Characterization of Deoxyribonucleases Induced by Poxviruses¹

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Increases in deoxyribonuclease activity assayed at alkaline pH can be observed in poxvirus-infected cells when native or denatured deoxyribonucleic acid (DNA) is used as substrate. The deoxyribonuclease assayable with native DNA as substrate, induced in HeLa cells by cowpoxvirus or vaccinia virus WR, can be separated from the corresponding enzyme present in normal cells by chromatography on diethylaminoethyl cellulose. In addition, the two enzymes induced in the virus-infected cells differ from each other in their chromatographic properties. The two induced enzymes have been further characterized with respect to properties of enzymatic reaction.

Several previous reports have demonstrated that, together with other early proteins, the activity of one or more enzymes with deoxyribonuclease activity is increased after infection of animal cells with poxviruses (9). These studies were carried out with various strains of poxviruses and different lines of cells. Conditions of assay varied with respect to pH and deoxyribonucleic acid (DNA) substrate used. Deoxyribonuclease activity assayed at alkaline pH is induced even in the absence of viral DNA replication, suggesting that the increased activity is due to the formation of early enzymes. Since no detailed characterization of these enzymes was carried out, it is not known whether one or more deoxyribonucleases are induced after infection with poxvirus. In this report we describe characterization and partial purification of the "alkaline" deoxyribonucleases induced in animal cells after infection with cowpoxvirus and vaccinia virus WR.

MATERIALS AND METHODS

Reagents. ³H-thymidine (New England Nuclear Corp., Boston, Mass.; specific activity: 27.8 mc/mg); pancreatic ribonuclease, pancreatic deoxyribonuclease (Worthington Biochemical Corp., Freehold, N.J.); deoxyadenosine monophosphate (Serva-Entwicklungslabor); diethylaminoethyl (DEAE)-SS cellulose (capacity 0.66 mvol/g; Serva-Entwicklungslabor); and Sephadex G-100 (pharmacia) were used.

Growth of cells and infection with virus. HeLa cells (S₈ clone) were grown on glass in Eagle's medium with 10% calf serum and transferred to spinner cul-

ture 48 hr before infection. HeLa cells were infected by procedure A described by Becker and Joklik (1). For infection, vaccinia strain WR and cowpox strain Brighton were used throughout. Purification of virus was carried out by the method of Joklik (7). All virus strains were tested for the absence of *Mycoplasma*.

Preparation of enzyme extracts. Mock-infected and infected cells were harvested by centrifugation, washed twice with phosphate-buffered saline, and frozen at -50 C until used. For preparation of extracts, the cells were suspended in 0.05 M tris(hydroxymethyl)aminomethane (Tris; pH 7.5) or 0.01 M Tris-hydrochloride (pH 8.0) and subjected to ultrasonic vibration (Branson Sonifier; 15 pulses of approximately 6 sec each). After centrifugation for 1 hr at 13,000 × g, the extracts were assayed for enzymatic activity. When the crude extracts were stored at 4 C, enzymatic activity did not decrease significantly over several days.

Isolation of DNA substrate. Radioactive DNA was isolated from HeLa cells or *Escherichia coli* B following a procedure described by Thomas et al. (16). Cells grown in the presence of ³H-thymidine were washed twice with phosphate-buffered saline and resuspended after freezing in SSC (0.15 M NaCl plus 0.015 M sodium citrate). After lysing the cells by the addition of sodium dodecyl sulfate (SDS) to a final concentration of 0.25%, they were incubated with Pronase (250 µg/ml) overnight at 37 C. The solution was then deproteinized by shaking gently with phenol saturated with SSC. Phenol was removed by extracting with chloroform. After addition of two volumes of ethyl alcohol, DNA was "spooled" from the solution and redissolved in 0.05 M Tris-hydrochloride (pH 7.4). After incubation for 30 min with pancreatic ribonuclease (heated for 10 min at 80 C) at a concentration of 50 µg/ml, the DNA was precipitated with ethyl alcohol, redissolved in 0.05 M Tris-hydrochloride (pH 7.4) and stored at 4 C over chloroform. The ratio of optical densities at 260 to

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230 to 280 nm for HeLa DNA was 1:0.45:0.54, and for *E. coli* DNA 1:0.49:0.54. DNA was denatured by heating at 100 to 150 μ g/ml in 0.05 M Tris-hydrochloride buffer (pH 7.5), at 100 C for 10 min and then cooling in an ice-water bath.

Other procedures. Protein was determined by the method of Lowry et al. (10). DNA was measured by Burton's modification of the diphenylamine reaction with deoxyadenosine monophosphate as the standard (3). Chloride concentration was determined argentometrically with a Kipp-Zonen Chlorometer.

Assay of deoxyribonuclease activity. Enzymatic activity was followed by determining the conversion of native or heat-denatured radioactive DNA to acid-soluble fragments. The standard reaction mixture contained in a total volume of 0.3 ml: glycinate buffer (pH 9.2), 10 μ moles; $MgCl_2$, 2 μ moles; nucleotide of native or denatured 3H -labeled DNA (2×10^4 to 1.4×10^5 counts/min), 6 to 9 μ moles. After incubation for 30 min at 37 C, the reaction mixture was chilled, and 0.1 ml of calf thymus DNA (90 mg/100 ml) was added as a carrier followed by 0.05 ml of 20% cold trichloroacetic acid. After standing at 0 C for 20 min, the reaction mixture was centrifuged for 10 min at $13,000 \times g$. A 0.2-ml sample of the supernatant fluid was added to 10 ml of Bray's solution and counted in a liquid scintillation counter. One unit of activity is defined as the amount of enzyme catalyzing the production of 1 μ mole of acid-soluble nucleotide in 30 min under standard conditions.

Endonuclease activity was assayed by the membrane filter assay described by Geiduschek and Daniels (5). The mode of enzymatic action was further studied by the method described by Birnboim (2).

RESULTS

Timecourse of induction of deoxyribonucleases. HeLa cells infected with cowpoxvirus or vaccinia virus WR show increased levels of deoxyribonuclease activity when assayed with native DNA at pH 9.2. Simultaneous assay of deoxyribonuclease activity under the same conditions with heat-denatured DNA as substrate revealed only slight increases in specific activities in infected cells. The kinetics of induction of both enzymatic activities confirm previous reports (8, 11, 12). Addition of mercaptoethanol during preparation of enzyme extracts did not result in a stimulation of enzymatic activity, which is in contrast to a deoxyribonuclease degrading native DNA at alkaline pH induced by herpes simplex virus (15). Blocking replication of viral DNA by the addition of fluorodeoxyuridine (FUDR) results in higher deoxyribonuclease activity assayed with native or denatured DNA after infection with either of the virus strains (8, 13; Fig. 1). Extracts prepared under these conditions therefore proved a better source of the enzymes. Of some interest is the observation that after infection with strain WR, in contrast to the infection with cowpoxvirus, deoxyribonuclease activity assayable with

FIG. 1. Induction of deoxyribonuclease activity after infection with cowpox and vaccinia WR in the absence of viral DNA replication. Cells were infected as described in the text. FUDR (10^{-6} M) was added at time of infection, and the cells were diluted postinfection in spinner medium containing 5% dialyzed serum. For assay of enzymatic activity, 1.5×10^7 cells were harvested at different times postinfection, washed with phosphate-buffered saline, and frozen at -50 C until used. Crude extracts were prepared in 0.05 M Tris-hydrochloride buffer (pH 7.9). Left half, native DNA; right half, denatured DNA. Cowpoxvirus (●), vaccinia virus WR (×), uninfected (□).

denatured DNA reaches a higher specific activity than with native DNA.

DEAE cellulose chromatography. Equal amounts of crude extracts prepared from mock-infected and infected cells were applied to and washed into a column of DEAE cellulose equilibrated with 0.01 M Tris-hydrochloride buffer (pH 8.0). Protein was eluted stepwise with Tris-hydrochloride buffer containing increasing amounts of KCl. Under these conditions, virtually all the deoxyribonuclease activity was retained on the column. Preliminary studies showed that, when assays were carried out with denatured DNA as substrate, enzymatic activity was found in all chromatographic fractions of normal and virus-infected cells in comparable amounts. On the other hand, when deoxyribonuclease activity in uninfected cell extracts was assayed with native DNA, most of the enzymatic activity could be eluted with 0.05 M and 0.1 M KCl. Chromatography of cowpox-infected cell extracts revealed in addition a new deoxyribonuclease activity which could be eluted with 0.2 M KCl. Mainly because of the low activity of "native" deoxyribonuclease in cells infected with vaccinia virus WR most of the chromatographic studies were carried out with FUDR-treated cell

FIG. 2. DEAE chromatography of HeLa cells mock-infected and infected with cowpoxvirus or vaccinia WR in the presence of FUDR (10^{-6} M). Cells were infected as in Fig. 1 and incubated for 9 hr. A 25-ml amount of crude extracts (3.5 mg/ml) was chromatographed on a DEAE cellulose column (0.75 cm² by 5 cm). Protein was eluted stepwise with 0.01 M Tris-hydrochloride buffer (pH 8.0) containing increasing amounts of KCl. The flow rate was adjusted to 20 ml/hr, and fractions of 3 ml were collected. Deoxyribonuclease activity was assayed in a volume of 0.05 ml. Conditions of assay were the same as described in the text. Native DNA (●), denatured DNA (×), uninfected (U), infected with cowpox (Cp), infected with vaccinia virus (WR).

extracts (Fig. 2). Extracts from cells mock-infected in the presence of FUDR behaved in the same way as uninfected cells. In cowpox-infected cells, a deoxyribonuclease activity (substrate, native DNA) eluted at 0.2 M KCl, the yield being about 20%. Most of the deoxyribonuclease activity assayed with native DNA from

cells infected with vaccinia virus WR in the presence of FUDR also eluted at 0.2 M KCl. Frequently, however, a small but significant amount of the induced "native" deoxyribonuclease was found to elute at 0.1 M KCl, which was considered an indication that this enzyme might differ with respect to chromatographic behavior from the corresponding one extracted from cowpoxvirus-infected cells. In cells infected with vaccinia virus WR in the presence of FUDR, a significant increase of deoxyribonuclease activity was also observed with denatured DNA, reaching a higher specific activity than with native DNA as substrate. This result is different from the observations made with cowpoxvirus (see Fig. 1 and 2). To clarify further whether "native" deoxyribonuclease from cowpox- or WR-infected cells can be separated by DEAE chromatography, the fractions containing "native" deoxyribonuclease from cowpox- or WR-infected extracts were rechromatographed on DEAE cellulose. Fractions containing the enzyme activity were pooled, diluted to 0.05 M KCl, and applied to a DEAE column (0.25 cm² by 2.5 cm) (Fig. 3). Cowpoxvirus-induced "native" deoxyribonuclease eluted as a single peak at a salt concentration of 0.16 M and can be separated from most of the enzymatic activity assayable with denatured DNA. "Native" deoxyribonuclease from vaccinia virus WR-infected cells eluted at a lower salt concentration (0.09 to 0.11 M); in addition, we found in the latter case a small amount of an unidentified deoxyribonuclease eluting at a higher salt concentration. The behavior of the two enzymatic activities on DEAE chromatography made it likely that "native" deoxyribonuclease from vaccinia WR- or cowpox-infected cells would differ also in other enzymatic properties. We accordingly studied the enzymatic properties of the two deoxyribonucleases degrading native DNA. These studies were carried out with the protein fractions eluted with 0.2 M KCl, because, at this stage of the purification, the enzyme activity was more stable than after rechromatography on DEAE cellulose.

Mode of enzymatic action. No endonuclease activity could be detected in either of the two deoxyribonuclease fractions, with the assay described by Geiduscheck and Daniels (5; Fig. 4). This confirms previous observations of McAuslan et al. for the cowpox induced "native" deoxyribonuclease (4). Since it cannot be completely excluded that DNA fragments are formed but nevertheless retained on the membrane filters by protein, we attempted to determine the mode of action of the two induced enzymes by still another method. Gel filtration of the reaction products of a deoxyribonuclease has been de-

FIG. 3. Rechromatography on DEAE cellulose of the deoxyribonuclease activity of extracts infected in the presence of FUDR (eluted with 0.2 M KCl). Fractions containing highest deoxyribonuclease activity were pooled, and 10 mg of protein after dilution to a salt concentration of 0.05 M KCl was applied to a DEAE cellulose column (0.25 cm² by 2.5 cm). Protein was eluted with a linear gradient from 0.1 to 0.4 M KCl in 0.01 M Tris-hydrochloride (pH 8.0). Volume of the fraction, 3 ml; ●, native; ×, denatured DNA. Assay of deoxyribonuclease activity was carried out under standard conditions with 0.1 ml of each fraction.

scribed as a method to distinguish between exonucleolytic and endonucleolytic cleavage of DNA (2). Even after considerable degradation, fragments of DNA besides mononucleotides remained which were of sufficient size to be excluded from the gel (Fig. 5). This elution pattern is very similar to that obtained with venom phosphodiesterase and indicates an exonucleolytic mode of action.

Effect of divalent cations. Degradation of "native" DNA by the enzymes from both sources was stimulated by Mg^{2+} . Under the standard assay conditions used, maximal activity was ob-

FIG. 4. Detection of degradation of native ³H-DNA by virus-induced deoxyribonuclease activity by membrane filter assay. The reaction mixture contained in 0.5 ml: 15 µmoles of glycinate buffer (pH 9.2); 3 µmoles of $MgCl_2$, 18 µmoles of nucleotide of ³H-labeled HeLa DNA and protein (0.2 M KCl fraction) as indicated. Time of incubation, 15 min. Cowpoxvirus (●), vaccinia virus WR (×), pancreatic deoxyribonuclease (▲).

FIG. 5. Chromatography on Sephadex G-100 of ³H-DNA at different stages of digestion with the induced deoxyribonucleases (0.2 M KCl fraction). Assays were carried out under standard conditions. Enzyme reactions were stopped by boiling the reaction mixture for 5 min and chilling. Chromatography was carried out on a column (0.5 by 15 cm) under the conditions described by Birnboim (2). Incubation mixtures with cowpox- and vaccinia-induced enzyme gave qualitatively the same results, and the results were similar to the results obtained with venom phosphodiesterase. Incubation: 0 min (●), 1 hr (×), 2 hr (■).

served at a magnesium ion concentration of 8×10^{-4} to 10^{-3} M. The cowpoxvirus-induced enzyme is stimulated by Mn^{2+} at low concentrations comparable to Mg^{2+} ion concentrations. Addition of Mn^{2+} at higher concentrations (above 15×10^{-5} M) did not result in a further stimulation. On the other hand, stimulation by Mn^{2+} , at least up to a concentration of 120×10^{-5} M, was observed for the deoxyribonuclease-degrading native DNA induced by vaccinia WR strain. The degree of stimulation depended somewhat on the amount of extract used in the assay.

Dependence on pH. Figure 6 shows the effect of pH on the rate of degradation of native DNA by the two enzyme extracts. Maximal activity for the enzymes induced by both virus strains was found at pH 9.2 by using glycinate buffer. In contrast to the cowpoxvirus-induced "native" deoxyribonuclease, the activity of the corresponding enzyme induced by vaccinia WR could not be determined reproducibly in Tris-hydrochloride buffer in the range between pH 7.0 and 9.0. Compared to the cowpox-induced enzyme, the activity seemed to decrease more slowly towards the neutral pH. By using phosphate buffer, the enzymatic activity could be studied at neutral pH.

Selective inhibition of native deoxyribonuclease by p-chloromercuribenzoate (pCMB). The two deoxyribonucleases are inhibited to different

extents by pCMB. The activity of the cowpox-induced deoxyribonuclease is rapidly inhibited by the addition of pCMB (Fig. 7). At a concentration of the inhibitor of 10^{-6} M, the enzymatic activity is reduced to less than 10% of the controls. In contrast, the vaccinia-induced enzyme at the same concentration is only inhibited by about 40%.

DISCUSSION

The results presented confirm previous reports on the induction by poxvirus infection of deoxyribonucleases degrading native or denatured DNA at alkaline pH. All virus strains so far investigated are capable—although to varying degrees—of inducing both activities. Using strain IHD for infection, we also observed increased deoxyribonuclease activity, when native or denatured DNA was used as substrate (6). The latter deoxyribonuclease can be clearly distinguished from the "acid" deoxyribonuclease discovered by McAuslan et al., which degrades denatured DNA but has been found to be a late function of poxvirus (14). Even though chromatography of the crude extracts on DEAE cellulose does not result in any

FIG. 6. Effect of pH on rate of reaction. The standard deoxyribonuclease assay was used except that the pH of the reaction was varied. Substrate: native HeLa DNA. The indicated buffers were added at a final concentration of $10 \mu\text{M}$ to 0.3 ml of reaction mixture. Glycinate buffer: X, cowpoxvirus infected; ▲, vaccinia virus WR. Phosphate buffer: cowpox infected (hollow X), vaccinia infected (Δ).

FIG. 7. Effect of p-chloromercuribenzoate on deoxyribonuclease activity. Degradation of native HeLa DNA was assayed under standard conditions. Vaccinia virus WR infected (●), cowpoxvirus infected (X).

significant increase of specific activity of deoxyribonucleases, possibly because of the instability of the enzymes (see also 4), it shows clearly that the increased nuclease activity from cowpox-infected extracts degrading native DNA is due to an enzyme eluting differently from the ion-exchange column than most of the "native" deoxyribonuclease from uninfected cells. Further, the induced "native" deoxyribonuclease from vaccinia virus WR-infected cells can be separated by chromatography on DEAE cellulose from the enzyme present in cowpox-infected cells. Enzymatic activity assayed with denatured DNA shows a substantial increase only in the case of cells infected in the presence of FUDR. No satisfactory separation of this activity from the corresponding deoxyribonuclease activity from normal cells has so far been achieved. It is not clear whether the same virus-induced protein can degrade native and denatured DNA or whether two different deoxyribonucleases with a preference for either native or denatured DNA are formed in the poxvirus-infected cell. Morrison and Keir have observed that the "alkaline" deoxyribonuclease-degrading native DNA induced by herpes simplex virus infection elutes also at pH 8.0 with 0.2 M NaCl from DEAE columns (15). An increase of enzyme activity directed against denatured DNA was also observed in herpes simplex-infected BHK cells. No purification of this enzymatic activity has been reported.

Because of the instability of the two "native" deoxyribonucleases under study and the lack of large amounts of infected cells, we have not been able so far to achieve a high purification which would be a prerequisite for a more definite study of the mode of action of the deoxyribonucleases. No endonucleolytic activity could be found with two different assay procedures, and this finding is supported to a certain extent by the observation that, after up to 30 min of incubation, thymidine monophosphate could account for 70 to 100% of the acid-soluble radioactivity.

Another unresolved question is whether the induced deoxyribonucleases are coded by the viral genome or whether they are derepressed or altered cellular enzymes. Should it turn out that the enzymes induced by the different virus strains studied in this report have an analogous mode of action, the observation that these proteins ob-

viously differ in several properties strongly favors the notion that we are dealing with virus-coded enzymes.

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