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Bacteriophage T7 DNA polymerase: Cloning and high-level expression

(thioredoxin/pBR322/synthetic DNA probe/T7 gene 5 protein/expression regulation)

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ABSTRACT **Phage T7 DNA polymerase consists of a 1:1 complex of the viral T7 gene *5* protein and the host cell thioredoxin. A 3.25-kilobase T7 DNA fragment containing the complete coding sequence of gene *5*, and the nearby genes *4.7* and *5.3*, was cloned in the *Bam*HI site of the plasmid pBR322. Transformation of the thioredoxin-negative (*trxA*$^-$) *Escherichia coli* strain BH215 with the recombinant plasmid pRS101 resulted in large overproduction of gene *5* protein corresponding to a level about 60-fold higher than in T7-infected cells. Transcription of gene *5* probably originates from a previously unknown *E. coli* RNA polymerase promoter located immediately upstream of the structural gene. Contrary to expectation, pRS101 could be maintained also in *E. coli trxA*$^+$ cells despite the *in vivo* formation of active T7 DNA polymerase. However, the expression of gene *5* was lower by a factor of 5–10 than in *trxA*$^-$ cells. Since the plasmid copy number in the two strains was the same, a gene dosage effect can be excluded. The observed difference suggests an autoregulatory interaction of T7 DNA polymerase holoenzyme on the expression of T7 gene *5*. The *trxA*$^-$ strain BH215/pRS101 is an excellent source of gene *5* protein and T7 DNA polymerase. After *in vitro* reconstitution of holoenzyme by addition of excess thioredoxin, highly active T7 DNA polymerase was purified to homogeneity by a simple antithioredoxin immunoadsorbent chromatography technique.**

Bacteriophage T7 has evolved a highly efficient and simple system for the replication of its linear duplex 40-kilobase (kb) DNA (1). A phage-induced DNA polymerase and a primase/helicase (gene *4* protein) can account for many of the events known to occur *in vivo* during replication of the T7 genome (2).

Isolation of *Escherichia coli* mutants unable to support the intracellular growth of phage T7 (3) demonstrated that the essential T7 DNA polymerase is a 1:1 complex of the viral gene *5* protein (80 kDa) and the *E. coli* thioredoxin (12 kDa) encoded by the *trxA* gene (4–6). The T7 DNA polymerase holoenzyme has three catalytic activities: 5′ to 3′ DNA polymerase, 3′ to 5′ double-stranded exonuclease, and 3′ to 5′ single-stranded exonuclease (7, 8). The gene *5* protein alone has single-stranded exonuclease activity, whereas both the DNA polymerase and double-stranded exonuclease activities of T7 DNA polymerase require the presence of the host subunit (7, 8). Addition of excess thioredoxin to the gene *5* protein *in vitro* will give reconstitution of T7 DNA polymerase (7, 8).

The role of thioredoxin as essential subunit of T7 DNA polymerase is still an enigma. Thioredoxin is a multifunctional small redox protein of known three-dimensional structure, with a cystine disulfide/cysteine dithiol located in its exposed active center (6). Only the reduced or dithiol form of Cys-32 and Cys-35 in thioredoxin is active in reconstitution of T7 DNA polymerase activity from gene *5* protein (9). Furthermore, other amino acid residues in thioredoxin play a critical role in T7 DNA polymerase activity—e.g., *trxA*7007 thioredoxin with Gly-92 exchanged to aspartic acid is inactive with gene *5* protein (10).

T7 DNA polymerase is an ideal system for studies of the structure and function of a DNA polymerase as well as protein–protein and protein–DNA interactions. Purification of the holoenzyme from T7-infected *E. coli* cells (7, 8, 11, 12) has so far only provided small amounts of pure protein. Based on the recently published complete nucleotide sequence of the T7 genome (13), we have developed a strategy for cloning and expression of gene *5*. Since expression of T7 DNA polymerase could be lethal to the host cell (14), we used a *trxA*$^-$ mutant (7004) lacking detectable levels of thioredoxin (6) to prevent *in vivo* formation of the holoenzyme. Subsequent transformation of *trxA*$^+$ host cells with the same hybrid plasmid showed fully viable transformed cells containing active T7 DNA polymerase with a much lower expression of gene *5*.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *E. coli* MC1061 [*araD139*, Δ(*ara, leu*)7697, Δ*lacX74*, *galU*$^-$, *galK*$^-$, *hsr*$^-$, *hsm*$^+$, *strA*] was obtained from B.-E. Uhlin (Umeå University, Umeå, Sweden). BH215, a *trxA7004* derivative of MC1061 (15), was obtained from J. Fuchs (University of Minnesota, St. Paul, MN). The plasmid pBR322 was from New England Biolabs.

Enzymes and Linker. Restriction endonucleases were purchased from New England Biolabs or Boehringer Mannheim. T4 DNA ligase, T4 polynucleotide kinase, and calf intestinal alkaline phosphatase were from Boehringer Mannheim. Thioredoxin was purified to homogeneity from *E. coli* SK3981 (16). Phage T7 DNA polymerase was prepared from $T7_{3,6}$-infected *E. coli* B cells (11, 12).

Phosphorylated, synthetic deoxynucleotide linker containing a *Bam*HI site (C-G-G-A-T-C-C-G) was from New England Biolabs.

Cloning. Phage T7 DNA was prepared from lysates of wild-type virus (17, 18). Preparation and cloning of DNA fragments was by standard techniques (19, 20). For isolation of the T7 DNA fragment containing gene *5*, T7 DNA was first digested to completion with *Pvu* II, yielding four fragments (13). A mixture of two fragments (10.7 kb and 11.5 kb) recovered after agarose gel electrophoresis was then further digested with *Aha* III. The gene *5*-containing fragment (3.25 kb) was isolated after agarose gel electrophoresis. A few additional *Aha* III sites not predicted by the T7 nucleotide

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Abbreviations: bp, base pairs; kb, kilobase(s).

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sequence (13), but located outside the fragment we isolated, were observed in the T7 DNA.

Hybridization. A synthetic oligodeoxyribonucleotide (C-A-A-C-T-T-G-C-C-G-G-A-A-C-G-C-A-G) complementary to positions 14,680–14,697 of the 1-strand of T7 DNA (13) was a generous gift from S. Josephson (Kabigen, Stockholm, Sweden). It was synthesized with the phosphoamidite method (21). This probe was 5'-end labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (19).

For screening of bacterial transformants, colonies were transferred from agar plates to Whatman 540 filters. Preparations of filters, hybridization, and autoradiography were accomplished as described (22). DNA restriction digests were hybridized directly in dried agarose gels after treating the gels as published (23). The final washing temperature in all hybridizations was 50°C.

Transformation Frequency, Resistance Level, and Plasmid Copy Number. To measure the efficiency of transformation, equal amounts of plasmid DNA were mixed with competent cells (19) and spread on selective plates to give 200–1500 colonies per plate. Resistance levels were determined by spreading plasmid-containing strains on selective plates containing increasing concentrations of ampicillin (0.05–8.0 mg/ml) to give 100–500 colonies per plate.

For the determination of plasmid copy number, cultures were grown and labeled with [^{3}H]thymidine as described (24). The washed cells were incubated at 37°C for 15 min in 1.1 ml Tes buffer (Tes = 0.05 M Tris·HCl/0.005 M EDTA/0.05 M NaCl, pH 8.0) containing lysozyme (1 mg/ml) and RNase (0.5 mg/ml). The lysate was sheared by being gently drawn 20 times in and out of a 1-ml pipette. Radioactively labeled covalently closed circular DNA and chromosomal DNA were separated by vertical dye/buoyant density gradients (25).

Enzyme Assays. DNA polymerase and exonuclease activities on single- and double-stranded DNA were determined as described elsewhere, with an incubation time of 15 min at 37°C (18). DNA polymerase assays of crude extracts contained 10 μM thioredoxin. One unit of DNA polymerase (exonuclease) catalyzes the incorporation (hydrolysis) of 10 nmol of total nucleotide into an acid-insoluble (acid-soluble) product in 30 min at 37°C (7, 18).

Protein Determination. The protein concentration in crude extracts was determined from the absorbance at 230 nm and 260 nm (26).

The concentration of purified T7 DNA polymerase or gene *5* protein was determined spectrophotometrically at 280 nm; the molar extinction coefficient for the holoenzyme, calculated from the predicted amino acid composition (13), is 144,000 $M^{-1}\cdot cm^{-1}$; the value for thioredoxin is 13,700 $M^{-1}\cdot cm^{-1}$ (6).

Electrophoresis. NaDodSO$_4$/polyacrylamide gel electrophoresis was carried out by using the discontinuous gel and buffer systems described by Neville (27).

Computer Analysis. A modified, previously published algorithm (28) was used in the search for potential promoter sites for *E. coli* RNA polymerase on the cloned T7 DNA fragment.

Purification of T7 DNA Polymerase and Gene 5 Protein. BH215/pRS101 cells were grown in a high-density fermenter and harvested in late logarithmic phase. Cells (10 g) were broken with a bacterial X-press and homogenized in 5 vol of 50 mM Tris·HCl, pH 7.5/0.1 M NaCl/10% sucrose/2 mM EDTA/5 mM 2-mercaptoethanol/1 mM phenylmethylsulfonyl fluoride. After centrifugation at 27,000 × *g* for 30 min, thioredoxin was added to a final concentration of 10 μM, and reconstituted T7 DNA polymerase was purified by antithioredoxin immunoadsorbent chromatography followed by phosphocellulose as described (11, 12). For experiments requiring gene *5* protein, thioredoxin was removed by gel filtration on a column of Sephadex G-75 at alkaline pH (18).

RESULTS

Cloning of a 3.25-kb T7 DNA Fragment in pBR322. A suitable fragment containing the entire coding sequence of gene *5* (nucleotides 14,353–16,465) can be isolated by digesting T7 DNA with the two restriction endonucleases, *Pvu* II and *Aha* III (Fig. 1). The predicted blunt-ended fragment has a size of 3247 base pairs (bp) corresponding to nucleotides 13,624–16,871 of the T7 DNA (13), and includes also the flanking genes *4.7* and *5.3*.

After isolation of the expected DNA fragment, synthetic *Bam*HI linkers were added by blunt-end ligation and the fragment was then ligated into the *Bam*HI site of phosphatase-treated pBR322 (Fig. 1). This ligation mixture was used to transform the *E. coli* strain BH215 (*trxA*$^-$). Transformants selected for ampicillin resistance were screened for the insert by colony hybridization with a ^{32}P-labeled synthetic oligonucleotide probe specific for gene *5*. Four of 70 clones contained the T7 DNA fragment; insertion of this fragment was confirmed by *Bam*HI recutting of the plasmids isolated from the positive clones.

Digestion of the recombinant plasmids with *Ava* I produced two fragments with sizes and probe hybridization patterns depending on the orientation of the T7 DNA insert (Fig. 1). The same orientation was found in all four clones, one of which was denoted pRS101 and chosen for further studies (Fig. 1). In the orientation of the T7 DNA insert in pRS101, transcription in the counterclockwise direction is needed for the production of gene *5* mRNA (Fig. 1). Only little transcription has been found to cross the *Bam*HI site of pBR322 in this so-called silent orientation (29–32). However, the number of four clones is statistically too small to draw any

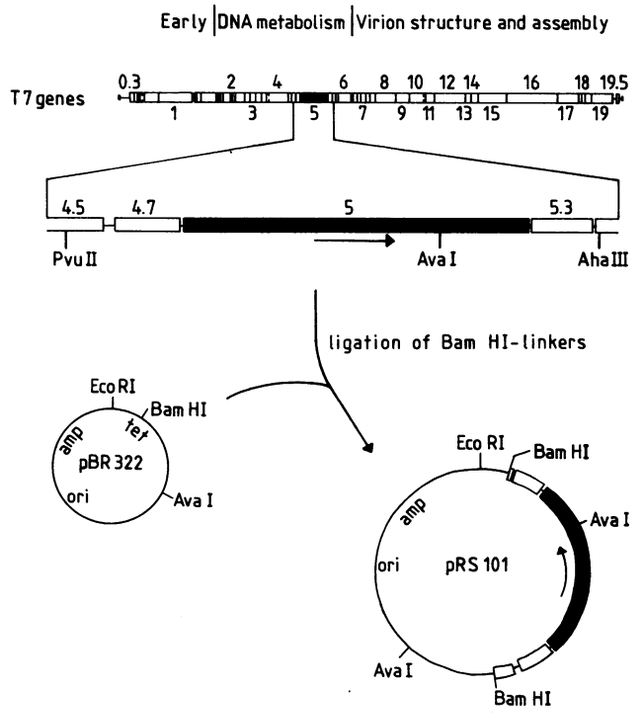

FIG. 1. Cloning of a 3.25-kb T7 DNA fragment containing gene *5* in pBR322. The phage T7 genome, the isolated DNA fragment, and the ligation of this fragment into the vector pBR322 are shown. In a normal T7 infection, only the early genes are transcribed by *E. coli* RNA polymerase (13). In the recombinant plasmid pRS101, DNA lengths are shown approximately to scale. All *Bam*HI and *Ava* I sites are marked. The arrow indicates the direction of transcription to produce mRNA for the cloned T7 genes.

definite conclusions about a possible preferential orientation of the insert.

Expression of the Cloned Gene *5*. To study if the presence of thioredoxin affects the transformation proficiency and/or the expression of the cloned gene *5* we also transformed MC1061, a *trxA*$^+$ counterpart, with pRS101. We observed no difference in transformation frequency (transformants per μg of DNA per viable cell) between the two host strains. Assays of DNA polymerase activity in the presence of excess thioredoxin (10 μM), using crude extracts of cells from cultures of BH215/pRS101 and MC1061/pRS101, revealed two unexpected results (Fig. 2). First, contrary to expectation, both extracts contained high DNA polymerase activity, indicating efficient expression of gene *5* from pRS101. Second, the DNA polymerase activity varied remarkably between the two systems with 5- to 10-fold higher activity in the *trxA*$^-$ host (BH215/pRS101).

As expected, there was no detectable DNA polymerase activity in extracts from BH215/pRS101 without the addition of thioredoxin to assays. In the presence of 10 μM thioredoxin the maximal specific activity of 1350 units/mg of protein was found in crude extracts of late logarithmic-phase cells (Fig. 2*A*). This value was about 60-fold higher than that observed previously in T7-infected cells (4, 11). Compared with the specific activity of pure T7 DNA polymerase (Table 1), the gene *5* protein level of BH215/pRS101 roughly corresponds to 5% of total soluble protein. In contrast, extracts of the *trxA*$^+$ host (MC1061/pRS101) showed T7 DNA polymerase activity without addition of thioredoxin (Fig. 2*B*), consistent with *in vivo* formation of T7 DNA polymerase from thioredoxin and gene *5* protein. Assays in the presence of 10 μM thioredoxin showed 3- to 4-fold higher activity, indicating that free gene *5* protein was present.

The large difference in expression of the gene *5* protein between the BH215/pRS101 and MC1061/pRS101 systems observed by DNA polymerase assays was confirmed by NaDodSO$_4$/polyacrylamide gel electrophoresis. As shown in Fig. 3 analysis of whole cells from a BH215/pRS101 culture showed a prominent band corresponding to gene *5* protein (80 kDa) of pure T7 DNA polymerase. This band was absent in cells containing pBR322 without insert and was much weaker in MC1061/pRS101, in agreement with the lower T7 DNA polymerase activity in these *trxA*$^+$ cells.

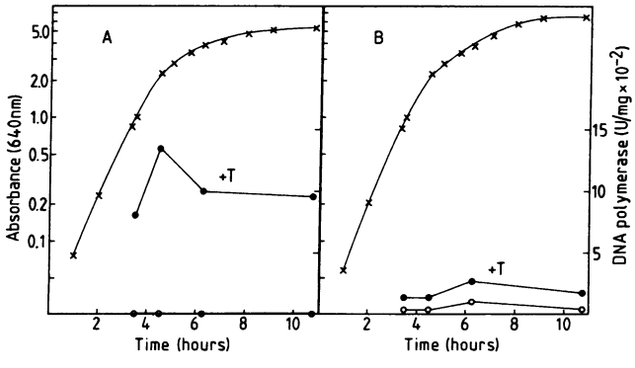

FIG. 2. DNA polymerase activity in cells from BH215/pRS101 (*A*) and MC1061/pRS101 (*B*) in different stages of growth. Parallel cultures of cells were grown in LB medium (11). Cell growth (×) was monitored at 640 nm in 1-cm (diameter) cells. Cells were harvested by centrifugation at 4°C and lysed by lysozyme and sonication (11) followed by preparation of crude extracts by centrifugation at 15,000 × *g* for 30 min. After dilution of the crude extracts by a factor of 200 (11), DNA polymerase activity was determined in an assay mixture either without (○) or with (●) addition of 10 μM thioredoxin (T). Enzymatic activity is expressed as units/mg of protein in crude extract.

Table 1. Enzyme activity of T7 DNA polymerase and gene *5* protein isolated from BH215/pRS101

Enzyme	Specific activity, units/mg of protein: DNA polymerase*	Double-stranded exonuclease†	Single-stranded exonuclease‡
T7 DNA polymerase	32,100	32,700	44,400
Gene *5* protein	<100	300	12,500

*Determined with heat-denatured salmon sperm DNA (18).
†Determined with native ^{3}H-labeled T7 DNA (18).
‡Determined with heat-denatured ^{3}H-labeled T7 DNA (18).

In parallel with the increase in the 80-kDa gene 5 band in stationary phase, a prominent band of about 40 kDa appeared. This band was also observed in untransformed BH215 cells in stationary phase (data not shown) and seems to be unrelated to gene *5* expression.

Potential Gene *5* Promoter in pRS101. In normal T7 infection, gene *5* is transcribed by T7 RNA polymerase (13); no promoter site for *E. coli* RNA polymerase has been described on the cloned T7 DNA fragment upstream of gene *5* (13). Since no strong promoter for counterclockwise transcription of pBR322 across the *Bam*HI site is known, we examined the nucleotide sequence around the linkage between pBR322 and the inserted T7 DNA fragment. No evidence for a promoter site was found. However, a computer search of the cloned T7 DNA fragment revealed a putative promoter site immediately upstream of gene *5* between nucleotides 14,293 and 14,321. In fact, a double promoter pattern was found, yielding a weak promoter site for *E. coli* RNA polymerase with either a 17- or a 15-base spacing between the −10 and the −35 box of the consensus sequence (33);

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14,290                                  14,320
  |                                       |
AGGTTTCTTGACAGTTGTTTCATATGAAGAGATTGTTAA
        −35 region              −10 region
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The significance level for a 17-base spacing was similar to that obtained for the T7 C promoter (13). The value for a 15-base spacing, weighed according to Harr *et al.* (28), was slightly lower. Such double promoters have been suggested to give higher expression than expected from their significance levels (28). Transcription of gene *5* in cells transformed with the plasmid pRS101 may start at this potential promoter sequence in the T7 DNA, but further studies on the level of mRNA are needed to establish if this sequence is used *in vivo*.

Expression of T7 Genes *4.7* and *5.3*. In addition to gene *5*, pRS101 contains the complete sequences of gene *4.7* (405 bp) and gene *5.3* (354 bp). Expression of gene *4.7* has been observed in T7-infected cells but nothing is known about the function of this small protein (34). Expression of the potential gene *5.3* found by T7 DNA sequence analysis (13) has not been described.

In NaDodSO$_4$/polyacrylamide gels of BH215/pRS101 and MC1061/pRS101 cells a weak band corresponding in size to that of gene *4.7* protein (15.2 kDa) was observed in cells harvested in stationary phase (data not shown). Expression of this protein and gene *5* protein is apparently not coregulated, a further support for the postulated localization of a promoter site for *E. coli* RNA polymerase directly upstream of gene *5*. Expression of this 15.2-kDa protein may instead be the result of read-through from a pBR322 promoter (32). The NaDodSO$_4$/polyacrylamide gels of cells containing pRS101 showed no evidence of the presence of the proposed gene *5.3* protein (13.1 kDa).

Possible Mechanism for the Thioredoxin-Mediated Regulation of Expression of T7 Gene *5*. The lower expression of T7

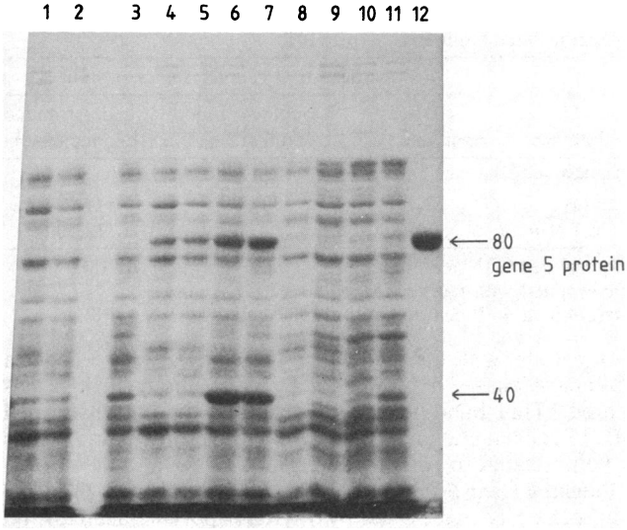

FIG. 3. $NaDodSO_4$/polyacrylamide gel electrophoresis (7.5% gel) of plasmid-containing cells. Cells from the same cultures as in Fig. 2 were harvested by centrifugation, lysed by boiling in $NaDodSO_4$, and applied to the gel. Each slot contained about 4.3×10^7 cells. Staining of the gel was with Coomassie brilliant blue R 250. Lanes 1–3, BH215/pBR322, cells harvested after 4, 6, and 9 hr, respectively. Lanes 4–7, BH215/pRS101, and lanes 8–11, MC1061/pRS101; the cells were harvested after 3.5, 4.5, 6.25, and 10.75 hr, respectively (see Fig. 2). Lane 12, 0.7 μg of T7 DNA polymerase from phage $T7_{3,6}$-infected *E. coli* cells. Numbers indicate sizes in kDa.

gene *5* from pRS101 in $trxA^+$ cells versus $trxA^-$ cells could result from differences in gene dosage—i.e., plasmid copy number and regulation of expression. Large amounts of the holoenzyme formed from gene *5* protein and thioredoxin within the $trxA^+$ cells might interfere with the processes involved in gene expression either by an overall effect on the complete recombinant plasmid or by a more specific effect on the cloned T7 DNA fragment. To differentiate between these alternatives, we performed a direct determination of the plasmid copy number. The same value was found for plasmids pBR322 and pRS101 and it was constant in the $trxA^+$ and $trxA^-$ host (data not shown). This clearly demonstrates that the observed large difference in expression of gene *5* is not a gene dosage effect. Rather, it implies a specific interaction of the *in vivo* formed T7 DNA polymerase holoenzyme in $trxA^+$ cells with the cloned T7 DNA fragment.

The $trxA^+$ and $trxA^-$ strains both showed about 2-fold higher ampicillin resistance when transformed with the hybrid plasmid pRS101 compared to pBR322. Further experiments are required to see if this is due to a possible transcription from the cloned T7 DNA fragment into the *bla* region of pBR322. The fact that both plasmids have the same copy number excludes also here a gene dosage effect.

Isolation and Characterization of T7 DNA Polymerase from BH215/pRS101. Thioredoxin was added in excess to extracts of BH215/pRS101 and the resulting T7 DNA polymerase was isolated by antithioredoxin immunoadsorbent affinity chromatography (12). The isolated T7 DNA polymerase was homogenous, as determined by $NaDodSO_4$/polyacrylamide gel electrophoresis. The pure enzyme was very active (Table 1) and had considerably higher specific activity than our previously purified T7 DNA polymerase (12). Also, the pure gene *5* protein, isolated by removal of thioredoxin from T7 DNA polymerase by gel filtration at pH 11.5 (18), displayed the expected single-stranded exonuclease activity but lacked DNA polymerase activity. The development of an improved purification method for gene *5* protein and T7 DNA polymerase and an enzymatic characterization will be published elsewhere.

DISCUSSION

We have cloned the gene *5* of T7 DNA polymerase on the plasmid pBR322 and obtained a high-level expression of active gene *5* protein in transformed $trxA^-$ cells. The gene *5* product is one of the major proteins in stationary-phase BH215/pRS101 cells. These cells are an excellent source of highly active gene *5* protein and reconstituted T7 DNA polymerase for further physicochemical and enzymic studies.

The gene for thioredoxin from *E. coli* (*trxA*) has been cloned on a pBR325 derivative and is very well expressed (16). Since the nucleotide sequence of the *trxA* gene has been determined (35), site-directed *in vitro* mutagenesis of both subunits in the T7 DNA polymerase is now possible and should help to clarify the role of each. The fact that thioredoxin from *E. coli* has known three-dimensional structure (6) together with the predicted amino acid sequence of gene *5* protein (13) should facilitate such investigations; in particular T7 DNA polymerase is also a prime candidate for x-ray crystallographic studies.

Our cloning of the 3.25-kb T7 DNA fragment into pBR322 showed several unexpected results. (*i*) The inserted gene *5* is efficiently expressed from the so-called silent orientation (29–32). We suggest that this is due to transcription of gene *5* in pRS101 from a previously unrecognized double promoter structure immediately upstream of gene *5*. (*ii*) The level of gene *5* expression of pRS101 was much lower in a thioredoxin-containing ($trxA^+$) strain. The formation of T7 DNA polymerase holoenzyme provides a mechanism for autoregulatory expression of gene *5*. Such autoregulatory interactions are known for many genes, including T4 DNA polymerase (36). (*iii*) We expected the expression of T7 gene *5* in $trxA^+$ cells to be lethal, since active T7 DNA polymerase is formed. For example, it is known that overproduction of DNA polymerases and ligases in *E. coli* (14, 37, 38) is lethal. The *polA* gene coding for *E. coli* DNA polymerase I could only be efficiently expressed after exchange of its own promoter for the inducible λP_L promoter (38). However, we observed no difference in viability of $trxA^+$ or $trxA^-$ cells with pRS101, although the former may contain about 10^4 copies of active T7 DNA polymerase per cell. Whether the absence of detrimental effects of pRS101 on cell growth in *E. coli* is a general feature of foreign DNA polymerases or reflects a specific property of T7 DNA polymerase remains to be determined.

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