β*, a UV-inducible Shorter Form of the β Subunit of DNA Polymerase III of Escherichia coli

II. OVERPRODUCTION, PURIFICATION, AND ACTIVITY AS A POLYMERASE PROCESSIVITY CLAMP*

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Rami Skaliter, Moshe Bergstein, and Zvi Livneh‡
From the Department of Biochemistry, The Weizmann Institute of Science, Rehovot 76100, Israel

Control elements located inside the coding sequence of dnaN, the gene encoding the β subunit of DNA polymerase III holoenzyme, direct the synthesis of a shorter and UV-inducible form of the β subunit (Skaliter, R., Paz-Elizur, T., and Livneh, Z. (1996) J. Biol. Chem. 271, 2278–2281, and Paz-Elizur, T., Skaliter, R., Blumenstein, S., and Livneh, Z. (1996) J. Biol. Chem. 271, 2282–2290). The protein, termed β*, was overproduced using the phage T7 expression system, leading to its accumulation as inclusion bodies at 5–10% of the total cellular proteins. β* was purified in denatured form, followed by refolding to yield a preparation >95% pure. Denatured β* had a molecular mass of 26 kDa and contained two isoforms when analyzed by two-dimensional gel electrophoresis. The major isoform had a pI of 5.45, and comigrated with cellular β*. Size exclusion high performance liquid chromatography under nondenaturing conditions and chemical cross-linking experiments indicate that β* is a homotrimer. DNA synthesis by DNA polymerase III* was stimulated up to 10-fold by β*, primarily due to an increase in the processivity of polymerization. It is suggested that β* functions as an alternative sliding DNA clamp in a process associated with DNA synthesis in UV-irradiated cells.

DNA-damaging agents in general, and UV radiation in particular, affect dramatically the physiology of Escherichia coli (1). These changes are regulated at the molecular level by several stress regulons, most notably the SOS and heat shock responses (2–4). The immediate response is a transient arrest of DNA replication (5–7), which provides time for the repair of damaged DNA. DNA replication then recovers in a process that requires SOS-inducible proteins (5–7), but its mechanism is largely unknown. During the post-UV recovery period mutations are formed, primarily at sites of DNA damage. This mutagenesis pathway is believed to occur by polymerization through DNA lesions, a process termed bypass synthesis (7–9).

A detailed biochemical analysis of in vitro replication of UV-irradiated or depurinated DNA with purified DNA polymerase III* (Pol IIII)1 holoenzyme (10–13) led us to concentrate on the β subunit of the polymerase and to conclude that it modulates UV mutagenesis in vivo (14) and bypass of UV lesions in vitro (15). The β subunit is the major processivity factor of Pol III holoenzyme (16). It is a homodimer that forms a ring structure (17), and once loaded on DNA by the γ complex it functions as a sliding DNA clamp that tethers the polymerase to the DNA, thus endowing it with high processivity (18–20). In companion studies (39, 40) we reported that in UV-irradiated E. coli cells a shorter form of the β subunit, termed β*, is produced, which corresponds to the C-terminal two-thirds of the β subunit. This study describes the overproduction, purification, and characterization of β* and its activity as an alternative processivity clamp for DNA polymerase III.

MATERIALS AND METHODS

Plasmids—Plasmid pSK11 that overproduces β* was constructed as follows. The source for the dnaN* gene was plasmid pUN222, which is similar to pUN234 (14) except that the dnaN gene was cloned opposite to lacP. It was constructed by doing the Sphi (1516)-Styl (3031) DNA fragment carrying the entire dnaN gene from plasmid pJS9 (21) into the SmaI site of plasmid pUC18. Plasmid pUN222 was digested with restriction nucleases EcoRI and Aval, to yield a 1125-base pair fragment, that was further digested with Hpal. This resulted in a 860-base pair fragment which carries the entire dnaN* gene, with its ATG codon at the 5′-terminus of the fragment. The ends of the fragment and of NdeI-decleaved plasmid pET3a (22) were filled-in with the Klenow fragment of DNA polymerase I and then the two DNA fragments were ligated to form plasmid pSK11. Plasmid pKT11 was derived from pSK11 by deleting a 134-base pair segment from the SacI site (2940) located downstream to the dnaN* terminator down to the BamHI site in the polylinker. This removed the re£ sequence downstream to the dnaN* gene.

Proteins and Chemicals—The β subunit was purified from E coli J13576 cells harboring plasmid pJS9 as described (21). DNA polymerase III* and single-strand DNA binding protein were purified as described by Lasken and Kornberg (23) and Weiner et al. (24), respectively. Affinity-purified antibodies against the β subunit and against β* were described in companion studies (39, 40). Restriction nucleases were purchased from Pharmacia Biotech Inc. and from New England Biolabs. T4 DNA ligase was the product of Stratagene, DNA polymerase I (Klenow fragment) and calf alkaline phosphatase were from U. S. Biochemical Corp., and chicken egg lysozyme was from Sigma. The sources for materials used are as follows: sodium deoxycholate, ethyl dimethyl carboximate, and N-hydroxysuccinimide, Sigma; nucleotides and phosphates, Pharmacia; radiolabeled nucleotides, Amersham Corp.; dimethyl sulfoxide, Pierce.

Purification of β* and Inclusion Bodies—E. coli BL21(DE3) cells harboring plasmid pKT11 were grown at 37°C in a 12-liter fermentor with constant stirring and aeration, to 100 Klett units. The cells were treated with 0.5 mM IPTG for 2 h to induce the synthesis of β*. The cells (18 g) were collected, resuspended in a equal volume of a buffer containing 50 mM TrisHCl, pH 7, 15% sucrose, and frozen in liquid nitrogen. The cells suspension was thawed at 10°C and its volume was increased to 100 ml with the same buffer. Cells were then disrupted by a 5-min sonication period in a Soniprep M5E sonicator equipped with a
medium tip at 34°C, and centrifuged at 25,000 rpm in a Beckman Ti-45 rotor for 1 h. The supernatant was discarded and the pellet was homogenized in water, spun at 15,000 rpm in a Ti-45 rotor for 0.5 h, and washed again with water. The pellet was homogenized and treated with chicken egg lysozyme (0.3 mg/ml) for 1 h at 22°C, followed by 2% sodium deoxycholate for 1 h at 22°C. Following centrifugation in a Beckman Ti-45 rotor at 15,000 rpm for 0.5 h, the pellet was washed once with a solution of 4 M urea in water for 0.5 h and twice with water. The washed inclusion bodies (final yield 200 mg) contained >90% pure β* as estimated by Coomassie Blue staining.

Denaturation—The inclusion bodies were denatured with a buffer containing 8 M urea, 50 mM Tris HCl, pH 7.4, 10 mM dithiothreitol, and 1 mM EDTA (buffer A). After 2 h of incubation at 4°C, the solution was spun at 40,000 rpm in a Beckman Ti-45 rotor at 4°C for 4 h. The supernatant contained the soluble β* as judged by SDS-PAGE.

Q-Sepharose Chromatography—Denatured β* were loaded on a 15-ml Q-Sepharose (Pharmacia) column equilibrated with 7 M urea, 50 mM Tris HCl, 1% sodium deoxycholate for 1 h at 2°C. Following centrifugation in a Beckman Ti-45 rotor for 1 h at 2°C, the column was washed with 2 column volumes of buffer B, and the proteins were eluted with 10 column volumes of a linear gradient of 0–500 mM KCl in buffer B. β* eluted at 150 mM KCl.

Phosphocellulose Chromatography—The peak fractions from the Q-Sepharose column were combined and fractionated on a phosphocellulose column (Whatman, P11) in buffer B. β* eluted in the flow-through.

Refolding of β*—The fractions containing β* (5 mg) were dialyzed against a concentration of 0.1 mg/ml protein and dialyzed against a refolding buffer containing 0.2 M KCl, 50 mM Tris-HCl, 0.5 mM EDTA, 10 mM β-mercaptoethanol, and 10% glycerol (buffer C). After dialysis was completed, the solution was cleared with a 2-h spin at 200,000 x g. The supernatant contained the soluble β*. About 40% of the β* (2 mg) remained soluble. The purity was greater than 98% as estimated by Coomassie Blue staining. β* was concentrated using Centriprep 10 (Amicon) and by dialysis against buffer C with 50% glycerol.

Refolding at concentrations higher than 0.1 mg/ml led to precipitation of β*.

Size Exclusion Chromatography—β* was analyzed by HPLC size exclusion chromatography on a TSK3000SW column (Tosoh) equilibrated with a buffer containing 50 mM Tris-HCl, pH 7.4 and 0.1 M KCl at a flow rate of 0.7 ml/min. The elution profile was monitored in parallel with a UV detector at 214 nm and by Western blot analysis, using anti-β antibodies. The column was calibrated with HPLC standards (Bio-Rad).

Chemical Cross-linking of Proteins—β* at a concentration of 10 μg/ml was incubated with 1 mg/ml dimethyl suberimidate (25) in a buffer containing 100 mM Tris-HCl pH 8.5 for 4 h at room temperature, after which it was fractionated by SDS-PAGE on an 8% gel. The cross-linked proteins were detected by Western blot analysis with anti-β antibodies. The β subunit (30 μg/ml in 50 mM Tris-HCl, pH 7.4) was cross-linked with 66 mM ethyl dimethyl carbodiimide and 13 mM N-hydroxysuccinimide for 4 h at room temperature.

Purification of β* from a Denaturing Polyacrylamide Gel—One mg of denatured β* was fractionated by SDS-PAGE (12.5% gel). The edges of the gel were cut out and stained with Coomassie Blue in order to localize β*. The gel region containing β* was cut out, and dialyzed against PAGE running buffer containing 10% glycerol. β* was electroeluted overnight at 70 V at 4°C. At the end a reverse voltage of 100 V was applied for 30 min order to detach the protein from the wall of the dialysis bag. The solution containing β* was cleared by centrifugation at 200,000 x g and dialyzed again overnight at 4°C against a renaturation buffer containing 50 mM Tris-HCl, 10% glycerol, 100 mM NaCl, 0.5 mM EDTA, and 1 mM dithiothreitol. The final dialysis was against the same buffer except that the glycerol was raised to 50%. The final concentration of β* was 0.1 mg/ml.

Standard Replication Assays—The synthetic oligonucleotide primer strand was annealed with a buffer containing 10 mM Tris-HCl, pH 7.5, 80 μg/ml bovine serum albumin, 5 mM dithiothreitol, 4% glycerol, 8 mM MgCl2, and 0.1 mM EDTA.

The standard replication mixture contained the annealing mixture supplemented with 0.5 mM ATP, 40 mM KCl, 50 μM dGTP, 50 μM dCTP, 0.15 pmol of DNA polymerase III*, 35 fmol (as circles) of primed M13mp8 ssDNA in a volume of 25 μl. β* (as a primer) and the β subunit (as a dimer) were added when required, usually at a final concentration of 200 ng. After a 15-min incubation the reaction was started by the addition of 50 μM dATP and 10 μM [α-32P]dCTP. The reaction was carried out at 30°C. Incorporation of [α-32P]dCTP into acid insoluble material was determined by trichloroacetic acid precipitation, and replication products were analyzed by alkaline agarose gel electrophoresis as described previously (26).

Processivity of DNA Polymerase III*—This was assayed under standard conditions as described above except that an excess of primed M13mp8 ssDNA (2.8 pmol) over the polymerase (0.05 pmol) was used, and SSB was omitted.

RESULTS

Overproduction of β*—Due to the low abundance of β*, we decided to overproduce it before attempting purification. We chose the phage T7 expression system (22), and the sequencing code of the dnaN* gene by precise tailoring of the ATG initiation codon of dnaN* to the Shine-Dalgarno and promoter sequences of the phage T7 φ10 gene in plasmid pET3a to yield plasmids pSK11 and pKT11. Pulse labeling with [35S]methionine of cells harboring plasmid pKT11 followed by SDS-PAGE and autoradiography revealed that, 30 min after addition of IPTG, β* was synthesized almost exclusively (Fig. 1). Based on densitometric scanning of Coomassie Blue-stained gels β* comprised up to 8% of the total protein (Fig. 2, lane 1). Immunoblots of extracts of overproducing cells were probed with polyclonal antibodies against the β subunit of Pol III, assuming that at least some antigenic determinants are
common to β* and the β subunit. Indeed, the overproduced protein reacted with the anti-β antibodies (Fig. 1).

Purification of β*—The overproduced β* protein was present in the cells as insoluble inclusion bodies. Those were washed extensively, solubilized in 8 M urea, purified by ion exchange chromatography on Q-Sepharose and phosphocellulose, and refolded (Fig. 2). The critical step in the purification procedure was the refolding step, and successful renaturation required that β* be present at a concentration of 0.1 mg/ml. Under these conditions, β* remained soluble and active (Fig. 2; see below). N-terminal protein sequence analysis revealed that the first 10 amino acids of the purified protein were Met Lys Arg Leu Ile Glu Ala Thr Gln Phe, as expected for β* (40). The presence of small amounts of the β subunit in the preparation of β* may interfere with the assay for β* activity. We examined the presence of contaminations of the β subunit in the purified β* preparation by Western blot analysis using anti-β subunit antibodies. Even at high amounts of β* no traces of the β subunit were detected (Fig. 3; detection limit was 1 ng of the β subunit).

Analysis of β* by Two-dimensional Gel Electrophoresis—The purified β* was analyzed by two-dimensional gel electrophoresis (isoelectric focusing and SDS-PAGE) according to O’Farrell (27). After separation the proteins were blotted onto a nitrocellulose membrane and probed with anti-β subunit antibodies. As can be seen in Fig. 4, β* had two major isoforms. The major form had a pI of 5.45 in agreement with the calculated pI value of 5.46. The significance of the two isoforms is not known yet. The relative abundance of the two isoforms varied in different preparations, most likely due to variations in the overproduction. The β subunit which was fractionated in the same gel as an internal marker had a pI of 5.15.

Determination of the Molecular Mass of Native β*—When analyzed by SDS-PAGE β* had a molecular mass of 26 kDa as expected from the size of the open reading frame in the dnaN* gene (Fig. 2). In order to estimate the molecular mass of native β*, we analyzed it by HPLC size exclusion chromatography, using a TSK 3000SW column. The elution profile was monitored with a UV detector at 214 nm, and the fractions were tested for the presence of β* by Western blot analysis. Fig. 5 shows the profile of elution from the HPLC column and the Western blot analysis of the protein peak (retention time of 27.4 min). Based on calibration with HPLC protein standards native β* has an
In addition to the monomeric and a putative subunit, the HPLC peak of Pol III holoenzyme that lacks the DNA clamp activity similar to the putative subunit, is 50–100-fold less active than the holoenzyme (15, 23). This is primarily due to the lower processivity of Pol III*, 8000 nucleotides per 8% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with anti-β antibodies. Lane 1, cross-linked β*; lane 2, cross-linked β subunit.

In order to further support its suggested trimeric structure, the β subunit and the HPLC peak of β* were each subjected to chemical cross-linking. These proteins proved to be remarkably resistant to cross-linking attempts by a variety of chemical agents. Successful cross-linking of β* was achieved with dimethyl suberimidate (25). The cross-linked products were separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and probed with anti-β antibodies. Lane 1, cross-linked β*; lane 2, cross-linked β subunit.

Fig. 6. Chemical cross-linking of β*. β* was cross-linked with dimethylsuberimidate, and the β subunit was cross-linked with ethyl dimethyl carbamidate and N-hydroxysuccinimide as described under "Materials and Methods." The cross-linked proteins were fractionated by 8% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with anti-β antibodies. Lane 1, cross-linked β*; lane 2, cross-linked β subunit.

apparent molecular mass of 75 kDa, representing most likely a trimeric form of β* (calculated molecular mass, 78 kDa).

In order to further support its suggested trimeric structure, the β subunit and the HPLC peak of β* were each subjected to chemical cross-linking. These proteins proved to be remarkably resistant to cross-linking attempts by a variety of chemical agents. Successful cross-linking of β* was achieved with dimethyl suberimidate (25). The cross-linked products were separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and probed with anti-β antibodies. Despite considerable efforts to reduce smearing, the treated proteins migrated as smeared bands; still the major result is clearly seen. The cross-linking of β* produced two forms in addition to the monomeric β*, a β* dimer formed most likely due to partial cross-linking, and a putative β* trimer representing fully cross-linked β* (Fig. 6, lane 1). The apparent molecular mass of the putative β* trimer was 107 kDa in this gel, higher than the calculated molecular mass of 78 kDa. To examine whether this discrepancy is due to an aberrant migration of the cross-linked protein in the gel we used cross-linked dimeric β subunit as a marker. The β subunit was cross-linked with ethyl dimethyl carbamidate and N-hydroxysuccinimide since dimethyl suberimidate was ineffective. As can be seen in Fig. 6 (lane 2) the only band in addition to the monomeric β subunit, presumably the β subunit dimer, had an apparent molecular mass of 107 kDa, identical to that of the putative β* trimer. Taken together with the HPLC data these result suggests that the native β* is a trimer.

β* Stimulates DNA PolymeraseIII*—Pol III*, a subassembly of Pol III holoenzyme that lacks the β subunit, is 50–100-fold less active than the holoenzyme (15, 23). This is primarily due to the lower processivity of Pol III*, 190 nucleotides per binding event, as compared to a processivity of >8000 nucleotides per binding event of the holoenzyme (16, 28, 29). Does β* have a DNA clamp activity similar to the β subunit, and can it stimulate Pol III*? As can be seen in Fig. 7, β* stimulated DNA synthesis by Pol III* up to 10-fold. The kinetics of this DNA synthesis was slow relative to that of Pol III holoenzyme (10), suggesting a slow initiation step and/or slow elongation. We next examined replication products by alkaline agarose gel electrophoresis (Fig. 8). In order to detect early replication products we preincubated all of the components of the reaction, except for dATP and dTTP, and then initiated polymerization by the addition of the two missing dNTPs. This allows the formation of polymerase-DNA complexes such that polymerization commences immediately upon addition of the dNTPs. Under these conditions, Pol III* synthesized DNA products not longer than several hundred nucleotides in length, which are hardly seen at the bottom of the autoradiogram. The longer products seen are most likely due to contaminating traces of the β subunit in the Pol III* preparation. They are visible here since the experiment was conducted with an excess of enzyme. Upon addition of β*, the amount and size of DNA products increased dramatically. The kinetics of elongation was fast, and full-length products were observed by 15 s. Thus, the addition of β* to Pol III* formed a polymerizing complex which behaved similarly to Pol III holoenzyme.

Can the activity observed be due to a contamination of the β subunit in our β* preparation? As shown in Fig. 3 no β subunit could be identified in immunoblots of β* preparations. However, in order to rule out completely this possibility, we fractionated the purified β* on an SDS-polyacrylamide gel, eluted the β* band and renatured it. This preparation of β*, which was resolved on the gel from any contamination of the β subunit, stimulated Pol III* (Fig. 9, lane 2 compared to lane 1 at each time point), although to a lesser extent than the original β* preparation (Fig. 9, lane 3 compared to lane 2 at each time point). Presumably the harsh treatment led to a considerable decrease in the activity of β*. Still, the results clearly show that the stimulation of Pol III* was caused by β*.

The Effect of β* on DNA PolymeraseIII*IsATPDependent—DNA synthesis by Pol III holoenzyme requires ATP (or dATP) for assembling a polymerase-DNA initiation complex (30). Does the effect of β* on Pol III* depend on ATP? To examine this issue we omitted ATP from the reaction mixture, and replacing dATP by dATPγS. dATPγS is incorporated into DNA by the polymerase, but in contrast to dATP it does not support the formation of a stable initiation complex between the primed DNA and the polymerase (13). As can be seen in Fig. 10, stimulation of Pol III* by either the β subunit or by β* was completely dependent on the presence of ATP. Notice that DNA synthesis in the presence of β* was 10-fold lower than in the presence of the β subunit.

β* Increases the Processivity of DNA Polymerase III*—In
order to examine whether the stimulation of Pol III* by β* occurred via an increase in the processivity of the polymerase, replication assays were performed with the template present at a 60-fold excess over the polymerase. This enabled to monitor the size of products that were replicated by the polymerase following a single binding event. Under these conditions, the length distribution of replication products remains unchanged with time, but their amounts increase. If processivity is affected, a change in the length of the replication products is expected. Under these conditions, Pol III* alone synthesized products of no longer than several hundred nucleotides in length, that were obscured by the unincorporated radiolabeled dNTP at the bottom of the autoradiogram (Fig. 11). In contrast, in the presence of β*, the polymerase synthesized long products, up to fully replicated DNA. Two major products were observed. The fully replicated DNA (7.2 kilobase pairs) and a shorter product of approximately 4 kilobase pairs, the result of a major pause site in the ssDNA template (Fig. 11). This was caused most likely by a secondary structure in the ssDNA, formed in the absence of SSB, that impeded the progression of the polymerase. Thus, similarly to the β subunit, β* conferred high processivity on Pol III*; however, the efficiency of the formation of processive polymerase complexes was lower.

**DISCUSSION**

Our results suggest that β* can act as an alternative polymerase clamp, and confer high processivity on Pol III* (Fig. 11). This activity requires ATP and is thus likely to involve loading of a Pol III*-β* complex on the primer template, similar to the case of Pol III holoenzyme (18, 19). The overall slow synthesis activity of this polymerase (Fig. 7) is likely to be due to a slower initiation stage, e.g. a slow loading process of β* on the DNA by the γ complex which is part of Pol III* (18, 19). Consistent with this possibility, when a period of preincubation was allowed, a
rapid phase of polymerization was observed, similar to that of Pol III*β2 (Figs. 8 and 10). A possible explanation is that the preincubation period allows the assembly of Pol III*β2 complexes, and thus upon addition of the dNTPs polymerization resumes immediately. According to this model the assembly of a Pol III*β2 complex on the primer-template is less efficient than the assembly of a Pol IIIβ2 initiation complex. However, once a Pol III*β2 complex is assembled on the DNA, it has a high processivity similar to that of Pol III holoenzyme.

It was recently shown by x-ray crystallography that the β subunit is composed of three structurally similar domains, and it dimerizes to form a hexagon-like ring (17). β* contains precisely two of the three domains of the β subunit (40). The fact that β* appears as a trimer suggests that it may form a β*2-like ring structure, composed of three two-domain proteins, forming an alternative DNA clamp. The three repeating domains of the β subunit are structurally very similar, although there is no significant homology at the amino acid sequence level (17). Although the overall structure of β2 may be similar to that of β2, it is expected to show significant differences in activity as compared to β2, since it lacks the N-terminal domain of the β subunit.

Polymerase clamps composed of homotrimeric clamp proteins are in fact more common than dimeric clamps. The gp45 gene of bacteriophage T4, which serves as an auxiliary subunit of T4 DNA polymerase (31), is an homologue of the β subunit (17). Indeed, gp45 forms a trimer in solution (32, 33) and is likely to form a β2-like sliding clamp. In eukaryotes, proliferating cell nuclear antigen (PCNA) serves as a processivity factor of DNA polymerase α, which is involved in DNA replication and repair (34–36). PCNA has usually a size of 29 kDa, and it forms a trimeric hexagonal ring-shaped structure very similar to that of the β subunit (37). Interestingly, it was recently reported that two PCNA genes were found in carrot, encoding putative PCNA subunits (37). Krishna, T. S., Kong, X. P., Gary, S., Burgers, P. M., and Kuriyan, J. (1994) J. Mol. Biol. 249, 660–6809.

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