

## Properties of the Relaxation Complexes of Supercoiled Deoxyribonucleic Acid and Protein of the R Plasmids R64, R28K, and R6K

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The presence of supercoiled deoxyribonucleic acid in the form of a relaxation complex is described for the antibiotic resistance plasmids R64, R28K, and R6K. The properties of these relaxation complexes indicate that they consist of a covalently closed circular deoxyribonucleic acid molecule associated with an activable, single strand-specific endonuclease.

Stable, extrachromosomal genetic elements in bacteria exist as covalently closed circular deoxyribonucleic acid (DNA) molecules (8). Among these, ColE1 (4), ColE2 and ColE3 (5), ColB (6), F1 (10),  $\Delta$  and  $\Delta\Delta$  (9), ColV2 (R. Leavitt and D. Helinski, unpublished data), and NR1 (C. F. Morris and R. Rownd, manuscript in preparation) also have been isolated as supercoiled circular DNA-protein complexes which, when treated with certain agents that affect protein structure, undergo a conversion from the supercoiled DNA form to the open circular (relaxed) form.

In this communication, a comparative study of the relaxation complexes of three R plasmids, R6K, R28K, and R64, purified from *Escherichia coli*, is reported. Some general properties of these R plasmids are given in Table 1. The *E. coli* strains J5-3 (R64) [Pro<sup>-</sup>Met<sup>-</sup>], RC85 (R6K) [Met<sup>-</sup>], and RC85 (R28K) were used as donors of the plasmids R64, R6K, and R28K, respectively, in conjugal transfer with the *E. coli* strain CR34 [Thr<sup>-</sup>, Leu<sup>-</sup>, Thy<sup>-</sup>, Lac<sup>-</sup>, Thi<sup>-</sup>] by a modification of the procedure of Nishioka et al. (14).

In all of the experiments, the cells were grown overnight at 37 C in M9 medium (16) containing glucose (0.2%), Casamino Acids (0.5%), and thiamine (2  $\mu$ g/ml). For the CR34 strains, thymine was added at a concentration of 2  $\mu$ g/ml. The overnight cultures were diluted 100-fold in 30 ml of the same medium and, in the case of the RC85 and J5-3 strains, supplemented with 300  $\mu$ g of deoxyadenosine per ml to

promote the incorporation of labeled thymine (3). [2-<sup>14</sup>C]thymine (New England Nuclear, 50 mCi/mmol) at a final concentration of 1  $\mu$ g/ml and [methyl-<sup>3</sup>H]thymine (New England Nuclear 10 Ci/mmol) at a final concentration of 10 ng/ml were used to label the cells. The diluted suspension was incubated in a rotary shaker at 37 C to a cell density of  $5 \times 10^8$  cells per ml, the cells were lysed, and the cleared lysates were prepared as described previously (12).

Figure 1 shows the relaxation of the R64 DNA-protein complex induced by different treatments. Cleared lysates containing tritiated R64 plasmid DNA and [<sup>14</sup>C]thymine-labeled, noncomplexed supercoiled and open circular DNA, prepared (12) by centrifugation in a CsCl-bouyant density gradient containing ethidium bromide from CR34 (R64), were mixed and exposed to different agents known to induce relaxation of plasmid complexes. Whereas the ratio of supercoiled to open circular DNA of the [<sup>14</sup>C]thymine-labeled noncomplexed DNA did not change after the treatment, a substantial fraction of the tritiated DNA from the cleared lysates shifted from the faster-sedimenting supercoiled DNA to the position expected for open circular DNA. This conversion was induced by treatments with sodium dodecyl sulfate (SDS), Pronase, ethidium bromide (300  $\mu$ g/ml), and incubation at 60 C for 30 min.

Table 2 summarizes the results shown in Fig. 1 and the results of similar treatments of the relaxation complexes of R6K and R28K. The results were independent of the host strain harboring the plasmids (Table 1). In all cases differentially labeled complexed and noncomplexed supercoiled and open circular DNA forms of the same plasmid were used as internal controls.

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TABLE 1. Characteristics of *R* plasmids<sup>a</sup>

R plasmid	Host strain <sup>b</sup>	Antibiotic resistance	<i>fi</i> character	<i>s</i> <sub>20,w</sub> (S)		Molecular wt (× 10)		
				Supercoiled DNA	Open circular DNA	Previously reported <sup>c</sup>	Calculated from supercoiled DNA <sup>d</sup>	Calculated from open circular DNA <sup>e</sup>
R64	J5-3 CR34	Tetracycline, streptomycin	<i>fi</i> <sup>-</sup>	89	61	76 (18) to 78.5 (19)	96.7	94.7
R28K	RC85 CR34	Ampicillin	<i>fi</i> <sup>-</sup>	68	50	44 (11)	51.6	56.2
R6K	RC85 CR34	Ampicillin, streptomycin	<i>fi</i> <sup>-</sup>	51	38	26 (11)	26.3	27.6

<sup>a</sup> Strains harboring the *R* plasmids were labeled with [<sup>3</sup>H]thymine, and cleared lysates were prepared. The cleared lysates were centrifuged to equilibrium in a CsCl gradient containing ethidium bromide. The fractions in the peak of supercoiled DNA were pooled and precipitated with ethanol after isopropanol extraction of the dye as described previously (12). The *s*<sub>20,w</sub> for supercoiled and open circular DNAs were determined in a 5 to 20% sucrose gradient [0.5 M NaCl, 0.005 M sodium ethylenediamine-tetraacetic acid, and 0.05 M tris(hydroxymethyl)aminomethane, pH 8.0] using an internal markers differentially labeled ColE1 supercoiled DNA (*s*<sub>20,w</sub> of 23S [1]) and bacteriophage MS2 (*s*<sub>20,w</sub> of 78.5S [15]).

<sup>b</sup> J5-3 (R64) and the *R* plasmid-containing RC85 strains have been described by Meynell and Datta (13) and Kontamichalou et al. (11), respectively.

<sup>c</sup> Numbers in parentheses indicate references.

<sup>d</sup> The following formula was used  $s_{20,w} = 0.034 M^{0.428}$  (1).

<sup>e</sup> The following formula was used  $s_{20,w} = 0.051 M^{0.386}$  (18).

The relaxation complex of the plasmid R28K (Table 2) is similar in its relaxation properties to the ColE2 plasmid that has been previously described (2). The supercoiled DNA of the R28K complex relaxed when treated with SDS, Pronase, or ethidium bromide (300 μg/ml), but it did not relax when heat treated (30 min at 60 C). Furthermore, the heat-treated R28K complex was insensitive to subsequent treatment with SDS. Similar heat inactivation of the DNA-protein complex has been reported for the ColE2 plasmid and the F1 sex factor (10).

The supercoiled DNA of the DNA-protein complex of the R6K plasmid (Table 2) was relaxed when treated with SDS or ethidium bromide. There was virtually no relaxation upon treatment with heat or Pronase. Furthermore, the Pronase or heat-treated relaxation complex of the R6K factor was not relaxable with SDS.

Two models have been proposed to explain the properties of a supercoiled DNA-protein relaxation complex (7); one postulates a pre-existing nick or gap in the supercoiled DNA, with the complex protein(s) functioning as a binder(s), whereas the other postulates that the complex consists of covalently closed supercoiled DNA and an inactive endonuclease or "nickase" that can be activated by the agents that induce relaxation. As previously indicated

for the ColE2 and F1 relaxation complexes, the evidence presented here favors the latter model for the R28K and the R6K complexes. The fact that heat treatment inactivates the R28K and the R6K complexes is directly compatible with the notion that an active enzyme is required to induce relaxation. Further evidence supporting the second model in the case of the R6K complex is the observation that treatment with a proteolytic enzyme or high salt concentrations (Y. Kupersztoch and D. Helinski, manuscript in preparation) removes the protein from the complex without affecting the supercoiled state of the plasmid DNA.

In the case of the R6K relaxation complex, it has been demonstrated that the nick or gap induced by activating the relaxation complex is specifically in the poly(UG)-binding strand (Y. Kupersztoch and D. Helinski, manuscript in preparation) as in the case of the complexes of the plasmids ColE1 (7) and ColE2 (2) and of the sex factor F1 (10). It also has been found that the nick introduced by relaxation of the R6K complex is at a specific site (Y. Kupersztoch, M. Lovett, and D. Helinski, manuscript in preparation), as is the case with the ColE1 plasmid (M. Lovett and D. Helinski, manuscript in preparation). Thus, in several respects the properties of the *R* plasmid relaxation complexes are similar to those reported for the

colicinogenic plasmids and the F1 plasmid. The different response of the DNA-protein complexes with respect to heat inactivation and relaxability with proteolytic enzymes may be due to different plasmid-coded protein components in the relaxation complexes of the various

plasmids. Alternatively, these differences may reflect variation in the nucleotide sequence at the DNA site of the various plasmids that interact with the protein components of the complex.

Finally, it should be stressed that the relaxa-

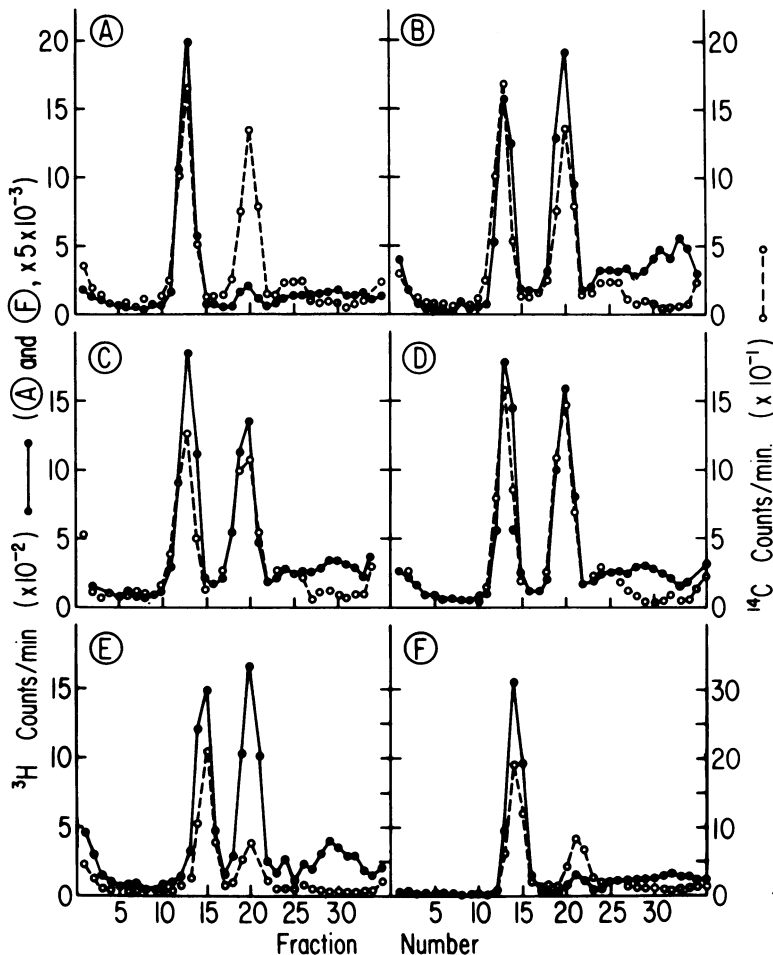


FIG. 1. Sedimentation analysis of the relaxability of the R64 DNA-protein complex. From a 30-ml culture of CR34 (R64) labeled with [ $^3\text{H}$ ]thymine, cleared lysates were prepared as described in the text. [ $^{14}\text{C}$ ]thymine-labeled supercoiled and open circular DNA of the same plasmid were added as internal controls. Treatments were performed in a final volume of 300  $\mu\text{l}$  of TES buffer [0.05 M NaCl, 0.005 M sodium ethylenediaminetetraacetic acid, and 0.05 M tris(hydroxymethyl)aminomethane, pH 8.0] containing 100  $\mu\text{l}$  of [ $^3\text{H}$ ]thymine-labeled cleared lysate and 100  $\mu\text{l}$  of [ $^{14}\text{C}$ ]thymine-labeled supercoiled and open circular DNA. After the treatments, 280  $\mu\text{l}$  of the reaction mixture was layered on a 5 to 20% sucrose gradient [0.05 M NaCl, 0.005 M sodium ethylenediaminetetraacetic acid and 0.05 M tris(hydroxymethyl)aminomethane, pH 8.0] and centrifuged in a Beckman SW50.1 rotor at 45,000 rpm for 45 min at 15 C. The gradients were collected from the bottom with a hollow needle, fractionated directly into 1-inch (ca. 2.54 cm) squares of Whatman no. 1 filter paper, counted as described previously (1). The DNA samples were treated as follows: (A) no additions (control); (B) 0.25% SDS, 10 min at 25 C; (C) 1.25 mg of Pronase (self-digested, 30 min at 37 C, concentration of 5 mg/ml in TES buffer prior to use), 10 min at 37 C; (D) as C and then 0.25% SDS for an additional 10 min at 25 C; (E) 300  $\mu\text{g}$  of ethidium bromide per ml, 10 min at 25 C; (F) 3  $\mu\text{g}$  of ethidium bromide per ml, 10 min at 25 C. More than 90% of the counts initially layered on the gradients were recovered after centrifugation. A through D and E and F are two independent preparations. (—) [ $^3\text{H}$ ]thymine-labeled R64 DNA from a cleared lysate. (-----) [ $^{14}\text{C}$ ]thymine-labeled, noncomplexed R64 supercoiled and open circular DNA.

TABLE 2. Induced relaxation of the complexes of the R plasmids by various treatments<sup>a</sup>

Treatment	Supercoiled DNA relaxed by treatment <sup>b</sup> (%)		
	R64	R28K	R6K
SDS, 0.25%	52	40	48
Ethidium bromide, 300 µg/ml <sup>c</sup>	50	70	49
Pronase, 1.25 mg/ml	40	60	5
Pronase, 1.25 mg/ml, followed by 0.25% SDS	40	61	18
Heat, 30 min at 60 C	70		2
Heat, 30 min at 60 C, followed by 0.25% SDS	70	5	3

<sup>a</sup> The complexes were prepared and the relaxation was assayed as described in the legend to Fig. 1, except that the R28K and R6K plasmids were centrifuged at 50,000 rpm for 60 min at 15 C. Pronase treatment was carried out as described in the legend to Fig. 1. In all experiments, differentially labeled supercoiled and open circular DNA of the same plasmid were used as internal controls. In the case of each plasmid, all of the treatments were carried out on the same preparation of relaxation complex.

<sup>b</sup> Percent relaxation =  $1 - \frac{\text{[treated] (counts of supercoiled DNA per minute)}}{\text{[untreated] (counts of supercoiled DNA per minute + counts of open circular DNA per minute)}} \times 100$ .

<sup>c</sup> The use of ethidium bromide as an inducer of relaxation of the R plasmids is based on the findings of D. Guiney (personal communication) on the ColE1 relaxation complex.

bility of most DNA-protein complexes by high concentrations of ethidium bromide must be taken into consideration when the dye-CsCl gradient centrifugation technique is used to estimate the number of copies of supercoiled plasmid DNA per cell.

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