of the suppressor mutations reside within gene 5 and each arises as a result of a single mutation that gives rise to a single amino acid substitution within gene 5 protein. Three of the suppressor mutations are located within the putative polymerization domain of gene 5 protein, and three are located within the 3' to 5' exonucleolytic domain. Each suppressor mutation alone is necessary and sufficient to confer the revertant phenotype. Based on the extensive homology between T7 gene 5 protein and the large fragment of *E. coli* DNA polymerase I, whose crystal structure is known, we speculate that thioredoxin binds gene 5 protein at each edge of cleft 2 such that the two proteins together clamp the duplex DNA into position.

The thioredoxin binding domain of bacteriophage T7 DNA polymerase confers processivity on E. coli DNA polymerase I

Mutations within the DNA sequence encoding a 76 amino acid insert located in the thumb region of T7 gene 5 protein affect the ability of thioredoxin to bind to gene 5 protein. This unique domain is subject to proteolytic attack, a phenomenon that is inhibited by the presence of thioredoxin. We have shown that insertion of this domain into the homologous site in E. coli DNA polymerase I results in a dramatic increase in the processivity of the chimeric DNA polymerase, a phenomenon that is dependent upon its binding to thioredoxin.

Structural Analysis of the Binding of T7 DNA Polymerase to a Primer-Template

We have analyzed the static binding of T7 DNA polymerase to primer-template complexes by (i) measuring the resistance of the DNA to challenge with exo- and endonucleases, (ii) nitrocellulose filter binding, and (iii) fluorescence spectroscopy. The gene 5 protein alone binds to an unmodified 21 base primer-25 base template with a half-life of less than one sec. The gene 5 protein-thioredoxin complex binds significantly more tightly, but the half-life for dissociation is still only several seconds. In contrast, when the gene 5 protein-thioredoxin complex is actively synthesizing DNA on M13 DNA, the half-life for dissociation increases to several minutes.

If the 3'-hydroxyl of the primer is replaced with a phosphate, the gene 5 protein-thioredoxin complex no longer binds to this primer, and the primer is resistant to the 3' to 5' exonuclease activity of the gene 5 protein. The lack of binding of T7 DNA polymerase to a 3'-phosphoryl terminated DNA is an important consideration in our attempts to obtain crystals of the gene 5 protein-thioredoxin complex bound to the primer-template; i.e., one can eliminate aberrant binding of the polymerase to the 3'-end of the template strand by using a 3'-phosphoryl terminated template strand in the complex. In addition, since the 3'-phosphoryl terminated DNA is resistant to degradation by the 3' to 5' exonuclease activity of the wild-type gene 5 protein-thioredoxin complex, one can form stable complexes of the wild-type gene 5 protein-thioredoxin with primer-templates in which the template has a 3'-phosphate.

When the primer annealed to a template is terminated with a 3'-ddNMP, it forms a tight complex with the gene 5 protein-thioredoxin complex; the half life for dissociation is two hours or longer. Such stable complexes are dependent on a high concentration (K_m = 250 μM) of the dNTP complementary to the next nucleotide in the template. The complex is temperature-sensitive; at 20 °C or 37 °C the half-life is two hours or 15 min, respectively. The complex is relatively insensitive to ionic strength; the half life in 350 or 500 mM NaCl is 20 or 10 min, respectively. These stable complexes are resistant to degradation from the otherwise very active 3' to 5' exonuclease activity of T7 DNA polymerase. In the absence of the next dNTP the primer is degraded within one sec, while in the presence of the next dNTP less than 10% is degraded after 12 hours. This stable complex is being used in our structural studies, including our attempts to obtain cocrystals of the native gene 5 protein-thioredoxin complex with a primer-template.

Topography of binding of T7 DNA polymerase to a primer-template

We have examined the interaction of the T7 gene 5 protein-thioredoxin complex with primer-templates of varying lengths to determine the minimum length of primer and template necessary to obtain stable binding. We have monitored binding by (i) measuring the resistance of the primer and/or template to challenge by exonucleolytic degradation, and (ii) nitrocellulose filter binding. Surprisingly, the length of the duplex region of the primer-template must be considerably longer for T7 DNA polymerase than that observed for a number of other DNA polymerases. The minimum size of the duplex region is 21 base pairs (primer of 21 nucleotides) for maximal binding affinity; the stability of the complex decreases with progressively shorter duplex regions. Optimal length of the 5'-single-stranded region of the template strand, where polymerization of nucleotides will occur, is four nucleotides; the half-life for dissociation of this complex is greater than two hours. With no exposed single-stranded DNA on the 5'-end of the template (i.e. a blunt end) the half-life is one minute. Thus the minimal primer-template length for maximal binding by gene 5 protein-thioredoxin consists of a 25 nucleotide template with a 21 nucleotide primer to yield a 4 nucleotide single-stranded 5' end of the template strand.

Crystallization of T7 DNA Polymerase

We have obtained crystals of the complex of T7 gene 5 protein and thioredoxin bound to a primer-template with a dNTP in the triphosphate binding site that diffract to Bragg spacings greater than 2.2 Å. In collaboration with Dr. Tom Ellenberger of our Department we have frozen the crystals and collected a native data set at the Cornell High Energy Synchrotron Source. We are currently attempting to obtain heavy atom derivatives. We have found that improved crystals can be obtained by using T7 DNA polymerase mutants having only a few amino acids lacking from the exonuclease domain. We expect to have structural data within the next few months

Effect of manganese ions on the incorporation of dideoxynucleotides

Incorporation of 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 . Substituting Mn^{2+} for Mg^{2+} reduces the discrimination against ddNMPs 100-fold for DNA polymerase I and 4-fold for T7

DNA polymerase. With T7 DNA polymerase and Mn^{2+}, ddNMPs and dNMPs are incorporated at the same rate. Mn^{2+} also reduces the discrimination against other analogs with modifications in the furanose moiety, the base, and the phosphate linkage. A metal buffer, isocitrate, expands the $MnCl_2$ concentration range effective in catalyzing DNA synthesis.

Molecular basis for discrimination against dideoxynucleotides

We have made use of hybrid DNA polymerases to identify those sequences within the polymerization site of T7 and *E. coli* DNA polymerase I that distinguish between deoxyribose and dideoxyribose in the nucleotide. Based on the regions identified in our *in vivo* selection procedure we constructed five hybrid molecules in which that segment of gene 5 protein was replaced with the homologous segment form *E. coli* DNA polymerase I. Although all of the hybrid polymerases had reduced activity, they could be screened for their ability to incorporate ddNMPs by assays of the enzyme in polyacrylamide gels. These studies identified the critical amino acid sequence and eventually defined a single residue (phenylalanine versus tyrosine) that accounts entirely for the several thousand-fold difference in the discrimination against ddNMPs by *E. coli* DNA polymerase I and Taq DNA polymerase as compared to T7 DNA polymerase. It is remarkable that the presence of a single hydroxyl group is sufficient to lead to the efficient incorporation of ddNMPs by all three polymerases. In a crystalline binary complex of dCTP and Klenow fragment the dCTP is located along the "O" helix, with the 3'-OH of the ribose located in proximity to the critical phenylalanine.

DNA polymerases that incorporate ddNMPs efficiently offer a number of advantages for DNA sequence analysis. Since discrimination against ddNTPs is sequence specific a major advantage of improving the rate of incorporation of ddNMPs relative to dNMPs is that it improves the uniformity of band intensities. A second practical advantage of the high efficiency of incorporation of ddNMPs is the requirement for smaller amounts of ddNTPs. This is particularly important for automated DNA sequencing procedures that use fluorescently labeled ddNTPs, since unincorporated nucleotides lead to a high fluorescent background. The elimination of discrimination against ddNTP is highly advantageous when Taq DNA polymerase is used in cycle sequencing.

DNA sequence analysis with the modified bacteriophage T7 DNA polymerase

The chemically or genetically modified gene 5 protein-thioredoxin complex has several properties that make it ideal for DNA sequencing. The enzyme is highly processive as a result of bound thioredoxin. By virtue of the modification the 3' to 5' exonuclease activity is reduced or eliminated. The enzyme does not significantly discriminate against dideoxynucleotides or nucleotide analogs used to eliminate band compressions. Moreover, the complete lack of discrimination against ddNMPs in the presence of Mn^{2+} results in uniform terminations of DNA sequencing reactions, with the intensity of adjacent bands on polyacrylamide gels varying by less than 10% . Consequently, ddNMP-terminated fragments have uniform radioactive intensity throughout the range of a few to thousands of nucleotides in length. There is virtually no background due to terminations at pause sites or secondary structure impediments.

Effect of pyrophosphorolysis on DNA sequence analysis

Pyrophosphorolysis is the reversal of polymerization whereby there is a nucleophilic attack on the 3'-terminal internucleotide linkage by inorganic pyrophosphate to release a dNTP. With T7 DNA polymerase, specific dideoxy-terminated fragments are sensitive to this reaction, resulting in their selective degradation and the loss of the corresponding band on the gel. An example of a sensitive site is CCATATddA with the template sequence of 3'-GGTATATAAAAT-5'. When

dITP is substituted for dGTP in the sequencing reactions, sensitive sites are more frequent, occurring once every 100 nucleotides. There is an inverse correlation between the strength of binding of T7 DNA polymerase to a given site and the sensitivity of that site. The fact that the pyrophosphorolysis reaction is strongest at sites where the polymerase binds with the weakest affinity suggests a mechanism for the interrelationship between pyrophosphorolysis and polymerization. Pyrophosphorolysis is eliminated by the inclusion of inorganic pyrophosphatase in the DNA sequencing reactions. Inasmuch as inorganic pyrophosphatase is highly specific for pyrophosphate and does not affect DNA sequencing reactions other than to prevent the degradation of specific fragments we believe that it should be used in all DNA sequencing reactions.

Novel strategies for DNA sequence analysis using modified T7 DNA polymerase

Incorporation of ddNTPs by T7 DNA polymerase is more efficient when Mn^{2+} rather than Mg^{2+} is used for catalysis. With T7 DNA polymerase and Mn^{2+}, ddNMPs and dNMPs are incorporated at virtually the same rate. Mn^{2+} also reduces the discrimination against other analogs with modifications in the furanose moiety, the base, and the phosphate. 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 gels varying by less than 10%.

Several applications exploit the uniform band intensities obtained with T7 DNA polymerase lacking exonuclease activity in the presence of pyrophosphatase and Mn^{2+}. 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. This method is appealing for use with capillary electrophoresis, where one is limited to all four sets of dideoxy-terminated fragments in a single capillary. We have also used equal band intensity to detect heterozygotic sequences in genomic DNA. Heterozygotic sequences can be detected since their bands have half the intensity of adjacent homozygotic sequences.

The DNA Binding Protein (Gene 2.5 Protein) of Bacteriophage T7

Single-stranded DNA binding proteins (*e.g. E. coli* SSB and T4 gene 32 protein) 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.

We have purified the gene 2.5 protein of phage 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 is non-cooperative. 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 molecules 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. *E. coli* SSB 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) interact with single-stranded DNA, whereas tryptophan residues do not.

T7 gene 2.5 protein interacts with T7 DNA polymerase as measured by affinity chromatography and fluorescence emission anisotropy. T7 DNA polymerase binds to a resin coupled to gene 2.5 protein and elutes at 250 mM NaCl. Steady state fluorescence emission anisotropy gives a dissociation constant of 1.1 μM for the gene 2.5 protein-T7 DNA polymerase complex, with a ratio of the proteins 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. *E. coli* SSB protein is most effective in stimulating the processivity and activity of T7 DNA polymerase on single-stranded DNA. T7 gene 2.5 protein is the second most effective, phage T4 gene 32 protein is significantly less effective, and *E. coli* recA protein inhibits T7 DNA polymerase. Gene 2.5 protein also interacts with the T7 gene 4 proteins as measured by affinity chromatography.

Gene 2.5 protein, like other single-stranded DNA binding proteins has an acidic carboxyl terminal domain that is thought to play a role in the interaction of these proteins with other proteins. Of the 21 carboxyl terminal residues in gene 2.5 protein, 15 are acidic. A truncated form of the gene 2.5 protein lacking the carboxy-terminal 21 amino acid residues no longer forms dimers nor physically interacts with T7 DNA polymerase or gene 4 protein. The truncated protein still has the ability to bind to single-stranded DNA. The mutant gene 2.5 protein, however, can not replace wild-type gene 2.5 protein for T7 DNA replication as measured by its inability to support the growth of T7 phage lacking gene 2.5.

We have shown that gene 2.5 protein stimulates the homologous pairing of complementary DNA strands: at 37 °C the rate is stimulated by a factor of 10,000 over spontaneous renaturation or by a factor of 100 over that observed at 68 °C in 1 M NaCl. The kinetics of homologous pairing are second-order. The reaction has no energy requirement and requires a saturating amount of protein to DNA. When the smaller of the two DNA fragments is 17, 170, or 3,000 nucleotides, gene 2.5 protein stimulates the rate of homologous pairing by a factor of <10, 500, or 10,000, respectively. A 1,000-fold excess of heterologous DNA has no effect on its ability to stimulate homologous base pairing. If one of the complementary strands is RNA gene 2.5 protein has no effect on the rate of homologous pairing. It has been reported that *E. coli* SSB protein and T4 gene 32 protein stimulate renaturation by increasing the second order rate constant, and *E. coli* recA protein stimulates renaturation by a first order mechanism. However, the extent to which these proteins stimulate this reaction is 10-fold less than that observed for the gene 2.5 protein.

In the presence of gene 2.5 protein single-stranded DNA is taken up into duplex DNA via a mechanism of random and bidirectional strand displacement or branch migration. In the presence of gene 2.5 protein, small fragments of DNA annealed to M13 DNA are displaced rapidly, while large regions of duplex are displaced less efficiently or not at all. It is intriguing that *in vivo* gene 2.5 protein may work in conjunction with other T7 replication proteins to provide a unidirectional mechanism for branch migration, a process which is likely to be important for recombination.

The Gene 4 Proteins of Bacteriophage T7; The Helicase/Primase System

The T7 gene 4 protein is found as two species of molecular weight 56,000 and 63,000. The two proteins arise from two different, in-frame initiation codons; they thus share carboxyl termini, while the 63-kDa protein has an additional 63 amino acid residues at its amino end. The 63-kDa protein catalyzes both helicase and primase activities, while the 56-kDa protein catalyzes only helicase activity. Both primase and helicase activities are dependent on the hydrolysis of a NTP to fuel the unidirectional movement of the proteins along single-stranded DNA. The helicase activity is being examined for its use in the sequencing of double-stranded DNAs and the primase as a means of stabilizing short oligonucleotide primers and in site-specific initiation of DNA synthesis.

Sequence analysis reveals a single 'A-type' NTP binding site (NBS) near the center of each gene 4 protein. We have used site-directed mutagenesis to alter the conserved Gly and Lys residues within this NBS. The NBS mutant gene 4 proteins do not complement a T7 phage lacking gene 4. Moreover, the mutations are dominant lethal: they block productive infection by wild-type phage. A NBS mutant 56-kDa protein binds NTPs but lacks the ability to hydrolyze them, and cannot bind to single-stranded DNA. Consequently, this mutant protein also lacks helicase activity. The mutant gene 4 proteins inhibit the NTP hydrolysis activity of wild-type gene 4 proteins in a stoichiometric manner. The inhibition constant (K_i = 22 nM) of this interaction may reflect the gene 4 oligomer dissociation constant in the presence of NTP and DNA. Analysis of the inhibition reaction indicates a linear mixed-type inhibition, and there is no preference for the 56-kDa protein binding to the 63-kDa protein or to another 56-kDa protein. The ability of the NBS mutant proteins to inhibit the activity of the wild-type gene 4 proteins shows that the active species is an oligomer, and that NTP hydrolysis is coordinated among each member of the oligomer.

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 initiation of the 56-kDa protein without affecting the sequence of the 63-kDa protein. Purified 63-kDa protein has primase, helicase, and single-stranded DNA-dependent NTPase activities. The constraints of primase recognition sequences, nucleotide substrate, and the effects of additional proteins on primer synthesis 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. Addition of a 7-fold molar excess of 56-kDa protein to 63-kDa protein increases the number of oligoribonucleotides synthesized by 63-kDa protein. 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 Bacteriophage T7

Due to its proximity to the T7 DNA polymerase gene (gene 5), and the high abundance of gene 5.5 protein, we undertook a study to determine its role in T7 growth. A T7 phage that is deleted for gene 5.5 is viable but DNA synthesis is reduced and the phage are sensitive to restriction by the phage λ *rex* exclusion system. Gene 5.5 protein has been overproduced from cells harboring the cloned gene. Gene 5.5 protein copurifies as a complex with the *E. coli* protein H-NS, a nucleoid protein that binds to double-stranded DNA and regulates transcription from a variety of *E. coli* promoters. *In vivo*, expression of gene 5.5 relieves the repression of the *E. coli proU* promoter by H-NS. Because gene 5.5 protein purified in the absence of H-NS aggregates we constructed a fusion protein of gene 5.5 protein and maltose binding protein. The fusion protein also copurifies with H-NS. Binding of the fusion protein to H-NS abolishes H-NS mediated inhibition of transcription by *E. coli* and T7 RNA polymerase.

One of the roles of gene 5.5 protein is to help the phage escape the restriction system encoded by the *Rex* exclusion system of phage λ. The *Rex* exclusion system is composed of two proteins, rexA and rexB, that together restrict the growth of a variety of phage, the best studied case being the restriction of phage T4 rII mutants. T7 phage that lack gene 5.5 are restricted by low levels of rexA and rexB, while even wild-type T7 is restricted by elevated levels of rexA and rexB. Mutations can be isolated in wild-type T7 that enable the phage to grow in the presence of the high levels of rexA and rexB; the mutations are all in gene 5.5 and change methionine 60 to an isoleucine. T7 gene 5.5 protein expressed from a plasmid enables T4 rII mutants, as well as T7 deleted for gene 5.5, to grow in the presence of rexA and rexB.

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