Breakdown of HeLa Cell DNA Mediated by Vaccinia Virus
(viral DNA/alkaline sucrose gradients)

J. RODNEY PARKHURST, A. R. PETERSON, AND CHARLES HEIDELBERGER*

McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wis. 53706

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ABSTRACT  Breakdown of HeLa cell DNA begins within 90 min after infection with vaccinia virus at a multiplicity of infection of 2 plaque-forming units per cell, and ends about 7.5 hr after infection. HeLa cell DNA is degraded to a uniform size of 1 to $2 \times 10^9$ daltons, as judged by alkaline sucrose sedimentation analysis. The rate of host-cell DNA degradation by vaccinia virus increased directly with the multiplicity of infection. Sedimentation patterns in neutral and alkaline sucrose gradients of viral DNA from infected cells, as well as from partially purified virions, indicated that two size classes of DNA were present. Class I DNA sediments like T4 DNA in neutral gradients and has a molecular weight twice that of T4 DNA in alkaline gradients. Class II DNA sediments as a molecule of lower molecular weight than T4 DNA in both types of gradients. Infection of prelabeled HeLa cells with vaccinia virus did not result either in formation of trichloroacetic acid-soluble radioactivity or, upon purification of the virions, radiactivity associated with class I DNA, indicating that vaccinia virus does not reutilize HeLa cell DNA.

Vaccinia virus (a DNA virus that replicates in the cytoplasm) inhibits the synthesis of host-cell DNA (1–3). The virions retain this capacity even after ultraviolet irradiation. Hence, the synthesis of viral DNA is not necessary for inhibition of host-cell DNA synthesis (3). Thus far, two nuclease, an endonuclease and an exonuclease, are among the five enzymatic activities that have been reported to be associated with vaccinia virions (4–8). Both nuclease activities are released by treatment with Nonidet (NP-40) detergent (6). Incubation of these activities in vitro with tritium-labeled lambda phage DNA results in formation of mononucleotides at pH 5, or a gradual reduction in the size of lambda DNA at pH 7.5 (7). The role of these nuclease in vivo is still obscure, but a recent report (9) stating that the virion-associated endonuclease is located within the cell nucleus shortly after infection may pinpoint its site of action. Furthermore, the results of an autoradiographic study were interpreted to suggest that HeLa cell DNA may be degraded and also reutilized for viral DNA synthesis (10), although other investigators have shown quite clearly that reutilization does not occur under normal conditions (1, 8).

The present work provides evidence that DNA from HeLa cells is degraded to a limited extent upon infection with vaccinia virus and delineates the kinetics of this host-cell DNA breakdown. We will also show that this breakdown occurs as an early event in the infection process, and that host-cell DNA does not appear to be reutilized for the synthesis of class I viral DNA.

MATERIALS AND METHODS

Cell Culture, Virus, and Mode of Infection. The basic techniques were the same as those described by Oki et al. (8). HeLa S3 cells and vaccinia virus (WR strain) used in these experiments were shown to be free of mycoplasma by a modified Hayflick method (11).

Labeling of HeLa Cells. Logarithmic phase HeLa cells (1 to $2 \times 10^8$ cells per ml) were cultured in medium composed of Eagle's minimum essential medium for suspension culture, 10% calf serum, 0.1% pluronic F18, and antibiotics (1% of a combination of 5000 units of penicillin and 5 mg of streptomycin per ml) with 0.1 $\mu$Ci/ml of [3-14C]dThd (54 Ci/mole) for 24 hr. Extracellular radioactive dThd was removed by centrifugation of the cells, followed by washing with phosphate-buffered saline (pH 7.3) and a second centrifugation. The pelleted cells were suspended in fresh medium and were grown for at least 2 hr before viral infection.

Labeled Vaccinia Virions were purified essentially as described by Fujitava and Heidelberger (12), except that the cell wash and hypotonic solutions used were those described by Dahl and Kates (13). In brief, partially purified virus was obtained by Dounce homogenization of infected cells, removal of nuclei, sonication of cytoplasmic fraction, concentration of virus by pelleting through 35% sucrose, followed by centrifugation of the virus once in a linear 25–50% sucrose gradient.

Labeling and Purification of Bacteriophage T4BO1'. Bacteriophage T4BO1' and its host Escherichia coli B23 were generous gifts from A. W. Kozinski. Radioactive phage was grown and purified by the method of Kozinski (14) and was labeled with 2.5 $\mu$Ci/ml of [3H]dThd (20 Ci/mole) or 0.4 $\mu$Ci/ml of [2-14C]dThd (54 Ci/mole).

Neutral Sucrose Gradients. 30-ml 5–20% linear sucrose gradients containing 0.5% Na dodecyl sulfate, 0.1 M NaCl, and 5 mM Tris·HCl (pH 7.5) were prepared in Beckman 320625 polycylommer tubes that had been boiled in 0.1 M EDTA (pH 10) for 1 hr. The final pH of the gradients was 7.7. 0.1 ml of purified labeled virions [8 $\times 10^9$ plaque-forming units (PFU) per ml, 80 particles per PFU] were mixed with 0.40 ml of a lysis solution containing 2 M Tris·HCl (pH 7.5), 0.40 M NaCl, 2 M EDTA, 2% deoxycholic acid, 4% sucrose, 2% mercaptoethanol, and 1% Na dodecyl sulfate. The final

Abbreviations: MOI, multiplicity of infection; PFU, plaque-forming units.

* American Cancer Society Professor of Oncology; to whom requests for reprints should be sent.
pH of the lysis solution was 7.15. The mixture was immediately layered on the top of the gradients and incubated for 15 min at room temperature before sedimentation was begun. Gradients were centrifuged at 22,500 rpm for 4 hr at 12° in a Spinco SW25.1 rotor in a model L ultracentrifuge. Fractions (about 33) of 0.9 ml were collected from the bottom of the gradient directly into scintillation vials.

Alkaline Sucrose Gradients. A published method (15) was used after modification. 30-ml 5-25% linear alkaline sucrose gradients were prepared containing 1.0 M NaCl, 1 mM EDTA, and 60 mM Na p-aminosalicylate with a final pH of 12.5. Isolated vaccinia virions or 1 to 3 X 10^6 HeLa cells were lysed for 30 min on top of the gradient, often together with reference bacteriophage T4, in an 0.4-ml layer containing 0.5 M NaOH and 5 mM EDTA. The gradients were centrifuged at 24,500 rpm for 3 hr at 20° in an M-60 International ultracentrifuge with an SB110 rotor. Fractions (about 33) of 0.9 ml were collected from the bottom of the gradients by insertion of a stainless steel tube. Trichloroacetic acid-insoluble material was collected on Gelman Type A glass-fiber filter discs, and the radioactivity was determined by scintillation counting. Recovery of greater than 90% of the radioactivity applied was obtained in these gradients.

Centroids: Calculation of S Value and Molecular Weight. The molecular weight and sedimentation coefficients for the DNA sediments in neutral and alkaline sucrose gradients were calculated by the method of Studier (16). For some experiments, the centroids were calculated for the sedimentation profiles according to the following formula: Centroid = \( \frac{\sum n_i f_i}{\sum n_i} \) where \( n_i \) is the net level of radioactivity in the \( i \)th fraction \( f_i \).

RESULTS

Kinetics of Breakdown of HeLa Cell DNA in Vaccinia-Infected Cells. The majority of DNA from uninfected HeLa cells sedimented in a band with its peak at fractions 9-11 of the alkaline sucrose gradients (Fig. 1A and B). The sedimentation constant calculated for the peak of this band was 155. In cells infected with vaccinia virus for 90 min, the fraction of DNA sedimenting in this region was reduced by half and the quantity of DNA with peaks at about fractions 19 (58 S) and 27 (41 S) increased concomitantly (Fig. 1A). A progressive time-dependent increase in the fraction of 41S DNA was observed up to 7.5 hr after infection (Fig. 1B), but the sedimentation profile of DNA from cells infected for 22.5 hr remained the same as at 7.5 hr after infection. No acid-soluble radioactivity was found in infected HeLa cells or medium up to 24 hr after infection (data not shown), thus confirming the findings of Kit and Dubbs (1). Sedimentation of uninfected HeLa cells labeled both with radioactive choline and leucine demonstrated that these precursors were not recovered in the DNA peak.

The centroid value, which includes all the fractions of a sedimentation profile, may be used to express the relative proportions of all species of DNA present (15). The centroids obtained from three separate experiments are graphed to show that the conversion of the DNA sedimenting at 155 S to DNA sedimenting at 41 S is virtually complete within 8 hr after infection (Fig. 2).

Effect of Increasing Multiplicity of Infection on HeLa Cell DNA Breakdown. The data shown in Figs. 1 and 2 were obtained with a constant MOI. Fig. 3 shows the extent of degradation of DNA in cells infected for 3 hr with increasing MOI. The sedimentation profile of DNA from cells infected with an MOI of 6 (25 particles per PFU) was similar to that from uninfected cells. However, as the MOI increased, the amount of DNA sedimenting as the control (155 S) decreased with a concomitant increase in the formation of the 41S DNA. The almost complete degradation of HeLa DNA (155 S)
DNA labeled of partially purified 3 by is shown. The standard 22.2 represent a fraction before infection not previously mentioned. Times after infection at an MOI of 2 were: 0.2, 0.5, 1.0, 1.5, 2.5, 4.5, 7.5, 11.5, 18.0, and 22.2 hr. The standard error of the mean (SEM) for these experiments is shown.

by 3 hr after infection at an MOI of 24 implies that the onset of the degradative process is an early event in infection.

HeLa Cell DNA Is Not Reutilized by Vaccinia Virus. When partially purified [3H]dThd-labeled virions were lysed and analyzed on alkaline or neutral sucrose gradients, reproducible bimodal sedimentation profiles of DNA were obtained (Figs. 4 and 5), showing that two DNA species are present. The most rapidly sedimenting species has been arbitrarily named class I and the slower sedimenting, class II DNA. The peak molecular weight of class I vaccinia DNA is 1.4 to 1.6 × 10^6 daltons in both alkaline and neutral gradients. This value indicates that class I vaccinia DNA is resistant to complete denaturation in alkali, which is consistent with Berns and Silverman's suggestion that the vaccinia genome contains at least one crosslink (17). Class II DNA is composed of fragments with an apparent molecular weight of about 10^5 in neutral as well as alkaline gradients. When purified vaccinia virions were incubated with DNase under appropriate conditions followed by lysis and sedimentation in alkaline sucrose gradients, class I viral DNA sedimented as previously observed, while class II sedimented at the top of the gradients, indicative of DNase sensitivity.

A comparison of the degradation patterns seen in Figs. 1 and 3 with the sedimentation profiles of viral DNA in Fig. 4 suggests that there is little or no reutilization of HeLa DNA by vaccinia virus. In order to test more rigorously whether the observed degradation of HeLa cell DNA was accompanied by its reutilization for viral DNA synthesis, we labeled the cells for 24 hr with 0.1 μCi/ml of [14C]dThd. After 24 hr, the cells were washed thoroughly to remove unincorporated radioactive dThd and allowed to grow in fresh medium for 2 hr before infection. Before infection the specific activity of the HeLa cell DNA was 5.3 × 10^4 dpm/μg. 22 hr after infection, virions were partially purified from the cells and subsequently lysed and sedimented in neutral and alkaline

Fig. 2. Centroid position against hours after infection of [14C]dThd-labeled HeLa cells by vaccinia virus. These data represent a replot of Fig. 1A and B and also include times of infection not previously mentioned. Times after infection at an MOI of 2 were: 0.2, 0.5, 1.0, 1.5, 2.5, 4.5, 7.5, 11.5, 18.0, and 22.2 hr. The standard error of the mean (SEM) for these experiments is shown.

Fig. 3. Alkaline sucrose sedimentation profiles of [14C]dThd-labeled DNA from HeLa cells infected with vaccinia virus for 3 hr at MOI of 0, (●); 6, (×); 12, (△); and 24 (▽). The superimposed arrows at 155 S and 41 S represent calculations. The arrow at 71 S represents the peak position of T4 bacteriophage DNA.

Fig. 4. Alkaline sucrose sedimentation of [3H]dThd-labeled DNA obtained from partially purified vaccinia virions after lysis. 1 hr after infection of HeLa cells with vaccinia virus, [3H]dThd was added to the growth medium. After an additional 22 hr, the tritium-labeled virions were purified. The superimposed arrows at 101 S and 27 S represent calculations. The arrow at 71 S shows the peak position of marker T4 bacteriophage DNA.
graduates. No radioactivity was found in the region corresponding to class I viral DNA, showing that HeLa cell DNA is not reutilized by vaccinia virus for the synthesis of this class of DNA. A small amount of radioactive DNA was found near the tops of these gradients, which sedimented in the region of degraded HeLa cell DNA and class II DNA. This radioactivity, 310 dpm/µg DNA, represents a reutilization of 0.01% of the total HeLa cell DNA, but had a specific activity 5.9% of that of the HeLa cell DNA. There is not sufficient information from these experiments to conclude whether there is a small amount of reutilization of HeLa cell DNA for the production of class II DNA, or whether degraded fragments of HeLa cell DNA are partially encapsulated by vaccinia virus.

**DISCUSSION**

The breakdown of HeLa cell DNA early after infection with vaccinia virus implies that either a viral enzyme (carried by the virion or synthesized early after infection) or the induction and/or activation of a cellular enzyme is responsible for this degradation. Our data do not allow us to distinguish conclusively between these possibilities. A recent report (9), indicating that a vaccinia virion-associated endonuclease is present within the cell nucleus shortly after infection, lends support to the hypothesis that the mechanism of the degradation of host-cell DNA may involve the release of a virion-associated enzyme capable of entry into the cell’s nucleus, which then degrades the host-cell’s DNA.

Vaccinia virus appears to be unique in the sense that it is the only DNA virus known to degrade host-cell DNA early in productive infection. Two other viruses whose DNA is replicated within the nucleus (Epstein–Barr and polyoma) also break down host-cell DNA, but under different conditions from vaccinia.

Infection of Raji cells (a nonvirus-producing cell line of Burkitt’s lymphoma) by Epstein–Barr virus results in degradation of cellular DNA (18). Thus far, we are not aware of similar findings in cell lines in which productive infection by Epstein–Barr virus occurs. Also, investigators (19) working with a polyoma virus-infected mouse-embryo cell line demonstrated that degradation of host-cell DNA occurs late in infection and only after its replication. The size of this degraded cellular DNA was reported to be about the same as that of the viral DNA.

Neither the mechanism of host-cell DNA degradation by vaccinia virus nor the significance of the resulting fragments is known. The onset of degradation is correlated in time with the first detectable viral DNA synthesis, and degradation is complete by the time encapsulation of viral DNA commences (2). Whether or not these correlations in time are coincidental remains to be determined.

It has been demonstrated that vaccinia virus does not reutilize HeLa DNA to synthesize viral DNA under normal conditions (1), although reutilization of host-cell DNA does occur when cells are intranuclearly irradiated with tritiated thymidine and then infected with vaccinia virus (8). In that special case the extent of reutilization was correlated with the specific radioactivity of the host-cell DNA and the consequent appearance of a DNase with a pH optimum of 9.2 (8). Two lines of evidence in the present work support the finding that vaccinia virus does not reutilize host-cell DNA. Breakdown of HeLa cell DNA does not result in the appearance of an intracellular DNA species of molecular weight comparable to that of class I viral DNA. Also, virions purified from HeLa cells labeled for 24 hr with [14]C]dThd before infection do not contain [14]C-labeled class I viral DNA.

Other investigators have found that DNA from vaccinia virions has an apparent molecular weight of 1.5 × 10⁶ (19, 20), to which class I DNA corresponds. Hence class I DNA, which survives DNase treatment of purified vaccinia virions, must be the viral genome. Class II DNA, which does not survive DNase treatment of virions, has not previously been reported.

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