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Elimination of naturally occurring crosslinks in vaccinia virus DNA after viral penetration into cells

(DNA replication/genome strand/single-stranded DNA/circular viral DNA/viral endonuclease)

BEATRIZ G. T. POGO

Department of Virology, The Public Health Research Institute of The City of New York, Inc., New York, New York 10016

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ABSTRACT **Vaccinia virus DNA, extracted from purified virus or from the cytoplasmic fraction of virus-infected cells very shortly after infection, was analyzed by sedimentation in alkaline and neutral sucrose gradients. The sedimentation properties of vaccinia DNA under denaturing conditions changed, immediately after penetration into the cell, from the characteristic circular viral DNA (crosslinked double-stranded linear DNA) to nicked circular DNA or to single-stranded molecules. This transition occurred at the time of uncoating of the virus and with a slight change in the DNA size, as judged by sedimentation in neutral sucrose. These results indicate that the crosslinks, that held the complementary strands of the genome together, are removed after penetration.**

When vaccinia DNA was incubated with the supernatant fraction of virus-infected cells, a similar change in the sedimentation properties of the DNA under denaturing conditions was observed. It is concluded that the endonuclease present in the supernatant of infected cells eliminated the crosslinks in the DNA, and that this enzymatic hydrolysis may be the mechanism by which crosslinks are removed prior to DNA replication.

The vaccinia genome is a linear double-stranded (ds) DNA molecule of about 122×10^6 daltons. The presence of crosslinks that hold together the complementary strands has been well documented (1–4). They seem to be located at both ends of the molecule in single-stranded (ss) regions, as judged by their sensitivity as ss nucleases (2, 3).

The biological significance of crosslinks in DNA molecules is not known. They may serve as recognition sites for DNA transcription, replication, or sizing; but for semiconservative replication to take place they must be removed, by an apparently enzymatic process.

Vaccinia virus contains at least two DNase activities in its core: one is an exonuclease that acts at acid pH, the other is an endonuclease that acts at slightly alkaline pH 7.8, and both exclusively attacking ss DNA (5, 6). Although these enzymes are expressed as late viral functions (7, 8), they may also be required early in infection. Thus, the endonuclease is released from the cores shortly after cell infection, and is active in the soluble fraction of infected cells (8, 9).

In this study, the possible involvement of virus-associated nucleases on viral DNA modifications prior to replication has been examined by following the fate of parental DNA after penetration of the virus into the cell.

MATERIALS AND METHODS

Virus and Cell Cultures. The IHD strain of vaccinia virus and L2 cells was used as described (10).

[^{3}H]Thymidine labeled virus was prepared by addition of 0.5 μCi/ml of [^{3}H]thymidine (specific activity 60 Ci/mol [Schwarz/Mann]) to the culture medium of infected cells from 1 to 5 hr after infection. At the end of this period, the isotope was removed and the cells were incubated with normal medium for an additional 18–20 hr. The cells were then harvested and the virus was purified by procedures involving freezing and thawing of the cells, and differential centrifugation, trypsin digestion, passage through discontinuous sucrose gradients and continuous density gradients of potassium tartrate for the extracts (11, 12). Viral cores were prepared as previously described (5, 6).

Infection of Cells by Virus Labeled with [^{3}H]Thymidine and Extraction of Viral DNA. To initiate synchronous infection, I added 10 plaque-forming units of ^{3}H-labeled virus per cell to monolayer cultures growing in 10-mm petri dishes. Adsorption of the virus was allowed to proceed for 1 hr at 4°. Unadsorbed virus was removed by three repeated washes with medium. When necessary, hydroxyurea from Sigma was added in a concentration of 5 mM to monolayer cultures containing 10^7 cells (13). At the time indicated in the data, the cells were scraped off the plates, washed with phosphate-buffered saline and resuspended in a solution containing 0.01 M Tris·HCl buffer, pH 7.8/1 mM $MgCl_2$/10 mM KCl (TMK). The cell suspension was maintained for 10 min at 4° and then disrupted with a Dounce homogenizer. The cell homogenate was spun down at $800 \times g$ for 3 min to sediment the nuclear fraction. The supernatant (cytoplasmic fraction), which contained 90% of the total [^{3}H]thymidine radioactivity, was then adjusted to 0.1% sodium deoxycholate, 0.5% sodium dodecyl sulfate, and 100 μg/ml of proteinase K (EM Laboratories, Inc.) was added; the mixture was incubated for 18 hr at 37°. At the end of this period an equal volume of buffer-saturated phenol was added, the solution was mixed gently with a glass rod for 5 min at 37°, and the mixture was centrifuged at 10,000 rpm for 30 min with a SS-34 rotor in a Sorvall RC2B centrifuge. The DNA in the aqueous phase was precipitated with 2.5 volumes of alcohol, collected as fibers with a glass rod or by centrifugation, and resuspended in a solution containing 0.01 M sodium citrate at pH 6.8 for further analysis. In some instances, treatment with 20 μg of RNase [DNase-inactivated (4,500 units per mg of protein, Worthington Biochem. Co.)] followed by another phenol extraction was performed. No major differences in the DNA obtained by these two procedures were observed.

Sedimentation analysis of DNA was performed by centrifugation at 20° in a SW41 rotor for 180 min at 40,000 rpm in neutral 5–20% (wt/vol) sucrose gradients containing 1 M NaCl, 0.05 M Tris·HCl (pH 7.8), 0.15% Sarkosyl NL97 (Geigy Industrial), and 0.001 M EDTA, or alkaline 5–20% (wt/vol) sucrose gradients with 0.9 M NaCl, 0.1 N NaOH, 0.15% Sarkosyl, and 0.001 M EDTA. Fractions were collected from the bottom, the DNA was precipitated with cold 10% trichloroacetic acid (TCA), the precipitates were collected on Millipore filters, and the radioactivity was determined by scintillation counting.

Preparation of Supernatant Fraction and DNase Assays. Cytoplasmic fractions were prepared from cells infected for 2 hr with unlabeled virus and from control cells, as described

Abbreviations: ds, double-stranded DNA; ss, single-stranded DNA.

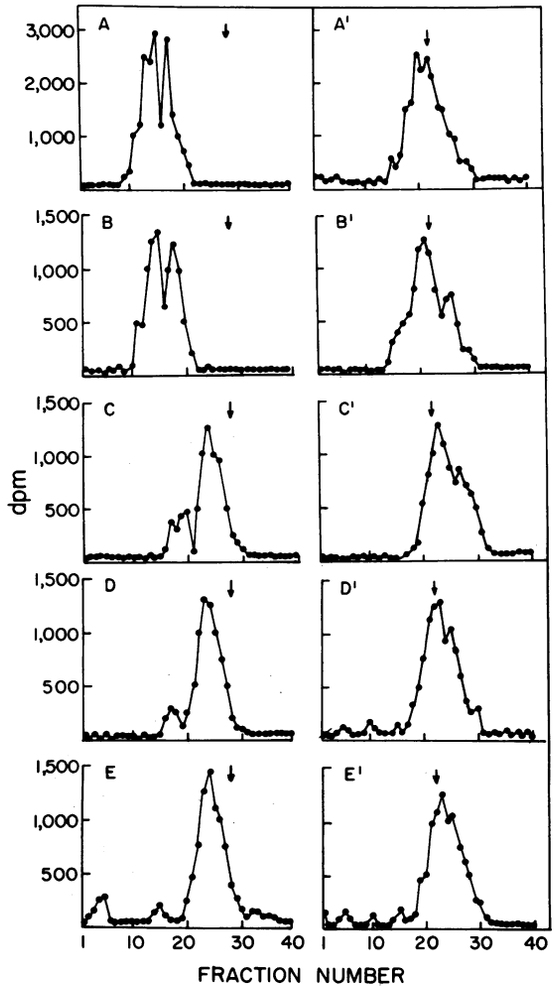

FIG. 1. Sedimentation analysis of DNA extracted from vaccinia virus and from the cytoplasmic fraction of virus-infected cells. (*Left panel*) Sedimentation through 5–20% alkaline sucrose gradients in a SW41 rotor at 40,000 rpm for 180 min. (*A*) DNA from virions; (*B*) DNA from the cytoplasmic fraction immediately after virus adsorption; (*C*) DNA from the cytoplasmic fraction after 45 min of infection; (*D*) DNA from the cytoplasmic fraction after 90 min; (*E*) DNA from the cytoplasmic fraction after 120 min. Arrow indicates the position of simian virus DNA (form I) in the same gradient. (*Right panel*) Sedimentation analysis of DNA through 5–20% neutral sucrose gradients in a SW41 rotor at 40,000 rpm for 180 min. (*A'*) DNA from virions; (*B'*) DNA from the cytoplasmic fraction after adsorption; (*C'*) DNA from the cytoplasmic fraction after 45 min of infection; (*D'*) DNA from the cytoplasmic fraction after 90 min; (*E'*) DNA from the cytoplasmic fraction after 120 min. Arrow indicates the position of T_4 DNA in the same gradient. The efficiency of assaying for ^3H was 25% in both experiments.

above. These fractions were further centrifuged at 10,000 × *g* for 30 min to sediment the larger particulate fractions. Aliquots of the supernatant fraction containing 50 μg of protein were incubated with 5 μg of [^{3}H]thymidine native vaccinia for 1 hr. At the end of this period, the samples were heated for 5 min at 100°, rapidly cooled in ice, and applied to the alkaline sucrose gradients. Aliquots were also used for determining the amount of DNase activity by using ss and ds ^{3}H-labeled *Bacillus subtilis* DNA as a substrate.

[^{3}H]DNA from *B. subtilis* was obtained from D. Dubnau, Department of Microbiology, Public Health Research Institute; T4 phage was a gift of M. Oishi, Department of Genetics, Public Health Research Institute; simian virus 40, forms I and III, were purchased from Bethesda Research Laboratories.

Table 1. Distribution of [^{3}H]DNA in alkaline sucrose gradients and measurement of DNA uncoating

Time (min) after infection	Distribution* (% of cpm)			Uncoating† (% [^{3}H]DNA sensitive to pancreatic DNase)
	106 S	91 S	67 S	
	66	34	—	0
0	58	42	—	0
45	2	27	71	20
90	3	10	87	40
120	5	5	90	56
120 + hydroxyurea	3	7	90	55

* Distribution (%) of vaccinia labeled DNA in the alkaline sucrose gradient peaks as calculated from the experiment represent in Fig. 1. Counts (80–90%) added were recovered. Virus alone gave 66 and 34% for the 106 S and 91 S species, respectively.

† Aliquots (0.1 ml) of cytoplasmic fractions from [^{3}H]DNA virus-infected cells, prepared as described in *Materials and Methods*, were incubated with 10 μg of pancreatic DNase and 0.2 ml of a buffer composed of 0.1 M Tris·HCl buffer at pH 7.8 with 10 mM $MgCl_2$ for 60 min at 37°. The reaction was stopped by addition of ice-cold 10% trichloroacetic acid and the precipitates that formed were collected on Millipore filters for radioactivity assays.

RESULTS

Sedimentation Studies of Vaccinia DNA. The extracted viral DNA sedimented in alkaline sucrose gradients as two species with sedimentation coefficients of ≅106 S and 91 S with respect to the marker simian virus 40 DNA (form I) (53 S) (Fig. 1*A*). These results are in agreement with data published by Geshelin and Berns (2) showing that the fast sedimenting material represents ss circular DNA of twice the molecular weight expected for the noncrosslinked complementary single strands of vaccinia DNA, while the slower sedimenting fraction consists of linear single strands of the same molecular weight of the faster material but with a single random nick. The proportion of these two DNA species varied in each experiment and these results suggested that one was derived from the other. In neutral sucrose gradients, vaccinia DNA sedimented as a broad peak at 67 S, identical to the marker T4 DNA of (120×10^6 daltons) (Fig. 1*A*).

A similar sedimentation pattern under denaturing conditions was observed for viral DNA extracted from cells immediately after adsorption (Fig. 1*B*). However, by 45 min after infection the sedimentation characteristics of parental DNA in alkaline sucrose had changed (Fig. 1*C*). Two sedimenting species could be observed, one rapidly sedimenting at 91 S (approximately 110×10^6 daltons), the other more slowly sedimenting at approximately 67 S (50–60 × 10^6 daltons) (14). The proportion of these two species varied in each experiment. In the experiment represented in Fig. 1*C*, 70% of the radioactivity sedimented as 67 S material, as expected for ss linear DNA molecules of between 50–60 × 10^6 daltons, derived from a noncrosslinked linear duplex by denaturation. This suggested that the crosslinks maintaining the two strands together even after denaturation had been broken and that the ss molecules sedimented at the expected sedimentation coefficient of between 60 and 70 S. The change in the sedimentation behavior of parental DNA was more striking as the infection progressed. Most of the DNA extracted from cells infected for 90 and 120 min sedimented between 60 and 70 S (Fig. 1*D* and *E*). Table 1 summarizes the distribution of different DNA species as a

Table 2. Susceptibility of viral DNA to the virion ss exonuclease

Source of DNA	Percent hydrolyzed	
	ss DNA	ds DNA
Virus	25	0 (5)*
Cells		
0 min after infection	27	0 (3)
120 min after infection	100	0 (3)

Five micrograms (1000 cpm) of native or heat-denatured DNA, extracted from virions and cytoplasmic fractions at 0 and 120 min after infection, were incubated with 10 μg of viral cores in 0.2 ml of a solution containing 0.1 M sodium acetate at pH 4.5, 0.5% Nonidet P-40, and 0.25% 2-mercaptoethanol. The reaction was allowed to proceed for 90 min at 37°, and was stopped by addition of ice-cold perchloric acid to a final concentration of 0.5 M. The precipitates were centrifuged, and the radioactivity was measured in the acid-soluble fraction.

* Number of experiments.

function of the time of infection. By 120 min, 90% of the viral DNA sedimented as 67 S. When hydroxyurea was added to the culture medium to prevent synthesis of progeny DNA, the parental DNA extracted after 2 hr of infection showed the same sedimentation characteristics as in Fig. 1*E*.

Even 2 hr after infection, only slight differences were observed when the parental DNA was sedimented in neutral sucrose gradients (Fig. 1*B'*–E') but, after 2 hr, the sedimentation characteristics in neutral sucrose indicated higher molecular weight components. These results, which are related to the onset of viral DNA replication, will be discussed in another communication (B. Pogo, manuscript in preparation).

Uncoating of Viral DNA. The uncoating of vaccinia DNA in infected cells occurs very shortly after penetration (15). To find out how this process is temporarily related to the removal of crosslinks in the parental DNA, I determined the sensitivity of viral DNA in cytoplasmic fractions to exogenous DNase after cell infection. The results are summarized in Table 1. After 45 min of infection, 20% of the viral DNA was uncoated, at 90 min 40% was uncoated, and at 120 min 56% was uncoated even in the presence of hydroxyurea. In these experiments, the maximum amount of uncoated DNA was 60% after 3 hr. It is concluded therefore that uncoating of the DNA and loss of the crosslinks occurred during the same period.

Susceptibility of Viral DNA to a ss Exonuclease. The changes in the sedimentation properties of vaccinia DNA after virus penetration were interpreted as removal of the crosslinks. However, the 67 S material could represent DNA molecules which have been broken and reduced to smaller components still containing the crosslinks. Structures of this kind will "snap back" to the duplex form after heat denaturation, and will not be completely digested by a ss exonuclease. To rule out this possibility, I extracted [^{3}H]DNA from virions and from cytoplasmic fractions after virus adsorption and 2 hr of infection, and heat-denatured and incubated the [^{3}H]DNA with viral cores under the conditions in which only the pH 4.5 DNase was active (5, 6). The results of these experiments are summarized in Table 2. As expected, the exonuclease did not attack the viral DNA in a ds configuration. When denatured viral DNA or DNA extracted from cytoplasmic fractions after virus adsorption was exposed to the exonuclease from the core, 25 and 29% of the radioactivity became acid soluble, respectively. By contrast, the DNA extracted after 2 hr of infection was completely hydrolyzed. These results clearly indicated that the

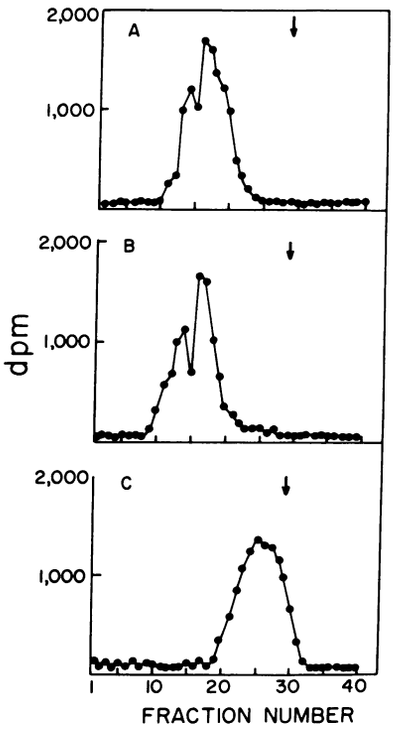

FIG. 2. Sedimentation of vaccinia DNA through an alkaline sucrose gradient after incubation with supernatant fractions from control and virus-infected cells. Conditions of centrifugation are as shown in Fig. 1. Arrow represents position of simian virus 40 DNA (form I) in the same gradient. (*A*) DNA from virions; (*B*) DNA from virions incubated with the supernatant from control cells; (*C*) DNA from virions incubated with the supernatant from virus-infected cells.

DNA from virions and from cells after adsorption, in which some of the molecules were nicked (Fig. 1*A* and *B*), contained crosslinks and were therefore partially digested by exonuclease. On the contrary, the DNA, extracted after 2 hr of infection, lacked crosslinks and was completely hydrolyzed by the enzyme.

Changes in Viral DNA Produced by the Virus Endonuclease. The changes in the sedimentation behavior of parental DNA after infection were interpreted as removal of crosslinks most probably by the action of an endonuclease. To substantiate this hypothesis, I used a soluble fraction obtained from cytoplasm of control and virus-infected cells as a source of such enzymatic activity. It has been shown (8, 9) that the virus-associated endonuclease active at pH 7.8 is released from the cores and can be detected in the soluble cytoplasmic fraction of infected cells. Native vaccinia DNA extracted from purified virions was incubated with the supernatant fraction of control and virus-infected cells for 1 hr at 37°, and then the mixture was heat-denaturated and sedimented through alkaline sucrose gradients. Fig. 2*A* shows that two species of vaccinia DNA sedimented at 106 S and 91 S, as did the DNA incubated with supernatant of control cells (Fig. 2*B*), whereas the DNA incubated with infected cell supernatant sedimented as one species at 67 S (Fig. 2*C*). These results indicated that an enzymatic activity present in infected cells was able to remove the crosslinks of the viral DNA and thereby allowed separation of the complementary strands under denaturing conditions. No significant acid-soluble radioactivity was released from the DNA during this incubation.

In another experiment, the type of DNase activity present in the supernatant was studied. Although the incubation with

Table 3. DNase activities in L cell supernatants

Source of supernatant	μg DNA solubilized/60 min	
	ss DNA	ds DNA
Control cells (pH 4.5)	0.10	0.52
Control cells (pH 7.8)	0.70	0.29
Virus-infected cells (pH 4.5)	0.14	0.61
Virus-infected cells (pH 7.8)	2.00	0.23

Fifty microliters of a supernatant fraction from control and virus-infected cells, prepared as described in *Materials and Methods* and containing 45 μg of protein, was incubated with 4 μg of native (ds) or denatured (ss) ^{3}H-labeled DNA from *B. subtilis* DNA (specific activity, 9000 cpm/μg) and 0.2 ml of a solution containing 0.1 M sodium acetate buffer at pH 4.5 or 0.1 M Tris·HCl buffer at pH 7.8 with 0.5% Nonidet P-40 and 0.25% 2-mercaptoethanol. The reaction was allowed to proceed for 60 min at 37°, and was stopped by addition of ice-cold perchloric acid to a final concentration of 0.5 M. The precipitates were pelleted by centrifugation at 10,000 rpm for 30 min at 4° and the radioactivity was measured in the acid-soluble fraction.

viral DNA was exclusively carried out at pH 7.8, I investigated the possibility that the other virus-associated DNase was also active. The results of these experiments are summarized in Table 3. When ss or ds ^{3}H-labeled DNA from *B. subtilis* was incubated with supernatant from control and infected cells at pH 4.5 and 7.8, I found that in the supernatant from control cells there was some detectable activity against ss or ds at either pH. By contrast, there was a considerable activity against ss at pH 7.8 in the supernatant from infected cells. When the products of degradation of this reaction were analyzed by DEAE-paper chromatography (16) it was established that they were mainly oligonucleotides (data not shown).

DISCUSSION

The results reported in this study indicated that the sedimentation properties of vaccinia DNA under denaturing conditions changed after penetration, from the characteristic circular DNA (106 S) or circular DNA with a nick (91 S) to ss molecules of the expected molecular weight (67 S). This transition occurring at the time of uncoating with a slight change in the size of the DNA, as judged by sedimentation in neutral sucrose, is interpreted as removal of crosslinks holding it together.

The elimination of crosslinks seems to be related to the process of viral DNA replication. Vaccinia DNA is synthesized in the cytoplasm of infected cells as ss fragments which are later ligated into larger molecules, without evidence for the formation of concatemers, between 2 and 5 hr after infection (ref. 17; B. Pogo, in preparation). For semiconservative replication of DNA to occur, the crosslinks that hold the complementary strands together have to be removed, thus facilitating the progress of the replicating fork. This process, as has been shown here, is under the control of an early function because it does not require DNA synthesis.

The results of the experiments in which native vaccinia DNA was incubated with supernatant obtained from control and virus-infected cells clearly indicated that the endonuclease present in the infected-cell supernatant was able to eliminate the crosslinks in the DNA. The absence of acid-soluble radioactivity after the incubation may suggest that few nucleotides have to be hydrolyzed to remove the crosslinks. Thus, it is worth emphasizing that only 5–10% of the total viral DNA is in ss form (B. Pogo, unpublished results) and these might be the sequences that the enzyme is able to recognize. The properties of this enzymatic activity present in infected cells are similar to those of the enzyme present in virus cores. Taken together these observations strongly suggest that the viral endonuclease is involved in the removal of crosslinks from the parental DNA after virus penetration.

The biological significance of crosslinks in DNA is not known. Naturally occurring crosslinks were found in SP_{82} bacteriophage (18) during T_7 infection (19) and more recently in yeast DNA (20). The participation of DNases in the elimination of crosslinks prior to replication also can be postulated in these systems.

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