Ultraviolet-Induced Cross-Links in the Deoxyribonucleic Acid of Single-Stranded Deoxyribonucleic Acid Viruses as a Probe of Deoxyribonucleic Acid Packaging

BERTOLD FRANCKE¹ AND DAN S. RAY

Molecular Biology Institute and Department of Zoology, University of California, Los Angeles, California 90024

Received for publication 15 February 1972

Deoxyribonucleic acid (DNA) from ultraviolet (UV)-irradiated ϕ X174 sediments in alkali at rates up to 1.7 times that of unirradiated ϕ X174 DNA and is observed as a condensed, cross-linked structure when examined in the electron microscope by the formamide spreading technique. This structure appears to result from multiple cross-links induced in the tightly coiled DNA contained within the spherical ϕ X174 capsid. In contrast, the DNA extracted after UV irradiation of the filamentous bacteriophage M13 is not strikingly altered in its sedimentation properties and appears by electron microscopy to be rod-shaped as a result of side-to-side association of the circular DNA. The differences in these UV-induced structures reflect the differences in the packaging of the single-stranded DNA in the two virions.

Bacteriophages ϕ X174 and M13 both contain circular single-stranded deoxyribonucleic acid (DNA) of approximately 2×10^6 molecular weight. The packaging of these nucleic acid molecules must be fundamentally different, however. The DNA of ϕ X174 is contained within a spherical virion, whereas that of M13 is contained within a filamentous virion. Two models have been proposed for the structure of the filamentous viruses. On the basis of electron microscopy and X-ray diffraction analysis, Marvin (7) proposed a model in which the DNA is encased with protein and then formed into a rod-shaped particle such as would result from pulling the encoated DNA ring taut and twisting it from opposite sides of the ring. Bradley, on the other hand, proposed a structure in which the viral coat forms a single cylinder containing the DNA in a central core (1, 2).

¹ In the hope that we might distinguish between the two models, we have attempted to cross-link M13 DNA by ultraviolet (UV) irradiation of the virion. DNA-DNA cross-linking might be more likely to occur in a structure like that of Bradley than in the one proposed by Marvin. For comparison, we also have investigated the cross-linking of the DNA in ϕ X174, a virion in which the single-stranded DNA would likely

have abundant DNA-DNA contact that would permit cross-linking of distant parts of the molecule.

MATERIALS AND METHODS

Bacteriophages. ϕ X am3, a lysis-defective mutant of ϕ X174 used for all experiments described here, and M13 wild type were grown and assayed as described previously (5, 9). Preparation of radioactively labeled phages has also been described earlier for ϕ X am3 (5) and M13 (4).

UV irradiation. Phage suspensions in 1.0 M NaCl, 0.01 M tris(hydroxymethyl)aminomethane (Tris), pH 8.0, 0.001 M ethylenediaminetetraacetic acid (EDTA) and about 15% sucrose were exposed at a 100-cm distance to a Westinghouse Sterilamp G 36 TSL in drops of ≤ 0.2 ml containing $\leq 10^{12}$ plaque-forming units (PFU)/ml. The irradiation was terminated by removing samples with a plastic pipette. Under these conditions, the surviving PFU of both ϕ X and M13 decreased by a factor of 10 in 20 sec.

DNA extraction. Viral DNA was prepared, after sodium dodecyl sulfate (SDS) and Pronase treatment of the phages, by two phenol extractions followed by one ethanol precipitation (5).

Centrifugation techniques. Gradients of 5 to 20% sucrose (w/v) of a total volume of 3.5 ml were centrifuged in a Spinco SW 56 rotor at 55,000 rev/min and 5°C. Neutral gradients contained 1.0 M NaCl, 0.01 M Tris (pH 8.0), and 0.001 M EDTA and were

¹ Present address: The Salk Institute, San Diego, Calif. 92112.

run for 2.5 hr. Alkaline gradients contained 0.25 M NaOH and 0.005 M EDTA and were run for 4 hr. Collection was from the bottom of the tube in 45 ± 2 fractions of 5 drops each. A sample of each fraction was spotted onto Whatman no. 3 filter paper discs, dried, and counted for radioactivity as described previously (5).

Electron microscopy. DNA samples were prepared for electron microscopy by the formamide spreading technique (12) as described by Davis, Simon, and Davidson (3). Specimens were examined in a Phillips 200 electron microscope at 60 kv.

RESULTS

When DNA from UV-irradiated ϕ X was extracted with phenol after SDS and Pronase treatment and analyzed by alkaline sucrose gradient sedimentation, the result shown in Fig. 1 was obtained. A considerable amount of DNA sedimented faster than the added marker DNA from unirradiated phage. An experiment similar to that shown in Fig. 1 with ϕ X labeled both in its DNA (32 P) and its protein (3 H) failed to demonstrate any protein label in the region of the alkaline gradient containing the fast sedimenting DNA (the experimental conditions were such that 0.2% of the protein label of the phage preparation could have been detected).

To eliminate any extraneous differences between preparations, ϕ X (32 P-labeled) and M13 (3 H-labeled) were mixed in a 1:1 ratio on the basis of PFU, and the DNA was extracted prior to (Fig. 2a, b) and after (Fig. 2c, d)

FIG. 1. Alkaline sucrose gradient sedimentation of DNA from UV-irradiated ϕ X174. DNA from irradiated 32 P-labeled ϕ X am3 (0.05% surviving PFU) was extracted (83% recovery) and mixed with 3 H-labeled DNA from unirradiated phage. Irradiation, extraction of DNA, and gradient centrifugation were as described in Materials and Methods. Direction of sedimentation in this and all other figures is from right to left. (●) 32 P counts/min, (○) 3 H counts/min.

UV irradiation. As a control, the DNA from irradiated phage (32 P- ϕ X or 3 H-M13) was cosedimented with its respective homologous DNA from unirradiated phage (3 H- ϕ X, 32 P-M13) under neutral and alkaline conditions (Fig. 3). The slight differences in sedimentation rates of the unirradiated samples (Fig. 2a, b) reflect the differences in molecular weight between the DNA species of ϕ X174 and M13 (1.7×10^6 and 2.0×10^6 daltons, respectively). The relative sedimentation rate of irradiated ϕ X DNA is dramatically increased in the alkaline gradient (Fig. 2d and 3b), and some broadening of the band and increase in sedimentation rate is apparent even in a neutral gradient (Fig. 2c and 3a). The incomplete unfolding of ϕ X DNA in alkali after irradiation was best documented in the alkaline gradient shown in Fig. 1. In this case, the DNA from irradiated ϕ X sedimented in

FIG. 2. Neutral and alkaline sucrose gradient sedimentations of DNA from a mixture of ϕ X174 and M13 before and after UV irradiation. ϕ X am3 (32 P-labeled) and M13 (3 H-labeled) were mixed in equal amounts of PFU. The mixture was split into two equal portions, and one portion was irradiated (ϕ X, 0.036% and M13, 0.048% surviving PFU). The DNA was extracted, with recoveries of 80% in both portions for both labels. Neutral (a) and alkaline (b) gradients before irradiation, neutral (c) and alkaline (d) after irradiation. Of a total of about 45 fractions collected, only those containing viral DNA are shown in this and the following figure. (●) 32 P counts/min, (○) 3 H counts/min.

FIG. 3. Neutral and alkaline sucrose gradients of DNA from irradiated ϕ X174 and M13, each cosedimented with its homologous, nonirradiated DNA. 32 P-labeled ϕ X am3 and 3 H-labeled M13 were irradiated separately (ϕ X, 0.060% and M13, 0.055% surviving PFU), and the DNA was extracted (>80% recovery in both cases). Both DNA preparations were mixed with marker DNA from their homologous unirradiated phages (3 H-labeled ϕ X and 32 P-labeled M13 marker DNA). ϕ X DNA in neutral (a) and alkaline gradients (b), M13 DNA in neutral (c) and alkaline (d) gradients. (●) 32 P counts/min, (○) 3 H counts/min.

alkali at rates up to 1.7 times that of DNA from unirradiated ϕ X. The usual form of ϕ X DNA in neutral gradients is more compact than that observed in alkali as evidenced by the 1.71-fold greater sedimentation rate in neutral 1.0 M NaCl as compared to alkaline medium of the same ionic strength (11). Thus, the faster sedimenting forms of DNA from irradiated ϕ X must have a radius of gyration in alkali equal to that of unirradiated ϕ X DNA in neutral 1.0 M NaCl.

In contrast, the sedimentation rate in alkali of DNA from irradiated M13 is only 1.04 times that of the DNA from unirradiated M13. In neutral gradients, the DNA from irradiated M13 appears to sediment slower than the unirradiated DNA by approximately 4% (Fig. 3c).

The most likely interpretation for the increased sedimentation rate of ϕ X DNA after UV irradiation of the whole virus is that cross-

linking of the DNA had occurred, preventing the complete unfolding of the single-stranded circle at alkaline pH. This would reduce the radius of gyration and increase the sedimentation rate of the DNA. Fractions of DNA from four regions of the gradient in Fig. 1 were pooled separately as indicated and examined by electron microscopy in the presence of formamide (Fig. 4). Whereas the molecules cosedimenting with the unirradiated marker DNA spread out largely as single circles (Fig. 4a), the faster sedimenting fractions (Fig. 4b-d) show increasing number of molecules in which distant regions of a circle lie in contact, giving rise to structures with one or more loops. This appearance is clearly different from the simple overlapping occasionally observed (Fig. 4a) and suggests the existence of cross-links in ϕ X DNA after UV irradiation.

Figure 5 shows electron micrographs of DNA extracted from M13 bacteriophage before (Fig. 5a) and after UV irradiation (Fig. 5b) and prepared under the same conditions as the ϕ X DNA shown in Fig. 4. The circular DNA from irradiated M13 phage particles appears to be cross-linked side-to-side at a small number of points. These structures are clearly different from those observed for the DNA from irradiated ϕ X.

To assess the biological significance of the cross-linking seen in ϕ X DNA, we have measured the kinetics of formation of the cross-linked form by alkaline velocity sedimentation and compared them with the kinetics of inactivation of the phage plaque-forming ability (Table 1). Only about 0.14% of the plaque-forming particles survived at the highest dose used (60 sec). This corresponds to an average of 6.6 lethal hits per molecule. At this level of survival, only 38.5% of the DNA appeared to be cross-linked, as judged by alkaline velocity sedimentation. The amount of cross-linking seen would account only for approximately 5% of the lethal UV photoproducts in ϕ X174.

DISCUSSION

Some of the viral DNA extracted from UV-irradiated ϕ X174 bacteriophages was unable to unfold completely under denaturing conditions, as demonstrated in alkaline sucrose gradient centrifugation and by electron microscopy in the presence of formamide. This observation is best explained by DNA-DNA cross-linking. The cross-links are likely to be DNA-DNA cross-links (8) rather than DNA-protein-DNA cross-links (10) since they are resistant to SDS, Pronase, and phenol extraction and to sedimentation in alkaline sucrose gradients. In addition,

FIG. 4. Electron micrographs of ϕ X174 DNA from the indicated regions of the alkaline sucrose gradient shown in Fig. 1. (a) Region 1, (b) region 2, (c) region 3, (d) region 4. Bar indicates 1 μ m.

TABLE 1. Kinetics of formation of the cross-linked form of ϕ X deoxyribonucleic acid (DNA)^a

Phage	UV irradiation (sec)	Surviving PFU (%)	Recovery of labeled DNA (%)	% Cross-linked DNA (by alkaline sedimentation)
ϕ X174	0	100	90.0	0
	17	13.1	97.5	9.2
	60	0.142	76.3	38.5

^a Abbreviations: UV, ultraviolet; PFU, plaque-forming units.

less than 0.2% of the coat proteins remain associated with the cross-linked DNA after extraction and alkaline sedimentation.

The amount of cross-linking seen in ϕ X174 DNA could account for only 5% of the biological inactivation, and it is therefore of minor significance in the UV inactivation of the virion. We have recently reported the use of UV-treated ϕ X174 to obtain partially formed parental replicative form DNA molecules (6). The inhibition of complementary-strand synthesis with increasing UV dose in this system was much greater than the appearance of cross-linked DNA in the infecting virus. It is therefore unlikely that the block in complementary-stranded

synthesis was due to the cross-linking, but rather must have occurred at the site of some other photoproduct, such as thymine dimers, not repairable when present in single-stranded DNA. The occurrence of DNA-DNA cross-linking must be borne in mind, however, when interpreting biophysical experiments involving UV treatment of the infecting virus particles, since a faster sedimentation rate in alkali can also be indicative of strand elongation.

The electron micrographs presented here suggest that some cross-linking also can be induced in the M13 virion. Its pattern is consistent with side-to-side cross-linking of the DNA at several points along the length of the DNA ring. The conformation of the DNA from irradiated M13 reflects the rod-shaped structure from which it was derived, whereas that of ϕ X174 reflects the spherical structure of ϕ X174. The interpretation of these results with regard to the two models of M13 structure depends crucially upon the chemical identity of the cross-links. In the absence of chemical analysis of the cross-links, the possibility that a small Pronase-resistant peptide might mediate the cross-linking cannot be excluded. Consequently, we cannot distinguish between the two models for M13 on the basis of these data. However, we can clearly distinguish the packaging of ϕ X174 DNA from that of M13

FIG. 5. Electron micrographs of (a) unirradiated M13 DNA and (b) DNA from phage irradiated to a survival of 0.13%. Bar indicates 1 μ m.

by this method and suggest that UV irradiation potentially may be a useful tool for approaching the conformation of nucleic acids in other structurally distinct complexes.

ACKNOWLEDGMENTS

We thank E. Cornell for her valuable technical assistance and Arleen Forsheit for instructing us on the formamide spreading technique.

This work was supported by grant GB 18074 from the National Science Foundation.

LITERATURE CITED

1. Bradley, D. E. 1967. Ultrastructure of bacteriophages and bacteriotocins. *Bacteriol. Rev.* **31**:230-314.
2. Bradley, D. E., and C. A. Dewar. 1967. Intracellular changes in cells of *Escherichia coli* infected with a filamentous bacteriophage. *J. Gen. Virol.* **1**:179-188.
3. Davis, R. W., M. Simon, and N. Davidson. 1971. Electron microscope heteroduplex methods for mapping regions of base sequence homology in nucleic acids, p. 413-428. *In* L. Grossman and K. Moldave (ed.), *Methods in enzymology*, vol. 21D. Academic Press Inc., New York.
4. Forsheit, A. B. and D. S. Ray. 1971. Replication of bacteriophage M13 VI. Attachment of M13 DNA to a fast-sedimenting host cell component. *Virology* **43**:647-664.
5. Francke, B., and D. S. Ray. 1971. Fate of parental ϕ X174 DNA upon infection of starved, thymine-requiring host cells. *Virology* **44**:168-187.
6. Francke, B., and D. S. Ray. 1971. Formation of the parental replicative form DNA of bacteriophage ϕ X174 and initial events in its replication. *J. Mol. Biol.* **61**:565-586.
7. Marvin, D. A. 1966. X-ray diffraction and electron microscope studies of the structure of the filamentous bacteriophage fd. *J. Mol. Biol.* **15**:8-17.
8. Opara-Kubinska, Z., Z. Kurylo-Borowska, and W. Szybalski. 1963. Genetic transformation studies. III. Effect of ultraviolet light on the molecular properties of normal and halogenated deoxyribonucleic acid. *Biochim. Biophys. Acta* **72**:298-309.
9. Ray, D. S., and R. W. Schekman. 1969. Replication of bacteriophage M13 I. Sedimentation analysis of crude lysates of M13 infected bacteria. *Biochim. Biophys. Acta* **197**:398-407.
10. Smith, K. C. 1967. Biologically important damage to DNA by photoproducts other than cyclobutane-type thymine dimers. *In* G. Silini (ed.), *Radiation research*. North Holland Publishing Co., Amsterdam.
11. Studier, F. W. 1965. Sedimentation studies of the size and shape of DNA. *J. Mol. Biol.* **11**:373-390.
12. Westmoreland, B. C., W. Szybalski, and H. Ris. 1969. Mapping of deletions and substitutions in heteroduplex DNA molecules of bacteriophage lambda DNA by electron microscopy. *Science* **163**:1343-1348.