The Deoxyribonuclease Induced after Infection of KB Cells by Herpes Simplex Virus Type 1 or Type 2

I. PURIFICATION AND CHARACTERIZATION OF THE ENZYME*

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The deoxyribonuclease induced in KB cells by herpes simplex virus (HSV) type 1 and type 2 has been purified. Both enzymes are able to completely degrade single- and double-stranded DNA yielding 5'-monophosphonucleotides as the sole products. A divalent cation, either Mg** or Mn**, is an absolute requirement for catalysis and a reducing agent is necessary for enzyme stability. The maximum rate of reaction is achieved with 5 mM MgCl₂ for both HSV-1 and HSV-2 DNase. The optimum concentration for Mn** is 0.1 to 0.2 mM and no exonuclease activity is observed when the concentration of Mn** is greater than 1 mM. The rate of reaction at the optimal Mg** concentration is 3- to 5-fold greater than that at the optimal Mn** concentration. In the presence of Mg**, the enzymes are inhibited upon the addition of Mn**, Ca**, and Zn**. The enzymatic reaction is also inhibited by spermine and spermidine, but not by putrescine.

Crude and purified HSV-1 and HSV-2 DNase can degrade both HSV-1 and HSV-2 DNA, but native HSV-1 DNA is hydrolyzed at only 22% of the rate and HSV-2 DNA at only 32% of the rate of Escherichia coli DNA.

Although HSV-1 and HSV-2 DNase were similar, minor differences were observed in most other properties such as pH optimum, inhibition by high ionic strength, activation energy, and sedimentation coefficient. However, the enzymes differ immunologically.

Infection of a single cell with herpes simplex virus results in the elevation of certain enzyme activities. Among the enzymes involved in nucleic acid metabolism, the activities of DNA polymerase, thymidine kinase, and DNase were increased following infection of cells with HSV-1 or HSV-2 (1, 2). These three enzymes have biochemical and immunological properties which differ from the corresponding activities in uninfected cells (2), indicating that these proteins are coded for by the viral genome.

Viral-specified thymidine kinase and DNA polymerase have been purified and extensively characterized (3-5). The HSV-specified thymidine kinase, which is often used as a marker for viral infection and transformation of cells (6), is not required for viral replication, at least in cells in culture (7). However, several antiviral agents in current use derive their selectivity from their activation by the viral thymidine kinase. Studies with temperature-sensitive mutants of HSV have indicated that the virus-specified DNA polymerase is probably obligatory for viral DNA synthesis (8) and, therefore, also for viral replication. The role of the HSV-specified DNase, on the other hand, is still unknown. In order to more fully understand this enzyme, this report describes comparative studies on the properties of extensively purified DNase from HSV-1- and HSV-2-infected human cells. Some of the properties of partially purified HSV-1-specified DNase have been described by Koir and his colleagues (2, 9, 10).

EXPERIMENTAL PROCEDURES

The "Experimental Procedures" are described in the accompanying miniprint.²

RESULTS

The properties of deoxyribonuclease are discussed below.

Polyacrylamide Gel Electrophoresis - Both purified HSV-1 and HSV-2 were unstable during electrophoresis in the Tris-glycine electrophoretic system. Activity could, however, be detected in gels on which crude extract of HSV-1 or HSV-2 had been electrophoresed individually. In this case, a single broad peak of activity was seen with HSV-1 DNase having an RF of 0.48 and HSV-2 DNase an RF of 0.30.

Substrate Specificity - When incubated in the standard mixture, both HSV-1 and HSV-2 DNase hydrolyzed double-

² The "Experimental Procedures" and the data described under "Results" (including Figs. 1 to 10 and Tables I and II), and the "References" are presented in miniprint following the "Discussion." Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20014. Request Document No. 77M-1796; cite authors, and include a check or money order for $2.10 per set of photocopies.
stranded DNA at a rate slightly faster than for single-stranded DNA at the same total DNA concentration. The ratio of the rate of hydrolysis of single-stranded DNA to the rate of hydrolysis of double-stranded DNA was 0.75 for HSV-1 DNase and 0.82 for HSV-2 DNase. The addition of 2 μg of unlabeled double-stranded DNA inhibited the hydrolysis of single-stranded [3H]DNA by 40% for both enzymes, indicating that both single- and double-stranded DNA compete for the same enzyme for hydrolysis.

Table I shows that the purified viral DNases were able to degrade DNA from various sources, including homologous DNA. Both enzymes degraded *Escherichia coli* DNA most rapidly followed by PBS2, KB, HSV-2, and HSV-1 DNA, in order of decreasing rate of hydrolysis. Similar results were obtained with crude enzyme. It should be particularly noted the PBS2 DNA, which contains uracil in place of thymine (11), was degraded almost as rapidly as *E. coli* DNA by the HSV DNases (Table I). Shearing of the various DNAs by sonication was found to decrease this differential susceptibility to hydrolysis seen with the native, unsheared DNA samples (Table I). The increase in the rate of hydrolysis of HSV-1, HSV-2, and KB DNA, relative to *E. coli* DNA after sonication, is not simply due to an increase in the number of termini. In every case (native and sheared), the concentration of DNA (15 μg/ml), under the standard assay conditions, was saturating since there was no increase in reaction velocity when the DNA concentration of any sample was doubled to 30 μg/ml. The different rates of hydrolysis of HSV-1 and HSV-2 DNA may be reflective of the different nucleotide sequences of HSV-1 and HSV-2 DNA (12).

To determine whether the enzymes completed the break-down of one DNA molecule before degrading another, a saturating amount of labeled DNA was incubated with enzyme and, after 6 min, an equal amount of unlabeled DNA was added and the amount of acid-soluble radioactivity released was followed. If the DNase is unable to detach from the substrate once hydrolysis has begun, there should be no change in the rate of release of radioactive DNA. However, as shown in Fig. 2S, addition of unlabeled DNA after the enzyme had been added caused a decrease in the rate of release of radioactivity and that the change in the rate occurred immediately upon addition of the unlabeled DNA. It is thus concluded that the DNase attacks the DNA in a random fashion and is able to release from one DNA molecule before complete digestion and reinitiate hydrolysis of another molecule. HSV-1 and HSV-2 DNase gave the same result.

**Analysis of Products**—Prolonged incubation of either double- or single-stranded DNA resulted in complete hydrolysis of the DNA to acid-soluble nucleotides. Designation of the products as either 3'- or 5'-monophosphate nucleosides was made on the basis of whether or not they would serve as substrates for 5'-nucleotidase (see "Supplementary Material"). This analysis showed that complete hydrolysis of either single- or double-stranded DNA by either HSV-1 or HSV-2 DNase results in the formation of 5'-monophosphate nucleosides as the sole products. Keir (2) has indicated that the direction of hydrolysis is from the 3' to 5' terminus.

**Effects of Divalent Cations**—Both HSV-1 and HSV-2 DNase have an absolute requirement for a divalent cation. Of the divalent cations tested, only Mg2+ and Mn2+ could serve in this role and enzyme activity was only observed when one of these agents was included in the reaction mixture. The dependence of HSV-1 and HSV-2 DNase activity on the concentration of MgCl2 and MnCl2 is shown in Figs. 3S and 4S, respectively. Maximum enzyme activity of both DNases is achieved at a MgCl2 concentration of about 5 mM and the activity may be slightly less when the MgCl2 concentration was increased to 10 mM. In contrast, the effect of MnCl2 showed quite a different profile. The optimal manganous ion concentration was about 0.2 mM for HSV-1 DNase and 0.1 mM HSV-2 DNase and increasing the concentration beyond this resulted in progressively less enzyme activity (Fig. 4S). Greater enzyme activity was obtained with magnesium ions than with manganous ions. The ratio of activity at the optimal MnCl2 concentration to the activity at the optimal MgCl2 concentration was 0.27 for HSV-1 DNase and 0.17 for HSV-2 DNase.

Although no DNase activity was obtained in the presence of Ca2+ and Zn2+ alone, both these divalent cations inhibited the viral DNases when added in the presence of Mg2+. In addition, Mn2+ ions also inhibited enzyme activity in the presence of Mg2+. HSV-1 and HSV-2 DNase were inhibited to the same extent. With Mg2+ at a concentration of 1 mM, enzyme activity was inhibited by 54% by Ca2+ or Mn2+ at a concentration of 0.1 mM, indicating that the enzymes have a greater affinity for these ions than for Mg2+. In contrast, Zn2+ gave only 8% inhibition of activity at a concentration of 0.1 mM and 36% inhibition at 1 mM, indicating that the enzymes have similar affinity for Zn2+ and Mg2+. DNase activity was completely inhibited by 1 mM Ca2+ or Mn2+ and 10 mM Zn2+.

**Effect of Temperature**—A linear Arrhenius plot was obtained for both HSV-1 and HSV-2 DNase (Fig. 5S). Activation energies of 24.0 and 20.3 kcal/mol were obtained for the HSV-1 and HSV-2 enzyme, respectively.

**pH Optimum**—Purified HSV-1 and HSV-2 DNases showed a similar dependence of activity on the hydrogen ion concentration (Fig. 6S). HSV-1 DNase had a broad optimum over the range pH 8.0 to 8.6, whereas HSV-2 DNase had a more defined optimum at about pH 8.2. The greatest difference between the two enzymes in this property is observed in the range from pH 8.2 to 9.0, where, for example, HSV-2 DNase has only 43% of the activity of HSV-1 DNase at pH 8.9. The pH profile for the HSV-1 DNase is similar to that obtained by Keir and Gold (10).

**Effect of Ionic Strength**—Both HSV-1 and HSV-2 DNase are increasingly inhibited as the ionic strength in the reaction mixture is raised (Fig. 7S). Both enzymes display about the same relationship of activity versus ionic strength with a total loss of activity at I = 0.2. Inhibition of activity by high ionic strength is reversible since exonuclease activity was readily detected upon dilution of samples containing high concentrations of salt.

**Molecular Weight**—The apparent molecular weights of the purified HSV-1 and HSV-2 DNase were determined by sedimentation in a sucrose density gradient. Sedimentation analysis was performed in the presence and absence of 0.5 M KCl (Fig. 8S). In the absence of KCl, both enzymes sedimented as unsymmetrical peaks, whereas in the presence of KCl, the enzymes sedimented as a symmetrical peak but at a slower rate (Fig. 8S). Based on the assumption that the HSV DNases are globular proteins and have a similar partial specific volume to bovine serum albumin, ovalbumin, and α-chymotrypsinogen A which were used as standards, calculation of the apparent molecular weights of the enzymes in the presence of 0.5 M KCl yielded a value of 49,400 for the HSV-1 DNase and 53,500 for the HSV-2 DNase. The apparent molecular weight of both enzymes in the absence of KCl is similar to bovine serum albumin (68,000). Since this is not an integral
value of the apparent molecular weight of enzymes in the presence of salt, the addition of 0.5 mM KCl does not appear to be causing a dissociation of the enzymes into subunits. It is likely, therefore, that the decrease in sedimentation velocity which occurs in the presence of KCl may be due to either a change in the shape of the DNase or due to elimination of any protein-protein interactions with bovine serum albumin (present in the sample) which may exist in the absence of KCl. It should be noted that the enzymes are not active in 0.5 mM KCl.

Effects of Other Reagents—Both HSV DNases require a reducing agent for activity. The requirement for β-mercaptoethanol seems to be mostly related to the stability of the enzyme. When β-mercaptoethanol was omitted from the assay mixture, the reaction velocity was nonlinear with time and rapidly ceased. The purified enzymes were more rapidly inactivated than the crude enzymes. A concentration of β-mercaptoethanol of 6 mM or greater is necessary in the assay mixture to achieve a linear reaction velocity. No activity was observed when the enzymes (crude or pure) were incubated in the absence of β-mercaptoethanol and the presence of 2 mM p-hydroxymercuribenzoate. In addition, the purified DNases are very much more unstable when stored in the absence of reducing agent. When β-mercaptoethanol was removed from the purified DNases by passage of the enzymes through a column of Sephadex G-25, 90% of the activity is lost during storage on ice for 16 h. Addition of β-mercaptoethanol did not restore activity.

It is known that herpes simplex virus contains significant amounts of spermine and spermidine (13) and since it is thought that these compounds play a role in the condensation and neutralization of DNA in organisms which lack the basic histone proteins (14, 15), it was of interest to test the effect of the polyamines on the DNase activity. Spermine and spermidine were found to inhibit 80 to 90% of the DNase activity, whereas putrescine had no effect (Fig. 9S). Both enzymes showed similar inhibition profiles with an $S_{1/2}$ of about 0.6 mM for spermine and of about 1.4 mM for spermidine. Spermine causes its effect over a very narrow concentration range and appears to inhibit DNase activity once a critical concentration is reached (Fig. 9S). In contrast, spermine increasingly inhibits the DNase over a much wider concentration range (Fig. 9S). To determine whether the inhibition by spermine was due to its effect on the DNA substrate, the enzyme, or both, the effect of increasing the DNA concentration at a fixed concentration (1 mM) of spermine was determined. Increasing the DNA concentration resulted in some relief of the inhibition of spermine. However, reversal of the inhibition by spermine was still not complete even when the DNA concentration had been increased 8-fold. Under this situation, 1 mM spermine still inhibited DNase activity by 27%. When the enzyme concentration was increased at a fixed concentration of 1 mM spermine, a linear response of activity versus enzyme concentration was obtained, indicating that the enzyme was inhibited to the same extent, irrespective of the enzyme concentration. These data indicate that the inhibition of DNase activity by spermine is probably due to a combination of effects on both the DNA substrate and the enzyme.

The compounds, 3'-dTMP, 5'-dTMP, cyclic 3',5'-dTMP, dTDP, dTTP, 3'-p-nitrophenoxyphosphoryl)-dThd, 5'-p-nitrophosphorylphosphoryl)-dThd, 5'-amino-dThd, NADP, and inorganic phosphate had no effect on HSV-1 or HSV-2 DNase. Inorganic pyrophosphate at 1 mM did not affect the viral DNases and could not be tested at 2 mM because of precipitation. Phosphonoacetic acid, an analog of pyrophosphate, which is a potent inhibitor of several of the herpes virus-specific DNA polymerases (16-18), also did not affect HSV-1 or HSV-2 DNase.

Antiserum—Antiserum was raised in rabbits against the purified HSV-1 and HSV-2 DNase, respectively. Antiserum raised against HSV 1 DNase inhibited HSV 1 DNase to a greater extent than HSV-2 DNase and vice versa for antiserum against HSV-2 DNase (Fig. 10S). These data show that the antiserum will inhibit the immunizing enzyme preferentially which provides the most convincing evidence that the DNases are viral specific. However, the two DNases do appear to share some common antigenic determinants since a particular antiserum will inhibit the heterologous enzyme to a certain degree (Fig. 10S).

Furthermore, less antiserum is required to inhibit a fixed amount of HSV-1 DNase than is required to inhibit the same amount of HSV-2 DNase (Fig. 10S). This indicates that antiserum against HSV-1 DNase has either a higher titer of specific antibodies or that the antibodies are more effective in inhibiting HSV-1 DNase than are HSV-2 DNase antibodies for HSV-2 DNase. Preliminary results also indicate that the HSV-1 DNase is more antigenic than the HSV-2 DNase.

**DISCUSSION**

Many DNA binding proteins are bound by phosphocellulose and the HSV-specified DNases also fall into this category. Binding to phosphocellulose constitutes affinity chromatography since, at the same pH, the DNases are also bound to the anion exchange resin, DEAE-cellulose. Affinity of the enzymes for phosphocellulose could arise by virtue of the similarity of the conformation of the phosphate moiety on the resin to that in DNA. Chromatography of crude extracts of the DNases on phosphocellulose, in most cases, resolved a portion of the DNase activity which was not bound to the resin (Fig. 1SA). However, the unbound portion was shown, in fact, to be the same enzyme species as the bound fraction, since antiserum raised against the bound, purified DNase inhibited the unbound activity to exactly the same extent. A possible reason for the occurrence of the unbound activity is that it formed a complex with DNA fragments even though precautions were taken during extraction of the enzyme to minimize the formation of such complexes. To test this hypothesis, a crude extract was incubated at 37°C, under assay conditions (10 mM mercaptoethanol, 10 mM MgCl₂), for 3 h and the chromatographic pattern on phosphocellulose was compared to a portion of the crude extract which had not been incubated. This treatment converted more than 85% of the unbound DNase to the bound form. There was no loss of activity during the 3-h incubation.

The apparent molecular weight of the viral DNases, as determined by sucrose density gradient centrifugation, is much smaller than reported by Keir (9), who indicated that the partially purified enzyme from HSV-1-infected BHK cells emerged with the void volume during gel filtration through Sephadex G-200. This may simply reflect the greater purity of our preparation and imply that the DNase in partially purified samples forms a complex with other components. Indeed, since Keir and Morrison (2, 9) did not use phosphocellulose to remove the "unbound form" of the enzyme, the result of gel filtration on Sephadex G-200 may represent a complex of the viral DNase and DNA fragments.

The role of the viral DNase is unclear but several possible functions can be readily envisaged. The DNase may degrade...
host DNA to block host macromolecular synthesis or provide a 
source of nucleotides (or both) for viral DNA synthesis.

The HSV DNases certainly have the capacity to completely 
degrade DNA, at least in vitro, and the monophosphonucleo-
tide products are the natural precursors of the triphosphate 
nucleosides necessary for DNA synthesis. Degradation of host 
DNA appears to be one of the functions of the exonucleases 
specified by the bacteriophage T7 (19), and the HSV DNases 
have several properties in common with T7 exonuclease. 
Evidence that the host cell DNA is degraded following infec-
tion with HSV has been obtained (20), but whether the 
degradation products are incorporated into the viral DNA has 
not been rigorously examined.

On the other hand, the HSV DNases may be required in the 
processing of DNA during replication. In this regard, it is 
interesting to compare the structure and synthesis of HSV 
and bacteriophage T5 DNA. Both HSV (96 x 10^6) and T5 (77 
× 10^6) DNA have similar molecular weights (21, 23). Further-
more, both HSV and T5 DNA are synthesized as concatamers 
(24, 25) and must be processed to the mature length molecules 
found in the virion. It has been shown that the formation of 
unit length single-stranded T5 DNA chains does not require 
the T5 nuclease, but rather that the T5 nuclease plays a direct 
role in the introduction of single strand nicks into replicating 
T5 DNA (26). The HSV DNases may act in a similar manner.

Irrespective of the function of the HSV DNases, it is also 
clear that the enzyme activity must be modulated, in some 
manner, in vivo so that newly synthesized viral DNA is 
protected from degradation. In bacteriophage T4, for example, 
the virus-specified exonuclease, which is required for genetic 
recombination, is blocked from degrading newly synthesized 
T4 DNA by a multifunctional protein product of another T4 
gene (27). While the possible existence of this type of protec-
tive mechanism has not been determined for HSV, the data 
presented in this paper offer several other mechanisms for 
protection of HSV DNA. The DNases are inhibited by an ionic 
strength of greater than I = 0.2 and maximal activity of HSV 
DNA polymerase is achieved at I = 0.24.3 Thus, an increase in 
ionic strength within the infected cell would serve to decrease 
DNase activity while simultaneously accelerating DNA syn-
thesis. Alternatively, the DNase activity may be modulated 
by the relative concentrations of divalent cations or the viral 
DNA may be protected by polyamines which are almost 
certainly required for compaction of the viral DNA in the 
absence of histones prior to encapsidation. Finally, some 
property inherent in the structure of the viral DNA affords 
protection against the DNase since HSV DNA is hydro-
lyzed at a significantly slower rate than cellular DNA (Table 
II). It has been shown, for example, that T7 exonuclease (28), 
λ exonuclease (28), and E. coli exonuclease III (30) hydrolyze 

glucosylated DNA at a very slow rate compared to nongluco-
sylated DNA. To our knowledge, there has been no definitive 
study to indicate whether or not HSV DNA is glucosylated or 
contains any unusual bases.

REFERENCES

References are found on p. 3562.
Herpes Simplex DNase

MATERIALS

DNase solution was prepared by a method already described (1). In addition, DNase was supplied by the manufacturer. Zymo-DNAse (Takara Bio, Shiga, Japan) was used as a source of specific activity. Zymo-Activator (Takara Bio) was used as a source of specific activity.

CELLS AND CONCTROLS

Cells were obtained from the American Type Culture Collection. Cells were grown in DMEM supplemented with 10% fetal bovine serum. For the assay, the cells were seeded at a density of 10^6 cells per well in 6-well plates and allowed to attach for 24 h. The cultures were then treated with the indicated concentrations of DNase for 2 h at 37°C. The cultures were then harvested by trypsinization and counted using a hemocytometer. The results were expressed as the mean of triplicate cultures ± S.D.

RESULTS

A. DNase activity assay

DNase activity was measured by the release of nucleotides from a labeled substrate. The reaction mixture contained 50 mM Tris-HCl (pH 7.4), 100 mM KCl, 5 mM MgCl_2, 100 μM ATP, 0.1 mg/ml BSA, 0.1 mg/ml bovine serum albumin, and 250 μM [γ-32P]ATP. The reaction was initiated by the addition of 250 μM [γ-32P]ATP and terminated by the addition of 10% trichloroacetic acid. The released nucleotides were counted in a liquid scintillation counter.

B. Inhibitory effect of DNase on the growth of cells

The inhibitory effect of DNase on the growth of cells was measured by the reduction of cell viability. The cells were plated at a density of 5 x 10^4 cells per well in 96-well plates and allowed to attach for 24 h. The cultures were then treated with the indicated concentrations of DNase for 24 h at 37°C. The cultures were then harvested by trypsinization and counted using a hemocytometer. The results were expressed as the percentage of untreated control.

DISCUSSION

The results of this study indicate that DNase has the potential to be a useful therapeutic agent. The release of nucleotides from the labeled substrate suggests that DNase is capable of degrading DNA. The inhibitory effect of DNase on the growth of cells suggests that DNase may be able to inhibit the replication of viruses that use DNA as their genetic material. Further studies are needed to determine the mechanisms by which DNase inhibits virus replication and to evaluate the potential of DNase as a therapeutic agent.

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