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Genetic Evidence for Multiple Nuclear Functions of the Herpes Simplex Virus ICP8 DNA-Binding Protein

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We have isolated several mutant herpes simplex viruses, specifically mutated in the infected cell protein 8 (ICP8) gene, to define the functional domains of ICP8, the major viral DNA-binding protein. To facilitate the isolation of these mutants, we first isolated a mutant virus, HD-2, with the *lacZ* gene fused to the ICP8 gene so that an ICP8–β-galactosidase fusion protein was expressed. This virus formed blue plaques on ICP8-expressing cell lines in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Mutated ICP8 gene plasmids contransfected with HD-2 DNA yielded recombinant viruses with the mutant ICP8 gene incorporated into the viral genome. These recombinants were identified by formation of white plaques. Four classes of mutants were defined: (i) some expressed ICP8 that could bind to DNA but could not localize to the cell nucleus; (ii) some expressed ICP8 that did not bind to DNA but localized to the nucleus; (iii) some expressed ICP8 that neither bound to DNA nor localized to the nucleus; and (iv) one expressed ICP8 that localized to the cell nucleus and bound to DNA in vitro, but the mutant virus did not replicate its DNA. These classes of mutants provide genetic evidence that DNA binding and nuclear localization are distinct functions of ICP8 and that ICP8 has nuclear functions other than binding to DNA. Furthermore, the portion of ICP8 needed for a nuclear function(s) distinct from DNA binding is the part of ICP8 showing sequence similarity to that of the cellular protein cyclin or proliferating cell nuclear antigen.

The major DNA-binding protein, or infected cell protein 8 (ICP8), is expressed by herpes simplex virus type 1 as a β or delayed early gene product. This gene product is required for viral DNA synthesis (7, 10, 29, 49, 51) and normal regulation of viral gene expression (16, 17). The functions and activities specified by ICP8 have not been completely established. The known properties of ICP8 include (i) the ability to bind to DNA in vitro and in vivo, (ii) the ability to localize to the cell nucleus, and (iii) the ability to promote assembly of nuclear structures involving viral and cellular DNA replication proteins.

ICP8 binds to single-stranded (ss) or double-stranded DNA in vitro (2, 21, 37, 42) and can be isolated in deoxynucleoprotein complexes from infected cells (25, 26). The latter observation has been interpreted to mean that ICP8 interacts directly with DNA in the infected cells. Because DNA binding by ICP8 molecules encoded by temperature-sensitive (*ts*) mutants is thermolabile in vivo (25, 26) or in vitro (42), this property may be an essential function of ICP8. The portion of ICP8 involved in DNA binding has not been precisely mapped. Some of the alterations in ICP8 molecules that are thermolabile for DNA binding are at residues 119, 348, and 450 (15). Because these sequence alterations render the protein conditionally defective, these changes may directly or indirectly affect the DNA binding site on ICP8. Leinbach and Heath (27) have reported that the carboxyl-terminal 69 kilodaltons of ICP8 can bind to DNA in vitro. Thus, the DNA-binding domain of ICP8 may map within this portion of the molecule, but a more precise mapping of this property is needed.

ICP8 contains the necessary signals for nuclear localization in the absence of other viral proteins (39), but these signals have not been mapped. One mutant protein, lacking residues 326 to 584, fails to localize to the nucleus (34), but it is not known whether the defect is due to lack of an essential localization signal or an altered conformation preventing nuclear uptake.

Cell biological studies of ICP8 nuclear localization have shown that ICP8 localizes to infected cell structures called prereplicative sites in the absence of viral DNA replication (12, 39). In these structures, ICP8 behaves as if it were bound to the nuclear matrix. Viruses expressing an altered ICP8 molecule fail to assemble prereplicative sites, and from these results, we concluded that ICP8 promotes the assembly of these structures containing DNA replication proteins (12). The portion(s) of ICP8 needed to promote assembly of prereplicative sites has not been identified.

To identify the portion of ICP8 needed for these functions and to attempt to separate the various functions of ICP8, we have devised a genetic system for the efficient transfer of mutations to the ICP8 gene in the viral genome. This report outlines the initial characterization of a set of viruses containing mutations specifically introduced into the ICP8 gene. The phenotypes of these viruses indicate that DNA binding and nuclear localization are genetically independent functions of ICP8 and that ICP8 specifies nuclear functions in addition to DNA binding.

MATERIALS AND METHODS

Cells and viruses. Vero cells were grown and maintained as described previously (23). The growth medium for the Neo^r cell lines S2 and B10 (see below) included 200 μg of the antibiotic G418 per ml during the first passage of cells after thawing or 500 μg of G418 per ml every five passages.

The herpes simplex virus type 1 wild-type strain KOS1.1 was propagated and assayed as described previously (23, 25). Mutant viruses were grown in ICP8-expressing S2 and

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TABLE 1. Complementation of ICP8 mutants by B10 and S2 cells[a]

Virus	Titer (10^9 PFU/ml)[b]					
	B10		S2		Neo^r	
	33.5°C	39°C	33.5°C	39°C	33.5°C	39°C
KOS1.1	1.7	1.7	0.7	2.0	1.3	1.8
*ts*13	4.3	3.1	2.3	4.0	1.0	<0.001
*ts*18	3.0	3.2	2.3	2.7	1.7	<0.001
*ts*HA1	2.3	3.7	3.7	1.7	2.4	<0.001

[a] Cultures of B10, S2, and Neo^r cells were infected with each virus and incubated at 33.5°C or 39°C.
[b] Plaque numbers were counted for 2 to 3 days.

B10 cell lines. For all experiments, monolayer cultures were infected with KOS1.1 or mutant virus at a multiplicity of 20 PFU per cell.

Isolation of ICP8-expressing cell lines. Vero cells were transformed with the plasmid pSG18-SacI (25, 40) or p8B-S (15) and pSVneo (45) essentially as described by Deluca et al. (13). After growth in medium containing the antibiotic G418 (a neomycin analog), 21 drug-resistant colonies were picked, grown into cultures, and screened for their ability to complement the growth of the ICP8 mutants *ts*13, *ts*18, and *ts*HA1 (10, 20). At the nonpermissive temperature, these *ts* mutants formed plaques in 7 of 21 cell lines derived from cultures receiving the ICP8 gene but not efficiently in Neo^r cells which were derived from cultures transfected with plasmid pSV2neo alone. The cell clone B10, derived from a culture transfected with plasmid p8B-S, and S2 cells, derived from a culture transfected with pSG18-SacI, yielded the highest levels of complementation and were chosen for further use (Table 1). KOS1.1 formed plaques in Neo^r cells as well as in B10 and S2 cells at both temperatures. The mutant viruses *ts*13, *ts*18, and *ts*HA1 formed plaques efficiently only at 33.5°C in Neo^r cells but formed plaques at the wild-type level at both temperatures in B10 and S2 cells. Southern blot hybridization was performed to determine the copy number of the ICP8 gene in these cell lines, and B10 and S2 cells contained approximately 1 and 10 copies per haploid genome, respectively (data not shown).

Plasmids. Plasmids p8B-S, pSV8, and pm1, as well as the nucleotide numbering system, were described previously (15, 47). The plasmid p8B-S was constructed by cloning a 5.9-kilobase-pair (kbp) *Bam*HI-*Sac*I fragment (map units 0.374 to 0.411), including the ICP8 gene promoter, into pUC18. The plasmid pSV8 was constructed by inserting a 5.5-kbp *Sma*I-*Sac*I fragment (map units 0.374 to 0.409) downstream of the simian virus 40 early promoter, and the plasmid pm1 was derived from plasmid pSV8 by changing codons 499 and 502 of the ICP8 gene from cysteine codons to glycine codons. Mutant ICP8 gene plasmids used in this study were derived from pICP8 or pSPICP8, in which a 5.5 kbp *Sma*I-*Sac*I fragment (map units 0.374 to 0.409) was inserted into pUC19 or pSP64, respectively. Plasmids pn10 and pn2 were generated by linearization of the plasmid spICP8 (which was achieved by partial digestion with *Sma*I) and subsequent insertion of a 14-nucleotide (nt) *Xba*I linker (5′-CTAGTCTAGACTAG-3′; New England BioLabs, Inc., Beverly, Mass.) containing stop codons in all three reading frames at nts 4084 and 3695, respectively. Therefore, pn10 encodes the first 1,160 amino acid residues, and pn2 encodes the first 1,029 amino acid residues of ICP8 as well as 4 additional amino acids, Pro-Ser-Leu-Asp, encoded by the *Xba*I linker sequence. Plasmid pd301 was generated by an internal in-frame deletion of a 2,001-base-pair (bp) *Not*I fragment (nts 1395 to 3396). Plasmids pd101 and pd102 were constructed in the following ways: the plasmid pSPICP8 was linearized by partial digestion with *Sma*I, and a 12-nt *Bgl*II linker, 5′-GGAAGATCTTCC-3′, was ligated in. A 1,642-bp deletion was generated by digestion with *Bgl*II (converted from a *Sma*I site at nt 652) and *Bam*HI (nt 2294) to yield plasmid pd101. Thus, pd101 lacks codons for residues 17 to 563 but has an insertion of one Arg codon encoded by the *Bgl*II linker sequence. An 1,188-bp deletion was generated by digestion with *Bgl*II (converted from *Sma*I at nts 652 and 1840) to yield plasmid pd102. Thus, pd102 lacks codons for residues 17 to 411 of the ICP8 coding sequence but encodes three additional amino acids, Arg-Ser-Ser, in the *Bgl*II linker sequence. Both plasmids pd101 and pd102 also contain a 14-nt *Xba*I linker at nt 4419, downstream of the ICP8 poly(A) signal. Because there are other *Sma*I sites around nts 4084 and 1840, both pn10 and pd102 were sequenced to determine the exact mutation sites.

Isolation of mutant viruses. The mutant virus HD-2, containing a *lacZ* insertion in the ICP8 gene, was isolated by the following steps. After deletion of a 780-bp *Xho*I fragment from plasmid pICP8, the plasmid was briefly digested with BAL 31, a *Bgl*II linker was added, and the *lacZ* gene (Pharmacia, Inc., Piscataway, N.J.) was inserted. The *lacZ* gene of pMC1871 contains no transcription promoter and lacks the first eight nonessential amino-terminal codons. The mixture of ICP8:*lacZ* plasmids was transfected with KOS1.1 DNA into B10 cells (22). After infection with the progeny virus, B10 or S2 cells were overlaid with medium 199: 1% calf serum containing 0.1% human immune serum for 1 to 2 days at 37°C. To detect β-galactosidase activity, the medium was then changed to 199 medium: 1.0% agarose containing 400 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml for 8 to 16 h. Recombinant viruses were identified as blue plaques at a frequency of about 0.1 to 0.5%. One mutant virus was plaque purified and designated as HD-2.

HD-2 served as the recipient virus to generate all mutant viruses in this study except *d*301. After cotransfection of infectious HD-2 DNA with mutated ICP8 plasmids, the potential recombinant viruses were first isolated as white plaques from the population of parental blue plaques in the presence of X-Gal and then selected for further analysis.

The mutant virus *d*301 was constructed by cotransfection of B10 cells with infectious KOS1.1 DNA and plasmid pd301, in which a 2,001-bp *Not*I fragment was deleted from the ICP8 coding sequence (Fig. 1). The progeny viruses from the marker transfer were tested for their ability to grow in B10 cells but not in Vero cells. One of the more then 300 viruses tested could grow in B10 but not in Vero cells.

Analysis of viral proteins. Cell monolayer cultures were infected with KOS1.1 or mutant virus and then labeled with [^{35}S]methionine and harvested as indicated in the text. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of infected-cell lysates was performed as described previously (23). After electrophoresis, gels were fixed, dried, and exposed to Kodak SB5 film, or the proteins were transferred by electrophoresis (30 V, 0.2 A overnight) to nitrocellulose filters for Western blot analysis. Detection of immune complexes on blots by a Western blot procedure involving a color reaction for the alkaline phosphatase activity was conducted as specified by the manufacturers (Promega Biotec, Madison, Wis.). The rabbit polyclonal serum 3-83 (24) or the mouse monoclonal antibody 10E-3 (41) (diluted 1:400) was used to detect ICP8. We used a densitometer to scan the

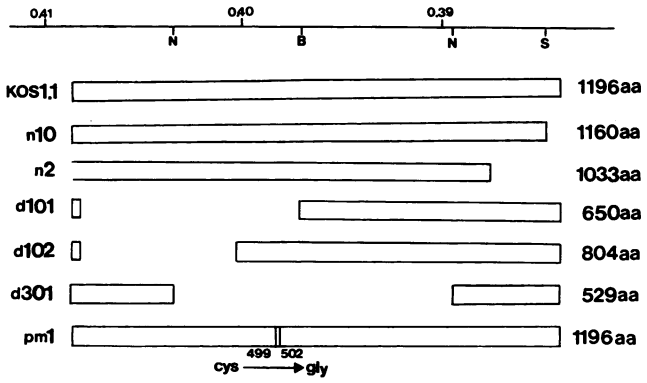

FIG. 1. Locations of the ICP8 gene nonsense (n), deletion (d), and point (pm) mutations used in this study. The location of the ICP8 coding region on the HSV-1 genome is shown at the top of the figure. Restriction sites shown are *Bam*HI, (B), *Not*I (N), and *Sal*I (S).

negatives of the color reactions of Western blots to quantitate the total amount of ICP8 present in a given extract. Various amounts of ICP8 were used to ensure that the assay was performed within the linear response range (results not shown).

ssDNA cellulose chromatography. ssDNA cellulose chromatography of infected-cell extracts was performed as described previously (21), except that flasks (75 cm^2) of infected Vero cells were labeled from 4 to 6 h postinfection with [^{35}S]methionine.

Analysis of viral DNA. (i) Preparation of DNA. Plasmid DNAs were prepared as described previously (11). Infectious viral DNA for marker transfer and marker rescue was purified from infected cells by centrifugation through NaI equilibrium density gradients as described previously (22). Viral DNA used for Southern blot analysis was purified by the following steps. Cells at a late stage of infection were frozen and thawed two or three times and were then sonicated for 30 s at 0 to 4°C. Cell debris were removed by centrifugation at 480 × *g*. The resulting supernatant was then subjected to centrifugation at 23,500 × *g*. The pellets were extracted with phenol-chloroform-isoamyl alcohol (24:24:1) three times. After ethanol precipitation, viral DNAs were dissolved in TE buffer.

(ii) Southern blot analysis. Viral DNAs were digested with appropriate restriction enzymes, separated by agarose gel electrophoresis, and transferred to nitrocellulose filters by the method of Southern (44). Plasmid DNAs used as probes for hybridizations were labeled as described previously (16).

(iii) Measurement of viral DNA synthesis. Monolayer cultures were infected with appropriate virus and labeled from 6 to 10 h with 20 μCi of [^{3}H]thymidine per ml, and total DNA was isolated by the method of Challberg (7). Viral DNAs were digested with the appropriate restriction enzymes and separated by agarose gel electrophoresis. After electrophoresis, the gel was treated with 1.0 M sodium salicylate for fluorography (8).

Indirect immunofluorescence. Indirect immunofluorescence was performed as described previously (38) with a 1:10 dilution of 793 anti-ICP8 monoclonal antibody (L. Pereira, unpublished results) and a 1:100 dilution of rhodamine-conjugated goat anti-mouse antibody for all mutant viruses except *n*2. A 1:30 dilution of anti-ICSP 11/12 polyclonal serum (36) and a 1:200 dilution of fluorescein-conjugated goat anti-rabbit immunoglobulin were used for the detection of *n*2 ICP8.

RESULTS

Construction of mutant viruses. (i) Strategies. To attempt to define the functional domains of ICP8, we constructed several different types of mutations in the cloned ICP8 gene (Fig. 1): (i) nonsense mutations (pn10 and pn2), (ii) internal deletions (pd301, pd101, and pd102), and (iii) a site-specific mutation (pm1) (15). We wanted to introduce the mutations into the ICP8 gene in the viral genome by marker transfer. However, the ICP8 gene is closed to ori_L, and ori_L sequences are always spontaneously deleted from plasmid clones (46, 49, 50). Thus, marker transfer of the ICP8 mutation from plasmids such as pSG18-SacI poses the risk of also transferring an altered ori_L. To avoid this potential problem, all of the mutated ICP8 plasmids used for marker transfer in this study were constructed from a plasmid in which the viral DNA sequences did not extend to ori_L. The viral sequence in the plasmid used starts at nt 437, only 170 bp upstream from the ICP8 coding sequences (Fig. 1 [15]).

Recently, the *Escherichia coli* β-galactosidase gene has been used to generate insertion mutant viruses that form blue plaques in the presence of X-Gal (6, 18, 19, 28, 35). To facilitate screening of recombinant viruses after marker transfer, a mutant virus which contains a *lacZ* gene insertion in the ICP8 coding region was constructed. This recombinant virus, designated as HD-2, formed blue plaques in the ICP8-expressing cell lines in the presence of X-Gal, but did not form plaques in Vero cells. To verify that the *lacZ* gene was inserted in the ICP8 gene of HD-2, a marker rescue experiment was performed with a cloned fragment containing only the ICP8 gene (nts 437 to 3995) from wild-type KOS1.1 DNA. The percent rescue ([titer of virus in Vero cells/titer of virus in B10 cells] × 100) for HD-2 was 19% (results not shown). Because the *lacZ* gene contains a stop codon, only the amino-terminal region of ICP8 was expected to be synthesized from HD-2. To map the ICP8:*lacZ* fusion region more precisely, the junction region was cloned from HD-2 DNA, and the DNA sequence indicated that the junction site was after nt 1450 (K. Baradaran, M. Gao, and D. M. Knipe, unpublished results). Thus, HD-2 encoded a fusion protein containing the amino-terminal 281 amino acid residues of ICP8 and the β-galactosidase protein and expressed under the control of the ICP8 gene promoter. When Vero cells were infected with HD-2, no wild-type ICP8 was detected, but a novel band of 145 kilodaltons, approximately the size predicted for the fusion protein, was observed (Fig. 2, lane 3). The ICP8–β-galactosidase fusion protein reacted with both the 3-83 rabbit polyclonal anti-ICP8 serum and a mouse monoclonal anti-β-galactosidase antibody (Promega Biotec) in Western blot analysis and in indirect immunofluorescence (data not shown).

(ii) Isolation of ICP8 mutant viruses. The HD-2 virus formed blue plaques in B10 cells in the presence of X-Gal. Mutated ICP8 gene plasmids cotransfected with HD-2 DNA yielded recombinant viruses with mutant ICP8 genes incorporated into the viral genome. Putative recombinant viruses were identified as white plaques in the presence of the parental blue virus plaques. With this approach, ICP8 gene mutations in the plasmids were introduced into the ICP8 gene in the viral genome. White plaques appeared at frequencies ranging from 2 to 39%. The frequency of white plaques in cells transfected with HD-2 DNA and pd101 or pd102 was not above the background. This was probably due

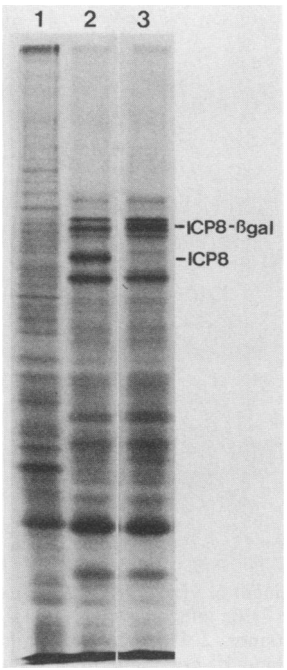

FIG. 2. Polypeptide profiles of HD-2- and KOS1.1-infected Vero cells. Cell monolayer cultures were infected at a multiplicity of 20 PFU per cell in the presence of 400 μg of phosphonoacetate per ml, labeled with [^{35}S]methionine from 5.5 to 6 h postinfection, and harvested at 6 h postinfection. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lanes: 1, mock-infected cells; 2, KOS1.1-infected cells; 3, HD-2-infected cells. The marks to the right indicate the positions of the fusion protein (ICP8-βgal; approximately 145 kilodaltons) and wild-type ICP8 (128 kilodaltons).

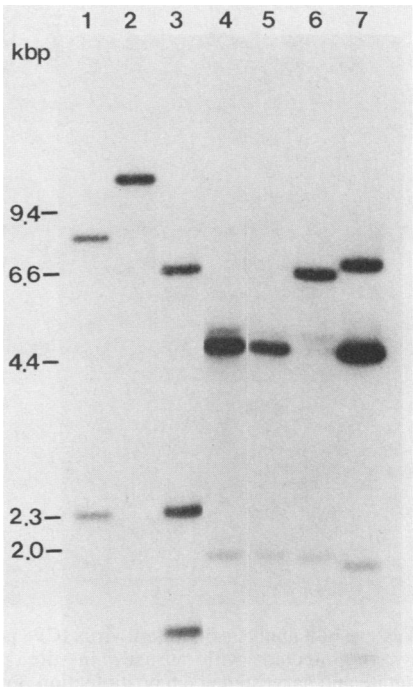

FIG. 3. Southern blot analysis of KOS1.1, HD-2, and *n*2 DNAs. KOS1.1, HD-2, and *n*2 DNAs were digested with *Bam*HI-*Xba*I (lanes 1, 2, and 3) and *Kpn*I (lanes 4, 6, and 5), and subjected to electrophoresis in parallel with *Kpn*I-digested pSG18-SacI (lane 7). Digested DNAs were separated on a 0.8% agarose gel, blotted onto a nitrocellulose filter, and hybridized to ^{32}P-labeled plasmid pICP8. The locations of molecular size markers are shown at the left.

to the limited amount of viral sequences available for recombination between pd101 or pd102 and HD-2 DNA.

The following types of analyses were performed to verify that the recombinant viruses contained the appropriate mutation as a result of marker transfer from the ICP8 gene plasmid constructs. The virus from each white plaque was plaque purified three times. Viral DNA was isolated, digested with appropriate restriction enzymes, and initially analyzed by agarose gel electrophoresis. The DNA fragment pattern was usually sufficient to discriminate the mutant DNA from HD-2 DNA. Southern blot analysis was performed to confirm the presence of mutations in viral DNA and the purity of the mutant virus populations. For example, the 8.2-kbp *Bam*HI G fragment of KOS1.1 DNA (Fig. 3, lane 1) was divided into 6.8- and 1.4-kbp fragments in *n*2 DNA by digestion with *Bam*HI and *Xba*I because of the *Xba*I linker (lane 3). The junction region of *Bam*HI G and V (2.3 kbp) was replaced by the *lacZ* gene in HD-2; therefore, digestion of HD-2 DNA with *Bam*HI and *Xba*I generated a 12.6-kbp fragment (Fig. 3, lane 2). Comparisons of *Kpn*I digests of KOS1.1 (Fig. 3, lane 4), HD-2 (lane 6), and *n*2 (lane 5) DNAs revealed that the patterns of KOS1.1 and *n*2 DNAs were identical but differed from that of HD-2 DNA because of the *lacZ* insertion (lane 6). In addition, because of our plasmid constructs, the ori$_L$-containing *Kpn*I fragments (1.85 kbp) of HD-2 and *n*2 DNAs (Fig. 3, lane 6 and 5, respectively) were identical to KOS1.1 (lane 4) and approximately 55 bp larger than the fragment in plasmid pSG18-SacI (lane 7), demonstrating the integrity of ori$_L$ in the mutant viruses.

Growth properties. All mutant viruses containing the mutations described in Fig. 1 failed to grow in Vero cells and required B10 or S2 cells for propagation. All of the mutant viruses isolated in this study grew to titers near wild-type levels in these ICP8-expressing cell lines. Because the ICP8 gene is the only known viral gene in the B10 and S2 cell lines, it is likely that the viral defects were due to lesions in the ICP8 gene. The plaque sizes produced by these mutant viruses were slightly smaller than those of KOS1.1. In all cases, the mutant viruses maintained their mutant phenotype after propagation in B10 and S2 cell lines in that the titers of all mutant viruses were between 10^8 and 10^9 PFU/ml in the cell lines but $<2.0 \times 10^3$ in Vero cells. This suggested that recombination between the mutant viruses and the wild-type ICP8 gene in the cell lines occurred at an insignificant frequency.

ICP8 expressed by mutant viruses. To confirm that the mutants expressed the predicted ICP8 polypeptides, extracts of ^{35}S-labeled mutant- and KOS1.1-infected Vero cells were analyzed by polyacrylamide gel electrophoresis (data not shown) and Western blotting (Fig. 4). The sizes of the ICP8 polypeptides specified by the mutant viruses were consistent with the expected mutational alterations. The 10E-3 mouse monoclonal antibody reacted with ICP8 polypeptides of mutants *pm*1, *d*101, *d*102, and *d*301 (Table 2) but not with those of *n*10 and *n*2 (data not shown). These results indicated that the 10E-3 monoclonal antibody reacts with an epitope contained, at least in part, within the carboxyl-terminal 36 amino acids of ICP8. These results also show that *d*101, *d*102, and *d*301 contain in-frame deletions.

Viral DNA replication. To assay the ability of each mutant

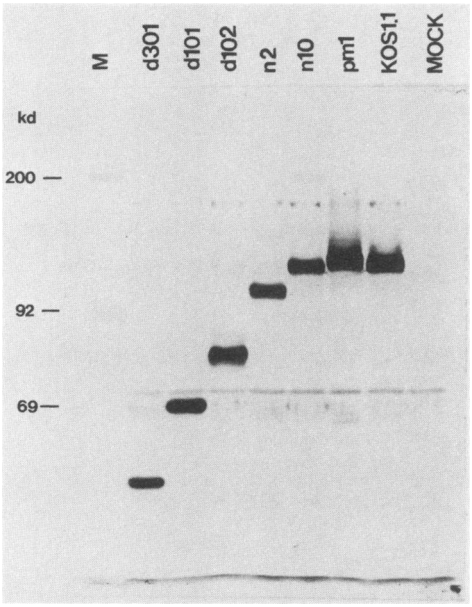

FIG. 4. Western blot analysis of mutant virus ICP8 polypeptides. Vero cells were infected with viruses in the presence of phosphonoacetate and harvested at 6 h postinfection. Proteins in the cellular extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose. The filter was then probed with polyclonal rabbit antiserum to ICP8 (3-83). Detection of the immune complex on the filter utilized a color reaction for akaline phosphatase activity. The locations of molecular size markers are shown at the left. kd, Kilodaltons.

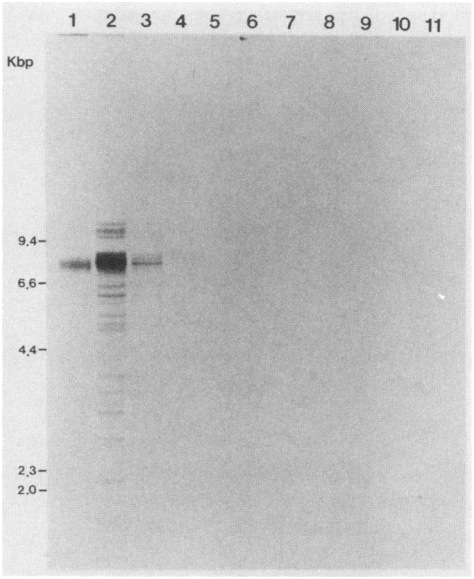

FIG. 5. Measurement of viral DNA replication. Vero cells were mock infected (lane 1) or infected with KOS1.1 in the absence of phosphonoacetate (lanes 2 through 4), or in the presence of phosphonoacetate (lane 5), *pm*1 (lane 6), *n*10 (lane 7), *n*2 (lane 8), *d*102 (lane 9), *d*101 (lane 10), or *d*301 (lane 11) and labeled with [^{3}H]thymidine from 6 to 10 h postinfection, and total DNA was isolated. Ten micrograms of each DNA sample (except in lane 3 [5 μg] and lane 4 [1 μg]) was digested with *Bam*HI and *Xho*I and separated by agarose gel electrophoresis. After electrophoresis, the gel was treated with 1.0 M sodium salicylate for fluorography.

virus to replicate its DNA, we infected Vero cells with the mutant viruses individually and labeled the cultures with [^{3}H]thymidine from 6 to 10 h postinfection. The cells were harvested, and DNA was isolated. Each DNA sample was digested with *Bam*HI and *Xho*I and subjected to agarose gel electrophoresis. For all ICP8 gene mutants, undetectable amounts of [^{3}H]thymidine were incorporated into viral DNA (Fig. 5, lanes 6 through 11) similar to DNA prepared from cells infected with wild-type virus in the presence of sodium phosphonoacetate (Fig. 5, lane 5), a compound that preferentially inhibits the herpes simplex virus type 1 DNA polymerase. Incorporation of [^{3}H]thymidine into wild-type viral DNA was detected when 1/10 the normal amount of total DNA was loaded (Fig. 5, lane 4). Therefore, we conclude that the viral mutants synthesized levels of viral DNA less than 10% of the wild-type level of DNA. In the mock-infected samples (Fig. 5, lane 1), a band of host DNA was seen. Thus, in mutant-infected cells, viruses could not promote viral DNA synthesis, but they still had the ability to inhibit incorporation of [^{3}H]thymidine into cellular DNA by 6 h postinfection.

TABLE 2. Solubility of ICP8 encoded by viral mutants

Virus	% of ICP8		Antibody[b]
	Supernatant[a]	Pellet	
KOS1.1	81	19	10E-3
*pm*1	12	88	10E-3
*d*101	22	78	10E-3
*d*102	33	77	10E-3
*d*301	42	58	10E-3
KOS1.1	77	23	3-83
*n*10	92	8	3-83
*n*2	10	90	3-83

[a] The supernatant and pellet fractions were defined as the samples obtained by centrifugation after DNase I treatment (21).

[b] The antibody used for the Western blots to visualize ICP8 was rabbit polyclonal 3-83 (24) or mouse monoclonal 10E-3 (41). The negatives of the color reactions of Western blots were scanned by densitometer.

DNA-binding properties of ICP8. (i) Solubility. Prior to studying the DNA-binding properties of mutant ICP8 molecules, the solubilities of the ICP8 polypeptides under standard extraction conditions were examined (Table 2). The solubility of the altered ICP8 polypeptides varied significantly. For example, *n*10 ICP8 was just as soluble as was wild-type ICP8, but for *n*2 and *pm*1, only approximately 10% was soluble.

(ii) Ability to bind to DNA. The DNA-binding properties of the soluble fraction of ICP8 molecules encoded by the mutant viruses were examined by chromatography on ss-DNA-cellulose columns. Because of the insolubility of some mutant ICP8 polypeptides, all extracts were clarified by centrifugation before application to the columns. The soluble fraction of the extracts was applied to the columns, and the proteins were eluted with increasing NaCl concentrations. Figure 6 shows the results for KOS1.1 and *pm*1 ICP8. Almost all of KOS1.1 ICP8 bound to the column (compare lanes 4 and 5 in Fig. 6A), and the majority of bound ICP8 was eluted at 0.5 M NaCl (lane 12). In contrast, the majority of *pm*1 ICP8 came out in the flowthrough (compare lanes 4 and 5 in Fig. 6B). The amount of each form of ICP8 eluted at each of the different NaCl concentrations from ssDNA cellulose columns was determined (Table 3). Mutant *n*10

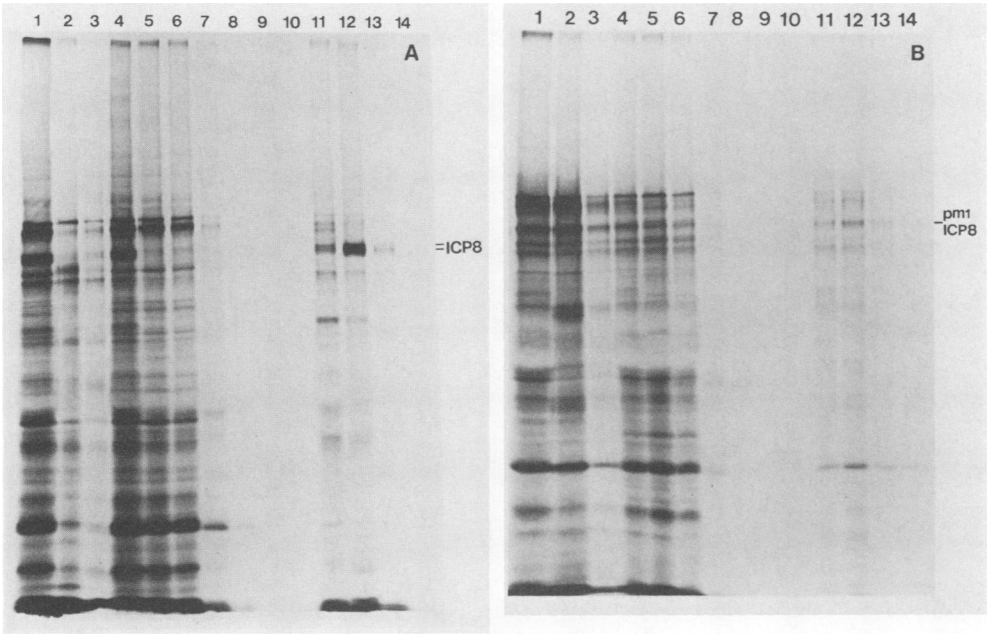

FIG. 6. ICP8 binding to ssDNA-cellulose. Vero cells were infected with either KOS1.1 (A) or *pm*1 (B) in the presence of phosphonoacetate and labeled with [^{35}S]methionine from 4 to 6 h postinfection. Various protein fractions resolved on ssDNA cellulose columns were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lanes: 1, total cellular lysate; 2, pellet from high-salt DNase extraction; 3, pellet after dialysis; 4, extract put on ssDNA column; 5 to 10, flowthrough and wash; 11, 0.3 M NaCl eluate; 12, 0.5 M NaCl eluate; 13, 1.0 M NaCl eluate; 14, 4.0 M NaCl eluate. Position of ICP8 (either KOS1.1 ICP8 or pm1 ICP8) is indicated on the right of either gel.

ICP8 bound to ssDNA-cellulose as efficiently and tightly as did wild-type ICP8. The lowest level of binding was observed with *d*301 ICP8 (21%) in which 56% of ICP8 was missing. The majority of the ICP8 encoded by amino-terminal deletion mutants *d*101 and *d*102 bound to DNA cellulose (72 and 75%, respectively). In contrast, the majority of *n*2 ICP8 was found in the flowthrough and wash fractions (54%). This result is consistent with the results of Leinbach and Heath (27) in which the carboxyl-terminal region, but not the amino-terminal region, of ICP8 synthesized in vitro bound to ssDNA cellulose columns. Therefore, we conclude that the portion of ICP8 from residues 564 to 1160 contains a region required for DNA binding.

Nuclear localization of ICP8 molecules encoded by viral mutants. Wild-type ICP8 was previously shown by immunofluorescence microscopy to localize into the nucleus in infected cells (14, 23, 38, 39). In the presence of a DNA synthesis inhibitor, ICP8 was found at the prereplicative sites (Fig. 7B; [39]) and during viral DNA replication, ICP8 was distributed in the nucleus as replication compartments (39). The cellular distribution of wild-type and mutant ICP8 molecules is shown in Fig. 7. The *n*10 ICP8 polypeptide, which lacks the last 36 amino acids from the carboxyl terminus and bound to an ssDNA cellulose column just as tightly as wild-type ICP8, remained within the cytoplasm (Fig. 7C). In contrast, the *pm*1 ICP8 polypeptide, which bound poorly to an ssDNA-cellulose column, was found predominantly in the nucleus (Fig. 7E). These results clearly demonstrate that the nucleus localization signal(s) of ICP8 is separate from the DNA-binding function.

The *d*101 ICP8 localized primarily within the nucleus (Fig. 7H) and bound to an ssDNA cellulose column (Table 3), but this virus did not replicate its DNA. The phenotype of this mutant provides genetic evidence that ICP8 has nuclear functions other than binding to DNA.

TABLE 3. Ability of mutant ICP8 to bind to ssDNA cellulose

Virus	% of ICP8:					
	In flowthrough and wash	Eluted at indicated NaCl concn				Bound
		0.3 M	0.5 M	1.0 M	4.0 M	
KOS1.1	2	23	67	8	<1	98
*pm*1	69	7	20	4	<1	31
*n*10	2	13	77	8	<1	98
*d*102	25	26	22	20	6	75
*d*101[a]	28	47	22	5	<1	72
*d*301[a]	79	6	13	2	<1	21
*n*2[a]	54	1	39	3	<1	46

[a] Data obtained from densitometry of the negatives prepared from the Western blots.

DISCUSSION

Phenotypic groups of ICP8 mutant viruses. On the basis of their phenotypic properties, we have classified the ICP8 mutant viruses into four groups (Table 4). The mutant ICP8 polypeptides of group A (*n*10 and *d*102) bound to ssDNA columns but failed to localize to the nucleus. The mutant ICP8 polypeptides of group B (*pm*1 and *d*301) localized to the nucleus but did not bind to DNA. The mutant ICP8 polypeptide of group C (*n*2) neither bound to DNA nor localized to the nucleus, and the mutant ICP8 polypeptide of group D (*d*101) not only localized to the nucleus but also bound to DNA.

It is not surprising that none of ICP8 mutant viruses could grow in the Vero cells because none of them promoted viral DNA replication. Failure to induce viral DNA synthesis by

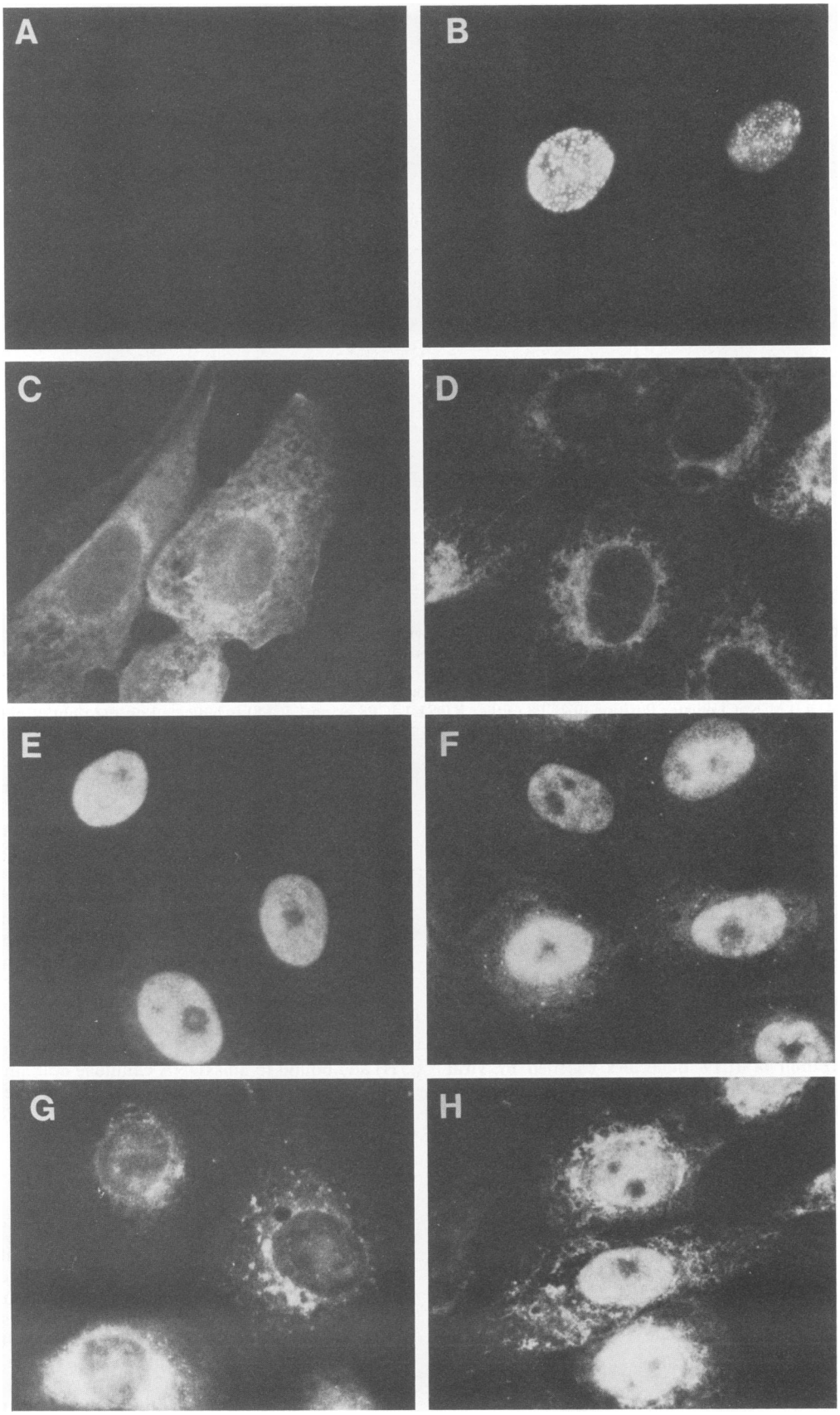

FIG. 7. Subcellular localization of ICP8 encoded by mutant viruses. Vero cells were infected with KOS1.1 or ICP8 mutant viruses. At 4 h postinfection, the cells were fixed, permeabilized, and incubated with either 793 anti-ICP8 monoclonal antibody and rhodamine-conjugated goat anti-mouse immunoglobulin (panels A through F and H) or anti-ICSP 11/12 polyclonal serum and fluorescein-conjugated goat anti-rabbit immunoglobulin (panel G). Immunofluorescence micrographs: A, mock-infected cells; B, KOS1.1-infected cells in the presence of phosphonoacetate; C, *n*10-infected cells; D, *d*102-infected cells; E, *pm*1-infected cells; F, *d*301-infected cells; G, *n*2-infected cells; H, *d*101-infected cells.

TABLE 4. Phenotypic classes of ICP8 mutant viruses

Group	Mutants	Growth on Vero cells	ssDNA binding[a]	Localization[b]
A	*n*10, *d*102	−	+	C
B	*pm*1, *d*301	−	−	N
C	*n*2	−	−	C
D	*d*101	−	+	N

[a] +, >50% binding.
[b] Mutant ICP8 molecules predominantly localized in the cytoplasm (C) or nucleus (N), as defined by indirect immunofluorescence.

the group A mutants could be attributed to the fact that ICP8 was excluded from the nucleus. However, we do not know whether restoration of nuclear localization would be sufficient for normal function of the protein. The group D mutant *d*101 exhibits a novel phenotype because it localized predominantly to the nucleus (Fig. 7H), and the majority of it can bind to ssDNA in vitro, but the mutant virus still fails to replicate viral DNA. This indicates that the role of ICP8 in viral DNA replication must be more than just simply binding to ssDNA. Matsumoto et al. (32) observed that the amino-terminal one-fifth of ICP8 shares some sequence similarity with rat proliferating cell nuclear antigen (PCNA), also known as cyclin (3) or the DNA polymerase-delta auxiliary protein (48). PCNA is a highly conserved protein whose synthesis is tightly associated with the cell cycle, occurring immediately before DNA synthesis (5, 30). PCNA, like ICP8, is a nuclear protein, and the intranuclear distribution of both PCNA and ICP8 is controlled by DNA synthesis itself or events triggered by DNA replication (5, 39). Moreover, the sites of nuclear localization of both PCNA and ICP8 have been found to correspond to sites of ongoing DNA synthesis (12, 31). In addition, both PCNA and ICP8 are required for DNA synthesis and can stimulate the activity of DNA polymerase (4, 33, 43, 48). Because the group D mutant virus *d*101 lacks the amino terminus of ICP8, it is conceivable that the amino terminus of ICP8 or a portion of it, like PCNA, has some nuclear function(s). This function(s) may involve interactions with different structural or functional elements in the infected cell nucleus, e.g., interaction with and stimulation of DNA polymerase (9).

The region required for DNA binding. In this study, mutant *n*10 ICP8 molecules bound to ssDNA in vitro as tightly and efficiently as wild-type ICP8 molecules did but failed to localize to the nucleus, suggesting that the absence of 36 carboxyl-terminal amino acids of ICP8 only affected nuclear localization, not DNA binding. This is in agreement with our previous finding that the abilities of ICP8 to localize to the nucleus and bind to the nuclear matrix are distinct from DNA-binding ability (25). Significant quantitative differences in DNA binding were observed when ICP8 was further truncated to residue 1029 (*n*2, 46%, compared with 98% for *n*10 polypeptide [Table 3]). This indicates that the region between residues 1029 and 1260 of ICP8 is required for efficient DNA binding. In addition, approximately 72 and 75% of *d*101 and *d*102 ICP8 polypeptides bound to ssDNA cellulose columns, respectively (Table 3). This demonstrated that the region from residues 564 to 1160 contains an ssDNA binding site. It should be noted that the majority of mutant ICP8 molecules which bound to ssDNA-cellulose eluted from the columns at a lower salt concentration (0.3 M NaCl) than that of wild-type ICP8 (0.5 M NaCl), suggesting that the amino terminus of ICP8 may be required for tight and efficient binding.

The mapping of the ssDNA binding domain of ICP8 must take into account not only the phenotypes of the deletion mutants but also the phenotypes of the *ts* mutants (15). Several interpretations of these results are possible. First, the amino-terminal portion of ICP8 is not absolutely required for ssDNA binding but may be involved in stabilizing the binding activity of the carboxyl terminus. This could explain the failure of the ICP8 molecules of *ts*13, *ts*18, and *ts*HA1 at the nonpermissive temperature to bind to DNA. Second, the amino terminus of ICP8 may be involved in the interactions with the carboxyl terminus of another molecule of ICP8, thus providing cooperative ssDNA binding. This is supported by the fact that mutant ICP8 polypeptides of *d*101 and *d*102 did not bind ssDNA-cellulose columns as efficiently as wild-type ICP8. It is also possible that ICP8 contains not only multiple binding sites but also an inhibitory region which could modulate the DNA-binding activities of this polypeptide.

It is interesting to note that the *pm*1 ICP8 polypeptide bound poorly to the ssDNA-cellulose column (31% binding [Table 3]), while the *d*101 polypeptide, which is missing the zinc finger structure motif (Cys-X_{2-4}-Cys-X_{2-15}-His-X_{2-4}-His), bound relatively well (72% binding). A similar observation was made for the DNA-binding domain of the simian virus 40 T antigen (1). Thus, this putative zinc finger cannot be an essential determinant of DNA binding for ICP8; however, it may still be involved in modulating the DNA-binding activity. It will be interesting to examine ssDNA-binding activities of ICP8 when either of these cysteines or adjacent cysteines are changed. Complete deletion of this region may lead the truncated *d*101 polypeptide to fold differently, allowing the binding region access to ssDNA.

The results of this study demonstrate that the nuclear localization signal(s) and ssDNA-binding domain(s) of ICP8 are separable. Analysis of these and additional ICP8 mutants will provide a more detailed definition of the ssDNA-binding domain(s) and its role in viral DNA replication and the nature of the other nuclear functions of ICP8.

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