Strand and Site Specificity of the Relaxation Event for the Relaxation Complex of the Antibiotic Resistance Plasmid R6K†

Yankel M. Kupersztoch-Portnoy,‡ Michael A. Lovett, and Donald R. Helinski*

ABSTRACT: Gentle lysis of bacterial cultures harboring the resistance transfer factor R6K permits the isolation of the plasmid DNA as a relaxation complex of supercoiled DNA and protein. The supercoiled DNA is relaxed to an open circular DNA form by treatment of the complex with sodium dodecyl sulfate (SDS) or ethidium bromide. Prior treatment of the complex with Pronase or heat treatment prevents the induction of relaxation of the supercoiled DNA by SDS. Preincubation of the complexed DNA with NaCl or CsCl at concentrations higher than 0.5 M disassociates the proteins from the DNA, rendering the supercoiled DNA molecule insensitive to further treatment with SDS or ethidium bromide. Upon the induction of relaxation a single nick is produced in the heavy strand of the DNA as defined by CsCl centrifugation in the presence of poly(U,G). This nick is present at a specific DNA site as indicated by a comparative analysis in alkaline sucrose gradients of the fragments produces upon digestion of supercoiled and relaxed DNA with the EcoR1 restriction endonuclease.

A variety of extrachromosomal genetic elements in bacteria have been isolated as relaxation complexes of supercoiled DNA and protein(s) (Helinski and Clewell, 1971). The characteristic property of these relaxation complexes is the conversion of the plasmid DNA from the supercoiled to the open circular (relaxed) form when they are exposed to ionic detergents or in some cases proteolytic enzymes. Detailed studies of the relaxation complexes of the colicinogenic factors E1 (ColE1) (Clewell and Helinski, 1970) and E2 (ColE2) (Blair *et al.*, (1971) and the F1 sex factor (Kline and Helinski, 1971) have revealed the strand specificity of the nick induced by the relaxation of the complex. Little is known, however, of the properties of relaxation complexes of R factors, plasmid elements that determine resistance to antibiotics. One such R factor, R6K, has been described by Kontomichalou *et al.* (1970) as a supercoiled DNA molecule that is 26×10^6 daltons in size. Its replication is under relaxed control, *i.e.*, there are approximately 13 copies per chromosome in the logarithmic phase of growth. This property together with the self-transmissibility of the plasmid provide a particularly advantageous system for the study of the mechanism of replication of a transmissible antibiotic resistance plasmid.

In this report the properties of the relaxation complex of the R factor R6K are described including evidence for the presence in this relaxation complex of a strand and site specific endonuclease that is activated by sodium dodecyl sulfate and ethidium bromide.

Experimental Section

Materials. Reagents and sources were as follows: Triton X-100 from Atlas Chemical; sodium dodecyl sulfate (SDS)[1] from Fisher Scientific; Sarcosyl N130 (sodium dodecyl sarcosinate) from the Geigy Chemical Co.; Pronase (B grade) and ethidium bromide from Calbiochem; EcoR1 restriction enzyme was a generous gift from Dr. Herbert Boyer; poly(uridylic acid; guanylic acid) (poly(U,G) U-G 1:10) from Miles Laboratory (lot 14-343-381); nitrocellulose filters (B-6, 24mm) from the Carl Schleicher and Schuell Co; [^{3}H]methylthymine (20 Ci/mmol) and [2^{14}C]thymine (55.2 Ci/mmol) from New England Nuclear Corp.

Bacterial Strains and Media. The *Escherichia coli* K12 strains utilized in this study were RC85 (R6K) *Met*$^-$ and CR34 *Thr*$^-$, *Leu*$^-$, *Thi*$^-$, and *Thy*$^-$. The CR34 strain harboring the resistance transfer plasmid R6K was constructed by conjugal transfer from RC85 (R6K) as described by Nishioka *et al.* (1969). The bacterial strains were grown in M-9 media (Roberts *et al.*, 1962) containing glucose (0.02%), casamino acids (0.5%) and thymine (2 μg/ml). In the case of RC85 (R6K) thymine was omitted and deoxyadenosine (300 μg/ml) added to enhance the incorporation of labeled thymine (Boyce and Setlow, 1962).

Preparation of Cleared Lysates. The lysing procdure was a modification of the method of Clewell and Helinski (1969). In a typical preparation, a 30-ml bacterial culture grown at 37° in the presence of [^{3}H]thymine (10 ng/ml) or [^{14}C]thymine (1 μg/ml) to a cell density of 5×10^8 cells/ml was harvested and washed two times with cold TES buffer (0.05 M NaCl-0.005 M EDTA-0.05 M Tris (pH 8.0)) by centrifugation at 4° for 5 min, at 12,000*g*. The pellet was resuspended in 1 ml of cold 25% sucrose in 0.05 Tris (pH 8.0), and the rest of the operations were carried out in an ice bath. Lysozyme (0.2 ml of a 5 mg/ml solution in 0.25 M Tris (pH 8.0)) was added, mixed gently, and incubated for 5 min; 0.4 ml of EDTA (0.25 M, pH 8.0) was then added and the incubation continued for another 5 min. The suspension was lysed by the addition of 1.6 ml of a solution

† From the Department of Biology, University of California, San Diego, La Jolla, California 92037. *Received August 5, 1974.* This work was supported by grants from the National Institute of Allergy and Infectious Diseases (AI-07194) and the National Science Foundation (GB-29492).

‡ Supported by Centro de Investigacion y Estudios Avanzados del Instituto Politecnico Nacional, Mexico, Apartado Postal 14-740, Mexico 14, D. F. Mexico.

[1] Abbreviations used are: SDS, sodium dodecyl sulfate; TES buffer, 0.05 M NaCl-0.005 M EDTA-0.05 M Tris (pH 8.0); TEM, 20 mM Tris (pH 8.0)-5 mM mercaptoethanol-1 mM EDTA.

of 0.2% Triton X-100, 0.0625 M EDTA, and 0.05 M Tris (pH 8.0). After 15 min on ice the suspension cleared and became viscous. Removal of the bulk of the chromosomal DNA was accomplished by centrifugation at 4° for 15 min at 40,000*g*. The supernatant, containing most of the plasmid DNA and a small amount of contaminating chromosomal DNA, is referred to as "cleared lysate."

Sucrose Gradient Velocity Centrifugation. The 5-ml neutral sucrose gradients with either 5–20% or 20–31% sucrose contained 0.05 M Tris, 0.005 M EDTA, and 0.5 M NaCl (pH 8.0); 200–300-μl samples were layered on each gradient. Centrifugation was performed in the Beckman SW 50.1 rotor at 45,000 rpm at 15° for 80 min in the case of the 5–20% sucrose gradients and 120 min in the case of the 20–31% gradients.

The alkaline sucrose gradients contained 1.0 M NaCl, 0.001 M EDTA, and 0.3 M NaOH. Centrifugation was performed at 45,000 rpm in an SW 50.1 rotor for 80 min (20–31% sucrose gradient) or 40 min (5–20% sucrose gradient) at 15°. For the separation of the linear and circular strands of R6K DNA, the centrifugation times were 240 min (20–31% sucrose gradient) and 120 min (5–20% sucrose gradient).

CsCl Equilibrium Centrifugation. Polyalomer tubes, used for these gradients, were boiled for 5 min in TES prior to use to minimize the nonspecific binding of the DNA to the wall of the tubes. Dye–CsCl equilibrium centrifugation was performed using the method of Radloff *et al.* (1967); 5-ml gradients contained 3.55 g of CsCl, 0.3 ml of 5 mg/ml of ethidium bromide in water, 3.8 ml of the DNA sample plus TES buffer, and 20 μl of 2% Sarkosyl in water. The gradients were centrifuged in a Beckman Ti50 rotor at 40,000 rpm for 36 hr at 15°. CsCl equilibrium centrifugation in the presence of the ribopolymer poly(U,G) was performed as described by Vapnek and Rupp (1970). Gradients contained 25 μl of 2% sarkosyl, 7.3 g of CsCl, and 4.8 ml of the DNA sample in 2 mM EDTA (pH 8.0). The refractive index was adjusted to 1.4024 by the addition of 2 mM EDTA. The gradients were centrifuged in a Beckman SW65 rotor at 35,000 rpm for 48 hr at 15°.

Gradient Collection. After centrifugation, the bottom of the tubes were punctured and fractions collected through a hollow needle. For analytical centrifugation the fractions were collected directly onto 1-in. squares of Whatman No. 1 filter papers. In preparative experiments the fractions were collected into microtiter trays and portions of the fractions were spotted on filter papers.

Concentration of the DNA Solutions. The DNA of the fractions were precipitated by treatment with two volumes of absolute alcohol and 0.1 volume of 3 M sodium acetate (pH 6.0). In the case of the CsCl–ethidium bromide gradients, the dye was removed with two extractions at 4° with 1 volume of CsCl saturated 2-propanol and the fractions were diluted with two volumes of TES buffer prior to the addition of the alcohol and sodium acetate. The solution was stored overnight at −20° and centrifuged at −20° for 30 min at 18,000*g*. The supernatant was discarded and the DNA precipitate was resuspended in 0.1 M Tris (pH 8.0) or 1:10 TES buffer. More than 80% of the counts initially present in the solution were recovered by this procedure.

Membrane Filter-DNA Binding Assay. The assay was a modification of the one described by Blair and Helinski (manuscript in preparation). S & S nitrocellulose filters were boiled for 5 min in H_2O, then washed five times with H_2O, and suspended in TEM buffer (20 mM Tris (pH 8.0)–5 mM mercaptoethanol–1 mM EDTA). Before use, each filter was washed by passing 15 ml of TEM buffer through it. Duplicate samples were diluted with 2 ml of buffer and filtered and the tubes were washed three times in each case with 3 ml of TEM buffer containing 2.0 M NaCl. The wash solution was passed through the same filter as the sample. The filtering apparatus was then washed three times with 5 ml of the same solution. In all the operations the filtration rate was about 2 ml/min. After the last wash passed through, the filter was dried under an infrared lamp and counted.

Digestion of DNA with EcoR1 Endonuclease. The standard incubation mixture contained 0.01 M $MgCl_2$, 0.02 M NaCl, 0.1 M Tris (pH 8.0), 0.01–1 μg of DNA, and 5–20 μg of EcoR1 endonuclease preparation in a total volume of 250 μl. Incubation was at 37° and the reaction was terminated by the addition of 5 μl of 0.25 M EDTA.

Counting of Radioisotopes. Samples were spotted or collected on 1-in. squares of filter paper, dried, washed with cold 5% trichloroacetic acid, 95% ethanol, and ether, and counted in a Beckman liquid scintillation counter. Membrane filters were counted directly without any washing. The scintillation cocktail contained 1.3 g of 2,5 diphenyloxazole/l. of toluene.

Results

Purification of the R6K Plasmid from CR34 (R6K). The molecular weight of the R6K plasmid DNA has been reported previously as 26×10^6 (Kontomichalou *et al.*, 1970). In order to assure that in the conjugal transfer of R6K from RC85(R6K) to CR34 the properties of the R6K plasmid were not altered, the antibiotic resistance pattern and the plasmid DNA of the presumptive CR34(R6K) strain were examined. The antibiotic resistance pattern acquired by the CR34(R6K) strain was the same as that reported for this plasmid by Kontomichalou *et al.* (1970), namely, streptomycin and ampicillin. For the physical characterization of the DNA, CR34(R6K) and CR34 were grown in the presence of [^{3}H]thymine as described in Materials and Methods and the cleared lysates were analyzed in preparative dye–CsCl gradients. As shown in Figure 1a, in the case of the DNA from the CR34(R6K) strain there is a satellite DNA peak in the position of supercoiled DNA in the dye–CsCl gradient. This peak is absent in the case of the DNA from the parent CR34 strain (Figure 1b). The peak fractions of the satellite peak were concentrated and analyzed by sucrose gradient velocity sedimentation. The results of this centrifugation are shown in Figure 1c. [^{14}C]Thymine labeled supercoiled ColE1 DNA and [^{14}C]uridine labeled bacteriophage MS2 were used as internal markers for the determination of the sedimentation coefficient of the plasmid DNA. Two discrete peaks were observed in this preparation; the major one with an $s_{20,w}$ of 51 S and a minor peak with an $s_{20,w}$ of 38 S. Analysis by electron microscopy showed that the main peak was composed of supercoiled DNA molecules and the 38 S region consisted of open circular DNA. This $s_{20,w}$ for the closed circular DNA form corresponds to a molecular weight of approximately 26.5×10^6 on the basis of a relationship between molecular weight and sedimentation coefficient of supercoiled DNA reported by Bazaral and Helinski (1968). The slower peak of DNA (Figure 1c) is probably the result of a small amount of breakdown of the faster (51 S) supercoiled DNA form to the open circular form (38 S) due both to the handling of the sample and to radioactive decay (Y. M. Ku-

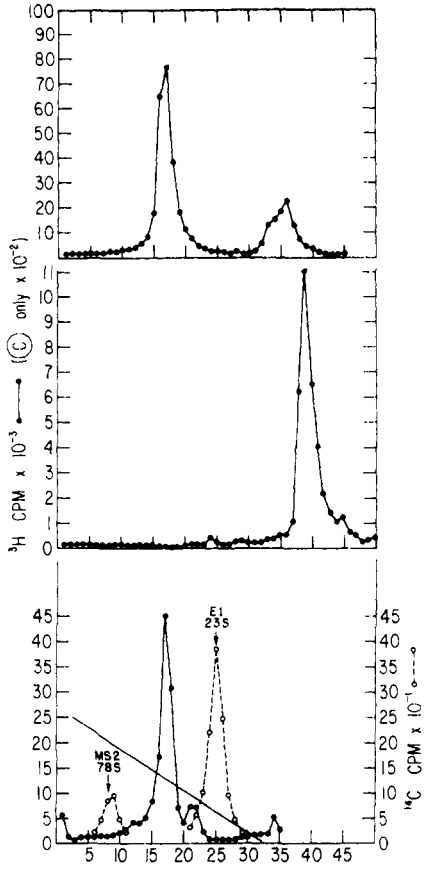

FIGURE 1: Characterization of the R6K plasmid in the CR34(R6K) strain. [^{3}H]Thymine labeled cleared lysates of CR34(R6K) and the parent strain CR34 were centrifuged to equilibrium in preparative dye-CsCl gradients. (a and b) CsCl-ethidium bromide gradients of the cleared lysates from CR34(R6K) and CR34, respectively. (c) Neutral sucrose gradient of the satellite DNA peak from a. The fractions of the gradient containing the satellite DNA in a (fractions 15–25 were pooled, 2-propanol extracted, and precipitated as described in Materials and Methods. The precipitated DNA was resuspended in a tenfold dilution of TES and mixed with [^{14}C]thymine labeled supercoiled ColE1 DNA and [^{14}C]uracil labeled bacteriophage MS2. The sample was then centrifuged in a 20–31% sucrose gradient as described in Materials and Methods.

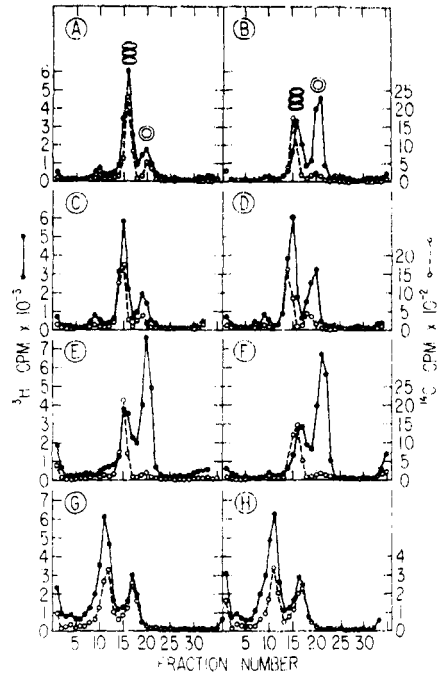

FIGURE 2: Sedimentation analysis of untreated and treated R6K relaxation complex. [^{3}H]Thymine labeled cleared lysates (100-μl portions) from CR34(R6K) were mixed with 100 μl of [^{14}C]thymine labeled noncomplexed supercoiled and open circular R6K DNA. The treatments described below were performed in a final volume of 300 μl of TES buffer. After the treatments, 250 μl of the reaction mixture was layered on a 5–20% sucrose gradient and centrifugation as described in Materials and Methods. (A) No treatment; (B) 0.25% SDS, 10 min at 25°; (C) 1.25 mg/ml of Pronase (self-digested 30 min at 37° at a concentration of 5 mg/ml in TES buffer prior to use), 10 min at 37°; (D) as in C then SDS (final concentration 0.25%) added for an additional 10 mm at 25°; (E) 300 μg/ml of ethidium bromide 10 min at 25°; (F) as in E then SDS (final concentration of 0.25%) for an additional 10 min at 25°; (G) incubation at 60°, 15 min; (H) as in G then SDS (final concentration of 0.25%) added for an additional 10 min. Treatments A–F and G and H were carried out on two different preparations. Centrifugation is from left to right. More than 95% of the counts initially layered on the gradients were recovered after centrifugation.

persztoch-Portnoy and D. R. Helinski, manuscript in preparation). The $s_{20,w}$ of 38 for the slower sedimenting DNA corresponds to a molecular weight of 27.6×10^6 according to the relationship between sedimentation coefficient of open circular DNA and molecular weight reported by Sharp *et al.* (1972).

Relaxation of the R6K DNA–Protein Complex. To demonstrate the existence of relaxation complex of R6K, cleared lysates of CR34 (R6K) were mixed with differentially labeled, noncomplexed supercoiled and open circular DNA, isolated by the dye-CsCl procedure, and the mixture was treated with the various agents known to induce relaxation of previously described plasmid relaxation complexes. As shown in Figure 2, treatment of the samples with SDS (Figure 2B) or ethidium bromide (Figure 2E) induces the conversion of approximately 50% of the DNA of the complex from a position of supercoiled DNA (as indicated by the ^{14}C-labeled supercoiled DNA marker) to that of open circular DNA. This conversion did not take place when the samples were treated with heat for 30 min at 60° (Figure 2G) or when they were incubated in the presence of Pronase (Figure 2C) for 30 min at 37°. Furthermore the supercoiled DNA after heat or Pronase treatments did not show any relaxation when it was subsequently incubated in the presence of SDS (Figure 2D and H, respectively).

Salt Dissociates the Protein from the DNA of Complexed R6K. A striking difference was found in the ratio of open circular DNA to total DNA in the ethidium bromide treated R6K complex (Figure 2E) and the complexed R6K DNA subjected to dye-CsCl equilibrium centrifugation (Figure 1a). If ethidium bromide induced relaxation of the complexed R6K DNA, a similar proportion of counts in the open circular DNA region should have been found in the dye-CsCl gradient and in the neutral sucrose gradient where the cleared lysate was treated with ethidium bromide (Figure 2E). The fact that a larger proportion of the counts appeared in the supercoiled DNA region in the dye-CsCl gradient than in the equivalent region in the ethidium bromide treated sample centrifuged in a neutral sucrose gradient suggested an effect of the CsCl on the induced relaxability of the complexed supercoiled DNA.

To analyze the effect of CsCl on the relaxability of supercoiled DNA in the R6K DNA-protein complex, cleared lysates were treated with CsCl before and after exposure to ethidium bromide. The results of these experiments (Figure 3) indicated that when the cleared lysates are exposed to CsCl prior to the addition of ethidium bromide, the DNA-protein complex was not relaxed (Figure 3a). If the order of

TABLE I: Ability of Complexed and Noncomplexed R6K DNA to Bind to Membrane Filters.[a]

Treatments	Cpm Applied		Cpm Bound		Fraction Bound	
	Relaxed Complex	Supercoiled	Relaxed Complex	Supercoiled	Relaxed Complex	Supercoiled
None	2132		1982		0.92	
None		18,313		1438		0.08
None	2132	18,313	1920	2311	0.91	0.12
Pronase 1.25 mg/ml 37°, 10 min	2132	18,313	88	1089	0.04	0.06

[a] [^{3}H]Thymine labeled R6K relaxed complex and [^{14}C]thymine labeled supercoiled R6K DNA were prepared as described in the text. The binding to membrane filters was carried out as described in Materials and Methods.

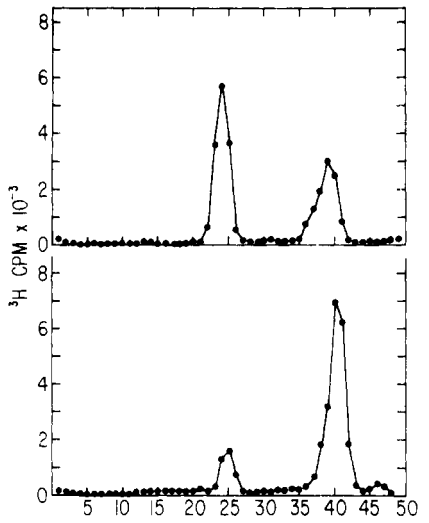

FIGURE 3: Effect of CsCl on the induction of relaxation of the R6K complex by ethidium bromide. A cleared lysate, prepared from a 30-ml culture of CR34(R6K) as described in Materials and Methods, was adjusted to a total volume of 7.6 ml with 1.10 TES buffer. The solution was divided in two equal fractions of 3.8 ml each. In a, 3.55 g of CsCl was added, followed by 0.3 ml of a solution of 5 mg/ml of ethidium bromide. In b, the order of addition of CsCl and ethidium bromide was reversed. The samples were centrifuged to equilibrium, and fractions were collected and counted as described in Materials and Methods. (a) CsCl before ethidium bromide; (b) ethidium bromide before CsCl. Recoveries of the counts applied were 98 and 95%, respectively.

additions is reversed (ethidium bromide before CsCl) the DNA of the cleared lysate banded mainly in the position of open circular DNA (Figure 3b). Similar results were obtained when the concentration of CsCl was 0.5, 1.0, 2.0, or 4.0 M. The results of this experiment are interpreted as an inactivation of the DNA-protein relaxation complex by the salt treatment. The salt inactivation of the complex could be due to a modification of the protein moiety of the complex rendering it resistant to the induction of relaxation; alternatively, the salt treatment could induce a disassociation of the protein-DNA complex to supercoiled DNA and protein(s). To distinguish between these alternatives a membrane filter assay for protein-DNA association was used. The principle of this assay is the ability of protein-DNA complexes to bind to membrane filter, while free DNA washes through the filter (Bourgeois, 1971). [^{14}C]Thymine labeled supercoiled R6K DNA was prepared from cleared lysates by ethidium bromide-CsCl equilibrium centrifugation as described in Materials and Methods. A [^{3}H]thymine labeled cleared lysate was induced to relax by the addition of ethidium bromide. The relaxed complex was centrifuged in a 20-31% sucrose gradient and the open circular DNA region pooled and concentrated as described previously. The membrane binding ability of the [^{14}C]thymine labeled supercoiled DNA and of the [^{3}H]thymine relaxed complex were assayed. As shown in Table I, the [^{14}C]thymine labeled supercoiled DNA from the dye CsCl gradient did not bind to the membrane filter in the presence of the [^{3}H]thymine labeled relaxed complex. The level of binding of the relaxed complex decreased from 91 to 4.2% after incubation with 1.25 mg/ml of Pronase for 10 min at 37°.

Similar high levels of membrane binding were obtained when the unrelaxed supercoiled DNA-protein complex was purified by centrifugation in sucrose density gradients containing 50 mM NaCl. No binding of the supercoiled DNA was observed, however, when the purification of the R6K DNA from cleared lysates involved Pronase treatment or was carried out by sucrose gradient centrifugation in the presence of 500 mM NaCl. From these data it is concluded that the exposure of the complex to salt (either CsCl or NaCl) dissociates the protein from the supercoiled DNA in the untreated relaxation complex. If the DNA of the complex is induced to relax, subsequent exposure to salt does not dissociate the protein from the open circular DNA. The protein in both the unrelaxed and relaxed complex, however, is removed by Pronase digestion.

Strand Specificity of the Nick or Gap in the Relaxed R6K Complex. [^{3}H]Thymine labeled and [^{14}C]thymine labeled R6K complex, relaxed by treatment with SDS, were purified by sucrose gradient centrifugation. The open circular region of each gradient was pooled, concentrated, Pronase treated, and centrifuged in alkaline sucrose gradients. The profiles of these centrifugations are shown in Figure 4A and B. Sedimentation of the relaxed R6K complex in 20-31% alkaline sucrose gradients resolved two symmetrical peaks of approximately the same size indicating that the relaxed molecules contained a single break in one of the two strands of the DNA double helix. The single stranded linear and circular regions of the [^{14}C]thymine labeled relaxed complex (fractions 16-30, Figure 4B) were mixed with single stranded circular (fractions 16-20, Figure 4A) or single stranded linear (fractions 23-28, Figure 4A) DNA from the [^{3}H]thymine labeled relaxed complex. The pooled fractions were neutralized and subjected to equilibrium centrifugation in CsCl in the presence of poly(U,G). The results of this experiment demonstrate that the [^{3}H]thymine labeled linear nicked strand is predominantly the heavy strand

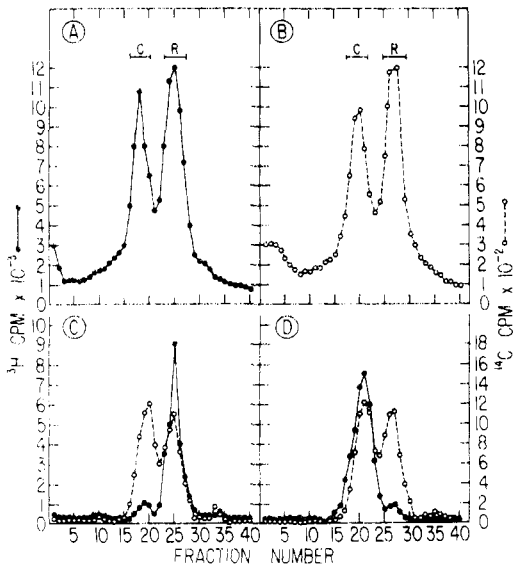

FIGURE 4: Strand specificity of the relaxation event. The R6K relaxation complexes from cleared lysates of RC85 (R6K) labeled separately with [^{14}C]thymine and [^{3}H]thymine were induced to relax by treatment with SDS as described in the legend of Figure 2 and purified in preparative 20–31% sucrose gradients. The DNA of the open circular region of each gradient was pooled and concentrated and Pronase treated as described in Materials and Methods and then centrifuged in 20–31% alkaline sucrose gradients. The fractions containing the separated strands were pooled as shown in A and B and analyzed by poly(U,G)–CsCl equilibrium centrifugation. (A and B) Alkaline sucrose gradient of [^{3}H]thymine and [^{14}C]thymine labeled complexes, respectively; (C) poly(UG)–CsCl equilibrium centrifugation of equal amounts of pools C and R from the [^{14}C]thymine labeled relaxed complex (frame B) mixed with pool C from the [^{3}H]thymine relaxed complex (frame A); (D) poly(UG)–CsCl equilibrium centrifugation of equal amounts of pools C and R from the [^{14}C]thymine labeled relaxed complex (frame B) mixed with pool R from the [^{3}H]thymine relaxed complex (frame A). The recovery of counts in the poly(UG)–CsCl gradients were greater than 85%.

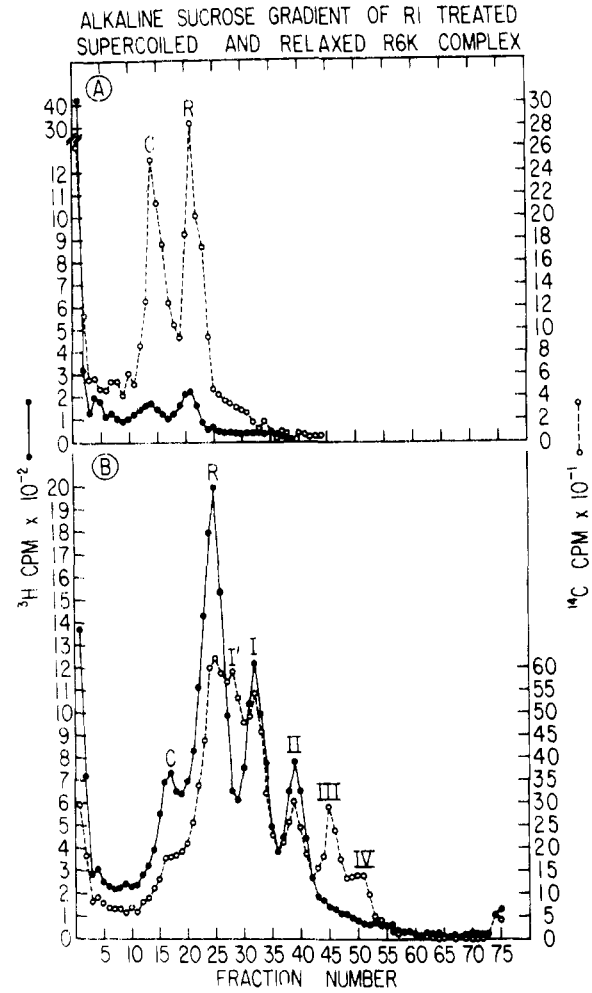

FIGURE 5: Alkaline sucrose gradient of untreated and EcoR1 treated supercoiled and relaxed R6K DNA. [^{14}C]Thymine labeled R6K complex, prepared and relaxed as described in the legend of Figure 4, and [^{3}H]thymine labeled supercoiled R6K DNA, prepared as described in Materials and Methods, were mixed, ethanol precipitated, and resuspended in 200 μl of 0.1 M Tris (pH 7.9)–0.01 M $MgCl_2$–0.05 M NaCl; 140 μl of this solution was incubated with 5 μl of EcoR1 enzyme for 30 min at 37° and 60 μl was incubated under the same conditions except without enzyme. The reaction was terminated by the addition of EDTA (final concentration of 0.025 M). The samples were layered on 5–20% alkaline sucrose gradients and centrifuged for 150 min at 50,000 rpm in the Beckman SW 50.1 rotor at 15°. Centrifugation is from left to right. (A) Untreated sample; (B) EcoR1 treated sample. The supercoiled R6K DNA pellets under these centrifugation conditions.

(Figure 4C), while the [^{3}H]thymine labeled circular strand is predominantly the light strand (Figure 4D). These results indicate that the nick or gap present in the R6K DNA after relaxation of the complex occurred specifically in one of the two strands of the R6K double helix; the strand exhibiting a higher affinity for poly(U,G).

Site Specificity of the Break Induced by the Relaxation of Complexed Supercoiled R6K DNA. To establish whether or not the break induced by the relaxation event of the R6K complex is at a specific site in the R6K DNA molecule, differentially labeled supercoiled DNA and ethidium bromide relaxed open circular DNA were incubated with the EcoR1 restriction endonuclease. Noncomplexed [^{3}H]thymine labeled supercoiled DNA was isolated from cleared lysates in a CsCl ethidium bromide gradient and further purified in a 20–31% neutral sucrose gradient. The supercoiled DNA region was precipitated and resuspended in 0.1 M Tris (pH 7.9). [^{14}C]Thymine labeled complexed R6K DNA was prepared from cleared lysates and purified in a preparative 20–31% neutral sucrose gradient containing 50 mM NaCl. The region of covalently closed supercoiled DNA was pooled and relaxation was induced by the addition of ethidium bromide. The DNA was precipitated as described in Materials and Methods, resuspended in TES buffer, and digested with Pronase (1.25 mg/ml), 15 min at 37°. The material was further purified by centrifugation in a 5–20% neutral sucrose gradient. The fractions containing the open circular DNA were mixed, precipitated, and resuspended in 0.1 M Tris, pH 7.9. The analysis of the digestion products of the incubation of differentially labeled supercoiled DNA and relaxed open circular DNA of R6K with EcoR1 restriction endonuclease is shown in Figure 5 and represented diagramatically in Figure 6. When the DNA samples were incubated in the absence of the enzyme, the circular and linear strands of the relaxed complex are clearly distinguished in the alkaline sucrose gradient, while greater than 98% of the unrelaxed supercoiled DNA sample pelleted to the bottom of the tube (Figure 5A). Digestion of the supercoiled DNA with the enzyme (Figure 6B) resulted in the generation of two single-stranded DNA peaks with S values of 30 S (peak I) and 25 S (peak II). The equivalent molecular weight of these DNA species (Studier, 1965) is 7.7×10^6 and 4.8×10^6, respectively. The sum of the molecular weights of these DNA molecules is similar to the estimated molecular weight of the single-stranded unit length of R6K (12.4×10^6). The fact that after 30-min digestion

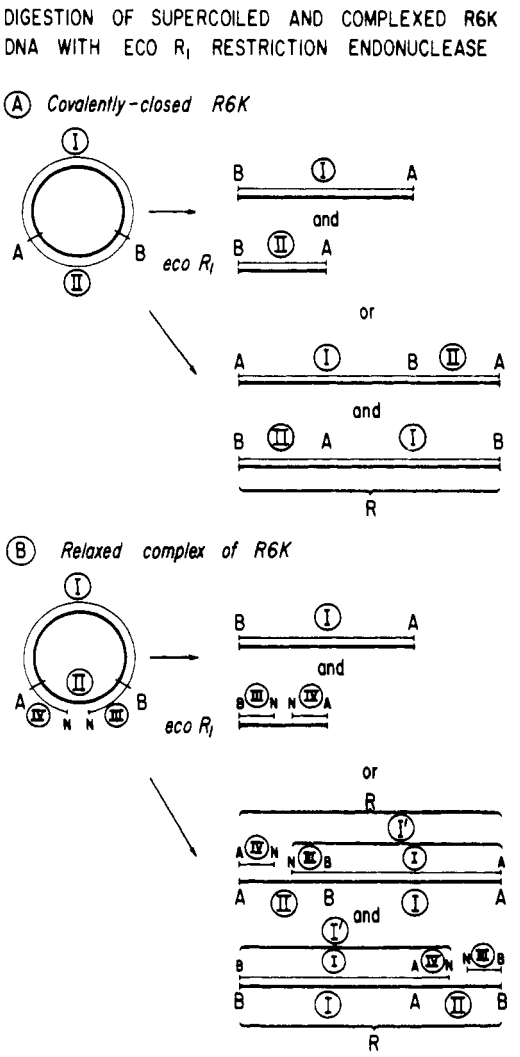

FIGURE 6: Fragments produced from the digestion of supercoiled and relaxed complex of R6K DNA with EcoR1 restriction endonuclease. The designations of the various fragments correspond to the designation of the peaks shown in Figure 5. N refers to the site of the nick in the relaxed complex.

there is a significant fraction of molecules with the sedimentation properties of single-stranded rods (Figure 5A and B) could be accounted for by the action of the EcoR1 restriction endonuclease on only one of the two sites in R6K DNA sensitive to the action of the enzyme as illustrated in Figure 6. With more active preparations of the enzyme, the relative ratio of unit length rods (Figure 5A and B) to peaks I and II (Figure 5B) was decreased.

Incubation of the relaxed R6K complex with EcoR1 endonuclease resulted in the formation of discrete digestion products designated in Figure 5B as R, I, I′, II, III, and IV. Peaks R, I, and II presumably were derived from the circular strand of the relaxed complex and, therefore, they correspond in size to the same segments obtained from the digestion of supercoiled circular DNA, namely 12.5×10^6, 7.7×10^6, and 4.8×10^6, respectively (Figure 6). Peaks III and IV, presumably, are the digestion products of the linear strand of the relaxed complex when the enzyme acts at both EcoR1 sites. Their sedimentation values of 20 S and 16 S correspond to molecular weights of approximately 2.8×10^6 and 1.6×10^6, respectively. These fragments presumably make up the 4.8×10^6 (peak III, Figure 5B) peak derived from the circular strand and, therefore, represent the fragments resulting from the site specific nick in the complexed R6K DNA (Figure 6). Peak I′ (Figure 5B) has a sedimentation value of 33 S equivalent to approximately 9.8×10^6 and, presumably, is derived from the linear strand of molecules of the relaxed complex where the EcoR1 restriction endonuclease cleaved only one of the two sensitive sites (Figure 6). These results indicate that the nick or gap in the R6K relaxed complex DNA molecule is site specific and is located in fragment II (4.8×10^6) derived from the R6K molecules.

Discussion

The physical properties determined for R6K DNA are in agreement with those reported by Kontomichalou *et al.* (1970). Under logarithmic growth conditions the R6K is isolated mainly as supercoiled DNA molecules of a molecular weight of 26×10^6. As shown in this communication, gentle lysis of *E. coli* cells harboring R6K permits the isolation of this plasmid as a complex of supercoiled DNA and protein that can be induced to relax with SDS or ethidium bromide. Of all the plasmid relaxation complexes described to date, the salt dissociability of the R6K complex is a unique property of this plasmid. This characteristic and the fact that Pronase treatment of the R6K complex does not induce the relaxation of the supercoiled DNA and renders the DNA insensitive to further treatment with SDS or ethidium bromide strongly support the endonuclease model proposed earlier for relaxation complexes (Clewell and Helinski, 1970). In this model the complex is considered to consist of covalently closed DNA associated with an endonuclease that is activated by certain agents that denature proteins. The properties of the R6K complex argue against the alternative model whereby a nick preexists in the DNA molecule and the supercoiled state is maintained by a binding protein(s) associated at the site of the nick. The fact that high salt concentrations dissociate the protein from the R6K DNA provides for the first time conditions that may release the associated protein from the complexed DNA in an active state with respect to the site-specific endonucleolytic activity. In the case of previously described relaxation complexes the agents that were effective in removing the protein from the complex also destroy or denature proteins.

The relaxation induced by ethidium bromide in the R6K complex and previously shown for the ColE1 complex (D. Guiney, unpublished observations) could either be a consquence of the unwinding of superhelical turns induced by the intercalating dye (Bauer and Vinograd, 1968) or a direct action of the dye with the protein moiety of the complex. It should be noted that two agents known to induce relaxation of the DNA-protein complex of the R6K factor, SDS and ethidium bromide, have been shown to cure R factors and certain other plasmids *in vivo* (Tomoeda *et al.*, 1968; Bouanchaud *et al.*, 1968). Experiments are in progress to analyze the possible induced relaxation of the complex *in vivo* by these agents as a possible mechanism for their curing of plasmids.

As illustrated in Figure 6, the proposal of two sites of differential sensitivity for the action of the EcoR1 restriction endonuclease in R6K DNA readily accounts for the products observed after digestion with this enzyme. The suggested different sensitivities of the two sites may be due to variations in the nucleotide sequence recognized by the EcoR1 restriction endonuclease (Hedgpeth *et al.*, 1972). The site specificity of the relaxation event is not a unique property of the R6K complex. In the case of the ColE1 and ColE2 com-

plex the relaxation event is also site specific (Lovett *et al.*, 1974a). Furthermore, the site of the nick in the case of the ColE1 plasmid appears to be located at the origin of ColE1 DNA replication (Lovett *et al.*, 1974b). Whether this characteristic is also shared by the R6K plasmid is currently under investigation.

Acknowledgment

We are indebted to Dr. Herbert Boyer for the generous gift of the EcoR1 restriction endonuclease.

References

Bauer, W., and Vinograd, J. (1968), *J. Mol. Biol. 33,* 141.
Bazaral, M., and Helinski, D. R. (1968), *Biochemistry 7,* 3513.
Blair, D. G., Clewell, D. B., Sherratt, D. J., and Helinski, D. R. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 210.
Bouanchaud, D. H., Scavizzi, M. R., and Chabbert, Y. A. (1968), *J. Gen. Microbiol. 54,* 417.
Bourgeois, J. (1971), *Methods Enzymol. 21,* 491.
Boyce, R. O., and Setlow, R. B. (1962), *Biochim. Biophys. Acta 61,* 618.
Clewell, D. B., and Helinski, D. R. (1969), *Proc. Nat. Acad. Sci. U. S. 62,* 1159.
Clewell, D. B., and Helinski, D. R. (1970), *Biochemistry 9,* 4428.
Hedgpeth, J., Goodman, H. M., and Boyer, H. W. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 3448–3452.
Helinski, D. R., and Clewell, D. B. (1971), *Annu. Rev. Biochem. 40,* 899.
Kline, B. C., and Helinski, D. R. (1971), *Biochemistry 10,* 4975.
Kontomichalou, P., Mitani, M., and Clowes, R. C. (1970), *J. Bacteriol. 104,* 34.
Lovett, M., Guiney, D., and Helinski, D. R. (1974a), *Proc. Nat. Acad. Sci. U. S. 71,* 3854.
Lovett, M., Katz, L., and Helinski, D. R. (1974b), *Nature (London) 251,* 337.
Nishioka, T., Mitani, M., and Clowes, R. C. (1969), *J. Bacteriol. 97,* 376.
Radloff, R., Bauer, W., and Vinograd, J. (1967), *Proc. Nat. Acad. Sci. U. S. 57,* 1514.
Roberts, R. B., Cowie, D. B., Abelson, P. H., Bolton, E. T., and Britten, R. J. (1962), *Carnegie Inst. Wash. Publ. No. 607,* 5.
Sharp, P. H., Hsu, M., Ohtsubo, E., and Davidson, M. (1972), *J. Mol. Biol. 71,* 471.
Studier, F. W. (1965), *J. Mol. Biol. 11,* 373.
Tomoeda, M., Inazuka, M., Kubo, N., and Hakamura, S. (1968), *J. Bacteriol. 95,* 1078.
Vapnek, D., and Rupp, W. D. (1970), *J. Mol. Biol. 53,* 287.

Proton Binding Changes in α-Chymotrypsin Dimerization†

Thomas A. Horbett and David C. Teller *

ABSTRACT: Proton binding changes occurring upon dilution of α-chymotrypsin solution were measured with a specially designed pH recording system. Correlation of these proton binding changes with disruption of α-chymotrypsin dimers during dilution shows that the ionization of certain groups on the enzyme is perturbed in the dimeric state. The pH dependence of the proton binding changes agrees closely with predictions made by K. C. Aune and S. N. Timasheff [(1971), *Biochemistry 10,* 1609] on the basis of the observed pH dependence of α-chymotrypsin dimerization. The observed proton binding changes during dimerization are directly predicted by a model in which the pH dependence of dimerization arises from competition between protons and other chymotrypsin molecules for the same sites on a chymotrypsin molecule.

Self-association reactions of proteins are routinely observed to be dependent on pH. Self-association of insulin (Steiner, 1952), lysozyme (Sophianopolis and van Holde, 1964), α-chymotrypsin (Steiner, 1954; Jandorf *et al.*, 1955; Aune and Timasheff, 1971; Aune *et al.*, 1971), β-lactoglobulin (Townsend *et al.*, 1960), and fibrin (Sturtevant *et al.*, 1955; Endres *et al.*, 1966; Endres and Scheraga, 1966) have all been shown to be pH dependent. This pH dependence implies that proteins preferentially associate when in a given protonated state. Since proton binding to the protein and protein self-association reactions involve the same equilibrating species, pH dependent protein self-association reactions may be examples of competing equilbria, as described by Wyman's "linked function" theory (Wyman, 1964). An alternative explanation for the pH dependence of reversible self-association of proteins can be based upon a combination of the electrostatic repulsion arising from two charged spheres (Verwey and Overbeek, 1948) together with the attraction of specific electrostatic groups (Timasheff, 1969).

Wyman's theory (1964) states that the change in the log of the equilibrium constant resulting from a change in the log of the activity of the ligand is equal to the difference in ligand binding by the two states described by the equilibrium constant. In the case of a dimerization constant which is a function of pH, the theory predicts that the binding of protons changes during the association reaction, resulting in

† From the Department of Biochemistry, University of Washington, Seatt, Washington 98195. *Received February 7, 1974.* The experimental work described in this paper was supported by U. S. Public Health Service Grant GM-13401.