Isolation and Function of the Gene *A* Initiator of Bacteriophage φX 174, A Highly Specific DNA Endonuclease

(cis-acting protein/DNA supercoils/replicative forms of DNA)

TIMOTHY J. HENRY* AND ROLF KNIPPERS†

Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft, Tübingen, Germany

ABSTRACT The gene *A* product of *Escherichia coli* bacteriophage φX 174, necessary for initiation of φX DNA synthesis, has been identified and purified to near homogeneity. The gene *A* protein is shown to be a highly specific DNA endonuclease which breaks one phosphodiester bond in the viral strand of either double-stranded, circular φX replicative form I (RFI), or in single-stranded, circular φX viral DNA; it is inactive against all other species of DNA tested.

The circular single-stranded DNA of bacteriophage φX 174 is converted to a double-stranded form soon after its penetration into the host cell (1). This synthesis of the complementary strand is catalyzed by host cell enzymes (1–3) and has been duplicated *in vitro* (4–6). The circular duplex DNA ("replicative form" DNA, "RF") is replicated semi-conservatively (7) *in vivo* to produce a number of daughter RF molecules via a rolling circle type of mechanism (8–10). These semi-conservative replication processes occur only if the product of the viral gene *A* is active (11, 12) and if the host cell replication machinery is intact.

The *in vivo* function of the gene *A* product has been most clearly delineated by Francke and Ray (13, 14). They showed that the majority of the phage DNA molecules in wild-type φX 174-infected cells contain a phosphodiester cleavage (a nick) at a specific site in the viral strand (RFII form of DNA), whereas in gene *A* amber mutant infected cells, essentially all φX-RF molecules remain as intact supercoiled circles (RFI). This result suggests either that (*a*) the gene *A* protein acts as a site-specific endonuclease, (*b*) the gene *A* protein is needed to activate such a cellular endonuclease, or (*c*) the A protein protects a particular region of the RF molecule so that, even though *random* nicks are made, only those in this region escape DNA ligase action and survive.

In this paper we describe an isolation procedure for the gene *A* protein. As suggested by the *in vivo* data, the purified gene *A* protein has a DNA endonuclease activity which specifically cleaves a phosphodiester bond in the viral strand of φX-RF or in single-stranded, circular φX viral DNA. Of the supercoiled DNA species tested, only φX 174 was attacked by this enzyme.

MATERIALS AND METHODS

Strains. Escherichia coli H514 (Endo I⁻, hcr⁻, Arg⁻, Thy⁻) (15) was obtained from Dr. H. Hoffmann-Berling. H514 was used for preparing ultraviolet-irradiated, φX-specific

Abbreviation: RF, replicative form.
* Present address: Sloan-Kettering Institute, 410 East 68th Street, New York, N.Y. 10021.
† Present address: Fachbereich Biologie, Universität Konstanz, 775 Konstanz, Germany.

proteins ([¹⁴C]- or [³H]amino-acid labeled) as well as for preparation of normal φX-infected cell extracts. The φX 174 amber mutant, am 3 (lysis⁻, gene *E*) was used as equivalent to φX-wild type. Am 3, am 86 (gene *A*), and *E. coli* HF 4714, the permissive host for φX amber mutants, were obtained from Dr. R. L. Sinsheimer.

DNA Substrates. [³H]φX-RFI was prepared from am-3-infected H514 essentially as described in ref. 16 (lysozyme–Brij lysis followed by isopycnic banding in propidium diiodide–CsCl). When double-labeled φX-RFI was made, [³H]thymidine (1 μCi/ml) and chloramphenicol (150 μg/ml) were added 5 min before infection with ¹⁴C-labeled am-3 φX phage; these cells were harvested for RFI purification at 15 min post infection.

φX 174-Specific Marker Proteins were prepared essentially as described by Godson (17) or Gelfand and Hayashi (23). The irradiated cells were incubated 15 min at 37° before infection with φX am 3 (multiplicity of infection 10–20). At the time of infection, a [¹⁴C]algal protein hydrolysate (Amersham/Searle) was added to 0.2 μCi/ml (2 × 10⁸ cells per ml). After 50 min, an excess of unlabeled casamino acids was added and incubation continued for 10 min. Labeled cells were washed and stored at −20° prior to being mixed with unlabeled, nonirradiated, φX-infected cells.

Purification of the A Protein. We determined by double-label experiments (sodium dodecyl sulfate gel electrophoresis of φX gene *A* mutant extracts versus wild-type) that the phage-specific protein of 56,000 daltons was the product of φX 174's gene *A*. Several recent reports (17–20) agree with this value for the molecular weight of the A protein in contrast to the lower values reported earlier (21–23). When the proper gene *A* mutant was used in double-label experiments, we did see a second ratio peak indicative of the second product of this gene (19, 47). In our gels, the small product migrated very close to the major capsid protein and was impossible to identify in singly labeled gels. We call the product of the entire gene *A* the "A protein" and the smaller fragment the "A' protein" (usage opposite that in refs. 19 and 47).

E. coli H514 cells were grown to 2 × 10⁸/ml. infected with φX am 3 (multiplicity of infection = 10) at 37°, and grown for 50 min. A liter of these cells was combined with an equal amount of ¹⁴C-labeled, ultraviolet-irradiated, φX am 3 infected cells (11 × 10⁶ cpm total). The combined cells, washed and adjusted to 2 × 10¹⁰/ml in TE were made 100 μg/ml in lysozyme. After 30 min at 0°, 1/10 volume of 5% Brij was added followed after 10 min by NaCl to 1 M. The clear, viscous lysate was centrifuged at 100,000 × *g* for 1 hr. Eighty to 95% of the A protein was recovered in the high-speed supernatant (Fig. 1*A* and *B*), whereas without the NaCl addition the A protein was found in the sediment (compare
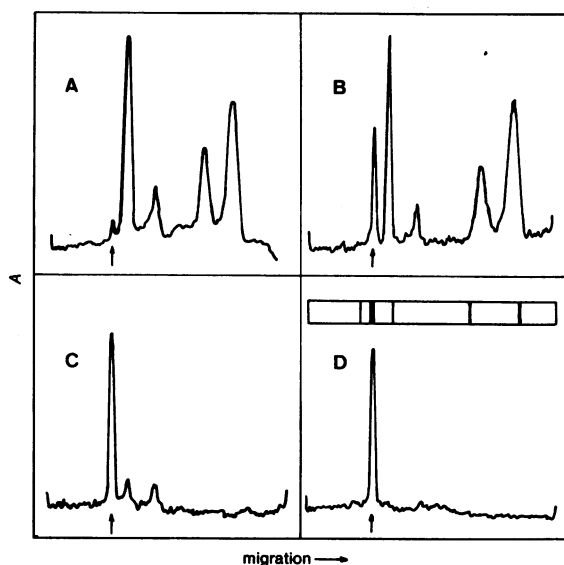
Fig. 1. Sodium dodecyl sulfate–polyacrylamide gel assay for A protein in various purified fractions. Panels A–D are densitometer tracings of autoradiographs made after electrophoresis of various fractions obtained during the purification of the gene A protein. Electrophoretic migration was from left to right. The *arrow* in each panel indicates the position of the A protein, 56,000 daltons. (A) Total extract; (B) 100,000 $\times$ g supernatant; (C) pooled fractions 41–61 from DEAE-cellulose column; (D) fraction 45 from the phosphocellulose column; D also contains a sketch of the Coomassie blue stainable bands present in this gel.

ref. 20). The supernatant was dialyzed versus 50 mM Tris· HCl, pH 7.5, 1 mM EDTA, 5 mM 2-mercaptoethanol, and applied to a column of DEAE-cellulose equilibrated with the same buffer. The A protein was quantitatively retained and eluted off of the column as a distinct peak of radioactivity at 0.23 M NaCl (Fig. 2A). At this point the A protein was 80% radiochemically pure (Fig. 1C), though sodium dodecyl sulfate gel electrophoresis showed many stainable bands. DEAE column fractions 41–61 were pooled and dialyzed versus 0.04 M sodium phosphate, pH 6.0, 10% glycerol, 1 mM EDTA, 10 mM 2-mercaptoethanol, and applied to a phosphocellulose column equilibrated with the same buffer. The column was eluted with a linear NaCl gradient; the radioactive protein eluted at about 0.7 M NaCl in a rather broad peak (Fig. 2B).

Gel electrophoresis of fraction 45 showed only five stainable bands, the most abundant corresponding exactly in its R_F value (56,000 daltons) to that of the single band seen in the autoradiograph (Fig. 1D). From the intensity of the stained band, we estimated a yield of approximately 130 μg per liter of cells, corresponding to about 3000 molecules of A protein per infected cell.

A final purification step was sedimentation of the phosphocellulose column peak fractions through a high salt, 5–20% sucrose gradient (10% in glycerol). There was exact correspondence of radioactivity and endonuclease activity across these gradients (data not shown), with the A protein sedimenting as a molecule with a molecular weight of 70,000. High salt was used wherever possible because it minimized the strong tendency of the A protein to aggregate and to stick to both plastic and glass tubes. When stored in 1 M NaCl, glycerol-phosphate buffer at either 0° or −20°, the A protein's enzymatic activity was stable for at least 6 months.

Endonuclease Assays. Radioactively labeled DNA (0.01–2 μg) was incubated at 37° with gene A protein (0.01–0.2 μg)
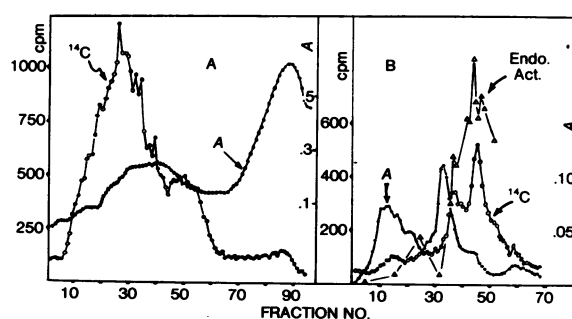

FIG. 2. (A) DEAE-cellulose chromatography of ϕX 174 infected cell extract. (B) Phosphocellulose chromatography of pooled fractions 41–61 from the DEAE column. Details in *Methods*. Phage-specific marker proteins = ^{14}C; nonradioactive protein and nucleic acids = A_{250} and endonuclease activity Δ. Endonuclease activity against ϕX-RF was measured by a filter binding assay after heat denaturation as described in ref. 43.

in 0.1–0.2 ml of buffer to give final concentrations of 50 mM Tris·HCl, pH 7.4, 10 mM MgCl₂, 5 mM 2-mercaptoethanol, and 0.5 mg/ml bovine serum albumin. The albumin had been heated at 75° for 1 hr to destroy any deoxyribonuclease activity. The endonuclease reaction with ϕX-RF required Mg^{++} and was not stimulated by the addition of 0.5 mM ATP. The reaction was stopped by the addition of EDTA to 0.1 M. Samples were layered (without prior denaturation) onto 5–20% alkaline sucrose gradients (1 M NaCl, 0.25 M NaOH, 50 mM EDTA) and sedimented for 2 hr at 50,000 rpm in the SW-50.1 rotor to separate RFI from smaller products or for 7 hr to resolve circular and linear strands. Samples were collected through the bottom of the tube into scintillation vials and counted in a Triton–toluene scintillator fluid. The activity of the enzyme was determined by comparing the percentage of material sedimenting slower than denatured RFI in the reaction mixture sample to that in a control incubated in buffer without enzyme.

RESULTS

Endonuclease Activity of the A Protein. No enzyme assays were carried out until the A protein was relatively pure. When fractions 37–47 from the phosphocellulose column were assayed for endonuclease activity versus supercoiled ϕX 174 RFI (Fig. 2B, open triangles), it was clear that an endonuclease activity was present whose activity closely paralleled the amount of A protein. When these fractions were tested for endonucleolytic activity against other supercoiled DNA molecules, no activity was detected (Table 1). Parallel controls with ϕX-RFI routinely gave an increase of 30–60% nicked RF. The colicin E1 supercoil (the gift of Dr. W. Goebel) listed in Table 1 had been grown in the presence of chloramphenicol and therefore contained ribonucleotides (24). The lack of endonucleolytic activity against this molecule thus shows that the A protein does not contain hybridase (RNase H) activity. Authentic *E. coli* hybridase (25) nicked approximately 25% of this Col E1 sample. Substrate specificity is convincingly demonstrated in the experiment shown in Fig. 3; since selectivity between ϕX 174 and SV-40 supercoils was maintained in both individual and mixed DNA samples, the lack of endonuclease activity on SV-40 supercoils cannot be due to some inhibitor in the SV-40 preparation or to an imbalance in the substrate–enzyme ratio.

The A protein does possess endonucleolytic activity against ϕX single-stranded DNA and, to a lower degree, against
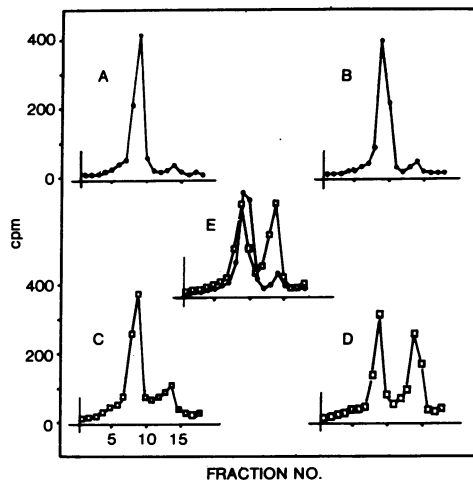
FIG. 3. Specificity of the endonuclease activity of gene *A* protein. Supercoiled, ^{32}P-labeled SV-40 DNA (1000 cpm, ≤ 0.2 μg) ● or ^{3}H-labeled ϕX-RFI (1000 cpm, $\leq$ 0.2 μg) □ was incubated with (panels *B,D,E*) or without A endonuclease (panels *A,C*) (0.05 μg of A per assay) in the assay buffer described in *Methods* for 1 hr at 37°. The incubated mixtures were sedimented on 5–20% alkaline sucrose gradients, collected, and counted as described in *Methods*. In one assay (panel *E*), the substrate was a mixture of ϕX-RFI and supercoiled SV-40. Sedimentation was from right to left.

single-stranded M13 DNA (Table 1). In the reactions with ϕX-RFI and ϕX viral DNA no small degradation products were ever seen. Thus the A protein does not have exonucleolytic activity. [Nor was any exonuclease activity demonstrable against ^{32}P-labeled T7 DNA, [^{3}H]RNA·fd DNA or poly(dA)·[^{3}H]poly(dT)].

The time-dependence of the endonucleolytic reaction against ϕX RFI supercoils is shown in Fig. 4. Note that the

FIG. 4. Time dependence of the endonucleolytic reaction. A 2-ml sample of assay mixture containing 15,000 cpm (14 μg) of ϕX-RFI was mixed with 20 μl (0.2 μg of A) of phosphocellulose column fraction 45 at 0°, transferred to 37° and sampled at the times noted. The 0.2-ml samples were added to 0.05 ml of 0.1 M EDTA to stop the reaction and then analyzed on alkaline sucrose gradients as described in *Methods*, except that these gradients did not contain NaCl; under these conditions, denatured RFI sediments at 34 S while the mixture of circular and linear strands from RFII sediments at 15 S. Top panels show the individual sucrose gradients for the times noted (min). The bottom panel shows the percent nicked (i.e., the percent of material in the denatured RFII peak) as a function of time. At 30 min, an additional 10 μl of enzyme (*E*) was added to the remaining 1.0 ml.

TABLE 1. *Nuclease activity of gene A protein on various substrates*

DNA substrate	% Nicked	
	Control	Change with enzyme
f1	15	5
f1	30	-7
f1	30	6
SV-40	5	0
PM 2	20	4
λ dv	38	-2
λ dv	47	5
Col E1*	7	3
ϕX RFI controls	5–15	30–60
ϕX single strand	0	37
M13 single strand	21	15

The DNA supercoils (top 9 lines) or single-stranded DNA (lines 10, 11) were incubated (*Methods*) with gene *A* endonuclease for 1 hr at 37°. Incubated mixtures were sedimented through alkaline sucrose gradients to separate denatured supercoils from nicked molecules. The background of nicked RF (2nd column) in the control sample (no enzyme) was subtracted from that in the treated sample to give the values in the 3rd column. ϕX-RFI controls were run in parallel with each assay to confirm the activity of the enzyme.

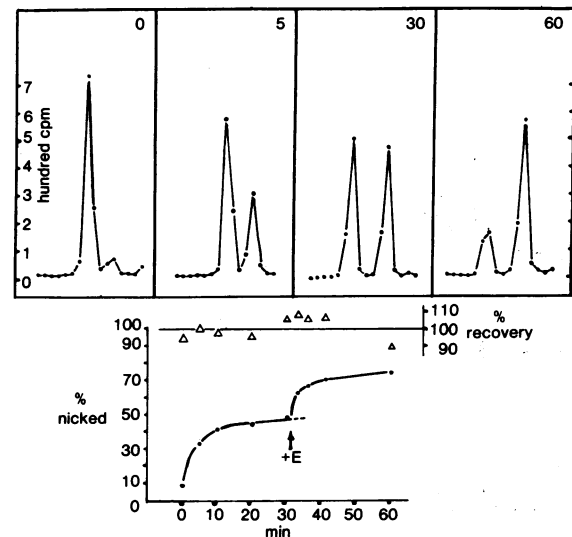* Grown in high levels of chloramphenicol; analyzed in a neutral sucrose gradient.

reaction ceases before completion at low enzyme levels. Since controls show that active enzyme is still present in assay mixtures incubated 1 hr at 37° in the absence of substrate, it appears this cessation is due to the failure of the enzyme to be reused (the ratio of enzyme molecules to substrate molecules in Fig. 4 is estimated at 1:1).

Although these kinetics are similar to those found for *E. coli* B restriction endonuclease (26), gene *A* endonuclease does not require *S*-adenosylmethionine and ATP like that enzyme. Moreover, labeled ϕX-RF is prepared by growth in the same host as that used to supply the enzyme extract, and thus should be protected by modification from resident *E. coli* restriction endonuclease. For this and other reasons, we feel that it is very unlikely that the activity we measure is such a contaminant.

The DNA Products of the Endonucleolytic Cleavage. When the reaction mixture of gene *A* protein treated ϕX-RFI was sedimented on a neutral sucrose gradient (Fig. 5), it was found that RFI (21S) had been converted to RFII (17S) with no indication of RFIII (the double-stranded linear, 15S, form). Thus the gene *A* endonuclease does not produce double strand scissions. Alkaline sedimentation of the reaction products showed only the collapsed supercoil (53S) and single-strands from the denatured RFII. There was no indication of fragments smaller than unit length single strands.

Extended alkaline sedimentation of the reaction products allows one to separate the linear and circular strands of denatured RFII (27). And by starting with a ϕX-RFI which is differentially labeled in its viral and complementary strands, it is possible to tell whether the single nick occurs randomly

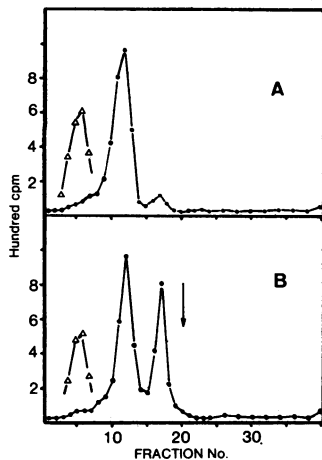FIG. 5. Neutral sucrose sedimentation analysis of the DNA products of the endonuclease reaction. A sample of [³²P]φX-RFI was incubated with (panel B) or without (panel A) gene A protein under the standard conditions described in *Methods* for 1 hr at 37°. The mixtures were then analyzed on 5–20% sucrose gradients containing 1 M NaCl, 50 mM Tris·HCl (pH 7.4), 10 mM EDTA. ³H-Labeled φX viral DNA (26S) was added as a marker Δ; the *arrow* in panel B marks the expected position (15S) for RFIII, the double-stranded linear form. Sedimentation was from right to left.

or in one particular strand of the RFI. The results of such an experiment where viral strands were labeled with ¹⁴C and complementary strands with ³H is shown in Fig. 6. It is clear that the linear (nicked) strands (14S) are exclusively of viral strand origin while the circular strands (16S) are complementary strands. This specific nicking of the viral strand of φX-RFI is exactly the result expected from the *in vivo* observations of Francke and Ray (13, 14).

DISCUSSION

The data presented show that *one* function associated with the gene A protein of φX 174 is an endonucleolytic one which specifically nicks the viral DNA strand of the φX-RF molecule. The most simple *raison d'être* for this nick is to initiate DNA synthesis by providing a 3′ OH primer terminus. φX DNA synthesis could then proceed via a rolling-circle type of mechanism to produce new RF molecules (8–10). Because of the mechanism that we envision for A protein action (following section), we feel that gene A protein functions not only to initiate DNA synthesis, but also to cut off a unit length viral strand after one round of replication has come close to completion. Thus we think gene A function is likely to be needed throughout infection for DNA synthesis. The only published experiments on this point (28) were equivocal. The analogous gene of bacteriophage fd (M13 and f1), gene *2*, is required for single-stranded viral synthesis as well as for RF formation (29, 30). In fact, the choice between viral or RF synthesis in f1 is determined entirely by the ratio of gene *2* to gene *5* protein (31).

Model for Enzymatic Action. Both φX single-stranded viral and RFI DNA are nicked by the enzyme. Because the most likely common element in both structures is the existence of a hairpin region, we feel that the nicking reaction probably occurs within the hairpin. That the gene A dependent nick takes place within a self-complementary region of the viral strand was strongly suggested by the work of Schekman and Ray (35) and Francke and Ray (13). They showed that linear viral strands obtained from the intracellular pool or from
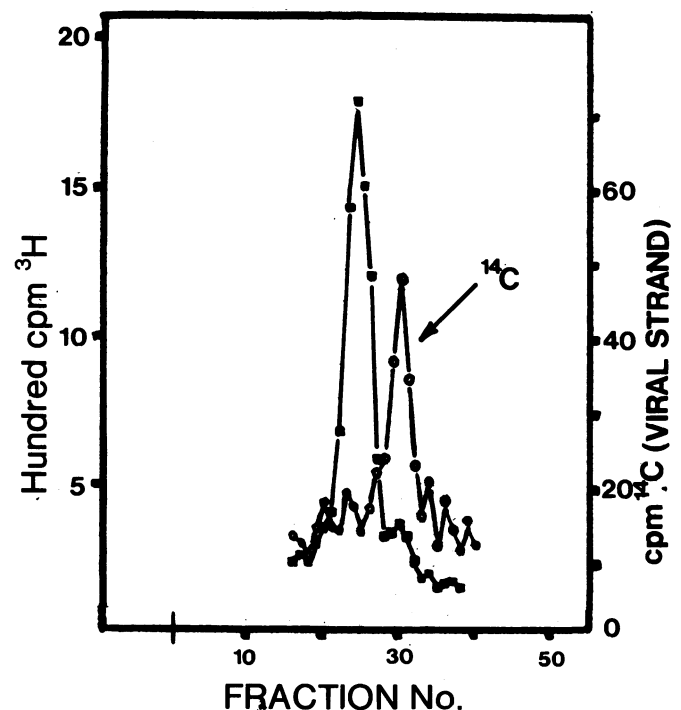

FIG. 6. Identification of the strand nicked by gene A endonuclease. Double-labeled φX-RFI (¹⁴C, viral strand ○; ³H, complementary strand ●) was used as the substrate for gene A endonuclease. A sample of the reaction mixture analyzed on a short alkaline gradient showed that 40% of the RFI had been nicked. The remaining reaction mixture was sedimented through a long alkaline sucrose gradient in order to separate circular (16S) from linear (14S, nicked) strands. Sedimentation was from right to left.

denatured RFII were 10% as infectious as intact, circular viral strands, whereas, linear strands generated by random nicking are essentially noninfectious. The preferred explanation is that the linear strands can circularize because of their complementary ends. Physical evidence for the existence of a hairpin structure comes from the demonstration of a partially DNase-resistant fraction in the viral strand and the RF (36, 37) and from electron microscopic visualization of a hairpin structure in the φX viral strand. Other evidence (38–40) suggests that the nick (and therefore the hairpin) and the origin of replication are actually within the A gene region.

The gene A catalyzed reaction might occur only when the hairpin region is "flipped out" of the RF. This would be dependent upon the amount of (superhelical) torsion in the molecule since torsion within covalently closed DNA molecules can be relieved either by the generation of superhelices or melting out a portion of the double helix into single-stranded regions which, if these regions are self-complementary, become hairpins. If the nick site were situated within the "body" of the hairpin, one would expect random nicking of both viral and complementary strands of the RF. However, the unpaired bases in the bend of the hairpin need not be identical in both strands and could provide the necessary distinction between viral and complementary strand.

This model would explain why small fragments of newly synthesized viral strand are not found *in vivo* even though the A gene endonuclease sensitive site is undoubtedly regenerated

by new DNA synthesis, i.e., why gene A endonuclease does not act prematurely. The original nick relaxes the RFI and the A protein is, therefore, incapable of nicking until at or near the end of one round of replication. We hypothesize that the signal for the end of one round of replication is the accumulation of superhelical torsion (in spite of the existence of a single-stranded region in the replication complex) to the point where the hairpin site in the gene A region flips out. This hairpin site would be recognized and nicked by gene A endonuclease and thus yield the observed linear single strands (35) and specifically gapped RFII (44). Perhaps host proteins (rep 3 protein?) participate in the maintenance or generation of superhelical torsion. Such an interplay between initiator proteins and those necessary for the generation of superhelices may also exist in polyoma-infected cells (41, 42). (See ref. 48 for similar reflections.)

Comments on the cis-Action and Chloramphenicol Insensitivity of the φX Gene A Function. Gene A protein acts only cis in vivo since gene A mutants cannot be rescued during multiple infection with a complementing mutant (1, 11). The large number of A protein molecules per cell found in our experiments suggests that cis-action is not likely to be due to compartmentalization. However, the highly hydrophobic, membrane-bound nature of the A protein (ref. 20, our observations) makes the estimation of functionally active, intracellular gene A endonuclease rather difficult. Dove has proposed a general mechanism for cis-acting proteins. It is that such proteins act only while they are nascent chains and are inactive after release from the ribosome. The observation of E. Linney and M. Hayashi (personal communication) that A protein‡ production and transcription are tightly coupled would seem to insure that A protein is synthesized in the vicinity of the DNA that codes for it and, since the A endonuclease sensitive site is probably within the A gene (38–40), in the vicinity of its substrate. This mechanism ensures efficient action of the A protein and explains how even a small amount of escape synthesis in the presence of chloramphenicol would be sufficient for the DNA initiation observed. Our isolation of active enzyme does not vitiate this type of mechanism since, as mentioned above, the intracellular activity of free A protein is difficult to assess.

Relationship to Other Initiator Proteins. The gene A protein of φX has been suggested to fit the classic definition of an initiator protein as set forth in the replicon hypothesis of Jacob et al. (32). That is, the presence of active A product positively controls the initiation of DNA replication. The φX gene A protein may be similar to the gene A product of bacteriophage P2 (33), to the O gene product of bacteriophage λ, to SV-40 gene A (45), to polyoma gene ts a (46), and to some protein rate-limiting for initiation in E. coli (34). Perhaps these gene products are also species-specific endonucleases.

1. Sinsheimer, R. L. (1968) *Progr. Nucleic Acid Res. Mol. Biol.* 8, 115–169.
2. Denhardt, D. T., Dressler, D. H. & Hathaway, A. (1967) *Proc. Nat. Acad. Sci. USA* 57, 813–820.
3. Wickner, W., Brutlag, D., Schekman, R. & Kornberg, A. (1972) *Proc. Nat. Acad. Sci. USA* 69, 965–969.
4. Olivera, B. M. & Bonhoeffer, F. (1972) *Proc. Nat. Acad. Sci. USA* 69, 25–29.
5. Schekman, R., Wickner, W., Westergaard, O., Brutlag, D., Geider, K., Bertsch, L. L. & Kornberg, A. (1972) *Proc. Nat. Acad. Sci. USA* 69, 2691–2695.
6. Wickner, R. B., Wright, M., Wickner, S. & Hurwitz, J. (1972) *Proc. Nat. Acad. Sci. USA* 69, 3233–3237.
7. Denhardt, D. T. & Sinsheimer, R. L. (1965) *J. Mol. Biol.* 12, 647–662.
8. Schröder, C. H. & Kaerner, H. C. (1972) *J. Mol. Biol.* 71, 351–362.
9. Gilbert, W. & Dressler, D. (1968) *Cold Spring Harbor Symp. Quant. Biol.* 33, 473–484.
10. Sinsheimer, R. L., Knippers, R. & Komano, T. (1968) *Cold Spring Harbor Symp. Quant. Biol.* 33, 443–447.
11. Tessman, E. (1966) *J. Mol. Biol.* 17, 218–236.
12. Lindquist, B. H. & Sinsheimer, R. L. (1967) *J. Mol. Biol.* 30, 69–80.
13. Francke, B. & Ray, D. S. (1971) *J. Mol. Biol.* 61, 565–586.
14. Francke, B. & Ray, D. S. (1972) *Proc. Nat. Acad. Sci. USA* 69, 475–479.
15. Dürwald, H. & Hoffman-Berling, H. (1968) *J. Mol. Biol.* 34, 331–346.
16. Sclair, M., Edgell, M. H. & Hutchison, C. A. (1973) *J. Virol.* 11, 378–385.
17. Godson, G. N. (1971) *J. Mol. Biol.* 57, 541–553.
18. Denhardt, D. T., Iwaya, M. & Larison, L. L. (1972) *Virology* 49, 486–496.
19. Linney, E. A., Hayashi, M. N. & Hayashi, M. (1972) *Virology* 50, 381–387.
20. Van der Mei, D., Zandberg, J. & Jansz, H. S. (1972) *Biochim. Biophys. Acta* 287, 312–321.
21. Benbow, R. M., Mayol, R. F., Picchi, J. C. & Sinsheimer, R. L. (1972) *J. Virol.* 10, 99–114.
22. Levine, A. J. & Sinsheimer, R. L. (1968) *J. Mol. Biol.* 32, 567–578.
23. Gelfand, D. H. & Hayashi, M. (1969) *J. Mol. Biol.* 44, 501–516.
24. Blair, D. G., Sherratt, D. J., Clewell, D. B. & Helinski, D. R. (1972) *Proc. Nat. Acad. Sci. USA* 69, 2518–5222.
25. Henry, C. M., Ferdinand, F-J. & Knippers, R. (1973) *Biochem. Biophys. Res. Commun.* 50, 603–611.
26. Eskin, B. & Linn, S. (1972) *J. Biol. Chem.* 247, 6183–6191.
27. Burton, A. & Sinsheimer, R. L. (1965) *J. Mol. Biol.* 14, 327–347.
28. Levine, A. J. & Sinsheimer, R. L. (1969) *J. Mol. Biol.* 39, 619–639.
29. Lin, N. S. C. & Pratt, D. (1972) *J. Mol. Biol.* 72, 37–49.
30. Tseng, B. Y. & Marvin, D. A. (1972) *J. Virol.* 10, 384–391.
31. Mazur, B. J. & Model, P. (1973) *J. Mol. Biol.* 78, 285–300.
32. Jacob, F., Brenner, S. & Cuzin, F. (1963) *Cold Spring Harbor Symp. Quant. Biol.* 28, 329–348.
33. Lindahl, G., Hirota, Y. & Jacob, F. (1971) *Proc. Nat. Acad. Sci. USA* 68, 2407–2411.
34. Lark, K. G. (1969) *Annu. Rev. Biochem.* 38, 569–604.
35. Schekman, R. W. & Ray, D. S. (1971) *Nature New Biol.* 231, 170–173.
36. Schaller, H., Voss, H. & Gucker, S. (1969) *J. Mol. Biol.* 44, 445–458.
37. Fiers, W. & Sinsheimer, R. L. (1962) *J. Mol. Biol.* 5, 424–434.
38. Baas, P. D. & Jansz, H. S. (1972) *J. Mol. Biol.* 63, 569–576.
39. Johnson, P. H. & Sinsheimer, R. L. (1973) *Fed. Proc.* 32, 491.
40. Benbow, R. M., Hutchison, C. A., Fabricant, J. D. & Sinsheimer, R. L. (1971) *J. Virol.* 7, 549–558.
41. Bourgaux, P. & Bouraux-Ramoisy, D. (1972) *Nature* 235, 105–107.
42. Cheevers, W. P. (1973) *Nature New Biol.* 242, 202–204.
43. Center, M. S., Studier, F. W. & Richardson, C. C. (1970) *Proc. Nat. Acad. Sci. USA* 65, 242–248.
44. Schekman, R. W., Iwaya, M., Bromstrup, K. & Denhart, D. T. (1971) *J. Mol. Biol.* 57, 177–191.
45. Tegtmeyer, P. (1972) *J. Virol.* 10, 591–598.
46. Francke, B. & Eckhart, W. (1973) *Virology* 55, 127–135.
47. Linney, E. & Hayashi, M. (1973) *Nature New Biol.* 245, 6–8.
48. Kato, A. C., Bartok, K., Fraser, M. J. & Denhardt, D. T. (1973) *Biochim. Biophys. Acta* 308, 68–78.

‡A' in their terminology.