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Intragenic Complementation among Partial Peptides of Herpes Simplex Virus Regulatory Protein ICP4

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Peptides of the herpes simplex virus type 1 regulatory protein, ICP4, which are translated from genes containing nonsense and deletion mutations retain specific biochemical properties and activities characteristic of the intact ICP4 molecule (N. A. DeLuca and P. A. Schaffer, J. Virol. 62:732–743, 1988). Mutant viruses expressing these peptides are deficient for viral growth in the absence of complementing wild-type protein supplied in *trans*, indicating that the mutant peptides are not functionally complete. In the present study we have demonstrated that certain pairs of mutants expressing partial ICP4 peptides complement each other. The complementation is shown at the level of transcription and results in enhanced virus growth. Among complementing pairs of ICP4 mutants is a virus expressing a peptide deleted for codons 185 to 309 (*d2*) and a virus expressing only the amino-terminal 774 amino acids (*n208*). By using a mobility-shift assay and by taking advantage of the specific DNA-binding properties of ICP4, it was demonstrated that novel ICP4-containing DNA-protein complexes were found when extracts from cells coinfected with complementing pairs of ICP4 mutants were incubated with target DNA. The novel complexes were shown to be a function of both mutant peptides in the coinfected cell, suggesting that complementation results from the multimerization of partial ICP4 peptides.

The major transcriptional regulatory protein of herpes simplex virus type 1 (HSV-1) is a 175-kilodalton polypeptide referred to as infected-cell polypeptide (ICP) 4 (2, 15). The ICP4 gene can be expressed in the absence of prior viral protein synthesis, and hence its product is defined as an immediate-early polypeptide (1, 15). From the study of virus strains containing temperature-sensitive (8, 28), deletion (4, 7), and nonsense (6, 7) mutations in the viral genes encoding ICP4, it has been demonstrated that ICP4 is required for at least two regulatory activities during productive infection, (i) the transcriptional stimulation of many HSV-1 early and late genes and (ii) the autoregulation of its own synthesis. These activities are consistent with the results of transient expression studies which show that ICP4 will transactivate a variety of HSV early and late gene promoters fused to reporter genes and negatively regulate expression from its own promoter (5, 9, 12, 19, 24, 25, 30).

Consistent with the nuclear function of ICP4 is its preferential localization to the nucleus of infected cells (2), where it accumulates in discrete subnuclear regions or compartments (16). These compartments are thought to be regions of ongoing viral DNA synthesis and gene expression. The ICP4 polypeptide is also modified by phosphorylation in a complex manner such that at least three phosphorylated forms are evident on one-dimensional sodium dodecyl sulfate-polyacrylamide gels (27, 35). More recently it has been shown that ICP4 will form specific protein-DNA complexes with DNA fragments representative of several HSV-1 genes (10, 17, 18, 23). While the proposed consensus for the ICP4-binding site is ATCGTCnnnnYCGRC (Y = pyrimidine; R = purine) (10), it has also been suggested that ICP4 will bind to nucleotide sequences which differ significantly from this sequence (22).

The mechanism of action of the ICP4 protein is unclear at present, although partial peptides of the ICP4 molecule which retain the ability to form specific DNA-protein complexes retain regulatory activity, and those which do not form such complexes are inactive (7). In this study it was shown that a virus expressing the amino-terminal 774 amino acids as a consequence of a nonsense mutation (*n*208) was able to participate in specific DNA-protein complexes and transactivate a number of HSV early genes (7). In transient assays this peptide was sufficient to transactivate an HSV-1 thymidine kinase-chloramphenicol acetyltransferase chimeric gene approximately half as well as the intact, 1,298-amino-acid protein (6). Despite the transactivity of the 774-amino-acid molecule, *n*208 is impaired for virus growth, apparently blocked at a stage subsequent to viral early gene expression (7). Therefore it was proposed that the carboxy-terminal region of the ICP4 molecule contains a domain which specifies a function required for full ICP4 activity.

Given these observations and the report that ICP4 exists as a multimer in vitro (21), we investigated the possibility that the functions missing in the truncated *n*208 molecule could be supplied in *trans* by a different ICP4 molecule, resulting in greater ICP4 function. The virus *d*2, which expresses an ICP4 protein possessing a deletion of codons 185 to 309, specifies the primary sequence missing in *n*208 (7). The *d*2 protein does not form a complex with DNA, nor does it specify regulatory activity. This study examines the intragenic complementation observed between the mutant ICP4 molecules *d*2 and *n*208. Initial experiments assess the levels of complementation following pairwise mixed infections of Vero cells with a number of ICP4 deletion and nonsense mutants. Subsequent experiments address the possible mechanism by which complementation may occur.

MATERIALS AND METHODS

Virus and cells. Procedures for the growth and maintenance of Vero and E5 cells were conducted as described previously (3). E5 cells express complementing levels of ICP4 and were derived from Vero cells as previously de-

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scribed (4, 6). The wild-type strain of HSV-1 (KOS) and all the ICP4 mutant strains of HSV-1 used herein were propagated on E5 cells except where specifically stated. The ICP4 nonsense mutants *n*6, *n*214, *n*208, *n*18, and *n*215 and deletion mutants *d*2 and *d*156 were previously described (7).

Complementation. Complementation experiments were performed in a manner similar to that described previously (31). Vero cell monolayers were coinfected with pairs of mutant viruses at a multiplicity of infection (MOI) of 5 PFU of each virus per cell. Similar monolayers were also singularly infected with each mutant virus at an MOI of 10 PFU/cell. Infected cultures were incubated at 37°C and were harvested at 18 h postinfection. The harvested cultures were frozen and thawed once, sonicated, and clarified of cellular debris. The clarified lysate was then assayed for total virus yield on E5 cells and for wild-type recombinants on Vero cells. Each complementation index [$AB/(A+B)$] was calculated as the ratio of the total virus yield of the mixed infection (AB) to the sum of the total virus yields of the corresponding single infections ($A+B$).

Transcription experiments. Nuclear run-on transcription analysis was performed by incubation of nuclei isolated from infected Vero cells with [^{32}P]GTP (New England Nuclear Corp.) and nonradioactive UTP, CTP, and ATP. The procedures for the isolation of infected-cell nuclei, in vitro RNA run-on reaction, and the subsequent isolation of the radioactively labeled RNA were similar to those previously described (7, 33). For each run-on reaction an 85-mm dish of Vero cells (5×10^8 cells) was placed on ice and rinsed with cold TBS (25 mM Tris hydrochloride, pH 7.4, 140 mM NaCl, 5 mM KCl) and then with RSB (10 mM NaCl, 10 mM Tris hydrochloride, pH 7.4, 3 mM $MgCl_2$). The cells were scraped into RSB containing 0.1% Nonidet P-40 plus 20 U of RNasin (Promega) per ml, dounce homogenized (four strokes), and concentrated by low-speed centrifugation. The nuclei were washed once with RSB plus 20 U of RNasin per ml, concentrated, and suspended in 20 μl of nuclei buffer (5 mM $MgCl_2$, 10 mM Tris hydrochloride, pH 7.5, 0.5 M sorbitol, 2.5% Ficoll). The nuclei were added to a transcription mix to yield a total volume of 100 μl containing final concentrations of 25% glycerol, 200 Ci of [α-^{32}P]GTP (New England Nuclear Corp; 800 Ci/mmol), 40 mM Tris hydrochloride (pH 8.3), 150 mM NH_4Cl, 7.5 mM $MgCl_2$, 0.5 mM ATP, 0.25 mM UTP, 0.25 mM CTP, and 200 U of RNasin per ml. The reaction was incubated at 30°C for 30 min, and then 20 U of DNase (DPRF, Cooper Biomedical) was added and incubation was continued for an additional 10 min. A 100-μl volume of proteinase K solution (20 mM Tris hydrochloride, pH 7.4, 4 mM EDTA, 2% sodium dodecyl sulfate, and 800 μg of proteinase K per ml) was then added, and the mixture was incubated at 37°C for 1 h. The reactions were then extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (24:1), ethanol precipitated, suspended in TE (10 mM Tris hydrochloride, pH 8.0, 1 mM EDTA), and passed over Sephadex G-50 to remove the unincorporated nucleotides.

The ^{32}P-labeled run-on RNA was used to probe nitrocellulose filters containing single-stranded recombinant M13 DNA specifying portions of the HSV-1 DNA sequences homologous and complementary to the indicated mRNA species. The M13-HSV constructs were obtained from David Knipe (Harvard Medical School). The conditions for hybridization, washing, and RNase treatment of the probed filters were exactly as previously described (33).

Immunofluorescence. Infected monolayer cultures were fixed, stained for indirect immunofluorescence, washed, and mounted as previously described (26). Where appropriate, the polyclonal antibody to ICP4 (provided by Richard Courtney, Louisiana State University Medical Center, Shreveport) (2) was used as a primary stain at a dilution of 1:10. The monoclonal antibody to ICP4, 58S, was used at a dilution of 1:30. Rhodamine isothiocyanate-conjugated goat anti-rabbit or anti-mouse antibodies (Cappel Laboratories, Cochranville, Pa.) were used as secondary stains at a dilution of 1:50.

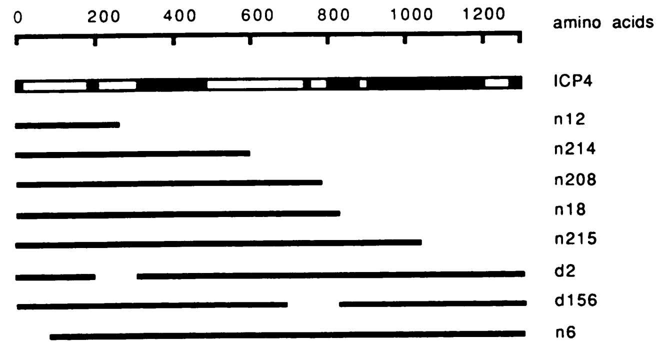

FIG. 1. ICP4 primary sequence expressed from mutant viruses. The wild-type ICP4 molecule is shown, indicating the regions most similar to the 140-kilodalton varicella-zoster virus analog (filled-in regions). This information is taken from McGeoch et al. (20). The ICP4 primary sequence expressed from mutant viruses used in this study is indicated by a solid line. The properties of the viruses have been previously described by DeLuca and Schaffer (7).

Complementation with plasmids. CV-1 cells were cotransfected with 2 μg of the ICP4 plasmids specified plus either 2 μg of pUC8 or the plasmid encoding the deletion mutant *d*2, as previously described. Twenty hours later, the transfected monolayers were infected with the ICP4 deletion mutant, *d*120 (4), at an MOI of 1 PFU/cell. At 18 h postinfection, the cultures were harvested, and clarified viral lysates were prepared as described above. The lysates were assayed on E5 cells for total yield and on Vero cells for recombinants. Recombinational rescue of *d*120 in these experiments was not observed. All of the plasmids used in these experiments were previously described (6).

Resolution of DNA-protein complexes. The procedure for the preparation of whole-cell extracts from infected Vero cells was as previously given (7). The conditions for the DNA-binding reactions and the electrophoretic resolution of DNA-protein complexes on polyacrylamide gels were also as previously given except that 3 μg of poly(dI · dC)(Pharmacia) was used instead of salmon testis DNA.

RESULTS

Complementation of ICP4 mutants. The ICP4 molecule synthesized by *n*208 (Fig. 1) localizes to the nucleus, is capable of forming specific DNA-protein complexes, and will transactive a number of early and late HSV-1 genes, yet is not sufficient to support virus growth. The virus *n*208 is impaired with respect to the synthesis of viral DNA and the expression of "true late," or γ_2, genes (7). The lack of expression of γ_2 genes may be a sole consequence of the DNA synthetic deficiency, due to the stringent requirement for viral DNA synthesis on late gene (γ_2) expression (14). Despite the lack of a large portion of the carboxy terminus (Fig. 1), the *n*208 molecule retains a number of physical and functional properties characteristic of the intact molecule, suggesting that the ICP4 structure may consist of separate domains specifying activities or properties collectively con-

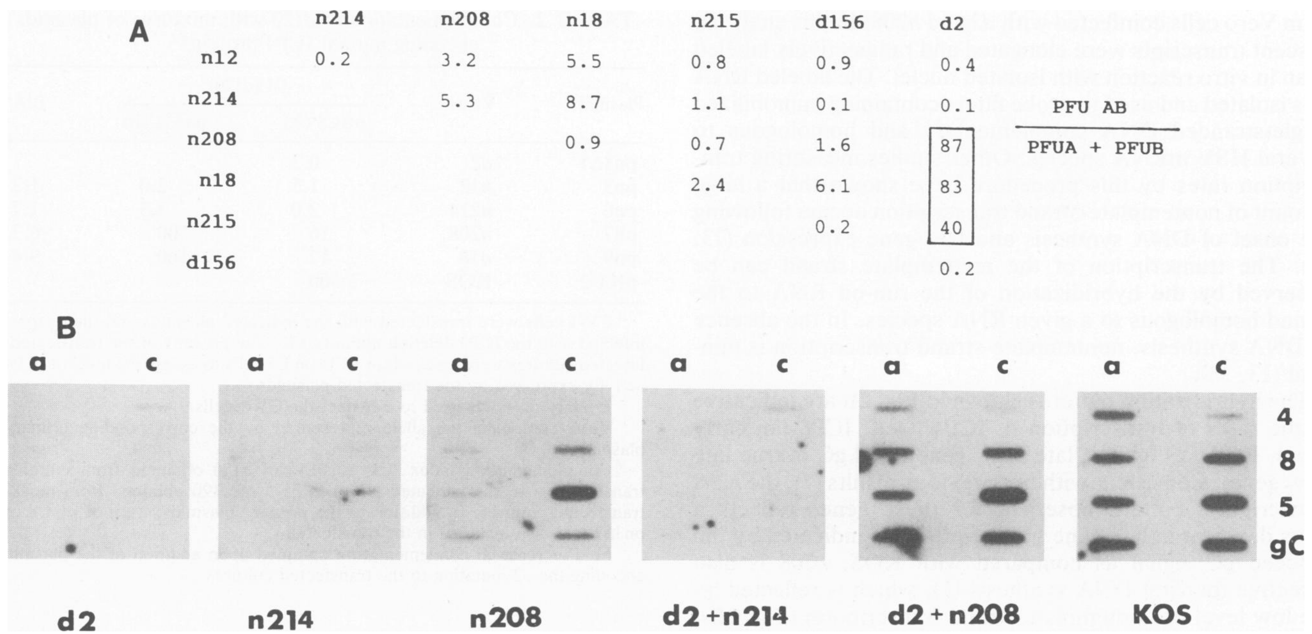

FIG. 2. Complementation between ICP4 mutants. (A) The indicated mutants were tested in a classic complementation experiment as described in Materials and Methods. Shown are the complementation indices for independent pairs of complementation tests. The indices included in the box represent values that we interpret as indicating that complementation has occurred. (B) Vero cells were infected with the indicated viruses at an MOI of 20 PFU/cell for the single infections and 10 PFU/cell per virus in the multiple infections. At 8 h postinfection, infected-cell nuclei were isolated and used in a nuclear run-on transcription experiment as described in Materials and Methods. The ^{32}P-labeled run-on RNA was used to probe filters containing single-stranded probes homologous (a) and complementary (c) to the mRNA for the indicated HSV gene product.

tributing to the function of ICP4 in viral infection. The ICP4 molecule specified by the virus *d*2, which lacks codons 185 to 309, contains the region not expressed in *n*208 (Fig. 1). Although the *d*2 peptide can localize to the nucleus of infected cells, it is not capable of binding to DNA in vitro and does not elicit regulatory activity when expressed from the viral genome (7). Given these observations, Vero cells were coinfected with these and other mutant viruses which express partial ICP4 peptides to determine whether the partial peptides could supply a function(s), in *trans*, resulting in enhanced virus growth by complementation. After one round of viral growth, the coinfected cultures were harvested and assayed on E5 cells for complementation. Complementation was considered positive if the ratio of the yields of the mixed infection to the sum of the yields from the corresponding single infections consistently exceeded a value of 10. This number, or complementation index, is an adaptation of a previously adopted convention determined by comparing information obtained from complementation tests using temperature-sensitive (*ts*) mutants of HSV-1 with the corresponding map locations of the mutations specifying the *ts* lesions (34). In the test shown in Fig. 2A, significant complementation was observed when any of the nonsense mutants, *n*208, *n*18, or *n*215, was coinfected with the deletion mutant *d*2. Mutants *n*18 and *n*215 specify ICP4 molecules that are larger than *n*208 and which exhibit similar activities (Fig. 1; 7). Mixed infections involving other pairs of mutants did not result in complementation after repeated trials.

It is unlikely that the elevated yields in the complementing pairs are due to recombination, given that (i) recombination frequencies are in most cases greater for many of the noncomplementing pairs (Table 1) and (ii) the recombination observed between *n*12 and any of the mutants *n*208, *n*18, or *n*215 did not result in complementation. Since the deletion in the virus *d*2 includes the locus of the nonsense mutation in *n*12 (Fig. 1), recombination between *n*12 and the other nonsense mutants would be expected to be at least as great as that between *d*2 and the nonsense mutants. In these experiments, recombinants were scored as PFU which did not require that ICP4 be supplied in *trans* and which therefore plaqued on Vero cells. Viruses isolated from these plaques have always contained at least one wild-type copy of the ICP4 gene (data not shown).

The complementation between *d*2 and *n*208 was also investigated at the level of transcription by measuring the rate of several representative HSV genes. This was performed by run-on transcription analysis with nuclei isolated

TABLE 1. Recombination between ICP4 mutants in infected Vero cells[a]

Mutant	Recombination[b] with mutant:					
	*n*214	*n*208	*n*18	*n*215	*d*156	*d*2
*n*12	ND	17	11	6.3	ND	ND
*n*214		2.1	3.3	3.8	ND	ND
*n*208			0.3	1.7	ND	3.8
*n*18				1.7	ND	5.1
*n*215					0.8	1.7
*d*156						ND

[a] The infected-cell lysates from the experimental shown in Fig. 2A were assayed for wild-type recombinants by the ability to form plaques on Vero cells.

[b] Numbers represent the ratio of the number of PFU on Vero cells to the number of PFU on E5 cells, multiplied by 100. ND, Not detected; no plaques were seen in duplicates of a 10^0 dilution of the infected-cell lysate.

from Vero cells coinfected with *d2* and *n*208. In this analysis, nascent transcripts were elongated and radioactively labeled in an in vitro reaction with isolated nuclei. The labeled RNA was isolated and used to probe filters containing immobilized single-stranded DNA complementary and homologous to several HSV mRNA species. Other studies measuring transcription rates by this procedure have shown that a large amount of nontemplate-strand transcription occurs following the onset of DNA synthesis and late gene expression (13, 33). The transcription of the nontemplate strand can be observed by the hybridization of the run-on RNA to the strand homologous to a given RNA species. In the absence of DNA synthesis, nontemplate-strand transcription is minimal (13, 33).

The hybridization patterns shown in Fig. 2B are indicative of the rates of transcription of ICP4 itself, ICP8 (an early gene), ICP5 (a "leaky" late or γ_1 gene), and gC (a true late of γ_2 gene). Consistent with our previous results (7), the *n*208 transcription pattern observed for these genes reflects a virus deficient in late gene transcription, as indicated by the reduced gC signal as compared with KOS. *n*208 is also defective for viral DNA synthesis (7), which is reflected by the low level of nontemplate-strand transcription (Fig. 2B). Earlier in infection (<6 h postinfection), transcription of the *d2* genome is limited to the immediate-early genes (7); however, at times beyond 6 h postinfection, transcription of the immediate-early genes declines. This has also been observed with an HSV-1 temperature-sensitive ICP4 mutant (13). At 8 h postinfection, the *d2* genome is transcriptionally silent compared with that of *n*208 (Fig. 2B). The hybridization pattern from cells coinfected with *n*208 and *d2*, however, is more permissive in nature than that from cells infected with *n*208 alone and resembles that observed with KOS. Therefore, the presence of the *n*208 and *d2* forms of ICP4 results in a transcriptional program more like that of the wild-type than does the presence of either of the two peptides alone. Also shown in Fig. 2B is the absence of complementation at the transcriptional level in cells coinfected with *n*214 and *d2*. This is consistent with the lack of complementation between *n*214 and *d2* as measured by enhanced growth (Fig. 2A).

To establish more firmly that the complementation seen in mixed infections is a consequence of the activities of the ICP4 proteins themselves, Vero cells were transfected with the plasmids encoding the mutated ICP4 genes used to construct the viruses described above, either alone or in combination. The transfected cells were then infected with the mutant virus *d*120. *d*120 contains 4.1-kilobase deletions in both copies of the viral ICP4, requiring ICP4 to be provided in *trans* for viral growth. At 18 h postinfection the cultures were harvested and assayed for complementation on E5 cells. As previously demonstrated (6), cells transfected with the plasmids encoding the *n*208 and *n*18 peptides resulted in slightly elevated levels of complementation. When the plasmid encoding the *d2* mutation was included with these plasmids in the transfection, the level of complementation was increased an additional six- to ninefold (Table 2). Plasmids encoding viral mutations that failed to complement *d2* (Fig. 1) also failed to complement in this assay.

Properties of complementing ICP4 peptides. The following experiments were performed to examine the mechanism of intragenic complementation observed above. Both experiments utilize the previously described observation that the monoclonal antibody 58S only recognizes ICP4 molecules expressing the 214 carboxy-terminal amino acids (7). Therefore, while the *d2* peptide is recognized by the monoclonal antibody, the *n*208 molecule is not.

TABLE 2. Complementation of *d*120 with mixtures of plasmids encoding mutant ICP4 proteins[a]

Plasmid[b]	Virus[c]	CI (*d*120)[d]		B/A[e]
		pUC8 (A)	pn3Δ1 (B)	
pn3Δ1	*d2*	0.3		
pn3	*n*12	1.5	2.0	1.3
pn6	*n*214	2.0	3.3	1.7
pn7	*n*208	16	100	6.3
pn9	*n*18	17	160	9.4
pK1-2	KOS	1,500		

[a] CV-1 cells were transfected with the indicated plasmids and then superinfected with the ICP4 deletion mutant *d*120. The progeny of the transfected-infected cultures were assayed for PFU on E5 cells to assess the level of ICP4 activity expressed by the transfected plasmids.

[b] Primary plasmids used to transfect the CV-1 cells.

[c] Virus containing the allele represented by the corresponding primary plasmid.

[d] Complementation index (CI) = PFU of *d*120 obtained from cultures transfected with the indicated plasmids/PFU of *d*120 obtained from mock-transfected cultures. In addition to the primary plasmids, 2 μg of pUC8 or pn3Δ1 was also included in the transfections.

[e] Fold increase in comlementation obtained upon addition of the plasmid encoding the *d2* mutation to the transfected cultures.

The first experiment examined the intracellular localization of the ICP4 peptides by indirect immunofluorescence in cells infected with *n*208 or *d2* alone and in cells coinfected with *n*208 and *d2*. The ICP4 in wild-type virus-infected cells was localized in discrete regions of the nucleus at late times postinfection (Fig. 3). These regions have been hypothesized to be the sites of viral DNA synthesis and late gene expression and have been termed replication compartments (16). By contrast, the *n*208 peptide stained diffusely within the nucleus of infected cells with the polyclonal antibody and did not react with the monoclonal antibody 58S (7) (Fig. 3). This localization pattern and the markedly reduced levels of viral DNA synthesis and late gene expression in *n*208-infected cells are observations consistent with the proposed role of the replication compartments seen for the wild-type peptide. The localization of the *d2* peptide differed from that of both KOS and *n*208 in two aspects. (i) While there was a strong nuclear component of fluorescence, significant levels of ICP4-specific fluorescence were also seen in the cytoplasm. Because the *d2* peptide lacks regulatory activity it is overproduced relative to *n*208 and KOS (7). Therefore, the cytoplasmic component may be a result of an excess of ICP4 leading to precipitation, or the saturation of possible cellular factors required for nuclear localization. (ii) The intranuclear distribution of the *d2* peptide was neither diffuse nor arranged into replication compartments characteristic of *n*208 or KOS. The fine structure of intranuclear *d2* shown in Fig. 3 has been observed with other ICP4 mutants which localize to the nucleus but do not possess DNA-binding properties (data not shown).

When cells were coinfected with the viruses *d2* and *n*208 and subsequently stained with 58S, the localization of the *d2* molecule was observed in the simultaneous presence of the *n*208 molecule. Under these conditions the localization of the *d2* molecule was more limited to the nucleus than it was in the absence of *n*208. While some *d2* nuclei in the mixed infection retained the staining pattern of *d2* alone, many nuclei showed staining patterns similar to that of KOS. High magnifications of several nuclei are shown in Fig. 3 for comparison. Since complementation between mutant vi-

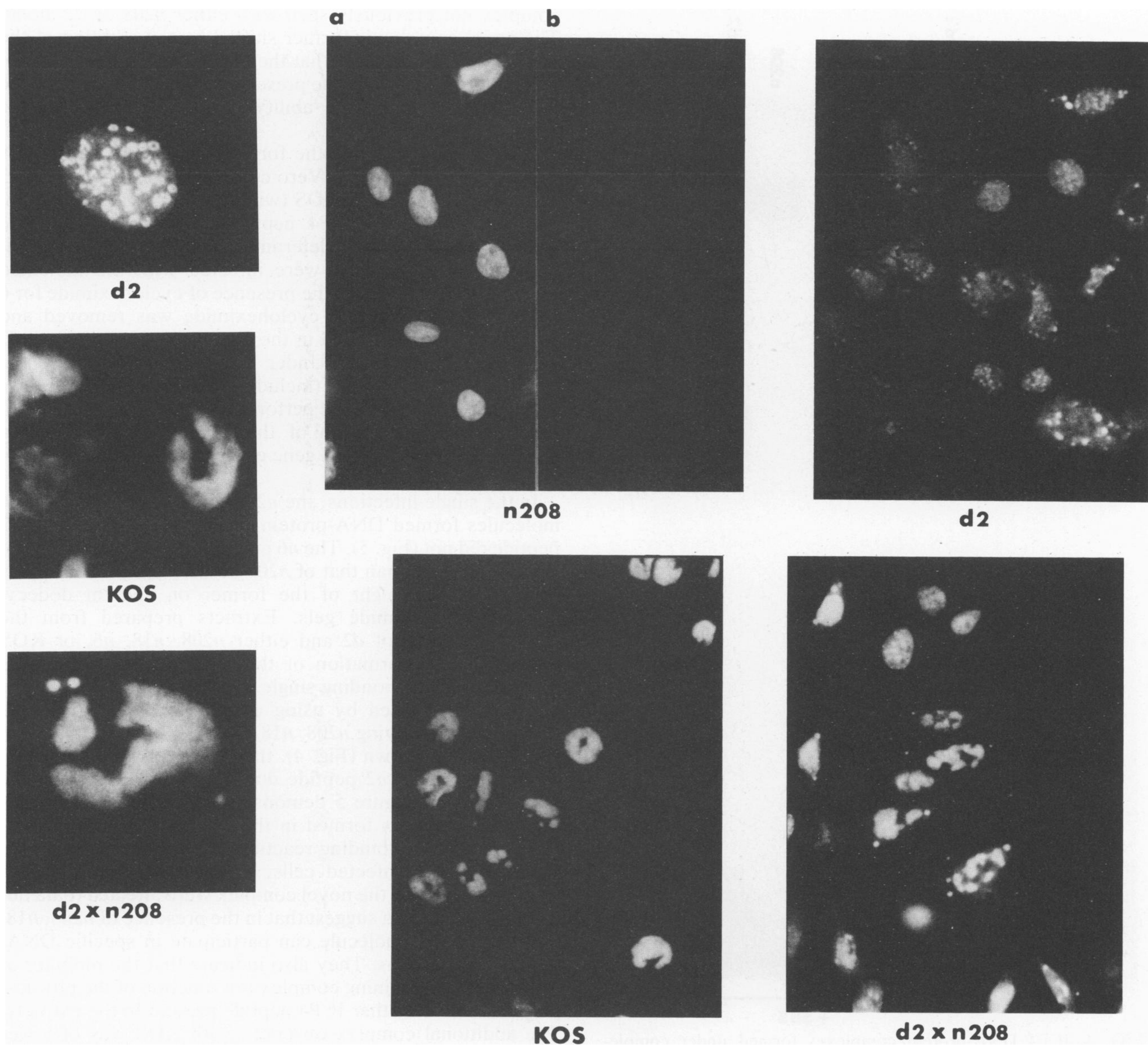

FIG. 3. Intracellular localization of the *d*2 molecule in infected and coinfected cells. Sparsely seeded Vero monolayers were infected at an MOI of 20 PFU/cell for the single infections and 10 PFU/cell per virus for the mixed infections. At 8 h postinfection the monolayers were fixed, permeabilized, and stained as described in Materials and Methods. For *n*208 the stain was the polyclonal antibody (2), followed by rhodamine isothiocyanate-conjugated goat anti-rabbit antibody (a), or the monoclonal antibody 58S, followed by rhodamine isothiocyanate-conjugated goat anti-mouse antibody (b). The remaining samples were stained with the monoclonal antibody preparation. To the left of the photographs showing populations of fluorescent nuclei are shown isolated nuclei at greater magnification to emphasize the change in the intranuclear distribution of the *d*2 molecule as a function of the presence of the *n*208 peptide.

ruses is rarely sufficient to yield wild-type levels of virus, it is not surprising that the staining pattern in the mixed infection was heterogeneous. A similar staining pattern was seen with the polyclonal antibody preparation which detects both *d*2 and *n*208 (data not shown). Therefore, the presence or the activity of the *n*208 peptide resulted in the more efficient nuclear localization of the *d*2 peptide and an intranuclear arrangement similar to that of the wild-type protein in some nuclei.

That the *d*2 peptide could be found in structures similar to the replication compartments observed for the wild-type peptide suggested that the *d*2 peptide may associate with DNA in the concurrent presence of the *n*208 peptide. We have previously shown that the *d*2 peptide extracted from *d*2-infected cells is unable to form specific DNA-protein complexes with a probe containing the start site of ICP4 transcription (7). This is also shown in the mobility shift assay of Fig. 4. Figure 4 indicates the position of the *n*208 and KOS DNA-protein complexes formed between a probe containing the start site of ICP4 transcription and proteins in extracts prepared from *n*208- and KOS-infected Vero cells. We have previously shown that these complexes contain the corresponding ICP4 peptides (7). The monoclonal antibody 58S further shifts the KOS ICP4-DNA complex but, consis-

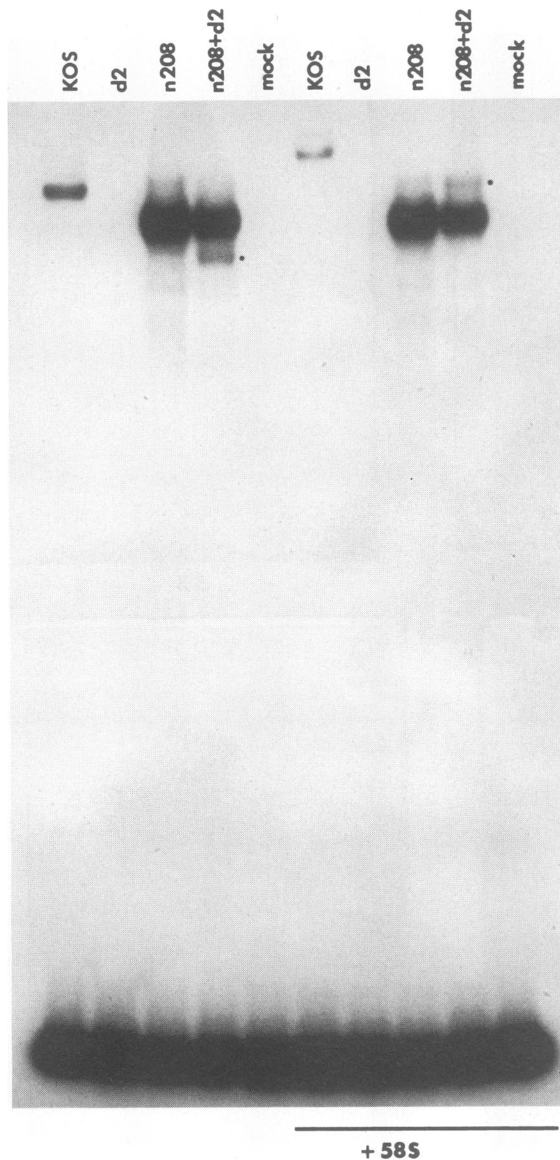

FIG. 4. ICP4 DNA-protein complexes formed under complementing conditions. Monolayers of Vero cells were infected with the indicated viruses as described in the legend of Fig. 3. At 8 h postinfection, extracts were prepared as previously described (7). A 3-μg sample of protein extract was incubated for 30 min at 22°C with approximately 1 ng of DNA probe (end labeled with ^{32}P to give 1.5 $\times 10^5$ to 3.0 $\times 10^5$ cpm/ng) and 3 μg of poly(dI · dC). The binding reactions were performed in duplicate. After the above incubation, 1 μl of a 1:10 dilution of 58S was added to one set, and incubation was continued for an additional 10 min. The resulting DNA-protein complexes were resolved from the unbound probe by electrophoresis as previously described (7). The probe used in this experiment was an *Eco*RI- to *Bam*HI fragment containing the HSV-1 sequences from −108 to +28 nucleotides relative to the transcription initiation site of the ICP4 mRNA. Dots indicate the position of the novel *d*2-containing complexes described in the text.

tent with previous results, fails to affect the mobility of the *n*208 complex. This complex can be further shifted by the addition of polyclonal ICP4 antibody (7). Incubation of extract prepared from *d*2- and *n*208-coinfected cells with the probe resulted in the formation of a complex having the mobility of the complex formed with *n*208 plus an additional complex not previously seen with either *n*208 or *d*2 alone. The novel complex is further shifted by the addition of the antibody 58S, indicating that the peptide *d*2 is present in the complex. Therefore, in the presence of the *n*208 peptide, the *d*2 peptide acquires the ability to participate in specific DNA-protein complexes.

To further investigate the formation of novel complexes involving the *d*2 peptide, Vero cells were coinfected with *d*2 and each of the strains KOS (wild type), *n*208, *n*18, and *n*6. The portion of the ICP4 peptide expressed from these viruses was previously determined (7; Fig. 1). Cycloheximide-treated Vero cells were infected with the indicated viruses and incubated in the presence of cycloheximide for 6 h, after which time the cycloheximide was removed and incubation was continued in the presence of actinomycin D for an additional 3 h. Under these conditions only the immediate-early proteins (including ICP4) are expressed (1, 15). This experiment was performed to eliminate the possibility that the formation of the novel complexes was a consequence of later viral gene expression and, hence, ICP4 regulatory activity.

In the single infections, the *n*208, *n*18, *n*6, and KOS ICP4 molecules formed DNA-protein complexes whereas the *d*2 peptide did not (Fig. 5). The *n*6 protein-DNA complex was of greater mobility than that of *n*208 despite the greater apparent molecular weight of the former on sodium dodecyl sulfate-polyacrylamide gels. Extracts prepared from the mixed infections of *d*2 and either *n*208, *n*18, *n*6, or KOS resulted in the formation of the DNA-protein complexes seen in the corresponding single infections. Additional complexes were formed by using extracts prepared from the coinfections involving *n*208, *n*18, and KOS, but not with *n*6. As previously shown (Fig. 4), the additional complex probably contains the *d*2 peptide due to its reactivity with the antibody, 58S. Figure 5 demonstrates the reactivity of the additional complex formed in the presence of *n*208. When 58S was added to binding reactions involving extracts from *d*2- and KOS-coinfected cells, the mobilities of both the KOS complex and the novel complex were affected (data not shown). These data suggest that in the presence of *n*208, *n*18, or KOS, the *d*2 molecule can participate in specific DNA-protein interactions. They also indicate that the mobility of the novel *d*2-containing complex is a function of the physical properties of the other ICP4 peptide present in the extracts. The additional complex observed with KOS was of lower mobility than that observed with *n*208 or *n*18, reflecting the difference between roughly 1,300 and 800 amino acids. The size difference between the *n*208 and *n*18 peptides is only 45 amino acids out of a total of 774 amino acids in *n*208. While this difference is discernible on sodium dodecyl sulfate-polyacrylamide gels, it is not resolvable in mobility-shift experiments. Consequently, the mobility of the novel complex would not be expected to differ between the two peptides.

DISCUSSION

The 175-kilodalton ICP4 protein of HSV-1 has been examined by genetic (3, 4, 6–8, 29) and biochemical (10, 11, 21) methods as well as by amino acid sequence comparison with the analogous protein from varicella-zoster virus (20). The sequence analysis of McGeoch et al. (20) has revealed continuous regions of amino acid sequence conservation with the varicella-zoster virus protein rather than dispersed similarities. The discrete regions of similarity may be indicative of functionally conserved domains of the polypeptide.

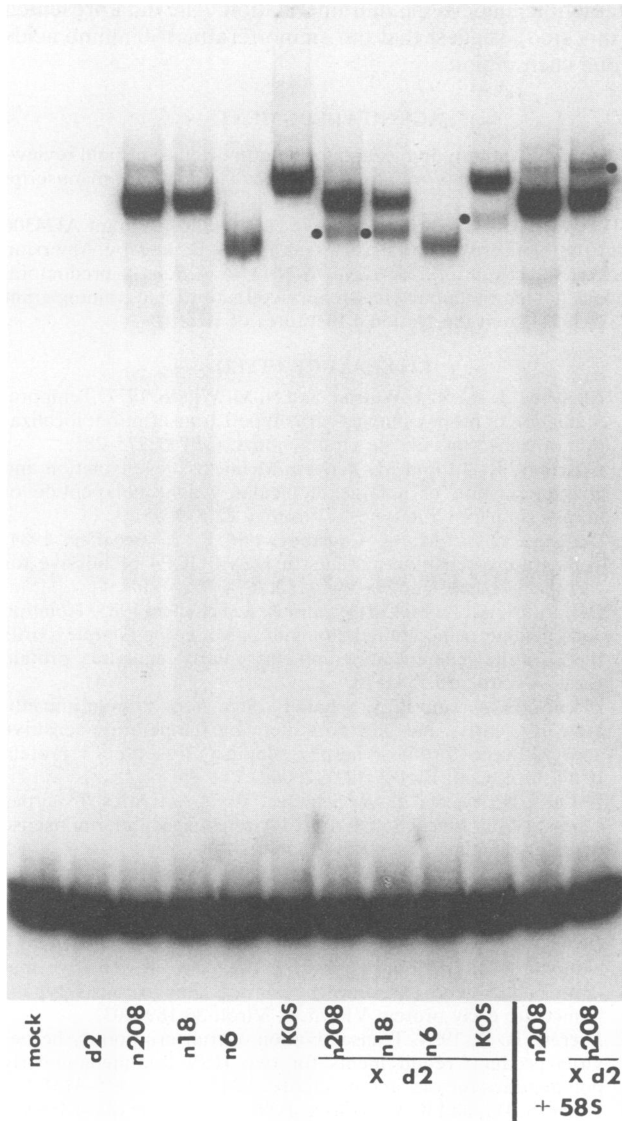

FIG. 5. ICP4 DNA-protein complexes formed under immediate-early conditions. Infected Vero cells were treated with cycloheximide (75 μg/ml) and actinomycin D (5 μg/ml) as described in the text. Extracts from cells infected with the indicated virus were used in a mobility shift experiment as described in the legend of Fig. 4 and in Materials and Methods. The indicated reactions were incubated with the 58S antibody, as previously described, prior to electrophoresis. The novel complexes formed with extracts from coinfected cells are indicated by dots.

Genetic analysis has revealed that activities and biochemical properties associated with the function of the intact ICP4 molecule can be separated, with partial ICP4 peptides expressed from mutant viruses (6, 7). One of these properties is the ability of ICP4 to form protein-DNA complexes with specific DNA sequences. This activity and the ability to regulate gene expression are properties inherent to the amino-terminal 774 amino acids of the ICP4 protein (7). We have previously located a sequence within this region responsible for nuclear localization (7) and recently have more finely defined domains contributing to DNA binding and transactivation (A. Shepard and N. DeLuca, manuscript in preparation). These domains correlate with regions of HSV-1 ICP4 which are similar to the varicella-zoster virus 140-kilodalton protein at the amino acid level. Therefore, the mutant virus *n*208, which specifies the first 774 amino acids (Fig. 1) of the 1,298-amino-acid wild-type protein, is sufficient to bind to DNA, autoregulate, and transactivate a number of early (β) and late (γ_1 only) genes (7). *n*208, however, still requires the wild-type ICP4 supplied in *trans* from ICP4-transformed cells for propagation. Therefore, the sequences carboxy terminal to amino acid 774 are required for full ICP4 function.

In the present study we have found that a portion of ICP4 containing the region missing in *n*208 can be supplied in *trans* with *n*208, resulting in complementation. The intragenic complementation between *d*2 and *n*208 results in elevated growth and enhanced transcription of the viral genome (Fig. 2). Viruses expressing larger nonsense peptides than *n*208 (i.e., *n*18 and *n*215) also are capable of the intragenic complementation observed with the virus *d*2 (Fig. 1 and 2). Complementation was not observed between *d*2 and a nonsense mutant (*n*214) specifying only the first 591 amino acids of ICP4.

Evidence from both genetic and sequence studies indicates that ICP4 is composed of discrete functional domains which presumably determine the function of ICP4 in viral infection. Therefore, it is conceivable that appropriate subsets of the molecule can individually contribute activities to reconstitute wild-type ICP4 function and, hence, viral growth. Such a mechanism would not require a direct interaction between complementing peptides. However, the ICP4 molecule expressed by the virus *d*2 can localize to the nucleus but does not participate in DNA-protein interactions in vitro, nor does it specify regulatory activities in transient assays or in the background of the virus (7). In view of the apparent null phenotype of *d*2, it is improbable that the *d*2 peptide provides an activity which acts in *trans* on its own. An alternative hypothesis involves the multimerization of the complementing pair of peptides. This mechanism requires that the multimeric heterocomplex results in a more functional quaternary structure than either of the homocomplexes alone. The best-characterized instance of intragenic complementation by this mechanism is the α-complementation observed in the *lacZ* system of *Escherichia coli*. In this system, a *lacZ* mutant containing a small deletion in the amino terminus and a mutant specifying a portion of the amino terminus spanning the deletion in the former can multimerize to form tetramers, resulting in the reconstitution of β-galactosidase activity (36).

Multimerization as a mechanism underlying complementation is consistent with previous results demonstrating that the wild-type ICP4 is isolated from infected cells as a homodimer (21). The immunofluorescence (Fig. 3) and the mobility-shift (Fig. 4 and 5) experiments presented herein are both consistent with the formation of heterocomplexes between complementing pairs of peptides. The immunofluorescence experiment (Fig. 3) demonstrated that the intracellular localization of the *d*2 molecule is dramatically affected by the simultaneous presence of the *n*208 molecule. In addition, novel DNA-protein complexes were formed when extracts were used from cells coinfected with *d*2 and either *n*18, *n*208, or KOS (Fig. 4 and 5). The monoclonal antibody 58S did not react with the *n*208 peptide but did react with the *d*2 peptide. Therefore, the presence of the *d*2 peptide in the novel DNA-protein complex was demonstrated by the reactivity of this complex with the 58S antibody, independent of the presence of the *n*208 peptide. Moreover, the mobility of the novel complex could be altered by substituting KOS for

*n*208 in the mixed infection (Fig. 5). The KOS and *n*208 molecules differ in electrophoretic mobilities (7), as do their corresponding DNA-protein complexes (Fig. 4 and 5). Taken together, these observations imply that the novel complexes contain ICP4 molecules from both viruses in the mixed infections. The multimerization of complementing molecules is implied by the ability to form heterocomplexes in the presence of DNA. We are currently investigating the quaternary structure of ICP4 peptides in the absence of DNA.

The function, activity, or structure that the *d*2 peptide provides in addition to that inherent in molecules such as *n*208 is of primary importance for the understanding of the intragenic complementation described in this study and of the function of ICP4 in general. One plausible hypothesis is that the *n*208 molecule, while able to bind to DNA as efficiently as the wild-type protein, is only one-half as active as the wild-type protein for transactivation (6, 7) and that association with the *d*2 peptide in part restores this activity. The inability of the *d*2 peptide (or others like it) to transactivate on its own may reflect the inability to bind to DNA. We have recently observed that a number of 2-amino-acid insertion and deletion mutations affecting amino acids 275 through 479 greatly reduce both DNA binding and transactivation regardless of the presence of the carboxy-terminal region of the protein (Shepard and DeLuca, manuscript in preparation). Therefore, the region of the ICP4 protein which confers specific DNA-binding activity in vitro is of primary importance for the function of ICP4. This would predict that the peptide expressed from *d*2 is inactive because it is altered in this important region. The association with *n*208 would complement this deficiency.

The lower abundance of the heterocomplexes relative to their homogeneous counterparts (Fig. 4 and 5) may reflect an absolute difference in relative quantity. Alternatively, the heterocomplexes formed with the *n*208, *n*18, or KOS molecules in the presence of *d*2 may possess a reduced affinity for the specific binding site relative to the corresponding homocomplexes. Heterocomplexes would be expected to exhibit a reduced ability to form a complex with DNA relative to the homocomplexes, reflecting the impaired DNA-binding domain of *d*2. This interpretation is consistent with observations from studies involving several multimeric DNA-binding proteins (32). The *d*2 molecule may also possess a structure which has a reduced capacity to multimerize.

ICP4 is of central importance to the regulation of viral gene expression and viral growth in vitro and in vivo. A full understanding of its function will be facilitated by a genetic examination of its structure, from primary to quaternary. The present study indicates how structure determined by regions of the primary sequence can contribute to full activity by multimerization. This study implies the existence of, but does not thoroughly examine, regions of the ICP4 molecule which are involved in the protein-protein interactions which result in multimerization. The increased mobility of the *n*6 protein-DNA complex relative to that of the smaller *n*208 molecule and the inability to detect a novel DNA-protein complex upon coinfection with *n*6 and *d*2 (Fig. 5) may suggest that the region missing in *n*6 is involved in multimerization. The *n*208 peptide which is capable of forming the novel complex with the *d*2 peptide possesses a primary structure which is completely contained within the *n*6 molecule with the exception of the first 90 amino acids (6). We are continuing to probe the structure and function of ICP4 by studying the intragenic complementation of a number of ICP4 alleles, and we are also determining regions of the ICP4 molecule which are involved in the protein-protein interactions underlying multimerization. The data presented in this study suggest that the amino-terminal 90 amino acids is one such region.

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