The herpes simplex virus type 1 alkaline nuclease is not essential for viral DNA synthesis: isolation and characterization of a lacZ insertion mutant

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Herpes simplex virus type 1 (HSV-1) encodes a novel enzyme activity, the alkaline nuclease, whose precise role in the viral replication cycle remains obscure. The alkaline nuclease gene corresponds to the UL12 open reading frame, which is predicted to encode a protein of 626 amino acid residues. We describe the isolation and characterization of a null mutant of the gene for the viral alkaline nuclease in which 917 bp from the N-terminal half of the gene (corresponding to residues 70 to 375) were deleted and replaced by the insertional mutagen ICP6::lacZ. The resulting mutant virus, AN-1, was propagated in helper cells (S22) which express the wild-type version of the alkaline nuclease gene. Mutant AN-1 growth in Vero cells is severely restricted, although small amounts of infectious virus are produced. On the other hand, wild-type levels of viral DNA and late viral proteins are expressed in virus AN-1-infected Vero cells. These results indicate that the HSV-1 alkaline nuclease gene product is not essential for viral DNA synthesis but may play a role in the processing or packaging of viral DNA into infectious virions. Possible roles in the viral infectious cycle will be discussed.

Introduction

Infection of mammalian cells with herpes simplex virus type 1 or type 2 (HSV-1 or HSV-2) results in the induction of a novel enzyme activity, alkaline nuclease (Hoffmann & Cheng, 1978; Keir & Gold, 1963). Alkaline nuclease purified from HSV-infected cells exhibits exo- and endonuclease activities and differs from host cell nucleases in its high pH optimum (Francke et al., 1978; Hoffmann, 1981; Hoffmann & Cheng, 1978, 1979; Morrison & Keir, 1968; Strobel-Fidler & Francke, 1980). Two lines of evidence indicate that alkaline nuclease is virus-encoded: the isolation of a temperature-sensitive (ts) mutant affecting the activity of the enzyme (Francke et al., 1978) and the demonstration that microinjection of HSV DNA fragments into Xenopus laevis oocytes results in the production of alkaline nuclease activity (Preston & Cordingley, 1982). HSV alkaline nuclease is a relatively abundant phosphoprotein in infected cells and has an apparent Mr of 85000 (85K) (Banks et al., 1983, 1985). The alkaline nuclease localizes to sites in the nucleus of infected cells which are distinct from the putative replication compartments in which HSV replication proteins ICP8 and DNA polymerase are found (de Bruyn Kops & Knipe, 1988; Knipe, 1989; Puvion-Dutilleul & Pichard, 1986; Randall & Dinwoodie, 1986).

The nuclease gene maps between 0-168 and 0-175 map units on the genomes of HSV-1 and HSV-2 (Banks et al., 1985; Costa et al., 1983; Preston & Cordingley, 1982; Wathen & Hay, 1984). The mRNA encoding alkaline nuclease has been mapped to a 2.3 kb mRNA which is a member of a family of unspliced 3'-coterminal mRNAs (Costa et al., 1983). The DNA sequence of this region reveals an open reading frame (ORF) encoding 626 amino acids, designated UL12 within the 2.3 kb mRNA (Costa et al., 1983; Draper et al., 1986; McGeoch et al., 1986, 1988b). The amino acid sequences of the HSV-1 and -2 nuclease genes are clearly related to sequences found in other herpesviruses, such as the Epstein–Barr virus BGLF5 gene (Baer et al., 1984) and varicella-zoster virus gene 48 (Davison & Scott, 1986).

The precise role of the alkaline nuclease in the viral life cycle is unknown. Studies with an HSV-2 ts mutant have been complicated by the fact that this mutant is somewhat leaky (exhibits residual nuclease activity at the non-permissive temperature) and may contain more than one mutation; investigators have reached different conclusions regarding whether the alkaline nuclease gene is essential for viral DNA synthesis (Franke & Garrett, 1982; Moss, 1986; Moss et al., 1979). To circumvent problems with potential by leaky ts mutants,
we have used an alternative approach for the isolation of mutations in targeted genes. This strategy consists of two components: (i) the isolation of host cell lines containing HSV DNA which are able to support the growth of potentially lethal null mutants and (ii) the construction of null mutants using an insertional mutagen consisting of the lacZ gene of *Escherichia coli* under the control of the regulatory elements of the large subunit of ribonucleotide reductase (ICP6). We have used this strategy to examine the roles of several HSV genes in DNA replication, including ribonucleotide reductase (Goldstein & Weller, 1988a), UL8 (Carmichael & Weller, 1989), UL9 (Carmichael et al., 1988) and UL52 (Goldstein & Weller, 1988b). In this report we describe the isolation of a deletion/insertion mutant in the alkaline nuclease gene. Our results indicate that the HSV-1 alkaline nuclease activity is not essential for viral DNA synthesis but may play a role in the processing or packaging of viral DNA into infectious virions.

**Methods**

**Cells and viruses.** African green monkey kidney cells (Vero; ATCC) were grown and maintained as described previously (Weller et al., 1983). S22 cells containing approximately five copies of the right-hand portion of the EcoRI D fragment (coordinates 0-086 to 0-194; sequence coordinates 12593 to 29338) of the HSV-1 genome (Carmichael et al., 1988) were grown in the presence of 250 μg/ml G418 (Geneticin; Gibco Laboratories). The KOS strain of HSV-1 was used as the wild-type virus. Viruses were propagated and assayed as described previously (Schaffer et al., 1978).

**Plasmids and bacteria.** The plasmid pSG10, containing the EcoRI D fragment of HSV-1 strain KOS (See Table 3 for coordinates), was described previously (Goldin et al., 1981); the construction of pSG10-BD2, containing a 3-1 kb BamHI–HindIII fragment (see Fig. 1 and Table 3), was also described previously (Weller et al., 1987). [DNA sequence information indicates that a BamHI site is present at position 24890 in HSV-1 strain KOS but not in strain 17 (L. Shao & S. K. Weller, unpublished data)]. Plasmid pBS-BD2 was constructed by inserting the BamHI–HindIII fragment from pSG10-BD2 into the vector Bluescribe (Stratagene). Plasmid pTZDN7, containing a 2.3 kb fragment of HSV-1 strain KOS (See Table 3), was also described previously (Goldstein & Weller, 1988b). Virus pellets were lysed by the addition of SDS and proteinase K to final concentrations of 0.1% and 100 μg/ml, respectively. Tubes were inverted gently and incubated at 37°C for 5 h. Samples were dialysed and concentrated, using an autoclaved Schleicher and Schuell Model UH020/2A microdialysis apparatus at room temperature, against four changes (60 min each) of 20% polyethylene glycol (PEG) 8000 (Sigma) in sterile Tris–EDTA (TE) buffer (Longmire et al., 1987). Sample volumes decreased approximately 10-fold during PEG dialysis. Desalting was accomplished by dialysis against two or three changes (30 min each) of sterile TE buffer at room temperature. DNA samples were carefully transferred (to avoid shearing) to sterile tubes and stored at -70°C.

**Isolation and analysis of viral and cellular DNAs.** Infectious virus DNA was isolated from purified virions as described (Goldstein & Weller, 1988b). Virus pellets were lysed by the addition of SDS and proteinase K to final concentrations of 0.1% and 100 μg/ml, respectively. Tubes were inverted gently and incubated at 37°C for 5 h. Samples were dialysed and concentrated, using an autoclaved Schleicher and Schuell Model UH020/2A microdialysis apparatus at room temperature, against four changes (60 min each) of 20% polyethylene glycol (PEG) 8000 (Sigma) in sterile Tris–EDTA (TE) buffer (Longmire et al., 1987). Sample volumes decreased approximately 10-fold during PEG dialysis. Desalting was accomplished by dialysis against two or three changes (30 min each) of sterile TE buffer at room temperature. DNA samples were carefully transferred (to avoid shearing) to sterile tubes and stored at -70°C.

**DNA to be analysed by restriction enzyme digestion was isolated as described above except that samples were extracted three to five times with phenol: chloroform: isooamyl alcohol (25:24:1) and precipitated with ethanol. Plasmid or viral DNA was digested with restriction endonuclease(s) as directed by the manufacturer, separated by agarose gel electrophoresis in Tris–borate–EDTA buffer and transferred to a GeneScreen-Plus nylon membrane (New England Nuclear) as recommended by the supplier. Recombinant DNAs used for probes for hybridization were labelled as described by Feinberg & Vogelstein (1983).**
Marker rescue and marker transfer. Marker rescue and marker transfer experiments were performed as described by Goldstein & Weller (1988b). For marker transfer of the deletion/insertion mutation into HSV-1 strain KOS DNA, 200 to 1000 infectious units (approximately 1 μg) of wild-type viral DNA were cotransfected with a 10-fold molar excess of the recombinant plasmid. The 7-kb insert containing the alkaline nuclease gene with the ICP6 lacZ insertion was excised from pAN-1 by digestion with NruI. This fragment was used with infectious HSV-1 strain KOS DNA to cotransfect S22 cells. When the c.p.e. was maximal, the virus was harvested and titrated on S22 cells. Plaques were stained simultaneously with neutral red and the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) as described (Goldstein & Weller, 1988a). Blue plaques were purified three times on S22 cells before stocks were made.

Synthesis of viral DNA in infected cells. Viral DNA synthesis was analysed as described previously (Aron et al., 1975) except that proteinase K was used instead of pronase. The non-permissive condition was growth in Vero cells and the permissive condition was analysed as described previously (Aron et al., 1975). Vero cells were infected with HSV-1 strain KOS or virus AN-1 at an m.o.i. of 10. At 12 h post-infection (p.i.), the cells were washed in NTM buffer (150 mM-NaCl, 50 mM-Tris-HCl pH 7.5, 2 mM-2-mercaptoethanol) and scraped into 2 ml NTM buffer. Cells from each plate were pelleted at 500 g for 10 min at 4 °C and cell pellets were resuspended in 200 μl of 20 mM-Tris-HCl pH 7.5 containing 5 mM 2-mercaptoethanol. Cells were disrupted by sonicating twice for 30 s in an ice water-cooled Heat Systems sonifier cell disruptor, and samples were adjusted to final salt concentrations of 4 mM-MgCl₂ and 100 mM-KCl. Cell debris was removed by centrifugation for 15 min at 1500 g. Nuclease assays were performed in 100 μl reaction mixtures containing 50 μl crude extract, 2.4 μg of 3H-labelled E. coli DNA (8.3 × 10⁸ c.p.m./μg), 20 μg unlabelled salmon sperm DNA, 50 mM-Tris-HCl pH 9.0, 2 mM-MgCl₂ and 10 mM-2-mercaptoethanol. Digestion of labelled DNA in HSV-1 strain KOS-infected extracts was complete in the presence or absence of the unlabelled DNA. Reactions were carried out at 37 °C and 14 μl aliquots were withdrawn at 0, 10 and 30 min. The release of acid-soluble 3H-labelled nucleotides was measured as described by Morrison & Keir (1968). Results are presented as 3H c.p.m. rendered acid-soluble from labelled high M, DNA. Each reaction contained 50 μg of cellular protein, corresponding to approximately 1.25 × 10⁹ cells.

Analysis of viral proteins. Vero or S22 cells were infected with HSV-1 strain KOS or mutant virus at an m.o.i. of 10 p.f.u. per cell. Cells were labelled at 5 h p.i. with [35S]methionine as described previously (Weller et al., 1987). At 16 h p.i. cells were harvested and the samples subjected to electrophoresis as described previously (Goldstein & Weller, 1988a).

Immunoblotting of HSV-infected cell extracts. Specific polyclonal antisera raised in rabbits against a fusion protein containing β-galactosidase and a portion of the alkaline nuclease gene were generously provided by Dr M. Challberg (NIH, Bethesda, Md., U.S.A.). Vero cells were infected with HSV-1 strain KOS at an m.o.i. of 5 p.f.u./cell. Cells were harvested at 12 h p.i., resuspended in 1 ml buffer containing 0.05 M-Tris-HCl pH 7.5, 0.15 M-NaCl, 0.1% SDS, 1% sodium deoxycholate and 1% Triton X-100 with five protease inhibitors (0.1 mM-TPCK, 0.1 mM-TLCK, 0.5 mM-PMSF, 250 kallikrein units/ml leupeptin and 2% aprotinin) and immediately boiled for 10 min. The extracts were then sonicated, clarified by centrifugation and stored at -20 °C. Aliquots (50 μl) were subjected to electrophoresis on 9% SDS-polyacrylamide gels (see above) at 40 mA. Proteins from the gels were electrophoretically transferred to nitrocellulose filters as described by Towbin et al. (1979). The nitrocellulose filters were then incubated with a 1:500 dilution of the specific antibodies described above and the immunoreactive protein was detected by staining with a goat anti-rabbit antibody conjugated to alkaline phosphatase (nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were purchased from Promega Biotech).

Results

Ts13, a mutant of HSV-2, possesses at least two distinct mutations, one affecting the induction of alkaline nuclease activity (mapping between 0.12 and 0.21) and one affecting virion thermostability (mapping between 0.64 and 0.70) (Chartrand et al., 1981; Moss et al., 1979; Timbury, 1971). Since revertants of ts13 which still contain the nuclease mutation (such as mutant 4-8) grew fairly well at 38 °C, it appeared that the HSV nuclease activity was not essential for viral growth (Moss et al., 1979). However, in subsequent experiments using higher non-permissive temperatures, it was discovered that these revertants were actually compromised for growth and DNA synthesis at 38.5 °C (Francè & Garrett, 1982), suggesting that whereas alkaline nuclease was not absolutely essential for viral DNA synthesis it appeared to be required for optimal DNA synthesis. Moss (1986) subsequently reported that mutant 4-8 was even more compromised in viral DNA synthesis and DNase activity if grown at 39.2 °C. Marker rescue of the ts nuclease lesion with the 16-7 kb EcoRI D fragment restored wild-type levels of viral nuclease activity, virus DNA synthesis and virus growth (Moss, 1986). These results led to the conclusion that HSV nuclease activity is essential for virus growth and viral DNA synthesis (Moss, 1986).

To extend these studies to HSV-1 and to provide further genetic evidence concerning the role of alkaline nuclease in viral DNA replication, we set out to isolate a null mutant in the HSV-1 alkaline nuclease gene which would express no residual activity under non-permissive growth conditions. We previously reported the isolation and characterization of a cell line (S22) which contains the DNA for the alkaline nuclease as well as other HSV genes at the left end of U₁ (Carmichael et al., 1988; Carmichael & Weller, 1989). S22 cells were made by cotransfection of Vero cells with pSV2neo and a clone containing the HSV-1 EcoRI D fragment (coordinates 0.086 to 0.194). Southern analysis has indicated that S22 cells contain approximately five copies of the DNA encoding the alkaline nuclease (Carmichael et al., 1988); thus S22 should be a suitable host cell for null mutants in the alkaline nuclease gene.
Isolation of a deletion/lacZ mutant in the alkaline nuclease gene

The plasmid pAN-1 was constructed such that 917 bp from the N-terminal half of the gene (corresponding to residues 70 to 375 of ORF UL12) was replaced by the insertional mutagen ICP6::lacZ (see Fig. 1). The ICP6::lacZ cassette contains the structural gene encoding β-gal under the control of the regulatory region of ICP6. Genes under the control of this regulatory region can be induced by the virion stimulatory protein, VP16, and also by the trans-inducing protein, ICP0 (L. Zhu & S. K. Weller, unpublished results). In this construct, β-gal can be induced to relatively high levels of expression (Goldstein & Weller, 1988b). Introduction of the altered alkaline nuclease gene in pAN-1 into the viral genome results in the disruption of the alkaline nuclease gene; a stop codon at the end of the lacZ gene ensures that the remaining portion of the alkaline nuclease is not expressed. Marker transfer experiments with pAN-1 and infectious wild-type DNA in S22 cells were carried out as described in Methods. The use of the lacZ gene as an insertional mutagen facilitated screening for recombinant virus because β-gal activity is readily detected by the formation of blue colour after staining with X-gal. Recombinant viruses (approximately 0.1%) were identified and two independently isolated recombinant viruses, AN-1 and AN-2, were plaque-purified on S22 cells. AN-1 was used in the current studies but AN-2 exhibited identical phenotypic and genetic characteristics.

Restriction analysis of AN-1

To confirm that AN-1 contains the ICP6::lacZ cassette at the appropriate position, DNAs from HSV-1 strain KOS, virus AN-1 and plasmids pBS-BD2 and pAN-1 were digested with BglII and HindIII and subjected to gel electrophoresis and Southern blot hybridization as described in Methods. Duplicate filters were hybridized to either the 32P-labelled BamHI–HindIII fragment from pBS-BD2 (Fig. 2a) or the 32P-labelled lacZ fragment (Fig. 2b). HSV-1 strain KOS and pBS-BD2 DNA (Fig. 2a, lanes 1 and 2) contain the wild-type 2.7 kb form of the BglII–HindIII fragment, whereas HSV-1 strain AN-1 and pAN-1 (Fig. 2a, lanes 3 and 4) contain the 6.0 kb version expected if the 917 bp deletion and the 4.2 kb insertion are both present. The band migrating at 3.6 kb in lanes 2 and 4 corresponds to the vector which hybridizes weakly to the probe. The origin of the band migrating a little faster than the 6.0 kb fragment of pAN-1 and virus AN-1 was detected. The 3.6 kb band in lanes 5 and 7 corresponds to the vector, as seen above. These results demonstrate that the ICP6::lacZ fragment of pAN-1 was inserted into the expected position of the genome.

Alkaline nuclease activity of AN-1

Extracts of infected Vero cells were assayed for alkaline nuclease activity as described in Methods. Table 1 shows that mock-, and virus AN-1- and AN-2-infected extracts do not exhibit significant amounts of alkaline nuclease activity, whereas HSV-1 strain KOS-infected extracts exhibit considerable activity under these reaction conditions. Furthermore, a recombinant of virus AN-1, AN-1-1b, generated during marker rescue analysis (described below), also exhibits wild-type levels of the enzyme. This result indicates that viruses AN-1 and AN-2 are incapable of inducing alkaline nuclease activity above levels obtained from mock-infected cell extracts and is consistent with the assumption that the deletion/insertion mutation has in fact disrupted the nuclease gene.

Growth properties of AN-1

The mutant AN-1 was tested for its ability to form plaques in untransformed Vero cells and in permissive
Table 1. Alkaline nuclease activity in Vero cells infected with HSV-1 strain KOS, viruses AN-1 or AN-2, or recombinant virus AN-1-lb

<table>
<thead>
<tr>
<th>Virus</th>
<th>3H liberated (c.p.m.)*</th>
<th>Percentage of wild-type activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected cells</td>
<td>61</td>
<td>3</td>
</tr>
<tr>
<td>AN-1</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>AN-2</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>KOS</td>
<td>1965</td>
<td>100</td>
</tr>
<tr>
<td>AN-1-lb†</td>
<td>1836</td>
<td>94</td>
</tr>
</tbody>
</table>

* 3H (c.p.m.) rendered acid-soluble from labelled DNA by crude extract corresponding to approximately 50 µg cellular protein (1-25 × 10⁶ cells) during 30 min at 37 °C. Approximately 2000 c.p.m. would be expected to be released for each reaction shown if degradation was complete.
† AN-1-lb is a recombinant virus obtained in a marker rescue experiment with infectious AN-1 DNA and the plasmid pBS-BD2.

Table 2. Plaquing efficiencies of HSV-1 strain KOS and viruses AN-1, AN-1-lb and AN1-2a on Vero and S22 cells*

<table>
<thead>
<tr>
<th>Virus</th>
<th>Vero cells</th>
<th>S22 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>KOS</td>
<td>2·9 × 10⁸</td>
<td>1·9 × 10⁸</td>
</tr>
<tr>
<td>AN-1</td>
<td>2·9 × 10⁹†</td>
<td>6·5 × 10⁷†</td>
</tr>
<tr>
<td>AN-1-lb§</td>
<td>1 × 10⁸</td>
<td>1·2 × 10⁸</td>
</tr>
<tr>
<td>AN-1-2a§</td>
<td>1·1 × 10⁸</td>
<td>8·5 × 10⁷</td>
</tr>
</tbody>
</table>

* Plaquing efficiencies were determined by titrating virus stocks on monolayers of the indicated cell line.
† Plaques could not be counted easily because they are extremely small and asymmetrical in shape. In six separate experiments, plaquing efficiencies of virus AN-1 on Vero cells ranged from 20- to 50-fold lower than that of the same virus on S22 cells.
§ Plaque sizes of virus AN-1 on S22 cells were slightly smaller than for HSV-1 strain KOS on S22 (see text).
¶ AN-1-lb and AN1-2a are recombinant viruses obtained in a marker rescue experiment with infectious AN-1 DNA and the plasmids pBS-BD2 and pTZDN7, respectively. Plaque sizes of both recombinants on Vero and S22 were identical to those of HSV-1 strain KOS.

S22 cells. As indicated in Table 2, the plaquing efficiency of virus AN-1 on Vero cells is considerably lower (20- to 50-fold) than on S22 cells. Plaques of virus AN-1 on Vero cells are very small compared to HSV-1 strain KOS (0-3 to 0-5 mm as compared to 1-6 mm) and have an unusual morphology, being asymmetrical instead of round. Table 2 also shows that virus AN-1 forms plaques efficiently on S22, although the plaque sizes are slightly smaller than those of HSV-1 strain KOS on S22 (1-3 mm compared to 1-6 mm).

To test further the growth properties of virus AN-1 in Vero and S22 cells, we measured the efficiency of virus production by single-step growth analysis (Fig. 3). Cells were infected at an m.o.i. of 2 p.f.u./cell and harvested at various times up to 44 h.p.i. Yields of virus were titrated in permissive S22 cells. The final AN-1 virus yield in S22 cells was reduced 200-fold compared to HSV-1 strain KOS in the same cells; thus, the mutant is somewhat compromised in its ability to produce virus even in permissive S22 cells. These results suggest that S22 cells cannot fully compensate for the mutation in virus AN-1. Yields of virus AN-1 in Vero cells were reduced approximately 4 × 10⁴-fold compared with wild-type virus in Vero cells, although small amounts of infectious virus are produced. Thus, virus AN-1 is severely compromised in Vero cells but can be propagated on S22 cells. The growth of virus AN-1 was also severely reduced in baby hamster kidney and human foreskin fibroblast cells (data not shown). These results suggest that the alkaline nuclease is required for efficient plaque formation and efficient production of infectious virus.

Genetic analysis

Marker rescue was performed to confirm that the growth defects observed in virus AN-1 were due to the mutation introduced into the nuclease gene. S22 cells were cotransfected with infectious virus AN-1 DNA and the plasmids shown in Table 3 and progeny virus was titrated in Vero and S22 cells. Parental virus AN-1 and recombinants could be distinguished on the basis of
Table 3. Marker rescue of virus AN-1

<table>
<thead>
<tr>
<th>Plasmid*</th>
<th>Coordinates†</th>
<th>Sequence coordinates‡</th>
<th>Marker rescue efficiencies§</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSG10</td>
<td>0-086-0-194</td>
<td>12593-29338</td>
<td>83</td>
</tr>
<tr>
<td>pSG10-B2</td>
<td>0-145-0-165</td>
<td>21655-24890</td>
<td>&lt;0.03‡</td>
</tr>
<tr>
<td>pBS-BD2</td>
<td>0-165-0-186</td>
<td>24890-27986</td>
<td>3.3</td>
</tr>
<tr>
<td>pTZDN7</td>
<td>0-165-0-180</td>
<td>24890-27265</td>
<td>0.25</td>
</tr>
<tr>
<td>pSG10-P5</td>
<td>0-129-0-151</td>
<td>19021-22479</td>
<td>&lt;0.03§</td>
</tr>
<tr>
<td>pSG10-P6</td>
<td>0-151-0-158</td>
<td>22479-23653</td>
<td>&lt;0.03§</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td></td>
<td>&lt;0.03§</td>
</tr>
</tbody>
</table>

* Plasmids were linearized, extracted twice with phenol-chloroform-isooamyl alcohol (1:1:48), and ethanol-precipitated before transfection.
† Coordinates are in the prototype orientation.
‡ Sequence coordinates are taken from HSV-1 strain 17 (McGeoch et al., 1988b).
§ Results are expressed as plating efficiencies, which were determined using the formula \[(\text{p.f.u./ml}_{\text{vero}})/\text{p.f.u./ml}_{\text{s22}}\] \times 10^3.
‖ Numbers in italics were considered negative for marker rescue.

plaque size and colour on Vero cells; only white, wild-type-sized plaques were counted in calculating marker rescue frequencies. Plasmids pSG10 and pBS-BD2 both efficiently rescued the lesion in virus AN-1; plasmid pTZDN7 also rescued the lesion but much less efficiently. The reason for the low marker rescue frequency of pTZDN7 is likely to be that the insert in the plasmid extends only 583 bp to the right of the position of the deletion in virus AN-1, thus reducing the region available for homologous recombination. These data are consistent with the conclusion that the growth defect in virus AN-1 is due to the insertion in the alkaline nuclease gene but we cannot rule out the presence of additional lesions because the smallest fragment capable of rescuing the lesion in virus AN-1 [the BamHI-BstEII fragment in pTZDN7 (24890 to 27265 in the HSV-1 strain 17 sequence)] overlaps the UL11 gene by 203 nucleotides on its left end and the UL13 gene by 315 nucleotides on its right end. Thus, it is theoretically possible that the growth defects in virus AN-1 are due to spontaneous mutations within the UL11 or UL13 genes also present within S22 cells. However, we consider this unlikely for two reasons. First, because virus AN-1 plaques are blue in the presence of X-gal, wild-type recombinants can be easily detected by screening for white plaques on Vero cells. The only plasmids which generated white plaques on Vero cells were pSG10, pBS-BD2 and pTZDN7 (data not shown). Twelve white plaques were picked from positive marker rescue plates, and all 12 grew on Vero cells. One white plaque each from the pBS-BD2 and pTZDN7 plates, AN-1-lb and AN-1-2a, respectively, was chosen for further study. AN1-lb and AN-1-2a were able to form large plaques on Vero cells with efficiencies identical to that of wild-type virus (Table 2). These results demonstrate that the loss of the lacZ gene (blue to white plaques) segregated perfectly with the restoration of growth on Vero cells. Second, because two independently isolated mutant viruses, AN-1 and AN-2, exhibited identical phenotypes, it is unlikely that viruses AN-1 and AN-2 both contain additional spontaneous mutations in UL11 or UL13.

Synthesis of viral DNA

The ability of virus AN-1 to synthesize viral DNA was measured by the incorporation of [Me-3H]thymidine. Wild-type- and mutant-infected Vero and S22 cells were labelled and total DNA was harvested as described previously (Aron et al., 1975). Cellular DNA corresponds to a peak (C) with a buoyant density of 1.690 g/ml and viral DNA corresponds to a peak (V) with a buoyant density of 1.725 g/ml. Symbols: [ ], c.p.m. \times 10^3; ●, buoyant density plot.

Fig. 4. Separation of viral and cellular DNAs by CsCl equilibrium centrifugation. (a and b) Mock-infected, (c and d) HSV-1 strain KOS-infected and (e and f) virus AN-1-infected Vero (a, c and e) or S22 (b, d and f) cells (10 p.f.u./cell) were exposed to 10 μCi [3H]thymidine/ml from 6 to 24 h p.i. Cell lysates were subjected to equilibrium centrifugation in neutral CsCl gradients as described previously (Aron et al., 1975). Cellular DNA corresponds to a peak (C) with a buoyant density of 1.690 g/ml and viral DNA corresponds to a peak (V) with a buoyant density of 1.725 g/ml.

Fig. 4 was corrected to show a clear separation of viral and cellular DNA peaks (C for cellular DNA and V for viral DNA), with a clear buoyant density gradient from 1.6 to 1.7 g/ml. This demonstrates the ability of viral DNA to synthesize in the presence of [3H]thymidine, with a buoyant density of 1.725 g/ml compared to the mock-infected peak at 1.7 g/ml. The viral DNA peak is distinct and shows a clear incorporation of thymidine into viral DNA strands.
There was no difference in the relative amounts of DNA synthesis when wild-type HSV-1 strain KOS virus was grown on Vero or S22 cells. Based on these results, it appears that the alkaline nuclease gene is not required for DNA synthesis.

Synthesis of viral proteins

Mutants that synthesize wild-type levels of viral DNA under non-permissive conditions would be expected to induce wild-type levels of late viral proteins. Vero and S22 cells were infected with virus AN-1 or wild-type HSV-1 strain KOS at an m.o.i. of 10 p.f.u. per cell, labelled with [35S]methionine and analysed by polyacrylamide gel electrophoresis. The patterns of protein synthesis of wild-type and mutant-infected S22 and Vero cells are very similar (Fig. 5). Late (γ) proteins ICP1/2, 1CP19/20 and ICP43/44 appear in all lanes. Thus, no defect in the synthesis of late proteins is apparent. AN-1-infected S22 and Vero cells both appear to be lacking a protein with an approximate $M_r$ of 85K, the expected size of alkaline nuclease (Banks et al., 1983). To confirm that virus AN-1-infected cells fail to produce the nuclease protein, virus AN-1- and HSV-1 strain KOS-infected cell extracts were used in an immunoblot analysis with antiserum raised against a β-gal:HSV-1 alkaline nuclease fusion protein (P. D. Olivo & M. D. Challberg, unpublished data). In HSV-1 strain KOS-infected S22 and Vero cells (Fig. 6, lanes 3 and 5, respectively), a major immunoreactive band was observed at a position corresponding to an $M_r$ of approximately 85K. In extracts from virus AN-1-infected S22 and Vero cells (Fig. 6, lanes 2 and 4, respectively), the 85K band was absent, however a band...
corresponding to an $M_r$ of 116K was observed. Since the antisemum was raised against a $\beta$-gal::alkaline nuclease fusion protein, this large band almost certainly corresponds to $\beta$-gal (116K), which would be expected to be produced by virus AN-1. Neither the 116K nor the 85K bands was observed in mock-infected S22 or Vero cells (Fig. 6, lane 1 and data not shown). These results suggest that virus AN-1 does not synthesize a protein corresponding to the wild-type alkaline nuclease protein, confirming the fact that the gene for this protein has been disrupted.

It is of interest that no band corresponding to the alkaline nuclease is seen in virus AN-1-infected S22 cells, either in the $[^{35}S]$methionine-labelled extracts on polyacrylamide gels (Fig. 5, lane 6) or on an immunoblot (Fig. 6, lane 2). In addition, alkaline nuclease activity has not been detected in S22 cells induced by superinfection with virus AN-1 (data not shown). These results suggest that alkaline nuclease may not be efficiently expressed in S22 cells; the significance of this finding will be discussed further below.

The results described above suggest that although virus AN-1 is severely compromised in plaque formation and production of infectious virus when grown on Vero cells, wild-type levels of viral DNA and proteins are synthesized in these cells. Thus the defect in virus AN-1 appears to act at a later stage than viral DNA synthesis and production of late proteins.

**Discussion**

We have described the isolation and characterization of a null mutant in the gene encoding the HSV-1 alkaline nuclease. The mutant AN-1 contains a 917 bp deletion of the N-terminal half of the gene (corresponding to residues 70 to 375 of the 626 residue ORF UL12) and an insertion of the ICP6::lacZ insertional mutagen. Virus AN-1 was propagated in helper cells (S22) which express the wild-type version of the alkaline nuclease gene and forms tiny, practically indistinguishable plaques on Vero cells. Interestingly, tiny plaques which are difficult to count were also reported for the HSV-2 mutant 4-8 under non-permissive growth conditions (Francke & Garrett, 1982; Moss, 1986). The plaque size and morphology of alkaline nuclease deficient (nuc-) mutants may indicate an unusual method of viral transmission in the absence of nuclease. In addition, the yield of virus AN-1 when grown on Vero cells was $10^5$-fold less than that of HSV-1 strain KOS. This result suggests that the mutant is severely compromised on Vero cells; however, the presence of a small amount of infectious virus produced suggests that a limited amount of virus production can occur in the absence of viral alkaline nuclease. Electronic microscopic studies suggest that a large number of empty capsids accumulate in cells infected with virus AN-1 and that, relative to wild-type virus, few mature extranuclear particles are produced (data not shown). Fig. 4 and 5 indicate that virus AN-1 is capable of inducing wild-type levels of viral DNA and protein synthesis. Taken together, these results suggest that viral alkaline nuclease is not essential for viral DNA replication but is required for the efficient production of infectious virus. Possible roles of the alkaline nuclease in the viral life cycle are discussed below.

A discrepancy exists between the conclusions reached by Moss (1986) and this study regarding whether the alkaline nuclease is required for viral DNA synthesis. One possible explanation for the discrepancy is that in addition to the nuclease lesion in mutant 4-8, an additional leaky ts mutation in one of the other replication genes in this region exists which is responsible for the observed defect in viral DNA synthesis. Although Moss performed marker rescue analysis on the mutation(s) in mutant 4-8, the rescuing fragment was quite large (16-7 kb) and contains the coding regions for several viral genes, including three genes essential for DNA synthesis, UL5, UL8 and UL9 (Carmichael et al., 1988; Carmichael & Weller, 1989; Matz et al., 1983; Zhu & Weller, 1988). Further genetic analysis will be required in order to fully explain this discrepancy.

The mutant AN-1 can form plaques efficiently on permissive S22 cells, indicating that complementation by the resident wild-type alkaline nuclease gene (present in approximately five copies per haploid genome) occurs. However, yields of virus AN-1 on S22 cells were decreased approximately 200-fold compared to HSV-1 strain KOS on Vero or S22 cells (Fig. 3). Thus, although the virus can certainly be propagated on S22 cells, virus growth is somewhat inefficient. One explanation for the inefficient growth may be relatively low levels of alkaline nuclease gene expression in S22 cells compared to HSV-1 strain KOS-infected cells; our inability to detect alkaline nuclease protein or activity in these cells (Fig. 6 and 7) supports this hypothesis. Banks et al. (1983) previously reported that alkaline nuclease is an abundant protein in infected cells. Taken together these data raise the interesting possibility that large quantities of alkaline nuclease may be needed for efficient infection. Attempts to correlate complementation of nuc- mutants with expression of the alkaline nuclease gene are currently in progress.

Although substantial information has been collected about its enzymic activities in vitro, intracellular localization and genetic properties, the physiological role of alkaline nuclease during the virus life cycle remains unclear. Based on its nucleolytic abilities, Hoffmann & Cheng (1978) proposed that the viral alkaline nuclease may be involved in the degradation of host cellular DNA.
to provide deoxynucleotide precursors for viral DNA synthesis. The reutilization of host DNA-derived dTTP for viral DNA synthesis has been reported; however, this is apparently not a major source of precursors for viral DNA (Nutter et al., 1985). In addition, the observation that substantial viral DNA synthesis (90% of wild-type levels) occurs in mutant-infected cells (Fig. 4) suggests that DNA precursors do not seem to be limiting, at least in exponentially growing cells in tissue culture. Further studies will be required to determine whether degradation of cellular DNA by alkaline nuclease occurs, and if so, whether this represents a substantial contribution to pools of precursor nucleotides. The presence of an active nuclease in the nucleus of infected cells raises the question of how it is modulated in vivo so that viral DNA is protected from degradation.

Although the alkaline nuclease exhibits endo- and exonucleolytic properties in vitro, especially at high pH, it is possible that its in vivo function might be different (Strobel-Fidler & Francke, 1980). Suggestions have been made that alkaline nuclease participates directly in viral DNA synthesis either as a helix-destabilizing protein (Hoffmann, 1981) or as a helicase or topoisomerase (Francke & Garrett, 1982). However, the observation that the alkaline nuclease does not localize to putative replication compartments (Puvion-Dutilleul & Pichard, 1986; Randall & Dinwoodie, 1986) and the demonstration in this study that in the HSV-1 mutant, AN-1, alkaline nuclease is not required for viral DNA synthesis, argue against a direct role for alkaline nuclease in viral DNA synthesis. The fact that alkaline nuclease is not required for origin-containing plasmid amplification (Puvion-Dutilleul & Pichard, 1986) and the existence in HSV-1 and in pseudorabies virus capsid proteins (Preston et al., 1980; Schaffer et al., 1983; Rixon et al., 1988), suggests that cis-acting signals that are required for cleavage and maturation, it follows that there must be trans-acting factors which recognize these sites and act to carry out cleavage and packaging. One approach to the identification of these functions is the isolation of mutants defective in these processes. Several mutants of HSV-1 representing at least five genes (UL6, UL26, UL28, UL32 and possibly UL54) which make but fail to process viral DNA at the non-permissive temperature have been isolated (Matz et al., 1983; Preston et al., 1983; Rixon et al., 1988; Sherman & Bachenheimer, 1987, 1988). In these experiments, no terminal fragments were observed following restriction enzyme digestion of viral DNA isolated from cells infected at the non-permissive temperature (Preston et al., 1983; Rixon et al., 1988; Sherman & Bachenheimer, 1987, 1988); this phenotype suggests that cleavage is completely blocked in these mutants at the non-permissive temperature. Some of these mutations have been mapped to genes encoding known or suspected capsid proteins (Preston et al., 1983; Rixon et al., 1988), and their existence in HSV-1 and in pseudorabies virus has been taken as evidence that maturation of viral DNA is closely correlated with the formation of full capsids (Ladin et al., 1980; Sherman & Bachenheimer, 1987, 1988). In contrast, AN-1 mutants do not exhibit a complete block of viral DNA processing; DNA from cells infected with virus AN-1 and digested with BamHI exhibits significant amounts of terminal fragments (M. R. Seghatoleslami & S. K. Weller, unpublished results).

It has also been proposed that the alkaline nuclease may play a role in the resolution of recombination intermediates or in some other step in recombination. If this were its role, a loss of alkaline nuclease activity could lead to inefficient processing or packaging of intermediates. Following synthesis of viral DNA in the form of large concatemers, maturation of viral DNA occurs and involves two steps: the processing of 'endless' replicative concatemers to unit-length molecules and the assembly of capsids containing a genome-equivalent of HSV DNA (Vlazny et al., 1982). Several lines of evidence suggest that viral genome maturation involves site-specific cleavage of viral DNA concatemers within the a sequence (Davison & Wilkie, 1981; Mocarski et al., 1985; Mocarski & Roizman, 1982; Spaete & Mocarski, 1985; Stow et al., 1983; Vlazny & Frenkel, 1981; Vlazny et al., 1982). Given the existence of cis-acting signals that are required for cleavage and maturation, it follows that there must be trans-acting factors which recognize these sites and act to carry out cleavage and packaging. One approach to the identification of these functions is the isolation of mutants defective in these processes. Several mutants of HSV-1 representing at least five genes (UL6, UL26, UL28, UL32 and possibly UL54) which make but fail to process viral DNA at the non-permissive temperature have been isolated (Matz et al., 1983; Preston et al., 1983; Rixon et al., 1988; Sherman & Bachenheimer, 1987, 1988). In these experiments, no terminal fragments were observed following restriction enzyme digestion of viral DNA isolated from cells infected at the non-permissive temperature (Preston et al., 1983; Rixon et al., 1988; Sherman & Bachenheimer, 1987, 1988); this phenotype suggests that cleavage is completely blocked in these mutants at the non-permissive temperature. Some of these mutations have been mapped to genes encoding known or suspected capsid proteins (Preston et al., 1983; Rixon et al., 1988), and their existence in HSV-1 and in pseudorabies virus has been taken as evidence that maturation of viral DNA is closely correlated with the formation of full capsids (Ladin et al., 1980; Sherman & Bachenheimer, 1987, 1988). In contrast, AN-1 mutants do not exhibit a complete block of viral DNA processing; DNA from cells infected with virus AN-1 and digested with BamHI exhibits significant amounts of terminal fragments (M. R. Seghatoleslami & S. K. Weller, unpublished results). It seems that cleavage can occur but production of infectious virions is not efficient. Thus, the block appears to be at a later stage than that in the previously isolated packaging mutants. One possible explanation lies in the frequency of appropriate cleavage sites within
the large head-to-tail concatemers. Current evidence suggests that L-S junctions exist which contain only one copy of the a sequence and that the a sequence must be duplicated either during, or just prior to, cleavage/packaging (Deiss et al., 1986; Deiss & Frenkel, 1986; Varmuza & Smiley, 1985; J. R. Smiley, personal communication). It is possible that the alkaline nuclease plays a role in the duplication of the a sequences and, if it is absent, cleavage can only occur at a small subset of junctions which already contain two copies of the a sequence. This could lead to inefficient packaging of viral genomes. The alkaline nuclease has been found in a complex of viral and cellular proteins which bind to the a sequence (Chou & Roizman, 1989). Further experiments will be required to determine whether the presence of alkaline nuclease in these complexes is fortuitous or reflects its actual involvement in maturation and/or cleavage events.

The results presented in this paper are consistent with either of the latter two proposals for alkaline nuclease function: in the processing of replication or recombination intermediates, or in the duplication or cleavage of the a sequences. Further experimentation will be required to distinguish between these possibilities and to determine whether alkaline nuclease also functions to degrade host DNA to provide precursors for viral DNA synthesis. No evidence to date indicates a sequence specificity for the in vitro nucleolytic activities of the alkaline nuclease. If a role in processing or packaging of intermediates is discovered, the issue of sequence specificity will have to be addressed. Alternatively, the essential role of alkaline nuclease during infection may not be enzymic; it is possible that alkaline nuclease plays a structural role in infection, perhaps during virus assembly. This would be consistent with the apparent requirement for large amounts of the protein for efficient virus production.

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