Herpes Simplex Virus Non-structural Proteins. IV. Purification of the Virus-induced Deoxyribonuclease and Characterization of the Enzyme Using Monoclonal Antibodies

By LAWRENCE BANKS, DOROTHY J. M. PURIFOY, P-F. HURST,1 R. A. KILLINGTON AND KENNETH L. POWELL*

Department of Microbiology, University of Leeds, Leeds LS2 9JT, U.K. and 1Department of Virology and Epidemiology, Baylor College of Medicine, Houston, Texas 77030, U.S.A.

(Accepted 8 July 1983)

SUMMARY

The alkaline nucleases induced by herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) have been purified from high salt extracts of virus-infected cells. The purification used three types of column chromatography and resulted in apparently homogeneous DNase preparations with good recovery. The enzyme from HSV-2-infected cells has been characterized. It had both exonuclease and endonuclease activity, each with an unusually high pH optimum. The enzyme had an absolute requirement for magnesium which could not be replaced by other divalent cations. Analysis of the sedimentation characteristics and electrophoretic properties of the purified enzyme indicated that it was composed of a single subunit of mol. wt. 85000. The purified HSV-2 enzyme was used as an immunogen to prime BALB/c mice which were used to prepare monoclonal antibodies. Three monoclonal antibodies were shown by several criteria to react with the enzyme. Thus, we were able to confirm that the 85K polypeptide did indeed have nuclease activity. This polypeptide was designated ICSP 22 in earlier studies and is a major polypeptide of virus-infected cells.

INTRODUCTION

Among the relatively few enzymes known to increase in activity after infection of cells by herpes simplex virus (HSV) is a deoxyribonuclease with an alkaline pH optimum (Keir & Gold, 1963; Keir, 1968). Partial purification and characterization indicated that the enzyme is an exonuclease specific for DNA (Morrison & Keir, 1968). More recently, the highly purified enzyme has been shown to possess both endo- and exonuclease activity and to have an active molecular weight of about 68000 (Hoffman & Cheng, 1978, 1979). Furthermore, Strobel-Fidler & Francke (1980) reported the polypeptide composition of their purified alkaline nuclease preparations and indicated that the enzyme was composed of up to four polypeptides of 70000 mol. wt. (70K) to 90K. There have also been reports of the physical map position of the DNase gene (Moss et al., 1979) and of its in vitro translation (Preston & Cordingley, 1982). The latter study showed that a major product of the area of the genome encoding the nuclease activity was an 85K protein. Finally, a mutant with at least one lesion in the DNase gene has been extensively studied (Francke et al., 1978; Moss et al., 1979; Francke & Garrett, 1982). The consensus from these reports is that the enzyme has an essential role in virus replication.

Since this virus enzyme is less well characterized than the other intensively studied HSV enzymes thymidine kinase and DNA polymerase, both of which have been associated with specific virus-induced polypeptides (Honess & Watson, 1974; Powell & Purifoy, 1977), we decided to study the enzyme in detail. In this report we describe a rapid method for purification of the alkaline nuclease. The enzyme obtained by this method is fully characterized and the preparation and use of monoclonal antibodies to the purified enzyme is described. The latter studies demonstrate conclusively that the 85K protein does have the enzymic activity of the purified protein.
**METHODS**

**Cells, medium and viruses.** Human epidermoid carcinoma No. 2 (HEp-2) and African green monkey kidney (Vero) cells used for all experiments were grown in Eagle's medium containing 10% calf serum and 0.025% sodium bicarbonate. The viruses used in this study were the 186 strain of HSV-2 (Rawls et al., 1968) and the HFEM strain of HSV-1 (Watson et al., 1966). Cells and virus were grown as described previously (Purifoy & Benyesh-Melnick, 1975).

**Infection and radiolabelling procedures.** Infection and radiolabelling were done as reported previously by Powell & Courtney (1975). Briefly, batches of 1.2 x 10⁶ HEp-2 cells growing in 80 oz glass roller bottles were inoculated with virus at a multiplicity of infection of 20 p.f.u./cell. Virus was allowed to adsorb to the cells for 1 h at 37 °C. At the end of the adsorption period, the remaining inoculum was decanted and the cell monolayers rinsed twice with fresh medium. Medium containing 3 μCi of ¹⁴C-labelled amino acid mixture (Amersham International) per ml was then added to the monolayers. Cells were harvested at the end of 18 h post-infection by scraping into the medium, and were subsequently collected by centrifugation at 2000 rev/min for 5 min. The cells were washed twice with phosphate-buffered saline (PBS) before being frozen at −70 °C as cell pellets.

**Enzyme extraction and purification procedures**

All procedures were done at 0 to 4 °C. The frozen infected cell pellets were thawed and suspended in extraction buffer (20 mm-Tris-HCl pH 7.5, 0.5 mm-dithiothreitol) at a cell concentration of 1 x 10⁶ to 3 x 10⁷ per ml. The cells were then subjected to ultrasonic disruption and extracted with high salt as described previously (Powell & Purifoy, 1977). The extract was dialysed overnight against several changes of DE buffer [50 mm-Tris-HCl pH 7.5, 0.5 mm-dithiothreitol, 0.2% Nonidet P40 (NP40), 20% glycerol]. After dialysis the extract was clarified by sedimentation at 40000 rev/min for 1 h; the supernatant fluid containing the nuclease activity was used for purification.

**DEAE-cellulose chromatography.** The next step in DNase purification was accomplished by chromatography on a column (2 x 20 cm) of DEAE-cellulose (DE52; Whatman) equilibrated in DE buffer. The cell extract was applied to the column, the column was washed with 2 vol. of DE buffer, and the DNase eluted with a 150 ml gradient of 0 to 0.3 M-KCl in DE buffer. Each fraction from the column was assayed for exonuclease activity and those containing the enzyme were pooled for further use.

**Phosphocellulose chromatography.** The peak of nuclease from the DEAE column was dialysed in DE buffer and applied to a column (2 x 15 cm) of phosphocellulose (P11 cellulose; Whatman). To prevent non-specific adsorption, the column was washed with bovine serum albumin (BSA) at 500 μg/ml in DE buffer and further washed with DE buffer before loading with enzyme. After loading, the column was washed with 2 vol. of DE buffer and further washed, and the nuclease was eluted with a 100 ml gradient of 0 to 0.4 M-KCl in DE buffer. Assays on the column fractions were done exactly as described for DEAE column fractions.

**DNA-cellulose chromatography.** Denatured DNA-cellulose chromatography was as described previously (Purifoy & Powell, 1976). Fractions containing the nuclease from the phosphocellulose column were pooled, adjusted to 500 μg/ml BSA, dialysed against two changes of low-salt buffer (50 mm-KCl), and applied to the DNA-cellulose column. Elution was achieved by the application of a gradient of 0.05 to 0.3 M-KCl in buffer. The column fractions were assayed for nuclease as described for the DEAE-cellulose column fractions. The purified protein was obtained from the eluate. The protein was pooled and dialysed against suitable buffers for each experiment.

**Biochemical tests on the nuclease activity**

**Assays for enzyme activity.** (i) The **alkaline exonuclease** activity was measured throughout the purification procedures. This method was a modification of the method of Morrison & Keir (1968) as reported previously (Purifoy & Powell, 1976). The standard assay mixture contained 50 mm-Tris-HCl pH 9.0, 2 mm-magnesium chloride, 10 mm-2-mercaptoethanol, 10 μg/ml [³H]thymidine-labelled native HEp-2 cell DNA (10000 ct/min/μg) and appropriate amounts of enzyme extracts. For individual tests this assay was modified as necessary. Such modifications are noted as the results are described. (ii) The **endonuclease** activity was detected using simian virus 40 form 1 DNA (SV40 DNA was a kind gift of Dr. J. Butel). Each sample of 0.8 μg of DNA was incubated with 0.5 μg of the enzyme as described above and then analysed by electrophoresis. The digested DNA was mixed with loading buffer (0.1% bromophenol blue, 1% SDS, 0.002 M-EDTA, 60% glycerol) and applied to slots in a 1% agarose gel. After electrophoresis the results were analysed by fluorography (Bonner & Laskey, 1974).  

**Protein assays.** These were done by the Lowry et al. (1951) method.

**Sucrose gradient sedimentation.** This was done by layering samples of enzyme or suitable marker proteins on the same or separate 5 to 20% (w/v) sucrose gradients made in Tris-HCl pH 9.0, 50 mm-KCl, 0.5 mm-dithiothreitol. The gradients were then centrifuged at 40000 rev/min in the SW56 rotor of a Beckman ultracentrifuge for 24 h. The gradients were fractionated by bottom puncture and the various markers assayed.
The DNase was detected either by enzyme assay or by assay for the trichloroacetic acid-precipitable radioactivity, the serum albumin or aldolase markers by the Lowry et al. (1951) assay, or lactate dehydrogenase (LDH) by enzyme assay. DNase was also run with LDH as an internal marker.

Polyacrylamide gel electrophoresis. This was done as described by Powell & Courtney (1975). The results were either analysed by autoradiography or by fluorography (Bonner & Laskey, 1974).

Serological tests

ELISA assays. Enzyme-linked immunoassays were done initially using detergent extracts of infected cells as antigen. Cells infected as noted above were resuspended in 0-1% NP40 (BDH) and 0 1% sodium deoxycholate. The suspension was sonicated in a water bath for 2 min and then clarified by centrifugation at 40000 g for 1 h. The supernatant from cells resuspended at about 10^6 cells/ml was used at an optimal dilution in the ELISA assay. Briefly, the extract was allowed to adsorb to the wells of 96-well plastic plates (Nunc) for 3 h at 37°C in bicarbonate–carbonate buffer pH 9.5 and then incubated overnight at 4°C. The wells were then washed three times with 0.05% Tween 20-0.85% sodium chloride (washing buffer). Suitable dilutions of tissue culture extracts from the cultures with antibody-producing clones were then added to the wells either undiluted or diluted in washing buffer with 5 mg/ml BSA. Antibody was allowed to absorb for 3 h at 37 °C when the plate was again washed three times with washing buffer. Each well was then treated with 0.2 ml of horseradish peroxidase-conjugated anti-mouse IgG (DAKO, Denmark) suitably diluted in washing buffer with BSA. After 1 h at 37 °C the wells were again washed with washing buffer and 0.2 ml of orthophenylenediamine solution (2 mg orthophenylenediamine dissolved in 200 μl methanol and 19.8 ml water and 20 μl 3% hydrogen peroxide) was added. After 1 h at 37°C the yellow colour which developed in positive wells was measured using a Flow Laboratories Titertek Multiscan reader.

Neutralization tests. Neutralization of DNase by antibody was measured using dilutions of each antibody incubated on ice for 12 h with purine enzyme prior to a standard enzyme assay.

Immunoperoxidase tests. Exonuclease-related antigens within infected cells were detected using this test. Coverslips were added to 60 mm Petri dishes before seeding them with Vero cells and allowing growth for 24 h. The cultures were infected with virus at about 5 p.f.u./cell and incubated for 6 h. Coverslips were washed with PBS, fixed with methanol in the dishes overnight at —20 °C and then rehydrated in PBS as required. The cells were reacted with primary antibody for 30 min at 20 °C and washed vigorously three times in PBS. Secondary rabbit anti-mouse IgG (peroxidase conjugate, DAKO) was added and allowed to react for 30 min at 20°C. After this incubation the cells were washed three times with PBS and the peroxidase-conjugated antibody which remained was detected with Hanker–Yates reagent (75 mg of Hanker–Yates reagent (Polysciences Ltd., Northampton, U.K.) dissolved in 50 ml 0.01% hydrogen peroxide in 0.1 M-Tris–HCl buffer pH 7.5). After 10 min of reaction the cells were washed in PBS, water, 50% and 75% ethanol and mounted for viewing.

Preparation of monoclonal antibodies

Inbred mice (BALB/c) were inoculated twice via the footpad with 2 to 3 μg of purified alkaline exonuclease in incomplete Freund’s adjuvant with a 14-day interval between inoculations. Three days prior to cell fusion, mice were given an intravenous boost of exonuclease via the tail vein. The cell fusion technique was essentially as described by Kennett et al. (1978). Spleens were aseptically removed from the immunized mice, chopped and passed through a stainless steel screen (60-mesh). The cells were collected in RPMI medium (Flow Laboratories) with 10% heat-inactivated foetal calf serum (FCS) and cell clumps were allowed to settle at room temperature for 5 min. The cell suspension was decanted into a 15 ml plastic centrifuge tube and underlaid with 2 ml FCS. Following sedimentation at 800 g for 10 min in an MSE centrifuge the cell pellet was treated with 5 ml ice-cold ammonium chloride (0-17 M) for 5 min and again underlaid with FCS and centrifuged. The cell pellet was resuspended in RPMI (at 37 °C) with 10% FCS and mixed with Sp2/0 Ag14 cells (Schulman et al., 1978; Killington et al., 1981) at a ratio of 10:1. The mixture of cells was washed twice in RPMI without FCS (at 37 °C) and mixed with 1 ml 30% polyethylene glycol (PEG) 1000 at 37°C in serum-free medium. After 1 min of PEG treatment the cells were pelleted by centrifuging at 800 g for 10 min and washed in RPMI with 10% FCS and again pelleted. Finally, the cells were resuspended in Hy-HAT medium (Kennett et al., 1978) and distributed into the wells of Linbro panels (Flow Laboratories) at 4 x 10^5 cells/well. After 24 h of incubation, the medium in the wells was diluted with a further 1 ml of Hy-HAT medium. After incubation at 37 °C in an atmosphere of 5% CO₂, the cells were found to produce hybrid colonies 5 to 8 days after fusion. Colonies of hybrid cells were transferred into new Linbro panel wells as soon as they were 2 to 3 mm in diameter. Once confluent, the cultures were transferred to 25 cm² plastic bottles in 5 ml of Hy-HAT medium. As soon as possible after initial isolation the hybrids were cloned by limiting dilution at < 1, 1, 2 or 5 cells/well in a 96-well tissue culture plate. The single cell origin of clones was ascertained by determining the cell multiplicity at early times after seeding wells. Clones were tested for the production of antibody at various times by the methods noted in the text and detailed in this section.
Production of ascitic fluid. BALB/c mice, 6 to 12 weeks old, were sensitized with pristane (2,6,9,14-tetramethylpentadecane, Aldrich Chemicals; 0.5 ml/animal) 1 week prior to injection with hybrid cells to produce ascites tumours. Generally, 10^7 cells were used and the mice were sacrificed when the growth of the tumours became obvious. The antibody was purified from ascitic fluid using DEAE-cellulose chromatography as described previously (Fahey & Horbett, 1959).

Immunoprecipitation. The antigen used for immunoprecipitation experiments was prepared from cells infected as described for enzyme purification but labelled with 1μCi/ml of a mixture of 14C-labelled leucine, isoleucine and valine (Amersham International). The cells were extracted with high salt as described for the enzyme experiments and the extract dialysed against PBS. Just prior to use the antigen was clarified by sedimentation at 100,000 g for 1 h. A 100μl amount of the antigen preparation was reacted with 50 μl of various dilutions of the antibody (4°C for 12 h). The resulting immune complexes were precipitated by addition of 75 μl of washed formaldehyde-fixed Staphylococcus aureus (a kind gift from Drs R. E. Randall and R. W. Honess). After 20 min at 20°C the cells and adhering complexes were collected by sedimentation at 2000 rev/min for 15 min. The pellet was washed six times in PBS, and the immune complexes were dissolved in disruption buffer and used for electrophoresis.

Immunoadsorbent chromatography. Five to 10 mg of monoclonal antibody was coupled to 1 g of cyanogen bromide-activated Sepharose 4B (Pharmacia) according to the manufacturers' instructions. An extract identical to that used for immunoprecipitation was applied to the column and was allowed to recycle overnight. The column was then washed extensively with PBS, 0.3 M-KCl and then 3.0 M-KCl. The final wash was with 3 M-potassium thiocyanate.

RESULTS

Purification of enzyme

Exonuclease activity reaches a maximum in HSV-infected cells at about 12 to 18 h post-infection (Powell & Purifoy, 1977). HEp-2 cells that had been infected with HSV-1 or HSV-2 for 18 h were therefore used for enzyme purification. The cells were extracted with high salt, and the extract was chromatographed on DEAE-, phospho- and DNA-cellulose as described in Methods. The purification obtained for a representative batch of HSV-2 enzyme is described in Fig. 1 and Table 1. The enzyme was efficiently extracted with high salt and bound to DEAE-cellulose. As can be seen in Fig. 1, the enzyme eluted from DEAE-cellulose as a single peak with 0.1 to 0.15 M-KCl. DEAE-cellulose-purified enzyme was applied to phosphocellulose to which it bound and from which it was eluted with 0.1 to 0.3 M-KCl. Finally, the enzyme was applied to a column of denatured DNA-cellulose to which it bound strongly, eluting with between 0.18 and 0.25 M-KCl. The procedure resulted in about 200-fold purification of the enzyme with good recovery (Table 1).

Polypeptide constituents of enzyme preparations

The polypeptide constituents of the enzyme at various stages of purification were examined by polyacrylamide gel electrophoresis (Fig. 2). The complicated profile of polypeptides present in whole cell extracts was reduced to a single polypeptide in the peak of enzyme activity from the DNA-cellulose column. This polypeptide was specifically labelled after virus infection of cells and it was the only polypeptide found by autoradiography of the stained gel. Thus, in the case of the HSV-2 enzyme the purified preparations contained a single polypeptide which was specific for virus-infected cells; this polypeptide was the ICSP 22 polypeptide we have described previously (Powell & Courtney, 1975). We shall return to the relation of the polypeptide to enzyme activity in a later section of the paper. In the case of the HSV-1 enzyme, a contaminating polypeptide was always present in even the best preparations (Fig. 2). This was of similar size to the second polypeptide described by Strobel-Fidler & Francke (1980) and may represent a breakdown product of the enzyme.

Properties of the purified enzyme

Divalent cation requirements

The effects of various divalent cations on the activity of the purified enzyme are shown in Fig. 3(a). Magnesium ions were absolutely required for optimal enzyme activity, with an optimum
Fig. 1. Purification of HSV-2-induced alkaline exonuclease. The figure shows the behaviour of the enzyme (assayed under standard condition, see Methods) during chromatography on (a) DEAE-, (b) phospho- and (c) DNA-cellulose. ——, DNase activity in each fraction; ·····, KCl concentration.

Table 1. Purification of enzyme

<table>
<thead>
<tr>
<th>Stage of purification</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Enzyme activity*</th>
<th>Fold purification</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cells</td>
<td>32</td>
<td>110·4</td>
<td>1073</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Cell extract</td>
<td>34</td>
<td>71·1</td>
<td>2712</td>
<td>2·5</td>
<td>160</td>
</tr>
<tr>
<td>Peak from DEAE-cellulose</td>
<td>50</td>
<td>55·5</td>
<td>2570</td>
<td>2·4</td>
<td>120</td>
</tr>
<tr>
<td>Peak from phosphocellulose</td>
<td>20</td>
<td>3·6</td>
<td>15236</td>
<td>14·2</td>
<td>45</td>
</tr>
<tr>
<td>Peak from DNA-cellulose</td>
<td>8</td>
<td>0·4</td>
<td>2 × 10^5</td>
<td>186·4</td>
<td>67</td>
</tr>
</tbody>
</table>

* Ct/min released per μg protein per 15 min at 39 °C.

Mg²⁺ concentration of 2 to 8 mM. The following cations, Ca²⁺, Zn²⁺ and Cu²⁺, could not replace Mg²⁺; whereas Mn²⁺ was minimally able to substitute for it, Mg²⁺ at high concentrations inhibited the activity of the enzyme (data not shown).
Purification of HSV-induced DNase

Fig. 2. The polypeptide constituents of nuclease preparations. (a) Dye-stained polypeptides present at each stage of the purification of the HSV-2-induced DNase. Stage 1 (lane 1) is the whole cell sample, stages 2, 3 and 4 the nuclease-containing fractions from DEAE-, phospho- and DNA–cellulose columns. (b) Autoradiograph of the radiolabelled polypeptides found in preparations 1 and 4. (c) Polypeptides present in a similar preparation of HSV-1-induced nuclease. In each case the samples were run on 8% single concentration polyacrylamide gels as described in Methods.

Effect of KCl on enzyme activity

Some KCl was present in purified enzyme preparations such that under the standard conditions about 10 mM-KCl was present in the assay. The optimal KCl concentration for DNase activity was 15 mM and increasing concentrations of KCl above this level gradually inhibited the enzyme (Fig. 3b).

Effect of reducing agents

The reducing agents 2-mercaptoethanol and dithiothreitol are commonly added to nuclease assays but neither had any effect on the activity of the herpes virus alkaline exonuclease, suggesting a lack of requirement for sulphydryl groups in this enzyme (Fig. 3c). However, in experiments similar to those reported by Hoffman & Cheng (1978) we found the enzyme to be sensitive to p-hydroxymercuribenzoate (50% inhibition at 1.9 mM p-OHMB).

Fig. 3. Properties of the HSV-2-induced DNase. The enzyme activity was measured under a variety of conditions. (a) The concentration of divalent cations was varied and the activity of the enzyme with each was determined: ■, Mg²⁺; △, Mn²⁺; ○, Cu²⁺; ●, Zn²⁺; □, Ca²⁺. (b) The effect of KCl on the enzyme was measured as in (a). (c) The effect of reducing agents on the enzyme activity was examined: ●, dithiothreitol; ○, 2-mercaptoethanol. (d) The effect of pH on the enzyme was measured: ●, single-stranded DNA; ■, double-stranded DNA as template. (e) Activity of nuclease on closed circular SV40 DNA: either (i) DNase 1 or HSV-2 DNase at pH 7 (ii) or pH 9 (iii) was assayed using this DNA; (iv) blank. (f) Determination of the size of active HSV-2 DNase. The enzyme was sedimented as described in Methods and the enzyme activity (●) in each fraction was determined. The purified enzyme was also radiolabelled; therefore, the radioactivity (▲) in each fraction was measured. The reference standards run in the gradient or in parallel runs are indicated above the enzyme peak: LDH, 140K; BSA, 69K.
**HSV DNase monoclonal antibodies**

---

![Graph (a) showing the effect of divalent cation concentration on the activity of HSV DNase monoclonal antibodies.](image)

![Graph (b) showing the effect of KCl concentration on the activity of HSV DNase monoclonal antibodies.](image)

![Graph (c) showing the effect of reducing agent concentration on the activity of HSV DNase monoclonal antibodies.](image)

![Graph (d) showing the effect of pH value on the activity of HSV DNase monoclonal antibodies.](image)

![Graph (e) showing the percentage of SV40 DNA hydrolyzed by HSV DNase monoclonal antibodies.](image)

![Graph (f) showing the fraction number distribution of LDH and BSA.](image)
Table 2. Isolation of monoclonal antibodies to the HSV-2 nuclease

<table>
<thead>
<tr>
<th>Polyclonal cells tested (%)</th>
<th>Nomenclature</th>
<th>Number of monoclones tested (%)</th>
<th>Number of positive monoclones</th>
<th>Antibody subtype*</th>
<th>Antibody titres†</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>Q</td>
<td>40</td>
<td>100</td>
<td>Q1, Q2-Q40</td>
<td>Q-IgG2a</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>22</td>
<td>100</td>
<td>V1, V2-V22</td>
<td>V-IgG2a</td>
</tr>
<tr>
<td></td>
<td>T2T</td>
<td>9</td>
<td>100</td>
<td>T2T1,T2T2-T2T9</td>
<td>T-IgG1</td>
</tr>
</tbody>
</table>

* Subtype determined by ELISA assay using rabbit anti-mouse IgG subtype sera (Miles Laboratories).
† Reciprocal of endpoint dilution by ELISA assay of selected monoclonal antibodies.

Effect of pH on enzyme activity

The effect of pH on the digestion of single- or double-stranded DNA is shown in Fig. 3(d). The pH optimum of the enzyme appears to be reached at a pH between 9 and 10, although an inhibiting pH value could not be reached. The pH optimum of the enzyme was the same for the degradation of either single- or double-stranded DNA. The results shown were obtained with Tris-buffered reaction mixtures. With other buffers of higher pKa, inhibiting pH values could not be reached (up to pH 10.5).

Exo- and endonucleolytic activities of the enzyme

As first reported by Hoffman & Cheng (1978) the enzyme has both exonucleolytic and endonucleolytic activity (Fig. 3(e)). SV40 DNA was rapidly degraded by the enzyme at pH 9-0, but very slowly at lower pH. The pH optimum for this activity is the same as that reported above for the exonuclease activity.

Sedimentation behaviour of the enzyme

The behaviour of the enzyme (from DNA-cellulose preparations) on sedimentation in 5 to 20% sucrose gradients is shown in Fig. 3(f). The enzyme sedimented slightly faster than BSA and slightly slower than lactate dehydrogenase. We estimate from these data a molecular weight for the active enzyme of about 80000. The behaviour of the enzyme did not alter when marker proteins were included in the same gradient. All the radioactivity in the final enzyme preparations co-sediments with the enzyme activity.

Monoclonal antibody studies

Preparation of monoclonal antibodies to the purified nuclease

Monoclonal antibodies to the HSV-2-induced nuclease were prepared as described in Methods using the purified nuclease derived from DNA-cellulose columns. After the fusion procedure, polyclones were allowed to develop. Supernatant fluids from wells containing such growing cells were tested by ELISA for antiviral antibody (Table 2). Polyclonal cells shown to be capable of antibody production were then cloned by limiting dilution. Each resulting monoclonal cell line was tested again for antibody production. In the results of the typical fusion shown in Table 2 all such monoclonal antibodies were positive by the ELISA assay with infected cell extracts, suggesting that the cells may have been homogeneous before cloning took place. Selected monoclones from these 71 were used in the subsequent experiments to characterize the alkaline nuclease.

Reaction of monoclonal antibodies with specific antigens

Table 3 summarizes the reaction of the three series of monoclonal antibodies with various antigen preparations. It is clear that each of the antibody preparations has similar properties, i.e. each reacts with infected and not with uninfected cell extracts and each reacts with purified enzyme preparations. Each is capable of the immunoprecipitation of an 85K polypeptide from virus-infected cells. The antibody preparations differ in that only one antibody (from the Q series) is capable of neutralizing enzyme activity. Ascitic fluid from control mice inoculated with SP2/0 Ag14 cells had no effect on the enzyme.
Use of monoclonal antibodies to locate the exonuclease in infected cells

Monoclonal antibodies from each series were used to detect the HSV-2 nuclease in infected cells using the indirect immunoperoxidase assay. In each case the nuclease was detected almost exclusively in the nucleus of infected cells (Fig. 4). This result differed from those obtained by cell fractionation (Morrison & Keir, 1968) which showed a much higher level (about 80%) of enzyme in the cytoplasm. The monoclonal antibodies showed no reaction with uninfected cells. Similarly, ascitic fluid from mice injected with SP2/0 Ag14 cells did not react with infected cells.

Use of monoclonal antibody to purify the DNase polypeptide

The reaction of monoclonal antibody with infected cell proteins was assessed using immunoadsorption columns. Analysis of a typical run of a Q1 monoclonal antibody column is

---

**Table 3. Reaction of monoclonal antibodies with specific antigens**

<table>
<thead>
<tr>
<th>Monoclonal antibody series</th>
<th>Reaction with infected cell extract*</th>
<th>Reaction with uninfected cell extract*</th>
<th>Reaction with purified HSV-2 nuclease*</th>
<th>Reaction with 85K polypeptide†</th>
<th>Neutralization of HSV-2-induced DNase activity‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>V</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>T</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Control §</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* ELISA test using the appropriate antigen: -, no reaction at 1/100 dilution; +, a reaction with at least 100-fold less antibody.
† By immunoprecipitation of infected cell extracts.
‡ Neutralization means the loss of 50% or more of enzyme activity on incubation with the monoclonal antibody.
§ Ascitic fluid from mice inoculated with SP2/0 Ag14 cells.
Fig. 5. Reaction of Q1 monoclonal antibody with HSV-infected cell polypeptides. Q1 antibody immobilized on Sepharose was allowed to react with infected cell polypeptides by the column technique (see Methods). Proteins which were not bound and passed through the column (b), and those which were bound to the column and released using 3 M-\(\text{NaCl}\) (a) were compared by polyacrylamide gel electrophoresis of the appropriate fractions. An autoradiogram of the gel is shown, with mol. wt. markers.

shown in Fig. 5. Although most of the infected cell proteins pass straight through the column, a small proportion is bound and elutes with high (3-0 M) salt concentrations. The material bound to the column consists entirely of the 85000 mol. wt. polypeptide found in virus DNase preparations (Fig. 5). The purification obtained using this method appears as good as that obtained using our three-column procedure (Fig. 2).

DISCUSSION

The preparation of a monoclonal antibody that precipitates an 85K protein from infected cells, neutralizes and reacts with purified alkaline nuclease enzyme in ELISA assays taken together with the data of Strobel-Fidler & Francke (1980) and of Preston & Cordingley (1982) demonstrate conclusively that the HSV-2 protein we have referred to previously as ICSP 22 [ICP 19 in the Honess & Roizman (1973) nomenclature for HSV-1 polypeptides] is the alkaline exonuclease. This raised several interesting points. ICSP 22 is a major polypeptide found in HSV-infected cells which can be identified even in stained polyacrylamide gels of such cells.
Thus, the virus not only makes an unusual exonuclease with a very high pH optimum (which may relate to the artificial nature of the in vitro assay), but also produces it in very large amounts. The maximum purification that could be obtained for such a major protein must be in the range of 200- to 500-fold. Thus, the early estimate by Hoffman & Cheng (1978) of a >1350-fold purification must be an overestimate.

The properties of the purified enzyme are remarkably consistent in the various preparations that have been reported. All the results show a very high pH optimum, an inhibition by high salt concentrations and a dependence on magnesium ions. There are still some discrepancies in the data. Firstly, the sizes reported for the active enzyme are different. Our data and those of Hoffman & Cheng (1978, 1979) point to an active enzyme of about the same size as the polypeptide chain that constitutes the enzyme molecule. Strobel-Fidler & Francke (1980), on the other hand, estimated an active size of the enzyme of about half this value. They suggested that the earlier estimate of the size of the enzyme was in error due to its binding to marker protein in the gradient. In the current work, we found that the enzyme had a consistent sedimentation rate in the presence or absence of marker protein in the gradient. Thus, it seems likely that the lower value obtained by Strobel-Fidler & Francke (1980) may be due to degradation of the enzyme.

Our results on the salt requirements of the enzyme demonstrate that it requires magnesium and this could be only partially substituted with manganese. Strobel-Fidler & Francke (1980) found a small amount of activity with this salt (Mn$^{2+}$), and Hoffman & Cheng (1978) found rather higher activity. This could obviously be due to variation in the properties of the salt preparations or the enzyme used.

Significant inhibition of the nuclease has been observed with KCl concentrations above 15 mM. This inhibition may help to explain the stimulation of HSV DNA polymerase activity which is observed on assay of crude cell lysates under high salt conditions but is not so marked with purified enzyme (Powell & Purifoy, 1977).

The preparation of monoclonal antibodies to the exonuclease should prove extremely useful in further studies of the enzyme. Thus, they already have confirmed that the 85K polypeptide has nuclease activity; they can now be used to recognize that part of the polypeptide which is the active site of the enzyme. They should also prove invaluable in the isolation and characterization of mutants with defects in the nuclease gene. The strong association of the nuclease with the cell nucleus and its unusual characteristics in vitro both suggest that the protein functions in association with other virus-induced proteins in the replication of virus DNA. The monoclonal antibodies reported here should also prove useful in the study of this complex.

This work was supported by a grant from the Medical Research Council. We would like to thank Ms S. Graham for technical assistance and Professor D. H. Watson for reading the manuscript.

REFERENCES


(Received 5 May 1983)