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Bacteriophage fd Gene II-Protein

II. SPECIFIC CLEAVAGE AND RELAXATION OF SUPERCOILED RF FROM FILAMENTOUS PHAGES*

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Bacteriophage fd gene II-protein was characterized as an endonuclease which specifically nicked supercoiled replicative form (RF) of filamentous phages in the viral strand. No other supercoiled DNAs tested were attacked by the enzyme, nor were doubly closed fd RF in the relaxed state nor phage fd single strands. Maximal activity was found at pH 8.5 and 80 mM KCl using fd RFI of physiological superhelicity. Mg^{2+}, but no other cofactor, was required for the cleavage reaction. A sealing activity was found to be associated with the enzyme. At a higher concentration of Mg^{2+} up to 40% of the reaction products were found as doubly closed relaxed fd RF. The protein was not found to be tightly attached to the cleaved strand.

Replication of bacteriophage fd double-stranded DNA is initiated by cleavage of the viral strand. Mutants in gene II of the phage are arrested after formation of the parental RF[1] (1, 2), suggesting that gene II-protein participates in the cleavage reaction of RFI which precedes replication. This paper will show that purified gene II-protein has the properties of a strand- and sequence-specific endonuclease and topoisomerase.

Similar enzymes are phage φX 174 *cis* A protein, which also requires supercoiled double strands to cleave the viral strand of φX 174 RF (3-5), and phage P2 gene A protein, which causes a strand-specific break, thereby initiating the replication of the phage (6). After cleavage φX *cis* A protein is assumed to bind tightly to the nicked viral strand at its 5'-end (7) and to promote in collaboration with the host-coded *rep* protein, the unwinding of the double helix necessary for replication (8).

EXPERIMENTAL PROCEDURES

Phage fd Gene II-Protein—The enzyme used is the homogeneous preparation described in the preceding paper (9). One unit is measured by the conversion of 1 pmol of fd RFI ($M_r = 4 \times 10^6$) to RFII and RFIV. The assay was performed with 0.4 μg of fd RFI in 20 μl of 20 mM Tris/Cl (pH 8.1), 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 20% sorbitol, and 4 μg of bovine serum albumin for 15 min at 30°C. The reaction was stopped with 10 μl of 60% glycerol, 60 mM EDTA, 3% Sarkosyl, and 0.1% bromphenol blue. The sample was loaded on a 1% agarose gel. The amount of gene II-protein for 50% conversion of the RFI band was determined by comparing the brightness of the separated bands in UV light after staining with ethidium bromide.

Other Enzymes—Escherichia coli DNA polymerase I was prepared as described (9). *E. coli* DNA ligase was purified from a hybrid overproducer (10). Nicking-closing enzyme, ω-protein, and antibody against ω-protein were kind gifts of Dr. H.-P. Vosberg (Heidelberg).

Supercoiled DNAs—Supercoiled DNAs from phages fd and φX 174-infected cells were prepared as described (9). ColE1 plasmid, PM2, and SV40 DNA were kind gifts of Dr. M.Q. Klinkert, Dr. H.-P. Vosberg, and T. Crefeld (Heidelberg), respectively.

Single-stranded fd DNA, T7 DNA, and λ DNA were extracted from purified phages by treatment with phenol.

Synthetic Phage fd RF—DNA synthesis on fd single strands (200 μg) was primed with oligonucleotides (8 μg) derived from fd RF or calf thymus DNA. DNA was synthesized by *E. coli* DNA polymerase I (6 μg) in 1 ml of buffer containing 100 mM KCl, 50 mM Tris/Cl (pH 8.1), 6 mM $MgCl_2$, 10 mM NH_4Cl, 50 μg of bovine serum albumin, 0.3 mM each of dATP, dCTP, dGTP, and dTTP (11). One of the triphosphates was labeled with ^3H or ^{32}P. For doubly labeled fd RF, ^{32}P-labeled fd ssDNA was used as template for the incorporation of ^3H-triphosphates into the complementary strand. After 3 h incubation at 20°C, NAD (0.1 mM) and *E. coli* DNA ligase (1 μg) were added and the mixture was further incubated for 2 h at 20°C. About 80% of the template DNA were converted into RFIV.

Negative turns were introduced into the relaxed covalently closed circles using calf thymus nicking-closing enzyme (topoisomerase) (3×10^4 units) and ethidium bromide (12). Phage fd RFIV (30 μg) was incubated in 1 ml of buffer containing 10 mM Tris/Cl (pH 7.5), 2 mM EDTA, 200 mM NaCl, 50 μg of bovine serum albumin, 1 mM spermidine/Cl and up to 10 μg of ethidium bromide for 1 h at 37°C in the dark. Ethidium bromide was removed from the DNA by extraction with 1-butanol. The superhelical density was analytically measured in an ethidium bromide-CsCl density gradient (13). The σ values were standardized with SV40 DNA as reference ($\bar{\sigma}^0 = -0.057$) (14).

Separation of Covalently Closed fd RF from fd RFII—DNA was exposed under controlled conditions to alkali and high salt, neutralized, and sucked through a nitrocellulose filter (11). For analytical purposes, the filters retaining the denatured DNA were counted, the passthrough containing cccDNA was precipitated with acid. It contained more than 97% of the DNA as doubly closed circles.

Sedimentation of DNA and Gel Electrophoresis—Velocity sedimentation in alkaline sucrose was performed as described (15). Agarose gels (1%) were run in 40 mM Tris/acetate (pH 8.1), 1 mM EDTA at 3 V/cm for 5 h. RFI, II, and IV were separated on a gel additionally containing 1 μg of ethidium bromide/ml.

RESULTS

Reaction Products of fd RFI with Gene II-Protein—Electrophoretically homogeneous gene II-protein (9) was incubated with fd RFI and the reaction product analyzed by equilibrium density banding in a CsCl-ethidium bromide gradient as described in Fig. 1. The fd RFI was converted into two distinct products. The peak at lower density consisted of RF open in one strand (RFII), the peak at higher density was found to be doubly closed relaxed RF (RFIV). This reaction reveals two activities of gene II-protein, an endonucleolytic activity and an additional sealing activity directed to the previously cleaved DNA. We will show that both activities are highly specific and therefore connected with gene II-protein.

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[1] The abbreviations used are: RF, replicative form DNA; RFI, covalently closed, supercoiled RF; RFII, RF with a discontinuity in the viral strand; RFIII, linear RF; RFIV, covalently closed, relaxed RF; ssDNA, single-stranded circular DNA; cccDNA, covalently closed circular DNA; dsDNA, double-stranded DNA.

Requirements for Enzyme Activity—Optimal ionic conditions in the reaction mixture were found to be 80 mM KCl and pH 8.5 at 30°C in the presence of Mg^{2+} (Fig. 2). The optima were broad. The rate of RF cleavage was considerably reduced at concentrations higher than 150 mM KCl and pH 9.5. Gene II-protein activity was diminished at temperatures below 25°C and above 37°C (Fig. 2). No activity was found in the presence of an excess of EDTA. Cofactors like ATP, *S*-adenosylmethionine, nicotinamide, or NAD did not influence the reaction. Spermidine inhibited gene II-protein at concentrations above 1 mM.

Effects of Various Divalent Cations on the Reaction Products—The distribution between RFII and the relaxed, doubly closed form RFIV was modulated by the concentration of Mg^{2+} in the reaction mixture. At low $MgCl_2$ endonucleolytic cleavage was preferred relative to the nicking-closing reaction. At high levels of Mg^{2+} the formation of RFIV was increased (Fig. 3). The overall reaction expressed in a minimum of residual RFI was found between 5 and 8 mM $MgCl_2$. We assume that a high concentration of Mg^{2+} keeps the nicked DNA in a configuration which is favorable for sealing.

Be^{2+} did not activate gene II-protein. Ca^{2+} and Ba^{2+} as divalent cations used at 5 mM did not allow the sealing reaction after cleaving of RFI (Fig. 4). Mn^{2+} led to cleavage of both strands resulting in linear DNA. In the presence of this cation phage ϕX RFI was partially cleaved to RFII (Fig. 4) and single-stranded DNA was degraded to small pieces (data not shown). We therefore assume that in the presence of Mn^{2+} the enzyme recognizes single-stranded areas in supercoiled

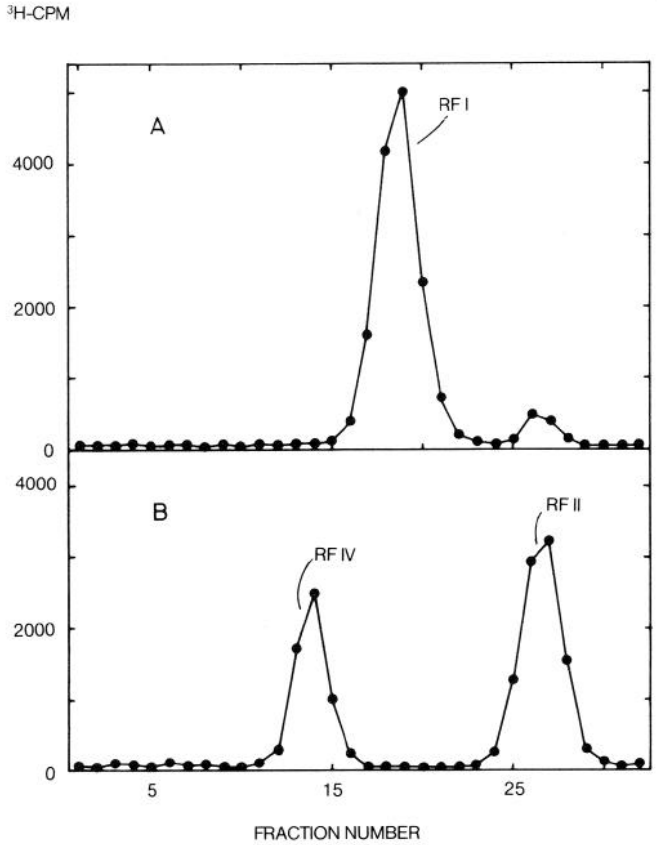


FIG. 1. **Analysis of the cleavage products of fd RFI by gene II-protein.** A 5-ml CsCl gradient (ρ = 1.60 g/ml) with 2 mg of ethidium bromide, 10 mM Tris/Cl (pH 7.5), and 1 mM EDTA was generated in a Beckman fixed angle rotor 50 Ti for 36 h at 20°C and 38,000 rpm. Samples in the gradient contained 500 ng of ^{3}H-labeled fd RFI (15,000 cpm) (*A*) or gene II-protein (0.2 unit)-treated ^{3}H-labeled fd RFI (*B*). The incubation was done in the presence of 10 mM $MgCl_2$ as described under "Experimental Procedures." The density increase in the figure is from right to left.

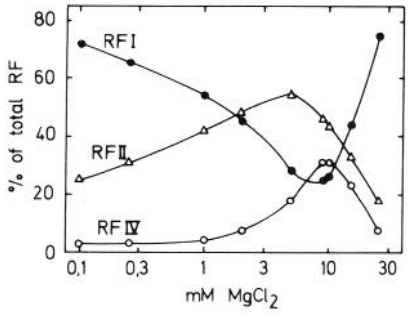


FIG. 3. **The influence of the Mg^{2+} concentration on the distribution of the RF forms generated by the reaction of gene II-protein with fd RFI.** The DNA was incubated as described under "Experimental Procedures" by adding increasing amounts of $MgCl_2$. Separation was on a 1% agarose gel with 5 mM $MgCl_2$. The bands were stained with ethidium bromide.

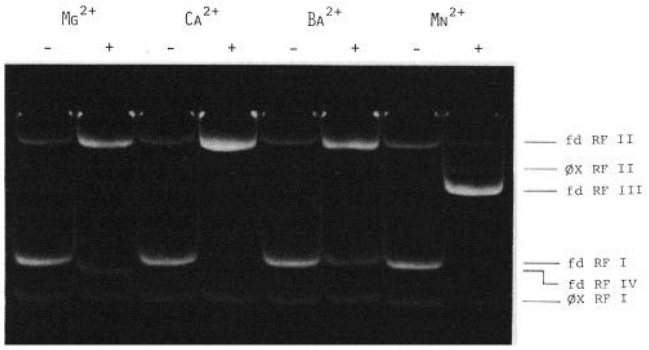


FIG. 4. **Effect of various cations on the activity of gene II-protein.** Phage fd RFI (400 ng), phage ϕX 174 RFI (50 ng) and gene II-protein (0.1 unit) were incubated as described "Experimental Procedures." Mg^{2+} in the assay was replaced by 5 mM of cation indicated in the figure. Separation of the products was on a 1% agarose gel with ethidium bromide. Assays were done with (+) and without (−) gene II-protein.

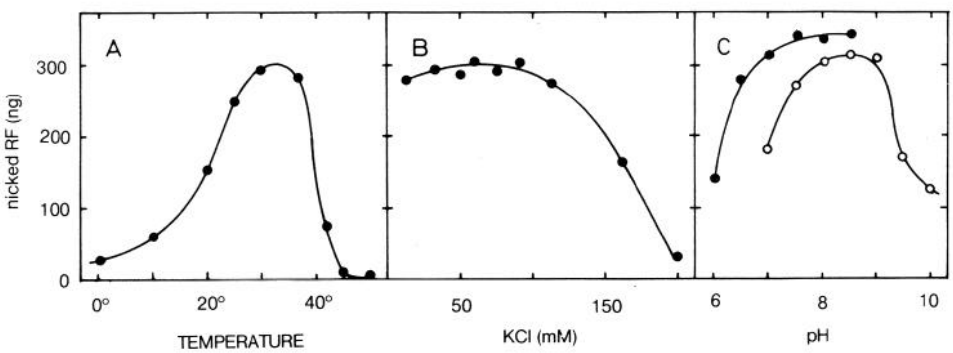


FIG. 2. **Optimal conditions for the cleavage reaction.** ^{3}H-labeled fd RFI (800 ng) and gene II-protein (0.1 unit) were incubated under variations of the standard condition. The filter-binding assay was used to measure the cleavage product RFII. *A*, temperature dependence: gene II-protein was added to the prewarmed incubation mixture; *B*, salt dependence for KCl; *C*, pH optimum in 50 mM imidazol/Cl (●——●) or 50 mM Tris/Cl (○——○). A contamination of RFII in RFI was subtracted as background.

DNA which leads to cleavage at those sites. In the presence of the other divalent cations investigated phage φX RFI was not attacked by gene II-protein (Fig. 4).

Substrate Specificity of Gene II-Protein—Several supercoiled DNA species were tested as substrates for gene II-protein. As shown in Fig. 5, supercoils from phage fd were attacked by gene II-protein whereas φX RFI, PM2, and SV40 DNA were not altered. Supercoiled DNA of phage f1 and M13 which are related to phage fd were a substrate for gene II-protein, and ColE1-plasmid did not react with the enzyme (data not shown).

The Influence of Superhelicity on the Cleavage of fd RF—Relaxed fd RF is not cleaved by gene II-protein (Fig. 6). This agrees with the above observation that RFIV is a final product of the gene II-protein reaction and is not further degraded (Fig. 1). Superhelical turns are apparently a prerequisite for activity of gene II-protein. In order to measure the influence of superhelicity on the cleavage reaction, synthetic fd RF of increasing negative superhelical density was prepared as described under "Experimental Procedures." Endonucleolytic cleavage was found maximal under conditions of superhelical density $\bar{\sigma}^0 = -0.075$ (Fig. 6). RFI isolated from infected cells had a $\bar{\sigma}^0$ value of −0.074, measured by equilibrium centrifugation (12). At low values of σ, *i.e.* an extreme number of negative turns in the RF, the gene II-product activity was found to decrease. In this case the character of the RF may approach a conformation found in ssDNA. Optimal gene II-protein activity corresponds to the physiological superhelical density.

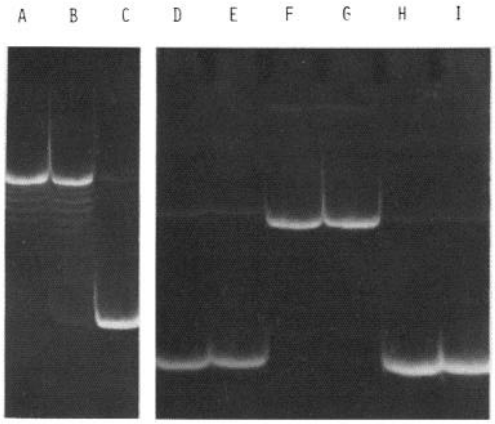


FIG. 5. **Substrate specificity of gene II-protein.** Various DNAs (250 ng each) were incubated with or without gene II-protein and analyzed on a 1% agarose gel as described under "Experimental Procedures." *Lane A*, fd RFI with 0.1 unit of gene II-protein; *B*, fd RFI with 0.05 unit of gene II-protein; *C*, fd RFI; *D*, φX 174 RFI with 0.1 unit of gene II-protein; *E*, φX 174 RFI; *F*, PM2-DNA with 0.1 unit of gene II-protein; *G*, PM2 DNA; *H*, SV40-DNA with 0.1 unit of gene II-protein; *I*, SV40-DNA. The lower bands represent Form I and the upper bands Form II and IV.

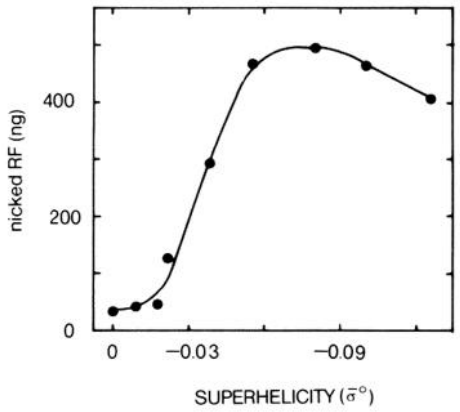


FIG. 6. **Dependence of gene II-protein activity on the superhelicity of fd RF.** Negative superhelical turns were introduced into synthetic ^{3}H-labeled fd RF as described under "Experimental Procedures." The σ values were estimated with an error of ±0.003. ^{3}H-labeled fd RF (1 μg) with a known superhelicity was incubated with 0.8 unit of gene II-protein.

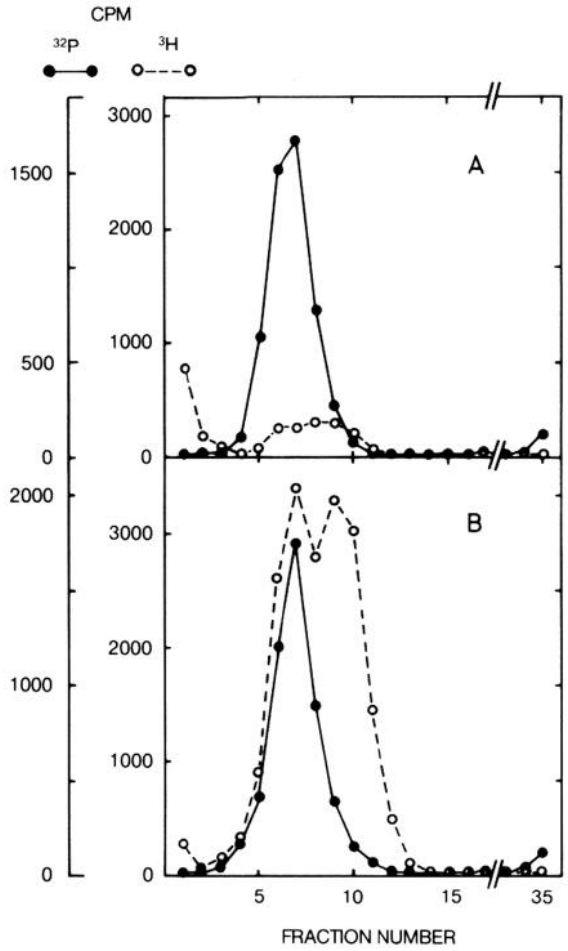


FIG. 7. **Phage fd single strands are not cleaved by gene II-protein.** Phage fd ^{32}P-labeled ssDNA (150 ng, 5000 cpm) (●——●) and ^{3}H-labeled fd RFI (300 ng; 30,000 cpm) (○- - -○) were incubated under standard conditions without gene II-protein (*A*) or with 0.2 unit of the enzyme (*B*). After addition of 20 mM EDTA, the samples were denatured with 0.2 M NaOH for 5 min at 50°C and layered on an alkali sucrose gradient and centrifuged as described under "Experimental Procedures." The direction of sedimentation in the figure is from right to left. Most of the RFI in *A* stuck to the bottom of the centrifuge tube.

Single-stranded fd DNA Is Not Cleaved by Gene II-Protein—Phage fd single strands were not found altered after incubation with gene II-protein (Fig. 7). Furthermore, single-stranded fd DNA did not disturb cleavage of RFI. When both DNAs were incubated in the same assay, the fd supercoils were efficiently cleaved, whereas the fd ssDNA remained intact (Fig. 7). The fd ssDNA was also not changed in the absence of RFI (data not shown).

Strand Specificity—Asymmetrically labeled fd RFI with ^{32}P in the viral strand and ^{3}H in the complementary strand was prepared as described under "Experimental Procedures." After cleaving with gene II-protein, the DNA was denatured in alkali and sedimented in an alkaline sucrose gradient (Fig. 8). The viral strand migrated as a full length linear molecule, whereas the complementary strand sedimented in front of the linear DNA at the position of circular fd DNA. This demon-

strates a selective cleavage of the viral strand in fd RFI.

Binding of Gene II-Protein at the Cleavage Site?—Under physiological conditions the nick was found to be a substrate for DNA-polymerase I (9). Ligase treatment after synthesis with DNA polymerase I resulted in the formation of RFIV (data not shown) which shows nick translation by the polymerase rather than strand displacement. Label could also be introduced at both ends of the cleaved DNA after alkaline treatment (16). Protein labeling (17) after the reaction of gene II-protein with fd RFI and subsequent sedimentation in sucrose did not produce radioactivity which co-sedimented with the DNA (data not shown). Furthermore, a filter-binding assay, designed to prove tight protein-DNA binding (18), did not indicate a covalent attachment of gene II-protein to the cleaved DNA strand.

Kinetics of the Cleavage Reaction and Heat Inactivation of Gene II-Protein—All fd RFI in the assay can be converted into RFII and RFIV when sufficient gene II-protein is present in the incubation mixture (Fig. 5). The cleavage reaction is diminished during the incubation period (Fig. 9*B*). This decay of activity can reflect a stoichiometric binding of the enzyme to the substrate, as it was inferred earlier from kinetics of phage φX *cis* A protein for the cleavage of φX RFI (3). Tight binding to the 5′-end of the cleaved DNA found for *cis* A protein (5, 7) could not be demonstrated for fd gene II-protein. Therefore, the reaction may be catalytic and the protein in solution slowly inactivated as demonstrated in Fig. 9*A*.

Formation of RFIV—In order to show that the described topoisomerase activity of gene II-protein is indeed associated with the enzyme, we have done various controls: (i) Phage φX RFI was not relaxed under the conditions applied for the formation of fd RFIV. Antibodies against ω-protein did not influence the relaxation of fd RF by gene II-protein but inhibited the nicking-closing reaction of ω-protein (19) against φX and fd RFI. (ii) Nicotinamide mononucleotide, an inhibitor of *E. coli* DNA ligase, did not prevent the sealing reaction with gene II-protein and randomly nicked fd RF could not be sealed by the gene II-protein preparation, but with *E. coli* DNA ligase.

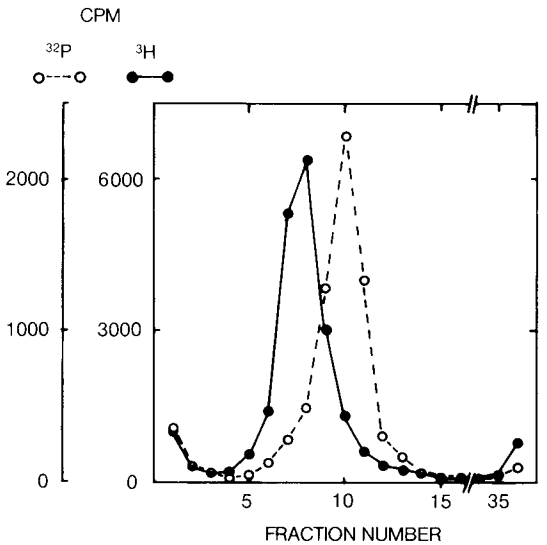


FIG. 8. **Alkaline sedimentation of asymmetrically labeled fd RF after treatment with gene II-protein.** The preparation of the fd RFI was as described under "Experimental Procedures." The viral strand was labeled with ^{32}P (10,000 cpm) (○- - -○), the complementary strand with ^{3}H (30,000 cpm) (●——●), the superhelicity was −0.08 according to the banding of the RF in ethidium bromide/CsCl. This fd RFI (120 ng) was treated with 0.1 unit of gene II-protein and processed as described in Fig. 7. The fast sedimenting label is circular fd DNA as judged by an external reference.

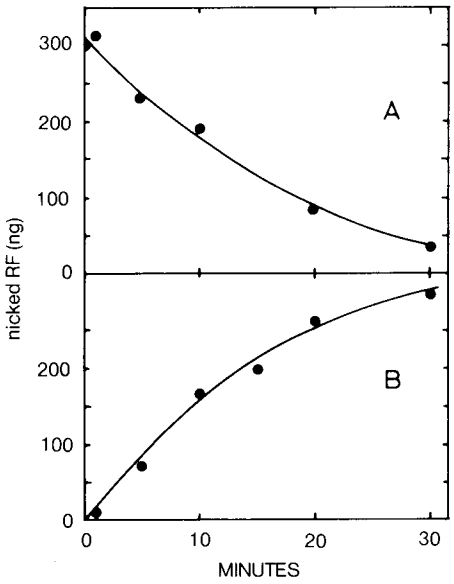


FIG. 9. **Heat inactivation and kinetics of fd RFI cleavage.** To measure the inactivation of gene II-protein, the enzyme (0.8 unit) was incubated in reaction buffer at 30°C without fd RFI (*A*). Aliquots (20 μl out of 130 μl) were added to 0.5 μg of ^{3}H-labeled fd RFI at the times indicated and further incubated for 30 min. Cleavage was determined with the filter-binding assay after denaturation. Kinetics of the cleavage reaction (*B*) was measured with 3.5 μg of ^{3}H-labeled fd RFI and 0.8 unit of gene II-protein at 30°C. Aliquots (20 μl out of 130 μl) were assayed for cleaved RF as described under "Experimental Procedures."

The formation of RFIV relative to RFII is independent on the enzyme concentration and the incubation time. The distribution between the two forms was also not influenced by treatment of the DNA after the reaction like the addition of phenol, EDTA, detergents, salt, proteinase K, and KOH. However, a small amount of doubly closed φX RFIV, which was found after reaction of φX RFI with *cis* A protein, could be converted into RFII by protease treatment (4).

The bacteriophage fd form IV resolved on agarose gels showed a spectrum of topoisomers as demonstrated by the ladder pattern on the gel (Fig. 5). This distribution is characteristic for the action of nicking-closing enzymes (20).

DISCUSSION

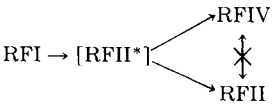
The data presented in this paper demonstrate that phage fd gene II-protein is a highly specific endonuclease which also shows a sealing activity at the cleavage site. The reaction only occurs with supercoiled RFI from filamentous phages. Phage fd single strands, relaxed fd RF, or supercoiled DNAs from other sources are not attacked by the enzyme. A single nick is found in the viral strand of the RF. Precise analysis revealed the nicking position in the sequence 5′-CTTT↓ATT-3′ in the intergenic region of the phage genome between nucleotides 5781/5782 (16). The adjacent nucleotide sequence can be drawn in a hairpin structure (21). From the optimum of superhelicity for the cleavage reaction it can be assumed that the recognition site for gene II-protein in fd RF has to be in a conformation which occurs neither in single strands nor in an undisturbed helix like in relaxed RF. The gene II-protein reaction can be altered by varying the Mg^{2+} concentration or by divalent cations other than Mg^{2+}. Ca^{2+} and Ba^{2+} limit gene II-protein to cleave fd RFI and do not allow subsequent sealing. Mn^{2+} extends the cleavage reaction to the formation of linear DNA. This situation resembles the action of a restriction enzyme specific for RFI of filamentous bacteriophages.

The nick in RFII is found in a region (16) where the origin of RF replication could be located *in vivo* (22). Besides fd

gene II-protein there are other proteins with the property to create an origin of replication by cleavage of DNA: the phage ϕX 174 *cis* A protein (4, 5, 7) and a host factor which acts in the presence of phage T7 proteins required for the replication of the phage (23).

The endonuclease activity of gene II-protein was demonstrated under physiological conditions and was found different from the relaxation complex observed for the colicin E1 plasmid which has to be induced by treatment with phenol or detergent (24).

In addition to the cleavage function, gene II-protein possesses a closing activity which was observed for one-third of the reacted RF molecules (Fig. 1). The constant ratio of RFII and RFIV formed reflects the intrinsic property of a relaxation intermediate (RFII*) which decays into the two DNA species:



The endonuclease and the nicking-closing activity may be involved in fd RF replication. It can be assumed that the nick created by gene II-protein is used to start unwinding of the duplex. This event also creates a start for DNA polymerase III holoenzyme to synthesize the viral strand. After one round of replication gene II-protein is supposed to circularize the full length viral strands.

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