The priB Gene Encoding the Primosomal Replication n Protein of Escherichia coli*

(Received for publication, December 21, 1990)

George C. Allen, Jr.† and Arthur Kornberg
From the Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

The gene encoding protein n of the Escherichia coli primosome has been discovered in the rpsF-rpsR-rpl operon and designated priB. The low copy number of PriB protein and the distinctive codon usage of its gene argue against its being a ribosomal protein. A strain which overproduces PriB was constructed and has been used to purify the protein to homogeneity. The overproduced protein behaves like that purified from wild-type cells.

The replication of φX174 single-stranded viral DNA to generate the duplex replicative form can be reconstituted in vitro using only Escherichia coli proteins (1). The roles of many of these proteins in both phage and host replication have been studied genetically as well as biochemically. These proteins include: DnaB, DnaC, DnaG (primase), DnaT, SSB, and DNA polymerase III holoenzyme. The replication of φX174 requires three additional host proteins: PriA, PriB, PriC (previously known as n’, n, and n”, respectively). The functions of these remaining proteins in phage replication have been explored (1). However, the limited amount of material and the lack of identification of their genes have prevented their further characterization and studies of their involvement in replication of the host chromosome. The recent isolation of the priA gene-encoding protein n’ (2, 3) has enabled the construction of a null priA mutant and provided insights into its role in replication (4).

In the φX ss to RF reaction, PriA acts at an early stage of primosome assembly by binding a specific hairpin on E. coli single-stranded DNA binding protein-coated φX ssDNA. This structure is then recognized and bound by proteins PriB and PriC. Formation of the primosome proceeds with the subsequent actions of DnaB, DnaC, DnaT, and primase (5). As a mobile complex, the primosome lays down the RNA primers that DNA polymerase III can elongate.

We report here the discovery of the priB gene that encodes the primosomal protein PriB. Identification of the gene has enabled us to the overproduce and purify PriB to homogeneity.

Materials and Methods

Strains, Reagents, and Proteins—The E. coli strain used was K38(HfrC(λ)) (6). The pGP1-2 and pT7-7 plasmids were kindly provided by Dr. S. Tabor (Harvard Medical School). Plasmid pCYB325 containing the rpsF-priB-rpsR-rpl operon was kindly provided by Dr. Katsumi Isono (Kobe University). Other sources were: unlabeled deoxynucleoside triphosphates and ribonucleoside triphosphates, catalase, and Superose-12 fast protein liquid chromatography column (Pharmacia LKB Biotechnology Inc.); [α-35S]dATP (Amer sham Corp.); bovine serum albumin, ovalbumin, carbonic anhydrase, lysozyme (Sigma); Bio-Rex 70 (Bio-Rad).

Buffers—Buffer A is 50 mM Tris-HCl (pH 7.5) and 10% (w/v) sucrose. Buffer R is 50 mM Hepes-KOH (pH 8.0 at 1 M), 20% (v/v) glycerol, 1 mM EDTA, 5 mM dithiothreitol.

Replication Assay for PriB Protein—The reconstituted φX ss to RF replicative assay was essentially as previously described (7) with the addition of potassium glutamate to 20 mM. One unit of replication activity promotes the incorporation of 1 pmol of nucleotide in 1 min at 30 °C.

Identification of the priB Gene—The PriB protein was purified to near homogeneity through the DNA-cellulose step by Dr. Philippe Tacchini (University of Geneva) as described (8). The N-terminal amino acid sequence derived from the priB start codon into which the $10 Shine-Dalgarno cassette was inserted. The plasmid, pGP1-2, contained the temperature-sensitive hfrC(h) (Ref. 12) and 1228 containing the priB ORF) subcloned into the BamHI site of pT7-7. Site-directed mutagenesis was used to create an NdeI site at the priB start codon into which the phi10 Shine-Dalgarno cassette was inserted. The plasmid, pGP1-2, contained the temperature-sensitive λ repressor gene, cI857, and the T7 RNA polymerase gene under control of the λ Pm promoter. Shifting the growth temperature from 30 to 42 °C derepresses the λ Pm promoter allowing expression of the T7 RNA polymerase that then transcribes the priB gene.

Construction of an Overexpression Vector—A plasmid, pRA47, expressing the priB gene from the T7 φ10 promoter and under translational control of the φ10 Shine-Dalgarno was constructed analogously to pRA45, as described (2). Briefly, the primary construction consisted of the 571-base pair Sau3Al fragment from the rpsF-priB-rpsR-rpl operon (nucleotides 657-1228 containing the priB ORF) subcloned into the BamHI site of pT7-6. Site-directed mutagenesis was used to create an NdeI site at the priB start codon into which the φ10 Shine-Dalgarno cassette was inserted. The plasmid, pGP1-2, contained the temperature-sensitive λ repressor gene, cI857, and the T7 RNA polymerase gene under control of the λ Pm promoter. Shifting the growth temperature from 30 to 42 °C derepresses the λ Pm promoter allowing expression of the T7 RNA polymerase that then transcribes the priB gene.

Other Methods—SDS-PAGE and Coomassie Brilliant Blue staining were performed as described (10). The protein concentration was determined by the Bradford method (11) with bovine serum albumin as a standard.

Results

Identification of the priB Gene—The N-terminal amino acid sequence derived from PriB protein was used to search the Swiss Protein database using the programs of IntelliGenetics (9). The primary construction consisted of the 971-base pair Sau3A fragment from the rpsF-priB-rpsR-rpl operon (nucleotides 657-1228 containing the priB ORF) subcloned into the BamHI site of pT7-6. Site-directed mutagenesis was used to create an NdeI site at the priB start codon into which the φ10 Shine-Dalgarno cassette was inserted. The plasmid, pGP1-2, contained the temperature-sensitive λ repressor gene, cI857, and the T7 RNA polymerase gene under control of the λ Pm promoter. Shifting the growth temperature from 30 to 42 °C derepresses the λ Pm promoter allowing expression of the T7 RNA polymerase that then transcribes the priB gene.

Other Methods—SDS-PAGE and Coomassie Brilliant Blue staining were performed as described (10). The protein concentration was determined by the Bradford method (11) with bovine serum albumin as a standard.

Results

Identification of the priB Gene—The N-terminal amino acid sequence derived from PriB protein was used to search the Swiss Protein database which contains potential proteins derived from ORFs as well as known protein sequences. This search identified the second ORF in the ribosomal operon containing rpsF (S6), rpsR (S18), and rpl (L9) (Ref. 12 and Fig. 1). Nineteen of 24 N-terminal amino acids of this ORF matched the analysis of PriB protein. The amino acid composition predicted from the ORF sequence is: Ala8, Cys5, Asp5, Glu5, Phe3, Gly8, His5, Ile2, Lys3, Leu7, Met3, Asn3, Pro3, Glu9, Arg, Ser10, Thr9, Val10, Trp3, and Tyr3. This composition is generally consistent with that of PriB protein except for...
previously overestimated values for Ser, Gly, and Ala (8). A strain bearing only the second ORF under control of the T7 promoter overproduced PriB activity 1200-fold (Table I).

Although located in a ribosomal protein operon, PriB is unlike ribosomal proteins in codon usage and abundance. Ribosomal protein genes exhibit a distinct codon bias for certain amino acids. The ribosomal protein genes which flank priB on either side (Fig. 1) share this codon usage pattern (12) whereas that of priB is clearly different (Table I). The abundance of PriB protein at 80 copies/cell (8) is comparable with that of many of the replication proteins rather than that of ribosomal proteins which number 1,000 to 10,000 copies/cell.

**Purification and Characterization of PriB**—PriB was purified from an overproducing strain using the αX ss to RF reconstituted replication assay. The purification from the soluble lysate was 11-fold with an overall yield of 71% (Table III). The final product was homogeneous as judged by SDS-PAGE (Fig. 2).

The PriB protein isolated from the overproducing strain was characterized to verify that it behaves like PriB derived from wild-type cells (8). The molecular mass calculated from the gene sequence predicts an 11.4-kDa protein. The overproduced PriB has an apparent mass of 12.2 kDa on SDS-PAGE (Fig. 2) as reported for the previously purified protein (8). The amino acid composition of purified, overproduced PriB matches well with that derived from the gene sequence (data not shown and see above). Based on the final step of the purification (Superose-12 chromatography), the native molecular mass of PriB was determined (Fig. 3). PriB elutes with an apparent mass of 25 kDa consistent with its being a dimer in solution (8). A titration of purified PriB protein shows a

**TABLE I**

| Plasmid  | Insert | PriB activity (x 10^3) |
|----------|--------|------------------------|
| pT7-6    |        | 2.4                    |
| pRA47 priB with T7 α10 SD | 2830 | 1180                  |

**TABLE II**

| Codon | All ribosomal proteins* | Only rpsF, rpsR, and rpl | priB |
|-------|-------------------------|--------------------------|------|
| Ala   | GCT 231 28 1            |                         |     |
| GCC   | 42 2 3                  |                         |     |
| GCA   | 132 10 1                |                         |     |
| GCG   | 76 5 1                  |                         |     |
| Ile   | ATT 58 3 5              |                         |     |
| ATC   | 183 17 0                |                         |     |
| Ala   | GYT 185 9 0             |                         |     |
| GCC   | 135 9 3                |                         |     |
| GGA   | 0 0 4                  |                         |     |
| GGG   | 8 1 1                  |                         |     |
| Lys   | AAA 273 17 1            |                         |     |
| AAG   | 97 6 3                 |                         |     |

*Proteins S1, S2, S5, S6, S8, S9, S10, S14, S15, S16, S18, S20, S21, L1, L5, L6, L9, L10, L11, L12, L13, L14, L15, L17, L18, L19, L24, L28, L30, and L33 are indicated under “all ribosomal proteins” (12).

**TABLE III**

| Purification of PriB |
|----------------------|

All operations were carried out at 0-4 °C unless otherwise noted. E. coli K38 (pGP1-2, pRA47) was grown in L broth containing 25 µg/ml ampicillin and 30 µg/ml kanamycin at 30 °C to A600 of 0.8. The cells were then induced by shifting to 42 °C for 15 min, then grown further for 2 h at 37 °C, harvested, resuspended in buffer A to A600 of 550, and frozen immediately in liquid nitrogen. A heat lysozyme lysis was carried out starting with 740 g of frozen cells. Dithiothreitol (1 M) was added to 10 mM, 0.5 M EDTA (pH 8.0) to 10 mM, 0.8 M spermidine HCl (pH 7.5) to 20 mM, 5 M NaCl to 100 mM, and ammonium sulfate (saturated at 4 °C) to 5%. The remainder of the heat lysis was as described (7) yielding Fraction I (670 ml). Ammonium sulfate (0.225 g/ml fraction I) was added; the precipitate was collected and resuspended in buffer A to 100 mM NaCl and applied to a 600-ml Bio-Rex 70 column (7.5 × 13.6 cm) equilibrated in buffer R + 100 mM NaCl at a rate of 1 column volume/h. The column was washed with 3 column volumes of buffer R + 100 mM NaCl and eluted with a 6-column volume linear gradient of buffer R + 100 mM to 1 M NaCl. Peak fractions were pooled (Fraction III, 990 ml). For gel filtration chromatography, 0.08% of fraction III was clarified in a Beckman TLA100.1 (15 min, 95,000 rpm) and applied to a 25-ml Superose-12 fast protein liquid chromatography column (HR 10/30) equilibrated in buffer R + 460 mM NaCl. The column was run at 0.2 ml/min, and 0.3-ml fractions were collected. Peak fractions were pooled (Fraction IV, 1.2 ml).

| Fraction | Total protein | Total activity | Specific activity | Yield |
|----------|---------------|----------------|------------------|-------|
| I. Lysate| 22,100        | 17             | 7.7              | (100) |
| II. Ammonium sulfate | 7,780 | 17 | 22 | 100 |
| III. Bio-Rex 70 | 2,670 | 14 | 52 | 82 |
| IV. Superose-12 | 1,420 | 12 | 85 | 71 |

*The values given for fraction IV are corrected for the fact that only 0.8 ml (0.68%) of Fraction III was used.*

**FIG. 1. The operon containing the priB gene.** The putative promoter (P), the ORFs (indicated by boxes), and the putative transcription terminator (t) were derived from the nucleotide sequence as described (12). The previously unidentified open reading frame has been designated priB, bp, base pairs.

**FIG. 2.**
The priB Gene Encoding Protein

requirement for approximately 1 dimer per ss φX circle replicated assuming that synthesis is processive once initiated (Fig. 4). Also as anticipated, the purified overproduced PriB is heat-stable (65% activity remains after 5 min at 100 °C) and is N-ethylmaleimide-sensitive (<3% activity remained after a 10-min treatment at 30 °C with 10 mM N-ethylmaleimide).

DISCUSSION

The priB gene is found in an operon flanked by ribosomal protein genes: upstream by rpsF and downstream by rpsR and rplI. Sequence analysis of this region identified a potential promoter and transcription terminator, and insertional mutagenesis indicated that these genes form an operon (12). The priB gene differs from its neighboring, ribosomal protein genes in two respects: 1) a 100-fold lower cellular abundance of its protein product and 2) distinctive codon preferences.

Control at the level of translational efficiency may be responsible for the low protein copy number.

The clustering of macromolecular synthesis genes for replication, transcription, and translation into operons has been reported in three other instances in *E. coli*; the *rpsU-dnaG-rpoD* operon that encodes ribosomal protein S21, primase, and the ε70 subunit of RNA polymerase (13); the *rplK-rplA-rplJ-rplL-rpoB-rpoC* operon encoding ribosomal proteins, L11, L1, L10, and L7/L12 and the β and β' subunits of RNA polymerase (14); and the *rpsM-rpsK-rpsD-rpoA-rplQ* operon that encodes ribosomal proteins, S13, S11, S4, and L17, and the α subunit of RNA polymerase (15). The benefits of organizing these genes as operons are still unclear particularly in situations where the final levels of the proteins encoded in the operon vary greatly. The possible advantage of linking the biosynthesis of RNA, DNA, and proteins by proximity of key genes deserves further attention.

The location of the priB gene within the *rpsF-rpsR-rplI* ribosomal protein operon suggests that it plays a critical role for *E. coli*. The other gene products encoded by these four operons are centrally involved in RNA, DNA, and protein metabolism. The particular aspect of these processes that priB contributes to is still unclear. With the identification of the *priA* and *priB* genes, *priC* is the only gene among those encoding ribosomal proteins, S13, S11, S4, and L17.

**Fig. 2.** SDS-PAGE analysis of PriB. The SDS-PAGE (15%) was carried out as described (10). Samples were applied directly in a total volume of 50 μl of loading buffer. Proteins (3.3 × 10^5 units) were visualized by Coomassie Brilliant Blue staining: Fraction I, 43 μg; Fraction II, 15.2 μg; Fraction III, 5.9 μg; and Fraction IV, 4.0 μg. Fractions are as described in Table III.

**Fig. 3.** Gel filtration of PriB and determination of native molecular mass. A, gel filtration on a Superose-12 fast protein liquid chromatographic column was the second and final chromatographic step (see Table III). B, the marker proteins, detected by monitoring UV absorbance (A_280 nm), are: catalase (cat, 232 kDa); bovine serum albumin (BSA, 67 kDa); ovalbumin (oval, 43 kDa); carbonic anhydrase (carb, 31 kDa); and lysozyme (lyso, 14.1 kDa).

**Fig. 4.** Titration of PriB protein in the φX ss to RF reaction. Nucleotide incorporation after 10 min at 30 °C was measured in a 25-μl reaction mixture containing 240 pmol (as nucleotide) of φX ss DNA (45 fmol of circle) and 0.042-0.42 ng (1.84-18.4 fmol) of PriB, as described under “Materials and Methods.”
encoding primosomal proteins that remains at large. With these genes in hand, the overproduction of the gene products will provide access to biochemical studies of primosomal assembly, structure, and function. Mutation as well as regulated usage of each of the genes will afford the means to elucidate their individual and collective roles in replication of the host chromosome, plasmids, and phages.

REFERENCES
1. Kornberg, A. (1982) Supplement to DNA Replication, pp. S105-S114, W. H. Freeman & Co., New York
2. Lee, E. H., Massi, H., Allen, G. C., Jr., and Kornberg, A. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 4620-4624
3. Nurse, P., DiGate, R., Zavitz, K., and Marians, K. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 4615-4619
4. Lee, E. H., and Kornberg, A. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3029-3032
5. Shlomai, J., and Kornberg, A. (1980) J. Biol. Chem. 255, 6794-6798
6. Tabor, S., and Richardson, C. C. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 1074-1078
7. Shlomai, J., and Kornberg, A. (1980) J. Biol. Chem. 255, 6789-6793
8. Low, R. L., Shlomai, J., and Kornberg, A. (1982) J. Biol. Chem. 257, 6242-6250
9. Brutlag, D. L., Clayton, J., Friedland, P., and Kedes, L. H. (1982) Nucleic Acids Res. 10, 279-294
10. Laemmli, U. K. (1970) Nature 227, 680-685
11. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254
12. Schnier, J., Kitakawa, M., and Isono, K. (1986) Mol. Gen. Genet. 204, 126-132
13. Burton, Z. F., Gross, C. A., Watanabe, K. K., and Burgess, R. R. (1983) Cell 32, 335-349
14. Bruckner, R., and Matzura, H. (1981) Mol. Gen. Genet. 183, 277-282
15. Post, L. E., Arfsten, A. E., Davis, G. R., and Nomura, M. (1980) J. Biol. Chem. 255, 4653-4659