Replication of Small Plasmids in Extracts of *Escherichia coli*

Involvement of the *dnaB* and *dnaC* Protein in the Replication of Early Replicative Intermediates

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Summary. The role of *E. coli dnaB* and *dnaC* protein in the replication of plasmid ColE1 and RSF1030 DNA was investigated in a soluble in vitro system (Staudenbauer, 1976a). Extracts from *dnaB* and *dnaC* mutants which are phenotypically DNA initiation- or DNA elongation-defective were examined for their replicative capacity. It was found that all mutants tested are deficient in the synthesis of supercoiled plasmid DNA. Deficient extracts of *dnaB* mutants could be partially complemented by purified *dnaB* wild type protein but required for full complementation *dnaC* wild type protein as well. The *dnaB* wild type protein could be replaced by a PldnaB analog (ban) protein complexed with a *dnaB* ts protein. Deficient extracts of *dnaC* mutants were complemented by purified *dnaC* wild type protein alone.

The in vitro plasmid replication cycle had been separated into an early and late stage (Staudenbauer, 1977). Analysis by CsCl velocity centrifugation of the plasmid DNA synthesized in mutant extracts indicates that the early stage, namely the synthesis of early replicative intermediates, proceeds in all *dnaB* and *dnaC* mutants tested. However, replication of the early intermediates during the late stage depends on both the *dnaB* and *dnaC* protein. These conclusions were confirmed using inhibitors of DNA synthesis.

Introduction

The DNA replication cycle of the small plasmids ColE1 and RSF1030 proceeds in vitro in two stages: (1) synthesis and (2) replication of early replicative intermediates (Staudenbauer, 1977). Stage I is sensitive to rifampicin and requires the addition of all four ribonucleoside triphosphates indicating an RNA-priming event at the origin of replication (Sakakibara and Tomizawa, 1974; Tomizawa et al., 1977). Synthesis of early replicative intermediates is catalyzed by DNA polymerase I (Staudenbauer, 1976b). Stage II is insensitive to rifampicin and depends only on the addition of ATP (Staudenbauer, 1977). Replication of early intermediates is catalyzed by the products of the *E. coli* replication genes *dnaE* (DNA polymerase III) (Staudenbauer, 1976b) and *dnaZ* (Staudenbauer, 1977).

The enzyme requirements for plasmid DNA replication were determined by complementation of *E. coli* mutant extracts with the corresponding wild type enzyme fraction. Therefore, additional proteins may be involved besides the ones already reported. For example, replication of early replicative intermediates is inhibited in *dnaG* mutants upon thermal inactivation indicating a *dnaG* protein-dependent reaction during stage II (Staudenbauer, unpublished results).

We were interested in the question whether in vitro plasmid DNA replication is dependent on the *E. coli* *dnaB* and *dnaC* functions for the following reasons. 1. Whereas it was found that in vivo ColE1 DNA replication is dependent on the *E. coli* *dnaC* function (Goebel, 1974; Collins et al., 1975), the effect of bacterial *dnaB* mutations is in controversy (Goebel, 1970; Goebel and Schrempf, 1972). 2. Temperature-sensitive (ts) *E. coli dnaB* and *dnaC* mutants exist which are defective phenotypically in *E. coli* DNA initiation or elongation (Beyersmann et al., 1971; Zyskind and Smith, 1977; Wechsler, 1975). The *dnaB* and *dnaC* proteins of these mutants might operate differently in plasmid replication.

Data presented in this paper show that replication of the early intermediates is dependent on both the *dnaB* and *dnaC* protein of all mutants tested. Replication also proceeds in the presence of the PldnaB analog (ban) protein (D'Ari et al., 1975). Synthesis of early intermediates, on the other hand, is independent of the *dnaB* and *dnaC* function.
The broth at 30°C. Preparation of cell-free extracts and ammonium sulfate fractionation have been described previously (Staudenbauer, 1976).

Materials and Methods

Bacterial Strains

The E. coli K12 strains used in this study are described in Table 1. Cells used for dnaB and dnaC complementation tests were grown in Difco antibiotic medium M-3 supplemented with 1% nutrient broth at 30°C. Preparation of cell-free extracts and ammonium sulfate fractionation have been described previously (Staudenbauer, 1977).

Assay of DNA Synthesis

The assay measured the incorporation of 3H-dTMP into acid-insoluble material (Staudenbauer, 1976b). Standard incubation mixtures contained final concentrations of 40 mM HEPES pH 8.0, 100 mM KCl, 10 mM magnesium acetate, 2 mM ATP, 0.4 mM each of CTP, GTP, and UTP, 0.05 mM NAD, 0.05 mM cAMP, 0.025 mM each of dATP, dCTP, dGTP, and 3H-dTTP (500 cpm x pmol⁻¹), 10 μg/ml plasmid DNA (ColEI or RSF 1030), plus extract (approximately 10 mg protein per ml assay mixture). Incubations were performed at 30°C for the times indicated.

Preparation of dnaB Wild Type and dnaBts Plbacin Protein

dnaB complementing activities were isolated from strains Q1610, Q1710, BT1071(Plbac crr), and Q1550 according to the method described (Lanka et al., 1978b). Fraction II (DEAE-cellulose) was used in all experiments. The specific activity of the dnaB (ban) protein from Q1610, Q1710, BT1071(Plbac crr) and Q1550 was 12.6, 67.0, 36.1 and ≤1.0 units dnaB/mg protein, respectively. One unit (U) of dnaC complementing activity incorporates 1 nmol of dTMP using dX174 DNA as template (Lanka et al., 1978b). The dnaB complementing activity from strain BT1071 (Plbac crr) contained Plbac and E. coli dnaBts protein in a molar ratio of approximately 2.6, as will be described elsewhere. Extracts of strain Q1550 always have a low specific activity which is lost on further purification (Lanka et al., 1978b). Extracts of Hfr H252 are inactive in dnaB complementation (S. Wickner, cited in Zyskind and Smith, 1977). However, Fraction II (DEAE-cellulose) contains an ATPase activity which can be inactivated by antibody directed against dnaB (Lanka et al., 1978a). This fraction was used in the experiment described.

Preparation of dnaC Wild Type Protein

The activity of dnaC protein was followed by using a complementation assay (Wickner et al., 1973). The E. coli dnaC PC22 receptor extract was prepared as described (Schuster et al., 1977). The initial purification steps are based on the fractionation scheme described for the isolation of dnaB protein (Lanka et al., 1978a). Starting with 358 g wet cell paste of strain BT1000, the DEAE-cellulose flow through contains the dnaC protein (289 mg protein, 1.35 units dnaC/mg). One unit (U) of dnaC complementing activity incorporates 1 nmol of dTMP using dX174 DNA as template. It was applied to a phosphocellulose column (Whatman P11, 2.6 x 9.5 cm), equilibrated with 20% glycerol, 50 mM Tris-HCl (pH 7.6), 1 mM MgCl₂, 1 mM EDTA, 1 mM DTT and 0.1% Brij-58. The dnaC protein was eluted with a linear gradient (0-0.4 M NaCl, 45 ml/h, 12 ml/fraction) at approximately 0.2 M NaCl. Fractions containing dnaC protein were concentrated by dialysis against 20% w/w polyethylene glycol 20,000 (Serva), 10% glycerol, 20 mM Tris-HCl (pH 7.6), 1 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA and 0.1% Brij-58 followed by a dialysis against this buffer (without polyethylene glycol and with 50% glycerol). This fraction was stored at -20°C (3.05 mg protein with 1.35 mg/ml and 49.2 U/mg). The dnaC complementing activity remains stable for at least half a year if it is stored in a Brij-58 containing buffer.

Results

Involvement of dnaB and dnaC Protein in Plasmid Replication

To investigate whether the dnaB and the dnaC protein are required for the replication of small plasmids,