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Strand-Specific Supercoiled DNA–Protein Relaxation Complexes: Comparison of the Complexes of Bacterial Plasmids *Col*E_1 and *Col*E_2

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ABSTRACT **Certain bacterial plasmids can be isolated as unique complexes of supercoiled circular DNA and protein. These complexes are distinguished by the conversion of the supercoiled DNA to the relaxed or open-circular DNA form upon treatment with ionic detergents, proteases, or alkali. This report demonstrates that the open-circular DNA resulting from the pronase-induced relaxation of the complexes of colicinogenic factors E_1 (*Col*E_1) and E_2 (*Col*E_2) possesses a strand-specific break. In each case this break is found in the heavy strand of the DNA as defined by CsCl centrifugation in the presence of poly-(U,G). In addition, the *Col*E_1 and *Col*E_2 complexes exhibit certain properties that are plasmid specific. Heat treatment, and to a lesser extent pronase treatment, inactivates the *Col*E_2 complex, making it insensitive to agents that formerly were capable of inducing relaxation (conversion of the DNA to the open-circular form). In contrast, the *Col*E_1 complex is not inactivated by these treatments. The potential role of these strand-specific relaxation complexes in DNA replication is discussed.**

It is possible, by gentle lysis of *Escherichia coli* cells with non-ionic detergents, to isolate several plasmids as complexes of supercoiled DNA and protein. These complexes, designated relaxation complexes, are characterized by the relaxation of the supercoiled DNA molecule to the open-circular form upon treatment with ionic detergents, proteases, or alkali. A relaxation complex was first reported and studied in detail in the colicinogenic factor E_1 (*Col*E_1) system (1), and similar complexes have been shown to exist for the plasmids determining the production of colicins E_2 and E_3 (2) and the episomes *Col*I_b (3, 4) and F_1 (5). In the case of *Col*E_1 (6) and F_1 (unpublished results) the relaxed, or open circular, DNA has been shown to possess a single break that occurs specifically in one of the two DNA strands.

This report is concerned with the properties of the relaxation complex of colicinogenic factor E_2 (*Col*E_2) and a comparison of these properties with those of the relaxation complex of the closely related *Col*E_1 factor.

Abbreviation: SDS, sodium dodecyl sulfate.

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MATERIALS AND METHODS

Reagents

Sources of most of the reagents have been described (1). Poly(U,G) was obtained from Research Products Division, Miles Laboratories (lot no. 343).

Strains

E. coli CR34, a thymine-requiring derivative of C600, requires thiamine, threonine, and leucine for growth. It was made colicinogenic for *Col*E_1 (K30) by D. Kingsbury by conjugal transfer from JC411 (*Col*E_1, Flac). CR34 was made colicinogenic for *Col*E_2 (P9) by the procedure of Smith *et al.* (7).

Media, growth, and labeling conditions

M9 medium, as described by Roberts *et al.* (8), was used. It contained 2 g of glycerol per liter, and was supplemented with Casamino acids (5 g), thiamine (250 mg), and thymine (2 mg). For the tritium-labeling experiments, 30-ml cultures contained from 0.3–0.6 mCi as [methyl-^{3}H]thymine; in certain cases the concentration of nonradioactive thymine was reduced to 1 mg/l to increase the specific activity of the DNA. For ^{14}C labeling, 0.01 mCi of [^{14}C]thymine, in a total of 23 μg of thymine, was added per 10 ml of media. For labeling with ^{32}P, a Tris-buffered medium that has been described (1) was used. 2.5 mCi of carrier-free [^{32}P]H_3PO_4 was added per 50 ml of culture. Cells were grown at 37°C with constant agitation to a cell density of approximately 5×10^8/ml before harvesting and subsequent DNA isolation.

Preparation and purification of complexed and noncomplexed DNA

ColE_1 and *Col*E_2 DNA–protein relaxation complexes were isolated and purified by a procedure described in detail elsewhere (1). The gently lysed cells were centrifuged at 46,000 $\times$ *g* for 20 min to remove the bulk of the chromosomal DNA. The resulting "cleared" lysate was further purified on a 15–50% sucrose gradient; the pooled DNA-containing fractions from this gradient are termed "purified complex." The purified complex could be stored in frozen aliquots at −20°C; it is relatively stable under these conditions for many weeks. In the experiments involving differentially labeled *Col*E_1 and *Col*E_2 DNA, the *Col*E_1- and *Col*E_2-containing cells were mixed and then lysed and purified together.

Noncomplexed *Col*E_2 DNA was prepared by a modification of the procedure outlined above. The Brij–deoxycholate lysate

was heated to 60°C for 20 min, then cooled on ice and centrifuged at 46,000 × *g* for 20 min. Supercoiled *Col*E$_2$ DNA was then isolated from the cleared lysate by the ethidium bromide–CsCl buoyant-density procedure of Radloff *et al.* (9). The heat treatment of the *Col*E$_2$ lysate inactivates the relaxation complex and results in an approximately 2-fold increase in the yield of noncomplexed supercoiled DNA from *Col*E$_2$ when compared to nonheated lysates.

Sucrose gradients

The sucrose gradients (30 ml, 15–50%) contained 0.05 M Tris (pH 8.0), 0.05 M NaCl, and 0.005 M EDTA. The 5-ml, 5–20%, neutral sucrose gradients contained the same buffer and 0.5 M NaCl. The 5–20% alkaline sucrose gradients contained 1.0 M NaCl, 0.001 M EDTA, and 0.3 M NaOH.

Strand separation and poly(U,G)–CsCl equilibrium centrifugation

Purified complex was prepared as described above, then treated with pronase and concentrated by pelleting in the SW65 rotor at 50,000 rpm, 15°C for 12 hr. The liquid was totally decanted, 0.4 ml of fresh buffer added, and the pelleted DNA allowed to resuspend while standing at 4°C overnight. A portion (0.3 ml) of this solution was layered on 5 ml of an alkaline 5–20% sucrose gradient and centrifuged in the SW65 rotor for 225 min at 55,000 rpm. 4-drop fractions were collected from the bottom of the tube and 10 μl of each fraction was counted to locate the two peaks containing the single-stranded linear and circular DNA elements, respectively. The appropriate fractions were then pooled. Poly(U,G)–CsCl equilibrium centrifugation was performed on each pooled fraction by a modification of the procedure described by Rupp (ref. 10 and personal communication). 0.15 ml of denatured, differentially labeled, marker DNA was added to a 0.1-ml portion of the pooled fraction from alkaline sucrose. This mixture was neutralized with 0.50 ml of 1 M Tris, pH 8.5. 0.05 ml of 2% Sarkosyl NL-30 was added to the neutralized DNA, and the entire solution was transferred to a polyallomer centrifuge tube containing 6.67 g of CsCl and 4.2 ml of glass-distilled H_2O. 0.025 ml of a 1 mg/ml solution of poly(U,G) was then added, the remainder of the tube filled with light mineral oil, and the material was centrifuged to equilibrium in a Beckman Spinco Ti50 rotor (36–48 hr at 44,000 rpm, 15°C). 15-drop fractions were collected directly on filter papers from a hole punched in the bottom of the tube.

The denatured, differentially labeled, marker DNA was prepared by heating supercoiled, noncomplexed DNA to 100°C in 0.1 N NaOH for 8–15 min. This treatment was sufficient to nick most of the supercoiled DNA. The single strands were then separated (from the supercoils) on an alkaline sucrose gradient and fractions containing single-stranded DNA were pooled. Radioisotopes were counted in a Beckman liquid scintillation counter as described (11), except that the scintillation fluid consisted of 1.3 g of 2,5-diphenyloxazole, per liter of toluene.

RESULTS

Comparison of the induced relaxability of the *Col*E$_1$ and *Col*E$_2$ complexes

It was of interest to determine whether the *Col*E$_2$ complex is similar in all respects to the relaxation complex previously described for *Col*E$_1$. To eliminate possible artifacts due to differences in lysis and purification, we labeled cultures of CR34 (*Col*E$_1$) and CR34 (*Col*E$_2$) with different isotopes, mixed the cultures, and isolated and purified the complexes together. The mixture of the two complexes was then exposed to various agents and the results analyzed by zonal centrifugation on 5–20% sucrose gradients. Similar experiments were also performed on the *Col*E$_2$ complex in the presence of differentially-labeled noncomplexed *Col*E$_2$ DNA.

While the *Col*E$_2$ DNA complex appeared to sediment slightly faster than the corresponding noncomplexed DNA (Fig. 1*A*), the two complexes sedimented at identical rates (Fig. 1*D*). Treatment of the complexes with sodium dodecyl sulfate (SDS) or 0.3 M NaOH consistently relaxed 5–10% more of the *Col*E$_1$ DNA than *Col*E$_2$ DNA.

A greater difference could be detected when a mixture of the two relaxation complexes was treated with pronase. After a 10 min incubation of the complexes with 1.25 mg/ml pronase in buffer at 25°C, only 49% of the *Col*E$_2$ DNA sedimented as relaxed DNA, while 74% of the *Col*E$_1$ DNA was converted to

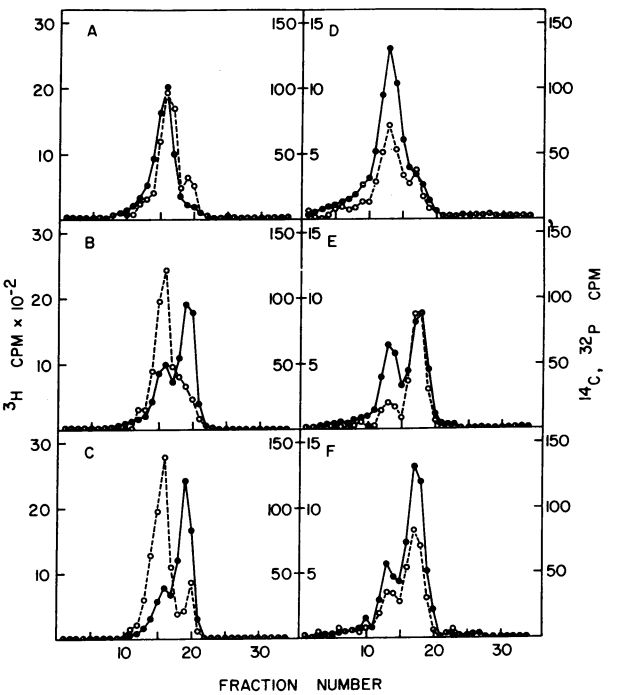

FIG. 1. Neutral sucrose gradient sedimentation of complexed and noncomplexed *Col*-factor DNA. (*A*), (*B*), and (*C*) contain a mixture of complexed and noncomplexed *Col*E$_2$ DNA. (*D*), (*E*), and (*F*) contain a mixture of *Col*E$_1$- and *Col*E$_2$-complexed DNA. Treatments described below were performed in a final reaction volume of 0.4 ml of buffer containing 0.1–0.2 μg of complexed DNA and the additions described below. After the incubation period at 25°C, 0.3 ml of each reaction mixture was layered on a 5 ml, 5–20% sucrose gradient and centrifuged in a Beckman Spinco SW50.1 rotor at 45,000 rpm for 150 min. Recovery of counts from the gradient, based on counts initially applied, was greater than 80% in each case. The DNA was treated as follows: (*A* and *D*) No addition (control), 20 min at 25°C. (*B* and *E*) 1.25 mg/ml pronase (self-digested 45 min at 37°C at a concentration of 5 mg/ml before use), 10 min at 25°C, then SDS in buffer added to a final concentration of 0.25%, and the mixture incubated an additional 10 min at 25°C. (*C* and *F*) 0.25% SDS, 10 min at 25°C. (●——●) ^{3}H-labeled *Col*E$_2$ DNA complex. (O- - -O) ^{32}P-labeled noncomplexed *Col*E$_2$ DNA (*A*, *B*, *C*) or ^{14}C-labeled *Col*E$_1$ DNA complex (*D*, *E*, *F*).

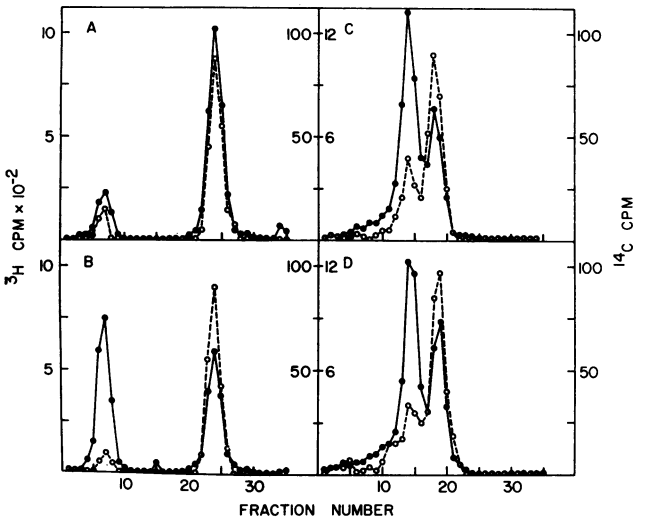

FIG. 2. Sucrose gradient sedimentation of *Col*E$_1$ and *Col*E$_2$ relaxation complexes. DNA was treated in a 0.4-ml reaction volume containing 0.1–0.2 μg of DNA in buffer and a 0.3-ml portion of each sample was layered on 5-ml neutral and alkaline sucrose gradients. Neutral sucrose gradients (*C*), (*D*) were performed as described in the legend to Fig. 1; the untreated control is shown in Fig. 1*D*. Alkaline sucrose gradients (*A* and *B*) were centrifuged at 45,000 rpm in an SW50.1 rotor for 100 min. The recovery was 80% or greater in each case. (*A*) No treatment (25°C, 20 min); (*B*) Heat treated, 60°C, 20 min; (*C*) Heat treated, 60°C, 20 min; (*D*) Heat treated, 60°C, 20 min, then made 0.25% in SDS and incubated 10 min at 25°C. (●——●) ^{3}H-labeled *Col*E$_2$ DNA, (○- - -○) ^{14}C-labeled *Col*E$_1$ DNA.

the slower-sedimenting form. Incubation with pronase for up to 40 min did not significantly increase the amount of relaxed *Col*E$_2$ DNA over that observed after a 10-min incubation. Treatment of the pronase-treated *Col*E$_2$ complex with 0.25% SDS generally resulted in a slight increase in the amount of relaxed DNA observed. However, the amount of DNA resistant to relaxation was greater after sequential pronase and SDS treatments of the complex (Figs. 1*B*, 1*E*) than when it was treated with SDS alone (Figs. 1*C*, 1*F*). Addition of a mixture containing 0.25% SDS and 1.25 mg/ml pronase in buffer to the *Col*E$_2$ complex resulted in the same amount of relaxation as was induced by SDS alone. Other proteases (trypsin, chymotrypsin, and pepsin) were tested, but all relaxed the *Col*E$_2$ complex without any detectable inactivation of the complex.

The effect of heat on the *Col*E$_2$ complex

Evidence for Inactivation of the Relaxation Complex. It was shown previously that relaxation was induced in the *Col*E$_1$ complex by heating of the purified complex to 60°C (1). The *Col*E$_2$ complex, however, was only relaxed to a very small extent by a similar heat treatment. Furthermore, the unrelaxed *Col*E$_2$ complex was now insensitive to further relaxation by any of the agents that normally induce relaxation in the unheated *Col*E$_2$ complex. The unheated control (Fig. 2*A*) showed a similar degree of relaxation upon alkali sedimentation for both the *Col*E$_1$ and the *Col*E$_2$ complex. In the heated sample (Fig. 2*B*), the *Col*E$_1$ complex was relaxed to the same extent as in the unheated control, but approximately 60% of the *Col*E$_2$ DNA now sedimented in the unrelaxed position despite exposure to alkali. Analysis of the heated *Col*E$_2$ complex on neutral sucrose gradients showed that approximately 30 to 40% of the *Col*E$_2$ complex was relaxed upon heating (Fig. 2*C*). Treatment of the heated *Col*E$_2$ complex with SDS (Fig. 2*D*) or pronase (not shown) caused only a slight increase in the amount of relaxed DNA. Complexed *Col*E$_2$ DNA which remains supercoiled after heat and pronase treatment behaves as normal supercoiled *Col*E$_2$ DNA upon ethidium bromide–CsCl density centrifugation.

Strand specificity of the nick in the relaxed *Col*E$_2$ complex

Sedimentation of the relaxed *Col*E$_2$ complex in alkaline sucrose indicated that the relaxed molecule contained only single interruptions in one of the two strands of the DNA double helix (Fig. 3*A*) (2). We undertook to investigate the strand specificity of the nick present in the relaxed *Col*E$_2$ complex using the strand-separation technique initially developed by Hradecna and Szybalski (12) to identify specific strands. With the assistance of W. Dean Rupp, it was possible to show that the complementary strands of *Col*E$_2$ DNA could be separated on the basis of their different affinity for poly(U,G). Denatured single-stranded *Col*E$_2$ DNA, when centrifuged to equilibrium in the presence of an excess of poly(U,G), separated into two bands, designated heavy and light, that contained approximately equal amounts of DNA. To test the strand specificity of the nick in the relaxed *Col*E$_2$ DNA, ^{3}H-labeled purified complex was prepared in the usual way. The complex was treated with pronase to induce relaxation and the open circular and linear single strands were isolated by alkaline sucrose gradient centrifugation (Fig. 3*A*). The circular and linear strands were then subjected to equilibrium CsCl centrifugation in the presence of an excess of poly(U,G). A differentially labeled mixture of both strands of *Col*E$_2$ DNA, obtained from randomly nicked *Col*E$_2$ DNA, was added as a marker. The results (Figs. 3*B*, 3*C*) indicated that the linear nicked strand (*pool R*, Fig. 3*A*) was predominantly (80%) the heavy strand, while the unnicked strand (*pool* C, Fig. 3*A*) is predominantly the light strand. The slight cross contamination observed in both cases is undoubtedly due in part to the overlap of the two bands in the alkaline sucrose gradient and a background level of random nicking by nucleases or other agents during purification. It could also represent a low extent of nonspecificity in the action of the complex. The results indicate that in most cases the nick present in *Col*E$_2$ DNA after relaxation of the complex occurs specifically in one of the two strands of the *Col*E$_2$ double helix, that strand exhibiting a higher affinity for poly(U,G) (the "Crick" strand) (12).

The strand specificity of the nick in *Col*E$_1$ DNA after relaxation was also tested on poly(U,G)–CsCl equilibrium gradients. The results shown in Fig. 3*D*, *E*, and *F* confirm the strand-specificity results of Clewell and Helinski, obtained by DNA–DNA hybridization (6). It is interesting to note, by comparing Fig. 3*B*, *C*, *E*, and *F*, that the strand nicked in both cases is the strand that binds the greatest amount of poly(U,G) and thus bands in the denser position. This is also true in the case of the F_1 complex. In addition, it appears that the light strand bands at a position of lower density when it is derived from relaxed complex than when it is derived from alkali-nicked noncomplexed DNA. The cause of this density shift is unknown and is presently under investigation.

The possibility that differential binding of poly (U,G) was an inherent property of the linear or circular structure of the single-stranded DNA was ruled out by the experiment shown

in Fig. 3*G*, *H*, and *I*. Noncomplexed supercoiled *Col*E$_2$ DNA, purified by ethidium bromide–CsCl density centrifugation was randomly nicked by heating in alkali. Single-stranded linear and circular molecules were then separated and centrifuged in the presence of poly(U,G) as described above. Both linear and circular single-stranded pools contained approximately equal amounts of heavy and light DNA, indications that the relative amount of poly(U,G) bound is not determined by the circularity or linearity of the DNA strand.

DISCUSSION

The results described here show that although plasmids can be isolated in the form of relaxation complexes with basically similar properties, these complexes can exhibit different properties dependent on the type of plasmid DNA involved. This may indicate that some part of the material in the complex is genetically specified by the DNA with which it is associated, or the different properties may involve the associations of different plasmid DNAs with different components specified by the bacterial chromosome. This could also reflect base sequence differences between the plasmid DNAs at the region of association of DNA and protein.

Two models have been proposed to explain the properties of a supercoiled DNA–protein relaxation complex (1); one postulated a preexisting nick in the supercoiled DNA, with the complex protein functioning as a "binder," while the other postulated that the complex consisted of covalently closed, supercoiled DNA and an inactive endonuclease or "nickase" that could be activated by agents that induce relaxation. This latter model was favored for the *Col*E$_1$ relaxation complex, and evidence presented here appears to favor this model for the *Col*E$_2$ complex as well. The fact that heat treatment of the *Col*E$_2$ complex *in vitro* renders it resistant to relaxation by proteolytic or denaturing agents is clearly compatible with the notion of an active enzyme. It is less likely that this treatment renders a linker, or binder, protein insensitive or inaccessible to agents that were previously capable of destroying it. Preliminary results also indicate that little if any of the protein remains in the nonrelaxed *Col*E$_2$ DNA after heat treatment, which would argue against a "binder model" (Blair, D. G., unpublished results). Such a model, however, cannot be completely ruled out by these observations. We have also considered the possibility that the protein in the *Col*E$_2$ complex possesses both binding properties and a ligase activity that is induced by heat to close a preexisting nick. This model seems unlikely in view of the heat sensitivity of known ligases (13, 14). In addition, attempts to interfere with the possible ligase action by heating the complex in the presence of large excesses of nicotinic acid mononucleotide and inorganic pyrophosphate showed no effect.

The demonstration of the strand specificity of the nick, or gap, in the relaxed *Col*E$_1$ and *Col*E$_2$ complexes is of considerable interest in view of the suggested role of these complexes in the duplication of supercoiled DNA molecules (1). The replication of a covalently-closed or supercoiled DNA molecule presumably requires as an initial step the introduction of a break in at least one strand of the double helix. This would release the topological restraints on the molecule and permit the unwinding of strands during replication. The relaxation complex conceivably could fulfill this function in plasmid replication through an activation of the proposed latent endonuclease in the complex. If this proves to be the

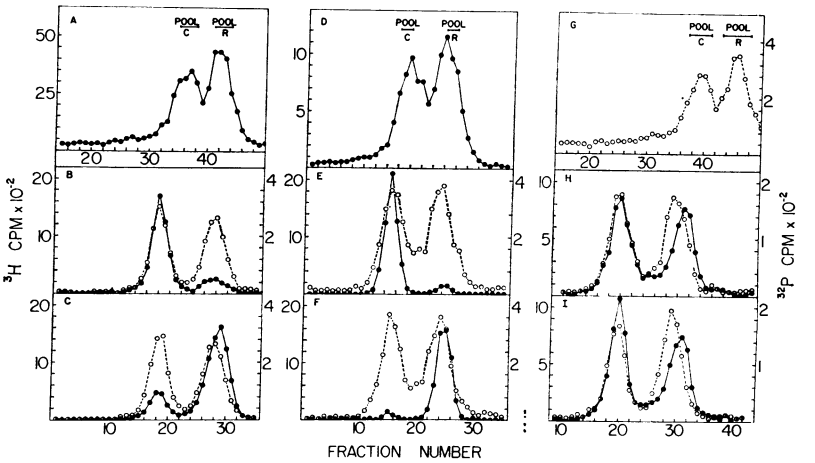

FIG. 3. Test of strand specificity of the nick in relaxed *Col*E$_1$ and *Col*E$_2$ DNA. Relaxed complexed DNA and randomly-nicked noncomplexed DNA were prepared as described in *Methods*. Single-stranded circular strands (*C*) and linear strands (*R*) were pooled from alkaline sucrose density gradients as shown; the pools were then centrifuged to equilibrium in the presence of an excess of poly(U,G) and a differentially labeled marker containing both DNA strands of *Col*E$_2$ DNA. In *B*, *C*, *E*, and *F*, the ^{32}P-labeled marker DNA was obtained from randomly nicked, supercoiled *Col*E$_2$ DNA. In *H* and *I* the ^{3}H-labeled marker DNA was obtained from relaxed *Col*E$_2$ complex. The gradients each contained approximately 0.1 μg of total DNA. The recovery of counts from the CsCl–poly(U,G) gradients was greater than 90%. (*A*) Alkaline sucrose density gradient, ^{3}H-labeled *Col*E$_2$ DNA (●—●); (*B*) CsCl equilibrium centrifugation containing *pool R* of (*A*), ^{32}P-labeled marker *Col*E$_2$ DNA (○- - -○) and poly(U,G); (*C*) CsCl equilibrium centrifugation containing *pool C* of (*A*), marker *Col*E$_2$ DNA and poly(U,G); (*D*) Alkaline sucrose density gradient, ^{3}H-labeled *Col*E$_1$ DNA (●—●); (*E*) CsCl equilibrium centrifugation containing *pool R* of (*D*), ^{32}P-labeled marker *Col*E$_2$ DNA (○- - -○) and poly(U,G); (*F*) CsCl equilibrium centrifugation containing *pool C* of (*D*), marker *Col*E$_2$ DNA and poly(U,G); (*G*) Alkaline sucrose density gradient, ^{32}P-labeled, randomly nicked, *Col*E$_2$ DNA (○- - -○); (*H*) CsCl equilibrium centrifugation, containing *pool R* of (*G*), ^{3}H-labeled marker *Col*E$_2$ DNA (●—●) and poly(U,G); (*I*) CsCl equilibrium centrifugation containing *pool C* of (*G*), marker *Col*E$_2$ DNA, and poly(U,G).

case, it would indicate that the initiating event of plasmid DNA replication involves both an activation of a latent endonuclease and an endonuclease-catalyzed nick, specifically in one of the two DNA strands. Gilbert and Dressler (15), in their discussion of the induction of the λ prophage, suggest that the strand nicked prior to replication may be the "Crick" or poly(U,G)-binding strand. The fact that the relaxation of the *Col*E$_1$, *Col*E$_2$, and F_1 complexes involves a nick or gap also in the poly(*U*,*G*)-binding, or heavy, strand may be entirely fortuitous or it may reflect some general property of the initial event of DNA replication. At the very least, it may indicate some common evolutionary origin for the relaxation complexes of *E. coli* plasmids and episomes.

We are indebted to W. Dean Rupp, who initially demonstrated for us the feasibility of separating the strands of *Col*E$_2$ DNA in CsCl–poly(U,G) equilibrium gradients.

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