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Natural Occurrence of Cross-Linked Vaccinia Virus Deoxyribonucleic Acid

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The molecular weight of native vaccinia deoxyribonucleic acid (DNA) is 1 to 1.17 times that of native T4 DNA. Sedimentation of denatured vaccinia DNA through alkaline sucrose gradients yields an apparent molecular weight greater than twice that of denatured T4 DNA, implying that the complementary strands of vaccinia DNA do not separate upon denaturation. When alkali-denatured vaccinia DNA is neutralized, it has the physical chemical properties of native DNA when tested by sedimentation through neutral sucrose gradients, banding in CsCl, and by hydroxylapatite chromatography. We conclude that almost all mature vaccinia DNA molecules contain a small number of naturally occurring cross-links.

The deoxyribonucleic acid (DNA) content of the vaccinia virus particle is approximately 2.75×10^{-16} g (4, 13, 22). This is now known to correspond to a single, linear duplex molecule with an approximate molecular weight of 1.5×10^8 (14, 26; C. C. Randall, L. G. Gafford, and J. M. Hyde, Fed. Proc., p. 364, 1967). We originally undertook a study of the single polynucleotide chains derived from the native DNA molecule by alkaline denaturation to learn whether the complementary strands were continuous or contained interruptions or alkali-labile linkages. Initial studies indicated that the complementary strands of the vaccinia DNA molecule were not separated at high *p*H (> 12), although denaturation had occurred. In this communication we present data which indicate that mature vaccinia DNA is naturally cross-linked.

MATERIALS AND METHODS

Vaccinia virus growth and purification. HeLa S3-1 cells were grown in suspension in Eagle medium (11) supplemented with 5% horse serum. Vaccinia virus (strain WR) was prepared and used to infect HeLa S3-1 cells as described previously (20, 25) in medium containing ^{3}H-thymidine (New England Nuclear Corp., Boston, Mass., > 15 c/mmole), 0.25 μc/ml. Vaccinia virus was purified by using the method of Joklik (15), except that the steps involving sonic oscillation and sedimentation through 36% sucrose were omitted.

T4 bacteriophage growth and purification. Bacteriophage T4 N98 was a gift from C. Merril. The phage was prepared by using *Escherichia coli* CR 63 growing in a modified "K" medium (8, 23) containing ^{14}C-thymidine (New England Nuclear Corp., 25 to 50 mc/mmole), 0.2 μc/ml, and purified by alternate low- and high-speed centrifugation (30).

DNA purification. Vaccinia DNA was purified using method 2-2 of Sarov and Becker (26), except that the virions were in 0.15 M NaCl plus 0.015 M sodium citrate (SSC), and the DNA was dialyzed against SSC overnight. In some cases the DNA solution was extracted with aqueous ether after the two phenol extractions. The 280- to 260-nm ratio of absorbance was 0.52, and the specific activity of ^{3}H DNA was 37,000 counts per min per μg. T4 DNA was extracted by the method of Mandell and Hershey (18). The 280- to 260-nm ratio of absorbance was 0.57, and the specific activity of ^{14}C-DNA was 7,500 counts per min per μg.

DNA denaturation and neutralization. DNA in 0.5 × SSC was denatured in 0.1 or 0.2 N NaOH. To neutralize DNA which had been denatured in the alkaline solution for 5 min at room temperature, an equivalent amount of HCl was added, in addition to sufficient tris(hydroxymethyl)aminomethane (Tris), *p*H 7.9, to make the final concentration 0.05 M.

Sucrose sedimentation. Zone sedimentation through preformed linear sucrose gradients (5% w/v to 20% w/v) was performed at 20 C in the model L preparative ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.) by using the SW 50L rotor. Neutral sucrose gradients contained 1 M NaCl, 0.05 M Tris (*p*H 7.9), 0.15% Sarkosyl (NL 97; Geigy Industrial Chemicals, Ardsley, New York), and 0.001 M (ethylenedinitrilo)tetraacetic acid (EDTA). Alkaline sucrose gradients contained 0.9 M NaCl, 0.1 M NaOH, 0.15% Sarkosyl, and 0.001 M EDTA. Fractions were collected by piercing the bottom of the nitrocellulose tube with a 26-gauge needle and collecting five-drop fractions in 10 ml of fluor [Liquifluor, 160 ml; Triton X-100 (Packard Instrument, Inc., La Grange, Ill.), 1,330 ml; toluene 2,510 ml], which contained 1 ml of water. Alkaline samples were neutralized with 0.18 ml of $SnCl_2$-hydrochloric acid solution (24) to reduce chemiluminescence. The samples were counted in a Beckman LS 250 liquid scintillation counter.

Isopycnic CsCl sedimentation. DNA was banded in CsCl in the model L preparative ultracentrifuge in the 40 rotor at 33,000 rev/min for 65 to 72 hr at 20 C. The CsCl solution contained 0.05 M Tris (*p*H 7.9) and 0.15% Sarkosyl in a final volume of 4.5 ml. The density was 1.695 g/ml. Fractions [0.1 ml, using a microburet (Micro-Metric Instruments Co., Cleveland, Ohio)] were collected and counted as described above. Refractive indexes were measured using an Abbé refractometer (Bausch & Lomb, Inc., Rochester, N.Y.).

Hydroxylapatite chromatography. Hydroxylapatite was prepared by method CPA of Main, Wilkinson, and Cole (17). A solution containing ^{3}H vaccinia DNA (0.05 to 0.1 μg), ^{14}C T4 DNA (0.3 μg), and unlabeled salmon sperm DNA (8 μg) as carrier was made 0.2 N NaOH and neutralized as described above. Unlabeled salmon sperm DNA (8 μg) was added as native carrier, and the DNA solution (0.4 ml) was diluted with 6.25 ml of 0.02 M KPO_4 (*p*H 6.8) which contained 1% formaldehyde. The dilute DNA solution was passed through a hydroxylapatite column (0.8 by 4.5 cm), which was then washed with 2 ml of 0.02 M KPO_4 (*p*H 6.8), which contained 1% formaldehyde, and eluted with a linear gradient from 0.02 M buffer (9 ml) to 0.50 M buffer (9 ml). The flow rate was 5 ml/hr, and 0.33-ml fractions were collected. Radioactivity assays were performed as described above.

RESULTS

Sedimentation of native vaccinia DNA through neutral sucrose. Purified ^{3}H native vaccinia DNA cosediments with ^{14}C native T4 DNA in a neutral sucrose gradient (Fig. 1). Sarov and Becker (26), using very low concentrations of T4 DNA, were able to effect a one-fraction separation of vaccinia (faster) and T4 DNA. Under most conditions, they report results identical to those shown in Fig. 1. Under our conditions, longer times of sedimentation do not effect any separation of vaccinia DNA and T4 DNA, and the distribution of T4 radioactivity obtained does not correspond to the skewed pattern which Burgi and Hershey (9) observed when the initial DNA concentration was too great. We added approximately 0.1 μg of vaccinia DNA and 0.5 μg of T4 DNA to our gradients.

Sedimentation of denatured vaccinia DNA through alkaline sucrose. ^{3}H vaccinia DNA and ^{14}C T4 DNA, which had been denatured together in alkali, were sedimented through 5 to 20% alkaline sucrose gradients (Fig. 2). The peak of denatured vaccinia DNA sediments faster than denatured T4 DNA. The ratio of distances traveled by the two peaks is 1.35. There is some trailing material in the vaccinia DNA peak, indicating size or configurational heterogeneity in the vaccinia DNA population. [The fact that the majority of the T4 single strands were intact, S = 71, had been calibrated by using closed circular simian virus 40 DNA, S = 53, (obtained from M. Martin) as a marker in alkaline sucrose.] To minimize possible damage to the DNA (i.e., single strand breaks), we lysed vaccinia virions directly in alkali on top of the gradient in the presence of T4 DNA (Fig. 3). In this gradient, the denatured ^{3}H vaccinia DNA sediments more homogeneously and 1.5 times as far as the denatured ^{14}C T4 DNA. These two runs represent the extremes observed in the ratio of distances traveled. In several experiments, denatured vaccinia DNA sedimented 1.35 to 1.45 times as far as T4 single strands. Vaccinia DNA, which has been treated with Pronase but not phenol extracted, displays slightly less trailing material when denatured and sedimented through alkaline sucrose; thus, increased manipulation of the DNA leads to more damage. The general formula for the determination of DNA molecular weight from sedimentation through sucrose in the presence of a marker of known molecular weight is given in formula 1, $M_1/M_2 = (D_1/D_2)^k$ (9), where M is the molecular weight of the species in

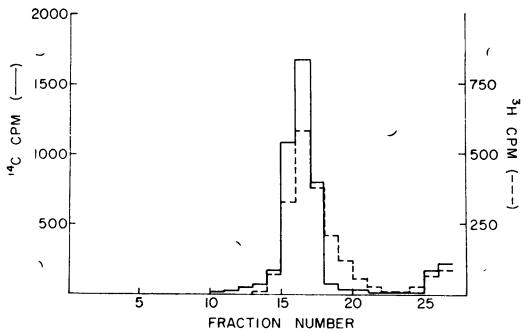

FIG. 1. *Sedimentation of native ^{3}H vaccinia DNA and native ^{14}C T4 DNA through a linear 5 to 20% neutral sucrose gradient. Centrifugation was for 140 min at 25,000 rev/min, 20 C.*

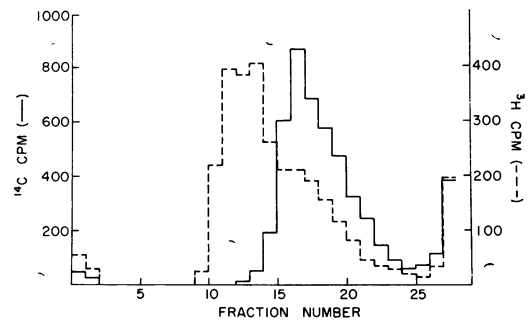

FIG. 2. *Sedimentation of denatured ^{3}H vaccinia DNA and denatured ^{14}C T4 DNA through a linear 5 to 20% alkaline sucrose gradient. Centrifugation was for 140 min at 25,000 rev/min, 20 C.*

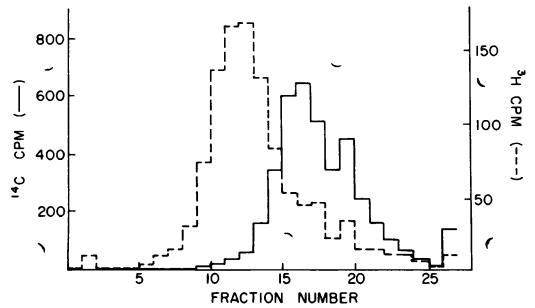

FIG. 3. *Sedimentation of denatured ^{3}H vaccinia DNA and denatured ^{14}C T4 DNA through a linear 5 to 20% alkaline sucrose gradient. The vaccinia DNA was released directly from the virions in alkali on top of the gradient. Centrifugation was for 140 min at 25,000 rev/min, 20 C.*

question and D is the distance traveled. The value of k has been determined to be 2.63, under the conditions of alkaline sucrose sedimentation employed here by Abelson and Thomas (1), and to be 2.5 by Studier (28), under similar conditions in the analytical ultracentrifuge. According to the data presented here and by Sarov and Becker (26), the molecular weight of native vaccinia DNA is 1 to 1.17 times the molecular weight of T4 DNA. If the data presented in Fig. 2 and 3 represented single vaccinia polynucleotide chains, they would have a molecular weight 2.4 (D_1/D_2 = 1.35 and k = 2.5) to 2.9 (D_1/D_2 = 1.5 and k = 2.63) times greater than single T4 polynucleotide chains. Because we know that the T4 single strands are intact, this is an untenable interpretation. Conversely, a closed, twisted circular DNA, when denatured, would sediment more rapidly through alkaline sucrose than at the rate that is observed for denatured vaccinia DNA (31). The data are consistent with cross-linked DNA molecules which would be denatured (i.e., lose their secondary structure) under alkaline conditions, but whose complementary strands would still be held together (2, 19, 27, 28).

Sedimentation of denatured vaccinia DNA through neutral sucrose. Alkaline solutions containing ^{3}H vaccinia DNA and ^{14}C T4 DNA were neutralized, and the DNA sedimented through neutral sucrose gradients. According to Studier (28), T4 polynucleotide chains sediment 3.3 to 3.7 times faster than native T4 DNA under these conditions and would have an $S_{20,w}$ 2.8 to 3.4 times that reported for native vaccinia DNA by Sarov and Becker (26). The complementary vaccinia strands, if held together by cross-links, would re-form the double helix on neutralization and have the same $S_{20,w}$ as the original native DNA. Thus, neutralized denatured T4 DNA should sediment 2.8 to 3.7 times faster than neutralized denatured vaccinia DNA which is cross-linked. The observed ratio for the two peaks is 3.2 (Fig. 4). Uncross-linked vaccinia single strands would sediment at least as fast as the T4 single strands. A large amount of the denatured T4 DNA sediments slower than the peak material. As Alberts and Doty (3) have shown, even smaller single strands are subject to extensive shear degradation during the required manipulations. The leading edge on the vaccinia DNA peak probably represents the same phenomenon, which would have a reverse effect; if there were very few cross-links along the molecule, large single-stranded fragments (molecular weight > 16 × 10^{6}) would sediment faster than native vaccinia DNA (28).

CsCl sedimentation. Native ^{3}H vaccinia DNA bands at a slightly lower density than ^{14}C T4 in CsCl (Fig. 5A). Vaccinia DNA and T4 DNA, which have been denatured in alkali, neutralized, and banded in CsCl, all in the presence of 1% formaldehyde (which prevents the reformation of hydrogen bonds) (7, 12, 29), both band as denatured DNA, although the vaccinia DNA peak is skewed toward a lighter density. No vaccinia DNA bands at the density of native DNA (Fig. 5B). If the formaldehyde is omitted, about 43% of the vaccinia DNA bands at the density of native DNA (Fig. 5C). Approximately 11% of the vaccinia DNA bands at an intermediate density, which would correspond to molecules which are partially native and partially denatured (7). In this preparation of vaccinia DNA, 10% banded at the density of denatured DNA before denaturation (Fig. 5A). In other preparations, this was not so. To minimize manipulation leading to single strand nicks along the vaccinia DNA molecule, virions were lysed directly in 0.2 N NaOH in the presence of T4 DNA. When this DNA solution was neutralized and banded in CsCl, 75% of the vaccinia DNA banded at the position of native DNA (Fig. 6).

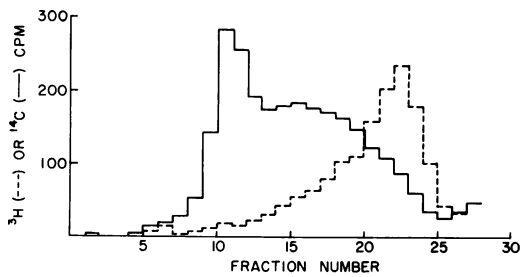

FIG. 4. *Sedimentation of neutralized, alkali-denatured ^{3}H vaccinia DNA and neutralized, alkali-denatured ^{14}C T4 DNA through a linear 5 to 20% neutral sucrose gradient. Centrifugation was for 70 min at 25,000 rev/min, 20 C.*

Hydroxylapatite chromatography. The third measurement of the extent to which alkali-denatured vaccinia DNA re-formed the original double helix upon neutralization was hydroxylapatite chromatography. Native DNA is retained on hydroxylapatite at salt concentrations which elute denatured DNA (5, 6). The separation of a mixture of equal amounts of native and denatured T4 DNA under our conditions is shown in Fig. 7A. Routinely, all material was retained on the column initially, and greater than 80% of both denatured and native DNA radioactivity was recovered in the peaks. A mixture of ^{3}H vaccinia DNA and ^{14}C T4 DNA was denatured in alkali, neutralized, and then chromatographed (Fig. 7B). The major peak of T4 DNA is eluted earlier than the major vaccinia DNA peak, which represents 88% of the total ^{3}H radioactivity recovered and whose position corresponds to that of native T4 DNA.

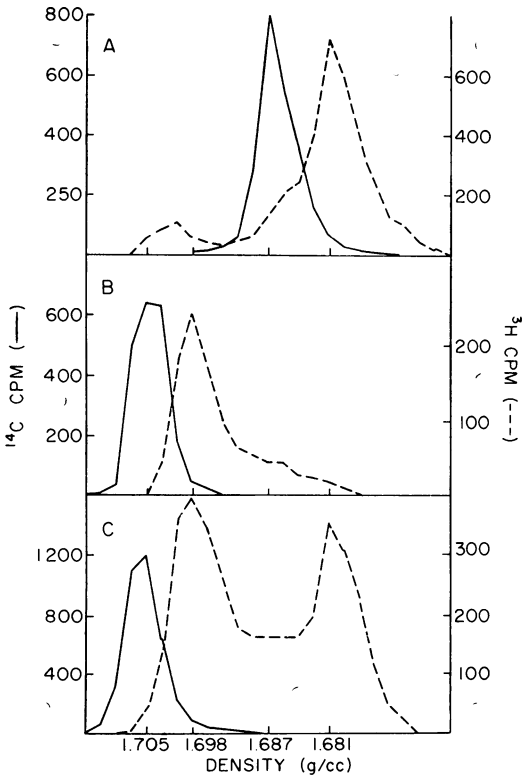

FIG. 5. *Isopycnic CsCl centrifugation at 33,000 rev/min, 20 C. (a) Native ^{3}H vaccinia DNA and native ^{14}C T4 DNA; (b) neutralized, alkali-denatured ^{3}H vaccinia DNA and neutralized, alkali-denatured T4 DNA. Denaturation, neutralization, and centrifugation were all done in the presence of 1% formaldehyde; (c) the same as 5B except that the formaldehyde was omitted.*

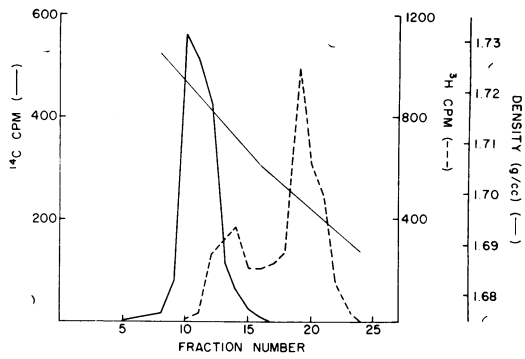

FIG. 6. *Isopycnic CsCl centrifugation of neutralized, alkali-denatured ^{3}H vaccinia DNA and neutralized, alkali-denatured ^{14}C T4 DNA. The vaccinia DNA was directly released from the virions by lysis in 0.2 N NaOH.*

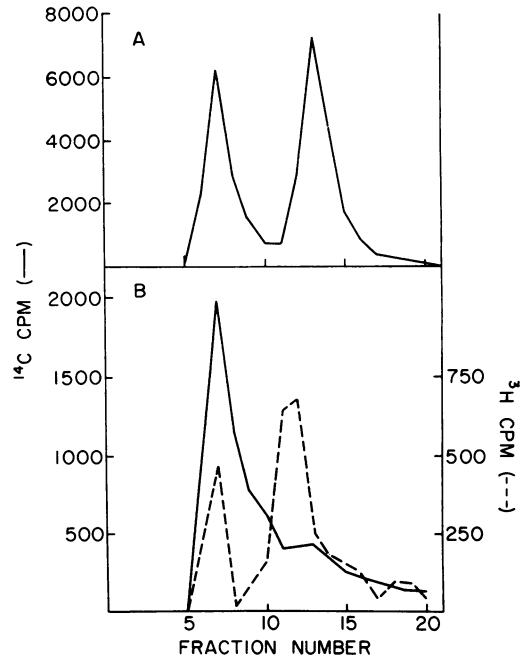

FIG. 7. *Hydroxylapatite column chromatography. (a) Mixture of equal amounts of native and denatured ^{14}C T4 DNA. The first peak is denatured DNA and the second peak is native DNA; (b) neutralized, alkali-denatured ^{3}H vaccinia DNA and neutralized, alkali-denatured ^{14}C T4 DNA.*

DISCUSSION

The main conclusion of this paper is that most, if not all, vaccinia DNA molecules contain naturally occurring cross-links. The data presented represent several virus preparations, all strain WR. Alkaline conditions, which denature T4

DNA and lead to separation of the T4 single strands, do denature vaccinia DNA but do not cause separation of the complementary single strands as shown by the data obtained from sedimentation through alkaline sucrose. The presence of cross-links would enable vaccinia single strands to re-form the double helix under conditions of rapid neutralization, which would not permit T4 single strands to do so. The predicted capability was confirmed by the results of experiments involving sedimentation through neutral sucrose, banding in CsCl, and hydroxylapatite chromatography.

We do not believe that the cross-links were introduced during the DNA purification. The vaccinia DNA was tested for cross-links after each step of the purification. Only the apparent number of single strand nicks increased with increased manipulation. Previous studies involving treatment of DNA with Pronase and phenol showed that these reagents did not introduce cross-links into DNA (3, 7, 29). Secondly, purified virions lysed directly in alkali contained cross-linked DNA. The NaOH did not induce cross-linking of T4 DNA. Alberts (2) has suggested that the shearing of DNA molecules during purification may generate cross-links by a mechanism involving ion pairs or free radicals in a molecule of sufficient size. This explanation would not be appropriate in the case of vaccinia DNA, because we were studying intact mature DNA molecules, almost all of which are apparently cross-linked.

An alternative explanation would be that vaccinia DNA contains multiple sequence duplications so that reannealing could take place rapidly. This seems unlikely because sonically treated vaccinia DNA (molecular weight, 10^6), which has been alkali-denatured and neutralized, does not re-form an amount of double helix which is detectable in CsCl or on hydroxylapatite (*unpublished data*). In addition, the complementary single strands would be separated in alkaline sucrose.

The number of cross-links per molecule has not been determined. The experiments described in the previous paragraph implied that there were no more than five cross-linked fragments out of a total of 150 derived from each original molecule (i.e., our limits of detection). The vaccinia DNA molecule was broken into five fragments of nearly equal size by passage through a 22-gauge needle. When this DNA preparation was denatured and sedimented through alkaline sucrose, only 20% of the DNA still sedimented as though it were cross-linked (*unpublished data*). Thus, it is possible that there is only one cross-link per molecule. Whether more than one cross-link could be present in a given localized area along the molecule is not known. Similarly, the chemical nature of the cross-links is not known. The cross-links are resistant to deoxycholate, Pronase (a proteolytic enzyme of broad specificity), phenol, ether, and high *p*H.

The reported ratio of plaque-forming units (PFU) to vaccinia particles has ranged from 1:4 to 1:100. It could be argued that, if 99 of 100 particles are not infectious, it is because their DNA is cross-linked. We cannot rule out the possibility that 1% of the molecules are not cross-linked. However, it is clear that, under our conditions of lysing the virus directly on top of an alkaline sucrose gradient, more than 90% of the DNA is cross-linked (Fig. 3). Thus, if such a preparation had a PFU-particle ratio greater than 1:10, the argument would not be valid. Our own preparations had a PFU-particle ratio of 1:15, which would not preclude the possibility that cross-linked molecules are not biologically active.

At present, the biological significance of DNA cross-links is uncertain. Such cross-links have been reported for the DNA of SP 82 bacteriophage, several bacteria, and calf thymus (2, 3, 10, 21). Alberts has reported that cross-links are present in *B. subtilis* DNA before, during, and after replication (2). B. Weiss has found that mature T4 and T7 DNA, which contain no cross-links, are cross-linked when added to extracts of infected cells (*in press*). Finally, Jungwirth and Dawid have noted that intracellular vaccinia DNA reanneals after denaturation under conditions which do not permit a large fraction of denatured cellular DNA to reanneal (16). However, the conditions they employed also would be expected to allow classical reannealing to take place.

If cross-linked vaccinia DNA molecules are biologically active, the implications with regard to DNA replication are clear. Assuming a semiconservative mechanism of replication, the cross-links would have to be dissolved at some point and then re-formed. Presumably the reactions would be under enzymatic control. Possible functions include the cross-link as a recognition point with regard to DNA replication, RNA transcription, and final DNA molecular sizing.

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