Single-Stranded Deoxyribonucleic Acid-specific Nuclease from Vaccinia Virus

ENDONUCLEOLYTIC AND EXONUCLEOLYTIC ACTIVITIES

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SUMMARY

A deoxyribonuclease purified from vaccinia virus was shown to have a specificity for single-stranded DNA. Undenatured HeLa cell DNA was digested at less than 2% of the rate of thermally denatured DNA. The low rate of digestion was probably due to single-stranded regions in the isolated HeLa cell DNA since no nicking of native T7 DNA was detected by alkaline sucrose gradient sedimentation. Evidence of endonuclease activity was obtained by sedimentation, gel filtration, and DEAE-cellulose chromatography of the digestion products. Exonucleolytic activity was also indicated by the high percentage of mononucleotides formed. The single-strand specificity and the endo- and exonuclease activities are similar to the previously described properties of the S1 nuclease from Aspergillus oryzae. The vaccinia deoxyribonuclease was also able to nick closed single-stranded circular DNA as well as closed superhelical double-stranded circles. The susceptibility of the latter probably resulted from the presence of weakly hydrogen-bonded regions in the DNA.

In the preceding paper (1) we reported the isolation of a deoxyribonuclease from vaccinia virus cores. The enzyme was extensively purified and the molecular weight was estimated to be 105,000. A polypeptide with a molecular weight of 50,000 was detected by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate suggesting the presence of two similar or identical subunits. The enzyme hydrolyzed denatured DNA and exhibited an optimum at pH 4.4. The specificity of the deoxyribonuclease for single-stranded DNA and evidence for both endonuclease and exonuclease activities are the subjects of this communication.

MATERIALS AND METHODS

Nucleic Acids—[32P]- and [3H]thymidine-labeled HeLa cell DNAs were prepared as described in the accompanying paper (1). Prior to labeling with [32P] (2 mCi/800 ml) the cells were incubated in phosphate-free media for 24 hours. The isolated DNA was filtered through a 10% agarose column (0.9 X 15 cm). The specific activities of the purified [32P]- and [3H]thymidine-labeled DNAs were 8633 cpm per nmole and 6226 cpm per nmole, respectively. T7 [3H]DNA provided by E. Paoletti and N. Nossal (National Institutes of Health) was purified as previously described (2); SV40 Form I and Form II [3H]DNAs, prepared as described by Sebring et al. (3), were obtained from M. Thoren (National Institutes of Health).

Nucleic Acid Denaturation—HeLa cell DNA was thermally denatured as described previously (1). T7 DNA and SV40 form II DNA were alkaline-denatured with 0.05 N NaOH at room temperature for 5 min and then neutralized with 0.1 M Tris-HCl, pH 7.4.

Sucrose Gradient Analysis of Digests of Deoxyribonuclease—Deoxyribonuclease digests of T7 native and denatured DNA were analyzed on 5 to 20% alkaline sucrose gradients containing 0.05 M NaCl, 0.1 M NaOH, and 1 mM EDTA and centrifuged at 190,000 X g for 2 hours at 23° in an SW 50.1 rotor. The digest of SV40 Form I DNA was analyzed on a 10 to 30% alkaline sucrose gradient containing 0.05 M NaCl, 0.3 M NaOH, 1 mM EDTA, and 0.015% Sarkosyl, and centrifuged at 200,000 X g for 16 hours at 10° in an SW 41 rotor. The digest of SV40 Form II DNA was analyzed either on a 5 to 90% alkaline sucrose gradient containing 0.5 M NaCl, 0.3 M NaOH, and 1 mM EDTA, or 0.015% Sarkosyl, and centrifuged at 180,000 X g for 80 min at 20° in an SW 65 rotor or on a 5 to 20% neutral sucrose gradient in 0.01 M Tris HCl, pH 7.5, 1 mM EDTA, and 0.1% sodium dodecyl sulfate.

DEAE-cellulose Chromatography of Deoxyribonuclease Digests—Enzymatic digests of HeLa cell [32P]DNA were analyzed on a DEAE-cellulose column as described by Tomlinson and Tener (4). The column (0.9 X 20 cm) was equilibrated with 0.02 M Tris-HCl-0.15 M NaCl, and 0.015% sodium dodecyl sulfate.

Gel Filtration of Digests of Deoxyribonuclease—Enzymic digests of [3H]thymidine-labeled HeLa cell DNA were analyzed on Superdex G-100 columns as described by Bimboim (5). The column (0.9 X 20 cm) was equilibrated with 0.015 M Tris-HCl, pH 7.0, 0.15 M NaCl, and 0.01% sodium dodecyl sulfate.

Enzymes—V1 nuclease from vaccinia virus was solubilized, purified, and assayed as described in the previous communication (1). Venom phosphodiesterase was obtained from Calbiochem.

Chemicals and Reagents—[3H]Thymidine (50.7 Ci per nmole) was purchased from New England Nuclear and [32P] was from Internation Chemical and Nuclear Co. Urea was obtained from Schwarz-Mann and 10% agarose (20 to 400 mesh) from Bio-Rad Laboratories. PEI-cellulose thin layer sheets were purchased from J. T. Baker Chemical Co.

1 The abbreviation used is: PEI-cellulose, polyethyleneimine-substituted cellulose.
Results

Specificity for Single-Stranded DNA—V. deoxyribonuclease, purified from vaccinia virions as described in the accompanying paper (1), exhibits a specificity for single-stranded DNA. Initially this was tested with [3H]thymidine-labeled HeLa cell DNA. Fig. 1 shows the enzymatic hydrolysis of thermally denatured and native DNA as a function of time. When hydrolysis of thermally denatured DNA plateaued at 45 min, approximately 6.4 nmol of nucleotide were rendered acid-soluble. In contrast, only 1% of the “undenatured” DNA or 0.15 nmol of nucleotide was acid-soluble during the same period of time. The plateau obtained with denatured DNA was not entirely due to residual duplex DNA or to a limit digestion of single-stranded DNA but to inactivation of the deoxyribonuclease since further hydrolysis occurred after addition of more enzyme (Fig. 1).

Since undenatured HeLa cell DNA was likely to contain some single-stranded regions produced during the isolation procedures, the single-stranded specificity of the deoxyribonuclease was tested more rigorously with a much smaller and uniform size DNA fragment produced during the isolation procedures, [3H]thymidine-labeled T7 DNA was denatured and treated as described under “Materials and Methods.” Equal amounts of denatured DNA (90 nmoles, specific activity 3500 cpm per nmole) were added to reaction mixtures containing 0.1 unit of enzyme. The reactions were stopped and analyzed on alkaline gradients as described under “Materials and Methods.” As controls native or denatured T7 DNA was added to parallel reaction mixtures in the absence of enzyme. All samples were incubated at 37° for 20 min. The reaction was stopped by the addition of NaCl and NaOH to final concentrations of 0.9 and 0.2 M, respectively. Samples were centrifuged at 215,000 × g for 16 hours at 25° in an SW 65 rotor. Under these conditions there was no significant difference in the sedimentation of the ribosomal RNA incubated in the presence or absence of enzyme (results not shown).

Analysis of Products of Limited Digestion by Sedimentation and Gel Filtration—Evidence of endonuclease activity was obtained by examining the products of limited digestion of alkaline denatured T7 DNA. Digested samples containing 0.4% and 7% acid-soluble nucleotides were analyzed on alkaline sucrose gradients. As a control, DNA incubated in the absence of enzyme was similarly analyzed. The results shown in Fig. 3 indicate a shift in the sedimentation of the DNA consistent with endonuclease action. Analysis of the products of limited digestion of thermally denatured HeLa cell DNA by gel filtration also provided evidence of endonuclease activity. Enzymatic digestion of the DNA was allowed to proceed until 6% of the nucleotides were acid-soluble. The product was then applied to a Sephadex G-100 column. DNA incubated for the same period of time without enzyme was similarly analyzed and eluted in the void volume. Approximately 18% of the digested DNA was shifted from the void volume of the column and eluted as a broad heterogeneous peak (Fig. 4A). More material was found in the total column volume in enzymatic digests which had a greater per cent of soluble nucleotides. However, this material did not coincide entirely with the [32P]ATP marker. As a control, the product of an exonuclease, snake venom phosphodiesterase, was analyzed on a similar column (Fig. 4B). In contrast to the previous results, the exonuclease product eluted in two peaks coinciding with the void volume and the [32P]ATP marker. Analysis of Digestion Products by Ion Exchange Chromatography—The size of the oligonucleotide products produced after ex-
FIG. 4. Gel filtration of denatured HeLa DNA after digestion with deoxyribonuclease. Purified deoxyribonuclease (0.05 unit) was added to standard reaction mixtures containing [3H]thymidine-labeled thermally denatured HeLa DNA (12.9 nmoles, specific activity 6226 cpm per nmole) and incubated at 37°C for 5 min. The sample was analyzed on a Sephadex G-100 column as described under "Materials and Methods." Fractions (0.45 ml) were collected and counted directly in a scintillation fluid containing toluene and Triton X-100. A second portion of DNA was digested with venom phosphodiesterase and analyzed on a similar column. [γ-32P]ATP was added to each sample as an internal marker. A, O-O-O, without enzyme; •-••, deoxyribonuclease reaction product; B, O-O-O, without enzyme; •-••, venom phosphodiesterase reaction product.

tensive digestion was determined by chromatography. Thermally denatured 32P-labeled HeLa DNA was incubated with purified vaccinia deoxyribonuclease for varying time intervals and the digestion product was analyzed on DEAE-cellulose columns in the presence of 7 M urea. Under these conditions, oligonucleotides are separated according to chain length. The products produced by digestion of [3H]thymidine-labeled HeLa DNA with an exonuclease, snake venom phosphodiesterase, or an endonuclease, pancreatic DNase I, were used as markers. As expected, 32P- and 3H-labeled peaks overlapped rather than coincided since at pH 7.5 there is partial separation based on nucleotide composition within peaks and only thymidine was labeled with tritium (4). A representative elution profile from an experiment in which approximately 80% of the thermally denatured HeLa cell [32P]DNA was rendered acid-soluble is shown in Fig. 5. Of the total 32P-labeled material recovered, 15% was mononucleotides, 5% was dinucleotides, 8% was trinucleotides, 7% was tetranucleotides, approximately 8% was pentanucleotides, and the remainder of the product was of higher molecular weight. The formation of thymidine monophosphate during digestion by V1 nuclease was analyzed further by thin layer chromatography on PEI-cellulose plates (Fig. 6). A significant peak of TMP was detected when 4% of the DNA was made acid-soluble. These results demonstrate that the deoxyribonuclease has both endonucleolytic and exonucleolytic activities.

pH Optimum of Endonuclease Activity—Since a pH optimum of 4.4 for the deoxyribonuclease was previously obtained using an acid solubility assay which may favor detection of exonuclease action (1), it was important to check the effect of pH using a specific endonuclease assay. Reaction mixtures containing identical amounts of deoxyribonuclease and denatured [3H]thymidine-labeled HeLa cell DNA were prepared at pH 3.7, 4.4, and 5.0. After 5 min of incubation at 37°C, the reactions were stopped and the samples were applied to Sephadex G-100 columns. The results (not shown) indicated that endonuclease activity was greatest at pH 4.4.

FIG. 5. Analysis of deoxyribonuclease digest on DEAE-cellulose column. Denatured HeLa cell [32P]DNA (30 nmoles, specific activity 8633 cpm per nmole) was incubated with V1 nuclease (0.4 unit) in 400 μl of the standard reaction mixture. At 17 min more enzyme (0.4 unit) was added and the sample was incubated for an additional 23 min. The venom phosphodiesterase digestion of [3H]thymidine-labeled DNA was described in Fig. 4. The reactions were stopped by boiling for 10 min. The samples were made to contain 7 M urea and analyzed on a DEAE-cellulose column as described under "Materials and Methods." Four-milliliter fractions were collected, of which 1-ml aliquots were counted directly in toluene-Triton X-100 scintillation fluid. O-O-O, venom phosphodiesterase; +--+, V1 nuclease. The number above each peak indicates the oligodeoxynucleotide chain length.

FIG. 6. Analysis of thymidine mononucleotide formation by thin layer chromatography on PEI-cellulose. Enzymatic digests of [3H]thymidine-labeled DNA containing no acid-soluble material (A), 2% (B), 4.2% (C), 8.0% (D), and 11.4% (E) were analyzed by thin layer chromatography on PEI-cellulose plates which were first washed with deionized water. The plates were developed with 0.5 M sodium formate, pH 3.4, and allowed to dry. Strips (1.5 cm) were cut and placed in scintillation vials to which 0.5 ml of 0.5 M LiCl and then a Triton X-toluene-Liquifluor scintillation solution were added.
Form II results in the production of linear and circular strands, respectively. 130 strands of denatured Form II DNA were enzyme dots not require the presence of free ends (Fig. 7). This molecule but contains a nick in one strand. Denaturation of closed circular molecules; Form II is also a double-stranded circle and counted in scintillation fluid.

SV40 Form I and Form II DNAs. Form I is a double-stranded action was stopped as previously described, immediately layered on an alkaline sucrose gradient, and analyzed as described under “Materials and Methods.” The samples were collected from the bottom and counted in scintillation fluid.

Fig. 8 (center). Sedimentation of deoxyribonuclease-treated SV40 Form I DNA in alkaline sucrose gradients. [3H]Thymidine-labeled SV40 Form I DNA (0.9 µg, specific activity 11,778 cpm per µg) was incubated in standard reaction mixtures in the presence of deoxyribonuclease. The product was then analyzed on an alkaline sucrose gradient and analyzed as described under “Materials and Methods.” The positions of Form I (63 S) and Form II and III (16-18 S) DNAs are indicated by arrows.

Cleavage of Circular DNA Molecules—The ability of the vaccinia deoxyribonuclease to cleave circular DNA was tested using SV40 Form I and Form II DNAs. Form I is a double-stranded closed circular molecule; Form II is also a double-stranded circular molecule but contains a nick in one strand. Denaturation of Form II results in the production of linear and circular strands which sediment in alkaline sucrose gradients at 16 and 18 S, respectively. Both strands of denatured Form II DNA were cleaved by vaccinia virus deoxyribonuclease indicating that the enzyme does not require the presence of free ends (Fig. 7).

Covalently closed circular molecules (e.g. SV40 Form I DNA) contain superhelical twists which result in unpaired or weakly hydrogen-bonded regions (6) in the DNA. These regions are nicked by single-stranded DNA-specific nuclease such as Neurospora crassa endonuclease (7). A preparation of native [3H]thymidine-labeled SV40 DNA, consisting predominantly of Form I but containing some Form II DNA, was incubated with vaccinia deoxyribonuclease. The product was then analyzed on an alkaline sucrose gradient. Under these conditions of centrifugation Form I DNA remains double-stranded and therefore sediments as a 53 S molecule while both Form II and Form III (a double-stranded linear molecule) sediment more slowly at 16 to 18 S (8). The results shown in Fig. 8 indicate that SV40 Form I DNA was nicked by the vaccinia deoxyribonuclease forming a structure sedimenting at 16 to 18 S. To determine whether the latter consisted of Form II or both Form II and Form III DNAs, the reaction product was analyzed on neutral sucrose gradients. Under these conditions Form I sediments at 21 S, Form II at 16 S, and Form III at 14 S (9). The results shown in Fig. 9 indicate that both Forms II and III were produced in the presence of vaccinia deoxyribonuclease.

**DISCUSSION**

A deoxyribonuclease purified from vaccinia virus was shown to have a specificity for single-stranded DNA. Undenatured HeLa cell DNA was digested at 2% or less of the rate of thermally denatured DNA. The low rate of digestion was probably due to single-stranded regions in the isolated HeLa cell DNA, since no nicking of native T7 DNA was detected by alkaline sucrose gradient sedimentation. Evidence of endonucleolytic activity was obtained by sedimentation, gel filtration, and DEAE-cellulose chromatography of the digestion products. Exonuclease activity was also indicated by the high percentage of mononucleotides formed. Although we cannot rule out the possibility that the endo- and exonucleolytic activities result from two separate enzymes, the pH optima determined by acid solubility and gel filtration were identical.

The deoxyribonuclease activity of intact vaccinia virus cores at low pH as well as of a purified deoxyribonuclease from infected cell extracts were previously thought to be exclusively exonucleolytic (10-13). However, the only documented evidence of this was based on nitrocellulose filter assays. Nevertheless, because of the possibility that the low pH endonuclease activity may be masked while in cores, we examined the products produced by limited digestion of denatured [3H]thymidine-labeled HeLa cell DNA at pH 4.4. Filtration of the products on Sephadex G-100 columns clearly indicated the production by intact cores of large oligonucleotide fragments. Thus, at pH 4.4 endonuclease activity can be demonstrated by both intact cores and purified V1 nuclease.

The sensitivity of Form I DNA to the vaccinia deoxyribonuclease was established using a limiting digestion reaction with purified deoxyribonuclease. Purified deoxyribonuclease was shown to degrade the linear and circular strands of denatured Form II DNA efficiently. The results shown in Fig. 9 indicate that both Forms II and III were produced in the presence of vaccinia deoxyribonuclease.

Unpublished results.
clcase probably results from the presence of unpaired or weakly hydrogen-bonded regions in the superhelical DNA (6). Both the N. crassa endonuclease (7) and the S1 nuclease (14) cleave superhelical DNAs. The latter enzyme also has a low but significant activity on linear double-stranded DNA (14). Further studies will be needed to determine whether the conversion of some Form II and Form III DNA by vaccinia deoxyribonuclease resulted from a low ability of the enzyme to nick double-stranded DNA or to recognize an interruption in the polynucleotide chain resulting from the cleavage of a single phosphodiester bond. It is possible that the exonuclease activity of the enzyme could recognize a single cleaved phosphodiester bond in the double-stranded DNA and thus make the gap wider. The endonuclease activity of the enzyme would then attack the opposite strand of the molecule, thus converting Form II to Form III.

The biological function of the vaccinia deoxyribonuclease and the reason for its presence in the viral core are unknown. The two strands of vaccinia DNA behave as if they are cross-linked, possibly at the terminal ends (15). Berns and Silverman (15) suggested that a single-stranded DNA-specific endonuclease would be needed for strand separation if replication were semi-conservative. Strand separation may then be a possible role of the deoxyribonuclease found in the core of the virus. Pogo and Dales (12) have suggested the possibility that deoxyribonucleases might be released from the virion after infection and inhibit host DNA synthesis.

Of the nucleases studied so far, the vaccinia V1 nuclease described here most closely resembles the S1 nuclease of Aspergillus oryzae (16). Both show specificity for single-stranded DNA, endo- and exonucleolytic activities, and pH optima of approximately 4.5 (1, 14, 16, 17). Although some ribonuclease activity may be associated with preparations of S1 nuclease, it is thought to be a contaminant (16). The molecular weight of the S1 enzyme is 60,000, while the V1 nuclease is composed of subunits with molecular weights of 50,000. The V1 nuclease has been used to discriminate between single- and double-stranded DNA for analytical and preparative purposes. Whether the V1 nuclease offers any practical advantages because of the absence of ribonuclease activity remains to be seen.

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