Relaxation Complexes of Plasmids ColE1 and ColE2: Unique Site of the Nick in the Open Circular DNA of the Relaxed Complexes

(Escherichia coli/supercoiled DNA/endonuclease/restriction enzyme/DNA replication)

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ABSTRACT The product of the induced relaxation of supercoiled DNA–protein relaxation complexes of colicinogenic factors ColE1 and ColE2 is an open circular DNA molecule with a strand-specific nick. Cleavage of the open circular DNA of each relaxed complex with the EcoRI restriction endonuclease demonstrates that the single-strand break is at a unique position. The site of the single-strand break in the relaxed ColE1 complex is approximately the same distance from the EcoRI cleavage site as the origin of ColE1 DNA replication.

Many of extrachromosomal circular DNA elements (plasmids) of Escherichia coli, differing in molecular weight, number of copies per cell, and in the properties they confer to their host, have been isolated as both supercoiled DNA–protein relaxation complexes and supercoiled DNA without associated protein (1). Treatment of the plasmid DNA–protein relaxation complexes in vitro with agents capable of altering protein structure induces the conversion of the supercoiled DNA in the complex to the open circular (relaxed) DNA form. The relaxation complexes of the colicinogenic factors, ColE1 (2) and ColE2 (3, 4), the sex factor F1 (5), and the R factor R6K (Kuperstock, Lovett, and Helinski, submitted for publication) have been studied in the greatest detail. Sodium dodecyl sulfate-induced relaxation of each of these complexes produces one strand-specific break. The properties of the relaxation complexes suggest that they consist of supercoiled DNA and a latent strand-specific endonuclease that is activated by treatment with certain protein-denaturing agents. In each case the broken strand is the poly(U,G) binding or “heavy” strand. More recently, it has been shown that the supercoiled ColE1 complex has three major protein components whose molecular weights are 60,000, 16,000, and 11,000 (Lovett and Helinski, manuscript in preparation). After the induction of relaxation with sodium dodecyl sulfate, the 60,000-molecular-weight-protein alone remains in association with the broken strand even under a variety of conditions that normally dissociate protein from DNA (Blair and Helinski, manuscript in preparation). The protein remains associated with the 5′ end of the broken strand (Guiney and Helinski, manuscript in preparation). It has been proposed that a covalent bond between the 5′ terminus of the DNA and the 60,000-dalton protein is formed upon relaxation of the ColE1 complex. The ColE2 relaxation complex has been found to exhibit similar properties upon treatment with sodium dodecyl sulfate.

These novel properties of plasmid DNA protein complexes have led to the suggestion that relaxation complexes are involved in the production of a single-strand cleavage in the initiation of replication and/or conjugal transfer of plasmid DNA. An involvement in such processes would most likely require that the relaxation event take place at a unique site in the DNA molecule. In this report, evidence will be presented from studies using the EcoRI restriction endonuclease that the strand-specific relaxation event takes place at a unique site on both the ColE1 and ColE2 plasmid molecules. The location of this site, at least in the case of the ColE1 plasmid, is approximately the same distance from the single EcoRI site as the origin of replication for this plasmid.

MATERIALS AND METHODS

Strains, Media, and Labeling Conditions. The E. coli K12 strains JC411 thy− (ColE1) and JC411 thy− (ColE2) have been described (6). M9 medium (7) containing 0.2% (w/v) glucose and 0.006% (w/v) of each of the required amino acids. For [3H]thymine labeling, cultures contained 1 μg/ml of nonradioactive thymine and 10–20 μCi/ml of [methyl-3H]-thymine. For [14C]thymine labeling, 10 μCi of [14C]thymine containing 27 μg of thymine was added to each 15 ml of medium. The amount of plasmid DNA present as relaxation complex was maximized by the addition of cyclic adenosine monophosphate (cAMP) to a final concentration of 2.5 mM (6). Under these conditions, about 80% of ColE1 DNA and 60% of ColE2 DNA are isolated in the form of relaxation complex.

Preparation of Complexed and Noncomplexed Plasmid DNA. Relaxation complexes of ColE1 and ColE2 were prepared as described by Clewell and Helinski (2), with the modification that crude lysates were made by the addition of Triton X-100 to spheroplasts (6) followed by centrifugation at 46,000 × g for 25 min to pellet most of the chromosomal DNA. The supernatant (cleared lysate) was centrifuged through 38-ml sucrose density gradients containing 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 50 mM NaCl for 16 hr at 4 °C at 25,000 rpm in a Beckman SW 27 rotor. Plasmid-containing fractions were pooled and precipitated with ethanol. After resuspension with Tris-EDTA–NaCl buffer containing 0.5% sodium dodecyl sulfate to induce relaxation, the DNA was centrifuged through 5-ml sucrose density gradients containing Tris-EDTA–NaCl buffer with 0.5 M NaCl for 140 min at 18 °C at 50,000 rpm in a Beckman SW 50.1 rotor to separate 238 supercoiled plasmid DNA from 17S open circular DNA. The open circular DNA was precipitated with ethanol and suspended in 0.1 M Tris-HCl (pH 7.5), 50 mM NaCl, 5–10 mM MgCl₂ for digestion with the EcoRI endonuclease.
For preparation of supercoiled ColE1 and ColE2 DNA, cells were grown in M9 glucose medium without the addition of cAMP. To minimize the amount of ColE2 DNA present as relaxation complex, cleared lysates of JC411 (ColE2) were heated at 60° for 20 min to inactivate the relaxation complex (4); Sarkosyl was added to a final concentration of 0.05% (w/v) and the lysate was centrifuged to equilibrium in a cesium chloride-ethidium bromide density gradient (8). Supercoiled ColE1 DNA was also prepared by the dye–buoyant density procedure. After centrifugation, the supercoiled DNAs were extracted four to six times with CsCl-saturated isopropanol (9) and then precipitated with ethanol. After resuspension with Tris–EDTA–NaCl buffer, the plasmid DNA was centrifuged through 5-ml 5–20% sucrose density gradients as described above, and the 23S supercoiled DNA was then precipitated with ethanol and suspended in RI buffer.

**Agarose Gel Electrophoresis of DNA.** Electrophoresis of DNA was performed in agarose gels set in cylindrical Plexiglas tubes of 6-mm internal diameter (15-cm lengths). Agarose of the indicated concentration was added to electrophoresis buffer (Tris–phosphate–dodecyl sulfate buffer (Loening) (11) containing 36 mM Tris, 30 mM NaHPO4, 1 mM EDTA, and 0.05% dodecyl sulfate (pH 7.7)), dissolved by autoclaving for 15 min, and poured into rubber-capped gel tubes. After the gels hardened, the rubber caps were removed, the top 5 mm of the gels were sliced off to provide a flat surface, and the bottoms of the gel tubes were covered with dialysis tubing. DNA samples were denatured with 0.1 volume of 1 M NaOH, as described by Hayward and Smith (11), before electrophoresis for analysis of single-stranded DNA. To assay radioactively labeled DNA, the gels were sliced into 1-mm discs and their radioactivity was determined in 10 ml of a toluene solution containing 4 g/liter of Omnifluor and 5% Protosol (New England Nuclear Corp.) after they were shaken on a rotary shaker for at least 2 hr at 37°.

**EcoRI Endonuclease Digestion.** The buffer for EcoRI digestion contained 0.1 M Tris–HCl (pH 7.5), 50 mM NaCl, and 5–10 mM MgCl2. EcoRI endonuclease was the generous gift of Herbert Boyer. Incubations were for 5–30 min at 37°.

**Reagents.** [methyl-3H]Thymine (40–60 Ci/mmol) and [2-14C]Thymin (46 mCi/mmol) were obtained from New England Nuclear Corp., Boston, Mass. Agarose was purchased from MCI Biomedical, Rockland, Md. cAMP was purchased from Sigma Chemicals, St. Louis, Mo. Sodium dodecyl sulfate was obtained from Gallard-Schlesinger, New York, N.Y.

**RESULTS**

**Cleavage of ColE1 DNA with EcoRI Endonuclease.** The EcoRI restriction endonuclease has been shown to cleave double-stranded DNA at a unique nucleotide sequence (12). By use of EcoRI cleavage sites as reference points, it has been possible to test whether the single-strand breaks found in the relaxed DNA form of the plasmid relaxation complexes are located at unique positions. If relaxation events break one DNA strand at a unique site on the plasmid DNA, EcoRI cleavage of the relaxed DNA should produce a double-stranded fragment of the DNA containing the strand-specific break which, when exposed to alkaline conditions, would yield one single-stranded fragment of unit size and two single-stranded fragments of less than unit size derived from the broken strand.

The product of EcoRI digestion of 23S supercoiled or 17S open circular ColE1 DNA derived from the induced relaxation of the ColE1 complex sediments as a linear 158 molecule in neutral sucrose density gradients (Fig. 1A and B). There are no detectable fragments of less than unit size (4.2 × 106 molecular weight), indicating that EcoRI cleavage produces one break in the ColE1 molecule. Analysis of ColE1 DNA cleaved by EcoRI, by electron microscopy and by electrophoresis in a 1% agarose gel with the EcoRI cleavage products of λ DNA (13) as molecular weight markers have confirmed that it is a linear molecule of unit size.

**Cleavage of ColE2 DNA with EcoRI Endonuclease.** EcoRI cleavage of supercoiled or open circular ColE2 DNA derived from the induced relaxation of ColE2 complex produces two fragments that sediment more slowly than intact ColE2 DNA in neutral sucrose density gradients (Fig. 1C and D). Electrophoresis in a 2.3% agarose gel with the Hm restriction endonuclease digestion products of simian virus 40 DNA as molecular weight references (14) showed that the small fragment of ColE2 DNA cleaved by EcoRI has a molecular weight of about 3.5 × 106. Electrophoresis in a 1% agarose gel with the EcoRI cleavage products of λ DNA as markers showed that the larger fragment has a molecular weight of 3.85 × 106.

**Site of the ColE1 Relaxation Event.** A portion of the EcoRI digest of relaxed ColE1 complex, shown in Fig. 1 to be a linear, unit-sized molecule upon neutral sucrose density gradient centrifugation, was analyzed in an alkaline sucrose
density gradient with an added marker of uncleaved, relaxed CoIE1 complex (Fig. 2A and B). The products of EcoRI cleavage of the relaxed CoIE1 complex are one fragment whose size is identical to linear single strands of CoIE1 DNA, termed fragment A, and two fragments of less than unit size, termed B and C. The size of fragments B and C has been determined by alkaline sucrose density gradient centrifugation, with the RI digestion products of λ DNA and supercoiled CoIE2 DNA as molecular weight markers. Correspondence between size and θ of the DNA fragments was determined by the equation of Studier (15) for denatured DNA in alkaline sucrose containing 1 M NaCl. The size of fragment B (16.3S) is $1.7 \times 10^6$ daltons and the size of fragment C (9.7S) is $4.0 \times 10^6$ daltons, or about 19% of unit size. Similar results were obtained where the RI digestion products of relaxed CoIE1 complex were examined by agarose gel electrophoresis after denaturation with alkali (Fig. 2C). The generation of two discrete fragments of less than unit size by EcoRI cleavage of relaxed CoIE1 complex indicates that the strand-specific break in the relaxed DNA takes place at one unique site on the plasmid DNA.

**Site of the CoIE2 Relaxation Event.** The products of RI cleavage of relaxed CoIE2 complex have been analyzed in a similar manner as those of CoIE1. A portion of the RI digest of a mixture of CoIE2 relaxed complex and CoIE2 supercoiled DNA (Fig. 1D) was analyzed in an alkaline sucrose density gradient (Fig. 3A and B). There are two fragments, A and D, that are derived from both supercoiled CoIE2 DNA and relaxed CoIE2 complex. Fragments A and D exhibit sedimentation coefficients (17S for A and 6.5S for D) that correspond to molecular weights of $1.9 \times 10^6$ and $1.8 \times 10^6$, respectively. There are two additional fragments, B and C, whose sizes are $1.7 \times 10^6$ daltons (16.2S) and $2.5 \times 10^6$ daltons (7.6S), which are derived only from the relaxed CoIE2 complex. The existence of fragments B and C indicates that the relaxation event occurs at one unique site on the larger of the two double-stranded fragments produced by RI cleavage of CoIE2 DNA. Alkaline-denatured relaxed CoIE2 complex, cleaved by EcoRI, was also examined by agarose gel electrophoresis with results similar to those found by alkaline sucrose density gradient analysis (Fig. 3C).

**DISCUSSION**

A feature of the plasmids studied in *E. coli* is their existence as relaxation complexes of supercoiled DNA and protein. In each case examined in detail the product of induced relaxation of the plasmid DNA is an open circular molecule with a single strand-specific break. This report has shown that the strand-specific break is located at a unique site on the CoIE1 and CoIE2 molecules with respect to an EcoRI endonuclease cleavage site. Recently, it has been shown also for the plas-
mids R6K (Kuperzstoch, Lovett and Helinski, manuscript in preparation) and pSC101 (Lovett, unpublished observations) that the site of the single-strand break in the DNA of the relaxed complex is unique. While bacterial restriction endonucleases have been shown to break both strands of a DNA duplex at a unique nucleotide sequence (12, 16, 17), no other endonucleases have been described whose action is the site-specific cleavage of one strand of a DNA duplex.

By the approach of Fareed et al. (18) for the determination of the origin and direction of replication of DNA of simian virus 40, it has been demonstrated, by EcoRI cleavage of replicating ColEl molecules isolated from intact E. coli cells, that ColEl DNA replication proceeds unidirectionally from an origin 18.3 ± 0.9% of unit length from one end of the EcoRI-cleaved molecule (Lovett, Katz, and Helinski, submitted for publication). The site of the nick produced by induced relaxation of the ColEl complex is approximately 19% from one end of the EcoRI-cleaved molecule. Although these data do not distinguish between the two ends of the EcoRI-cleaved ColEl DNA molecule, it is most probable that the site of the nick in the DNA of the relaxed complex and the position of the origin/terminus are located approximately the same distance from the same end of the molecule. Location of the relaxation event at or near the origin/terminus of ColEl replication suggests an involvement of the protein components of the relaxation complex in either the initiation or termination of replication, possibly satisfying a requirement for a nicking event(s) at the origin/terminus. It is possible that relaxation complexes are an integral feature of the organization of E. coli plasmid DNA molecules as replicons; for every replicon there may be a requirement for one relaxation complex and one or more relaxation event(s) at the origin or terminus of replication. Conversion of supercoiled M13 DNA (RF I) to the open circular form (RF II), required for both RF I and viral strand synthesis, involves the action of the M13 gene 2 product (19–21). Nicking of øX174 DNA by the gene A product has also been suggested as a requirement for DNA replication (22). It is possible that the products of the M13 gene 2 and øX174 gene A are the bacteriophage equivalents of plasmid relaxation complex proteins acting at the origin of DNA replication.

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