JOURNAL OF VIROLOGY, Nov. 1985, p. 558–570
0022-538X/85/110558-13$02.00/0

Vol. 56, No. 2

Isolation and Characterization of Deletion Mutants of Herpes Simplex Virus Type 1 in the Gene Encoding Immediate-Early Regulatory Protein ICP4

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Received 3 June 1985/Accepted 7 August 1985

Using Vero cells tranformed with the wild-type gene for ICP4 as the permissive host cell, we isolated herpes simplex virus type 1 (HSV-1) mutants containing deletions in both copies of the ICP4 gene. The mutants, d120 and d202, contained deletions of 4.1 and 0.5 kilobases, respectively, in each copy of ICP4. ICP4 mRNA synthesized in d202-infected Vero cells was 0.5 kilobases smaller than that synthesized in cells infected with the wild-type virus. No ICP4 mRNA was detected in d120-infected Vero cells. d120 and d202 specified polypeptides that reacted with ICP4 antiserum and were smaller than the wild-type ICP4 polypeptide by 130 and 30 kilodaltons, respectively. The only other HSV-1 gene products detectable on infection of Vero cells with d120 and d202 were ICP6 (of the early kinetic class of HSV-1 polypeptides), ICP0 (immediate early), ICP22 (immediate early), and ICP27 (immediate early). Immediate-early polypeptides ICP0 and ICP27 were expressed to a higher level in Vero cells infected with an ICP4 temperature-sensitive (*ts*) mutant (*ts*B32) at 39°C, indicating immediate-early stimulatory activity associated with the *ts* ICP4 polypeptide. In addition, the patterns of complementation of d120, d202, and *ts*B32 in ICP4-transformed cells also demonstrated inhibitory activity associated with the *ts* form of the ICP4 polypeptide.

The viral genes expressed initially after infection of susceptible cells with herpes simplex virus type 1 (HSV-1) have been designated immediate-early (or α) genes (3, 19). These genes are operationally defined as those transcribed in the absence of prior viral protein synthesis (3, 19). Five immediate-early mRNA species are translated into the polypeptides ICP0, ICP4, ICP22, ICP27, and ICP47 (19, 50). Functional immediate-early polypeptides are required for efficient expression of early (β) or late (γ) viral genes (20).

The functional roles of individual immediate-early gene products in the regulated expression of HSV-1 genes have been examined by several methods. First, temperature-sensitive (*ts*) mutants of HSV-1 containing mutations in the genes for ICP4 and ICP27 have been isolated and characterized (11, 35, 37, 40). Phenotypic analysis of these mutants has shown that ICP4 and ICP27 perform essential replicative functions and are involved specifically in modulating HSV-1 gene expression (11, 36, 40, 49). Second, reconstruction experiments involving cotransfection of plasmids containing the immediate-early genes for ICP4 and ICP0 and suitable test genes indicate that both stimulate expression of HSV-1 early genes in *trans* (12, 30, 39). Finally, deletion mutants in the gene for ICP22 have been shown to be capable of growing in Vero cells, a cell line which is routinely used as a laboratory host for HSV. The involvement of the immediate-early polypeptides ICP22 and ICP47 in HSV-1 gene expression, however, remains obscure.

The most extensively studied immediate-early gene of HSV-1 is that encoding the 175-kilodalton polypeptide ICP4. A large number of temperature-sensitive mutants in the gene for ICP4 exist. Collectively, they define HSV-1 complementation group 1-2 (43). A common characteristic of *ts* mutants in this group is that they induce dramatic overproduction of immediate-early polypeptides at the nonpermissive temperature. Based on this observation and the results of temperature-shift experiments, it has been postulated that ICP4 is required for negative regulation or attenuated expression of immediate-early genes (11, 36). Other features of cells infected with most ICP4 *ts* mutants at the nonpermissive temperature are underrepresentation or absence of early and late polypeptides and absence of viral DNA (11, 36). The basis for these phenotypic properties is that wild-type ICP4 is required for the accumulation of stable transcripts from early and late genes, and the products of early genes are required for viral DNA synthesis. A subset of *ts* mutants in group 1-2 induce the synthesis of appreciable levels of early polypeptides and viral DNA at the nonpermissive temperature (8). The absence of detectable infectious virus in such mutants is attributable to the absence of late polypeptides. Based on the cumulative evidence, it is thought that ICP4 is (i) required for the expression of early and late genes, (ii) autoregulatory, and (iii) involved in the inhibition of expression of other immediate-early genes. Furthermore, it appears that the mechanism of action of ICP4 involves modulation of gene expression at the level of accumulation of stable transcripts.

As just stated, our current understanding of the activities of and requirements for wild-type ICP4 is based primarily on the phenotypic properties of ICP4 *ts* mutants at the nonpermissive temperature. The conclusions drawn from studies of these mutants rest in part on the assumption that the temperature-sensitive forms of the ICP4 polypeptide have no influence on HSV-1 gene expression. This may not, in fact, be the case. Recently, it has been shown that ICP0 can act in *trans* to stimulate expression from early promoters in a cotransfection assay (30, 39). This presents an interesting paradox in that most ICP4 *ts* mutants overproduce immediate-early polypeptides (including ICP0) and yet fail to express early polypeptides. Therefore, either cotransfection experiments do not reflect an event occurring in viral infec-

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TABLE 1. Growth of HSV-1 *ts* mutants on 2-6 and Vero cells

Virus	Complementation group	Defective gene	Virus grown on[a]	
			2-6 Cells	Vero cells
KOS			8.0×10^6	3.7×10^6
*ts*B32	1-2	ICP4	7.8×10^5	2.1×10^2
*ts*B21	1-2	ICP4	6.0×10^4	5.0×10^0
*ts*L14	1-2	ICP4	1.1×10^5	1.7×10^2
*ts*Y46	1-5	ICP27	2.5×10^1	1.0×10^1
*ts*J12	1-9	gB	4.0×10^2	3.6×10^2
*ts*A24	1-1	ICP8	3.0×10^1	3.0×10^1

[a] 2-6 and Vero cells were infected with the indicated virus multiplicity of infection of 1.0 PFU per cell and incubated at 39°C for 18 h. The cultures were then harvested and assayed on Vero cells at 34°C.

tion or, more interestingly, the *ts* form of the ICP4 polypeptide has an inhibitory effect on early gene expression or the *trans* activity of ICP0 or both. In addition, the isolation of *ts* mutants in the structural gene for ICP4 which exhibit distinctly different regulatory phenotypes (8) suggests that the *ts* form(s) of ICP4 may exhibit activities capable of modulating gene expression to a greater or lesser degree.

To better define the role of ICP4 in the life cycle of HSV-1 and to answer questions regarding the possible regulatory activities of *ts* forms of this protein on HSV-1 gene expression, we isolated deletion mutants of HSV-1 which induce the synthesis of truncated forms of the ICP4 polypeptide. Because ICP4 performs an essential replicative function, we developed a system for screening and propagating ICP4 deletion mutants by constructing cell lines expressing the wild-type ICP4 gene. Such a system has proven invaluable for the study of deletion mutants in the major transcriptional regulatory protein of adenovirus, EIA (22). Although cells transformed with HSV-1 genes have been shown to complement *ts* mutants (7, 41), such cells have not been used to date to isolate deletion mutants of HSV-1. In the present study, two ICP4 deletion mutants were isolated and used to address the following questions. (i) What is the polypeptide phenotype of cells infected with a mutant lacking a full-length ICP4 polypeptide, and (ii) does this phenotype differ from that of *ts* mutant-infected cells in which a full-size yet temperature-sensitive form of the ICP4 polypeptide is produced?

MATERIALS AND METHODS

Cells and viruses. Procedures for the growth and maintenance of African green monkey kidney cells (Vero, ATCC P130; CV-1, ATCC P27) were previously described (51). Nero cells are an uncloned population of G418-resistant Vero cells generated by transformation with pSV2neo.

The wild-type KOS strain of HSV-1 and the KOS-derived temperature-sensitive mutants (Table 1) were propagated and assayed as previously described (8). *ts*B32, *ts*B21, and *ts*L14 are mutants in the structural gene for ICP4 (8, 11).

Plasmids. pSV2neo contains the bacterial gene for neomycin resistance under control of the simian virus 40 early promoter (46). pKXC (Fig. 1) contains the *Xho*I C fragment of KOS and was constructed as described previously (10). pKBZ and pKX2 (pKEB-X2) are diagrammed in Fig. 1 and were constructed as described previously (8).

Plasmids pKX2-Δ*Sac*I and pKX2-P4 were constructed from pKX2 in the following manner. pKX2 was partially digested with *Sac*I, religated with T4 DNA ligase, and transformed into *Escherichia coli*. Ampicillin-resistant clones were screened only for deletion of the 0.5-kilobase (kb) *Sac*I fragment (Fig. 1) to yield pKX2-Δ*Sac*I. pKX2-P4 was constructed by insertion of the 220-base-pair *Eco*RI-to-*Sma*I fragment (−110 to −330 [Fig. 1]) into the *Eco*RI site of pKX2 in the proper orientation. The *Sma*I site at −330 was modified with *Eco*RI linkers. DNA-modifying enzymes and *Eco*RI linkers were obtained from New England BioLabs, Inc. (Beverly, Mass.) and used as prescribed by the manufacturer.

Nucleic acid isolation. Purified HSV-1 (14) and bacterial plasmid DNAs were isolated as described previously (8). Cytoplasmic RNA was isolated from Nonidet P-40 extracts of Vero cells free of nuclei, as previously described (24).

Transformation of Vero cells. pSV2neo confers resistance to the antibiotic G418 (geneticin; GIBCO Laboratories, Grand Island, N.Y.) when integrated into cultured mammalian cells (46). Vero cells were cotransformed with pSV2neo and HSV-1 ICP4 as follows. pSV2neo (0.5 μg) was coprecipitated with 5.0 μg of pKXC in the presence of 15 μg of salmon testes DNA (Sigma Chemical Co., St. Louis, Mo.) in a total volume of 1.0 ml by the procedure of Graham and van der Eb (16). Freshly trypsinized Vero cells (4×10^6) were added to the precipitate and incubated at 37°C with continuous shaking for 30 min. The cell-DNA mixture was suspended in 20 ml of culture medium and transferred to two 85-mm petri dishes. After a 4-h incubation at 37°C, the adsorbed cells were subjected to glycerol (15%) shock and incubated for an additional 2 days at 37°C. The cells were then trypsinized and seeded into 85-mm petri dishes at a density of 5×10^3 cells per cm^2. After the cells had adsorbed to the dish, the medium was removed and medium containing 1.0 mg of G418 per ml was added. After 5 to 10 days, the G418 concentration was lowered to 400 μg/ml. After 12 to 18 days, individual G418-resistant colonies were isolated, amplified, and subsequently screened for ability to support replication of *ts*B32 at 39°C in a plaque assay. Approximately 20% of the G418-resistant clones exhibited detectable complementing activity. One cell line, 2-6, was retained for further study.

Cell lines 1-2, 1-5, and 4-2 were generated in a similar manner, except that pKX2-P4 rather than pKXC and d120 and d202 were used to generate and screen G418-resistant clones, respectively. Among pKX2-P4-transformed cells,

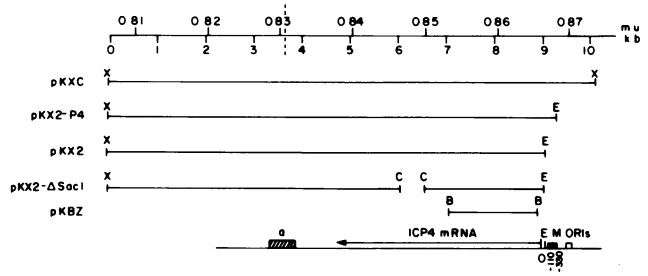

FIG. 1. HSV-1 DNA inserts in plasmids used in this study. The region of the HSV-1 genome in the I_S orientation (17) between map units (m.u.) 0.806 and 0.877 containing ori$_S$ (47), the ICP4 upstream regulatory sequences (closed box) (23), the ICP4 message (50), and the a sequence (27) is shown with respect to the HSV-1 inserts in pKXC, pKX2-P4, pKX2, pKX2-Δ*Sac*I, and pKBZ. The derivation of these plasmids is described in Materials and Methods. The relevant restriction sites shown are *Eco*RI (E), *Sma*I (M), *Bam*HI (B), *Sac*I (C), and *Xho*I (X). The dashed line represents the L-S junction of the viral genome.

approximately 40% of the G418-resistant clones demonstrated complementing activity.

CAT assays. The transfection of HSV-1–chloramphenicol acetyltransferase (CAT) chimeras and in vitro assays for CAT were performed by a modification (10) of the procedure of Gorman et al. (15). The construction of pIE3CAT, ptkCAT, and p5CAT was described previously (10). These plasmids contain the CAT gene under control of HSV-1 immediate-early, early, and late promoters, respectively.

Electrophoresis. Lysates of [^{35}S]methionine-labeled infected cell cultures were prepared and electrophoresed on sodium dodecyl sulfate (SDS)-polyacrylamide gels as described previously (8). DNA fragments generated by restriction endonuclease digestion were separated on horizontal agarose gels. Cytoplasmic RNA was separated by electrophoresis on agarose gels containing 2.2 M formaldehyde by the procedure of Goldberg (13).

Blot hybridization. The treatment of DNA restriction fragments in agarose gels, subsequent transfer to nitrocellulose, and hybridization were performed by the method of Southern (45). Formaldehyde gels containing separated RNA were washed with water, soaked for 30 min in 50 mM NaOH-10 mM NaCl, soaked for 30 min in 0.1 M Tris hydrochloride (pH 7.5), and then soaked for 30 min in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). RNA was then transferred to nitrocellulose by the procedure of Southern (45). RNA blots were hybridized to denatured probe in 50% formamide–5× SSC–10 mM Tris hydrochloride (pH 7.5)–1× Denhardt solution–25 μg of salmon testes DNA per ml–10% dextran sulfate at 42°C for 18 h. For Northern blot hybridization, ^{32}P-labeled probe was NaOH (0.5 M) denatured in the presence of 1 mg of salmon testes DNA. The solution was neutralized by addition of 0.2 M Tris hydrochloride (pH 7.5)–0.5 M HCl. Sequential washes of 3 and 0.1× SSC were performed at 50°C. Probes were prepared by nick translation (25) in the presence of [^{32}P]dGTP and [^{32}P]dCTP (Amersham Corp., Arlington Heights, Ill.).

Western blot analysis. Western blot analysis of gel-separated SDS peptides was conducted as previously described (2, 48), with the following modification. Before reaction with antibody, the nitrocellulose was incubated overnight at 4°C with BLOTTO (21). The antibody reaction was performed in 10 ml of BLOTTO–0.1 ml of ICP4 rabbit antiserum. The filter was then stained with ^{125}I-labeled protein A (Amersham). Rabbit ICP4 antiserum (6) was the generous gift of Richard Courtney (University of Tennessee, Knoxville).

RESULTS

Derivation of a cell line expressing ICP4. ICP4 performs an essential replicative function. To propagate viral mutants deleted in the ICP4 gene, we constructed cell lines which express the wild-type gene for ICP4. Viable cells expressing this gene have been isolated and characterized previously (7, 32), ruling out the possibility of inherent cytotoxicity of the resident ICP4 gene or its product.

Vero cells were cotransfected with plasmids pSV2neo and pKXC (Fig. 1). After 2 weeks, G418-resistant colonies were isolated, amplified, and screened for ability to support replication of *ts*B32 at 39°C. *ts*B32 is a temperture-sensitive mutant in the structural gene for ICP4 (11). Approximately 20% of the G418-resistant lines isolated were capable of complementing *ts*B32. The cell line designated 2-6 demonstrated the greatest complementing activity and therefore was chosen for further study. As well as all other cell lines to be described in this study, 2-6 cells are indistinguishable from Vero cells with respect to morphology and growth rate.

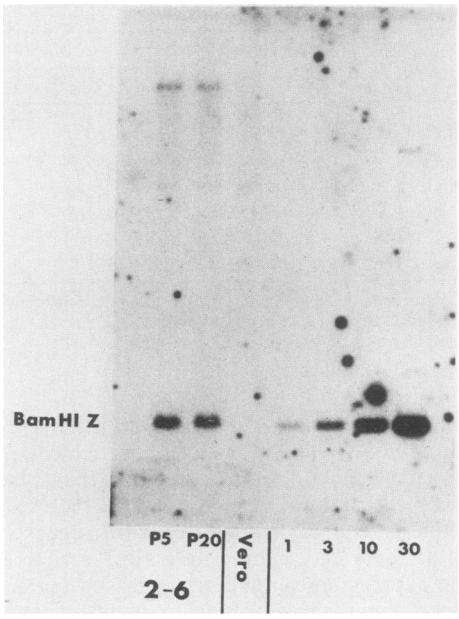

FIG. 2. Southern blot analysis of HSV-1 DNA in 2-6 cells. Electrophoretically separated *Bam*HI digests of 2-6 cell DNA (10 μg) (from passages 5 and 20) and Vero cell DNA were transferred to nitrocellulose paper and probed with the ^{32}P-labeled *Bam*HI Z fragment (1.84 kb) from pKBZ (Fig. 1). We also included *Bam*HI-digested pKBZ (7.8 kb) to visualize 1 (25 pg), 3 (75 pg), 10 (250 pg), and 30 (750 pg) copies of viral DNA per 3×10^9 base pairs of cellular DNA.

To demonstrate the presence of ICP4 coding sequences in 2-6 cells and to determine the approximate number of copies of the ICP4 gene present, we compared DNA from 2-6 cells with Vero cell DNA by Southern blot analysis (Fig. 2). The probe used in this experiment was the purified *Bam*HI Z fragment obtained from plasmid pKBZ (Fig. 1). This fragment contains approximately 50% of the coding sequence for ICP4. The standards present on the right half of the gel indicate that 2-6 cells contain approximately five copies of the *Bam*HI Z fragment per 3×10^9 base pairs. Moreover, 2-6 cells appear stable with respect to the integrity of these sequences over 15 passages (Fig. 2).

2-6 cells express biologically active ICP4. 2-6 cells contain approximately 5 copies of the *Bam*HI Z fragment per haploid equivalent or 10 copies per cell. This does not necessarily imply that all five copies serve as templates for the production of ICP4. The measure of ICP4 expression used in this study was the complementation of ICP4 *ts* mutants at 39°C. Table 1 shows the growth characteristics of various HSV-1 *ts* mutants on 2-6 and Vero cells at 39°C. *ts*B21, *ts*B32, and *ts*L14 each specify a thermolabile ICP4 polypeptide (8, 11). *ts*A24, *ts*J12, and *ts*Y46 contain mutations in ICP8 (51), gB (9), and ICP27 (40), respectively. In this experiment, 2-6 and Vero cells were infected with 1 PFU of the indicated mutant per cell at 39°C. The cultures were harvested at 18 h postinfection and assayed on Vero cell monolayers at 34°C. Only mutants defective in ICP4 grew to substantially higher levels (3 to 4 orders of magnitude) in 2-6 than in Vero cells. However, either complementation was not complete or the mutant forms of ICP4 were able to interfere with the action of the complementing wild-type ICP4 polypeptide, since the yield of mutant virus was 1 to 2 orders of magnitude below

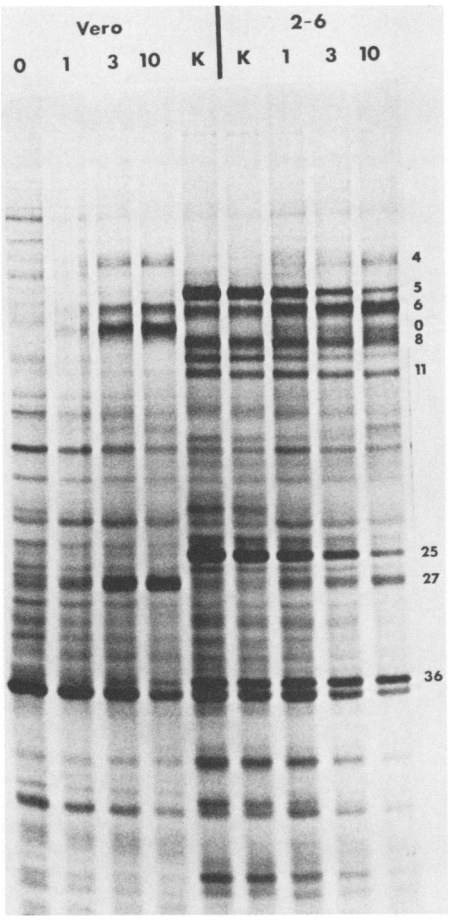

FIG. 3. Synthesis of viral polypeptides in *ts*B32-infected Vero and 2-6 cells. Monolayers of 2-6 and Vero cells were infected at the indicated multiplicities of infection with *ts*B32 at 39°C. Cells were also infected with KOS at a multiplicity of infection of 3 PFU per cell (K). [^{35}S]methionine was present during incubation from 5 to 16 h postinfection. At 16 h postinfection, total cell lysates were prepared for SDS-PAGE. The infected-cell polypeptides were electrophoretically separated on a 9% polyacrylamide gel cross-linked with N,N'-dicyclohexylcarbodiimide (28). Infected-cell polypeptides representative of the α (ICP4, ICP0, and ICP27), β (ICP6, ICP8, ICP11, and ICP36), and γ (ICP5, ICP19-20, and ICP25) kinetic classes are labeled for reference (28).

that of KOS in 2-6 cells. The diminished yield of ICP4 *ts* mutants in 2-6 cells at 39°C relative to wild-type virus was not significantly reflected in the 39-34°C plating efficiencies of the mutants on 2-6 cells. The plating efficiency (39-34°C) of wild-type virus on 2-6 cells was near 1.0, whereas that of the mutants ranged from 0.3 to 0.8. The plaque sizes of the mutants on 2-6 cells at 39°C, however, were substantially smaller than that of KOS (data not shown).

An alternative means of assessing the extent of complementation of ICP4 *ts* mutants in 2-6 cells is to compare the synthesis of viral polypeptides in 2-6 and Vero cells at 39°C. In Vero and other cell types, *ts*B32 characteristically overproduces immediate-early polypeptides and fails to synthesize early and late polypeptides at 39°C (11). This is presumably due to the lack of functional ICP4 at this temperature. The complementation of *ts*B32 by wild-type ICP4 synthesized in 2-6 cells, as reflected by the synthesis of early and late viral gene products, was both substantial and multiplicity dependent (Fig. 3). At a multiplicity of 1 PFU per cell, the polypeptide profile of *ts*B32 in 2-6 cells resembled that of the wild-type virus. At multiplicities greater than 1 PFU per cell, however, characteristics of the mutant phenotype were clearly evident and became more pronounced with increasing multiplicity. A similar multiplicity effect has also been observed for *ts* mutants in the HSV-1 DNA-binding protein in transformed cells (41). 2-6 cells contain approximately 10 copies of the *Bam*HI Z fragment per cell, yet this number was not sufficient to restore completely the wild-type phenotype at the multiplicities of infection tested. Three possible explanations exist for this observation. (i) Only a small fraction of the integrated ICP4 sequences serve as templates for the expression of ICP4. (ii) ICP4 mRNA is not expressed as efficiently when its template is integrated into the cellular genome as in the viral genome. (iii) The mutant ICP4 polypeptide interferes with the action of wild-type ICP4 present in transformed cells. Data presented below support the third possibility.

Derivation of a cell line expressing ICP4 but lacking ori$_S$. The fragment of HSV-1 DNA used to generate 2-6 cells contains, in the following order, the *cis*-acting packaging sequences (a) (27) derived from the joint region or termini of the viral genome, the gene for ICP4 (26, 28, 38), the regulatory sequences for expression of ICP4 mRNA (5, 23), and an origin of viral DNA synthesis (ori$_S$) (47) (Fig. 1). As shown previously (7), passage of ICP4 *ts* mutants in such cells resulted in the generation of *ts*$^+$ recombinants. In the present study, the number of recombinants generated in complementation tests after amplification of a single *ts*B32 plaque isolate in 2-6 cells was approximately 1% (data not shown). Because this level was well below the yield of *ts* mutant virus generated by complementation in these cells, it was felt that wild-type recombinants would not seriously interfere with the isolation of deletion mutants. Therefore, 2-6 cells were used to generate and screen putative deletion mutants in ICP4, as described below. To obtain recombinant-free stocks of the ICP4 deletion mutants, however, it was necessary to isolate ICP4-transformed cell lines which would not give rise to wild-type recombinants. Consequently, the deletion mutants generated as described below were used to screen G418-resistant cell lines isolated after cotransfection of pSV2neo and pKX2-P4 (Fig. 1). pKX2-P4 contains the a sequences, ICP4, and the regulatory elements for ICP4 expression but lacks ori$_S$. It was anticipated that deleting ori$_S$ would reduce, if not eliminate, the generation of wild-type recombinants. Recently, it has been demonstrated that this is indeed the case (7). Among the cell lines isolated after cotransfection with plasmids pKX2-P4 and pSV2neo, approximately 40% were able to support growth of the deletion mutants. Three were retained for further analysis. The three lines, 1-2, 1-5, and 4-2, contained three, three, and five copies, respectively, of the ICP4 coding sequence per haploid equivalent of DNA (data not shown).

Isolation of deletion mutants in 2-6 cells. A plasmid containing a 0.5-kb deletion in the ICP4 coding sequences was constructed from pKX2 by partial *Sac*I cleavage and religation (Fig. 1). The deleted ICP4-containing plasmid pKX2-Δ*Sac*I was first assayed for ability to express ICP4 activity before attempts to introduce the deletion into the viral genome. This was done by cotranfecting CV-1 cells with either pKX2 or pKX2-Δ*Sac*I and HSV-1–CAT chimeric genes and measuring the levels of expression of the HSV-1–CAT chimeras induced by wild-type ICP4 and the deleted form of the protein. In this way the effect of the 0.5-kb deletion on ICP4 activity could be assessed. We and others have shown that ICP4 alone can suppress immediate-early

CAT chimeric gene expression (10) and simultaneously enhance early and some late gene expression in *trans* (10, 12, 30). Here, immediate-early-CAT (pIE3Cat) expression was suppressed whereas early-CAT (ptkCat) and late-CAT (p5Cat) expressions were enhanced by wild-type ICP4 (Fig. 4). The CAT signal generated from extracts of cells cotransfected with the deleted ICP4 plasmid resembled that derived from extracts of cells cotransfected with pBR322. Therefore, the 0.5-kb internal deletion abolished the *trans*-acting activity of ICP4.

pKX2-Δ*Sac*I was then linearized and coprecipitated with infectious KOS DNA, and 2-6 cells were cotransfected with the mixture. Individual progeny plaques from this transfection were isolated and screened for ability to replicate in 2-6 cells and not in Vero cells. Of 224 plaque isolates, 2, designated d120 and d202, exhibited impaired ability to replicate in Vero cells.

Stocks of d120 and d202 generated in 2-6 cells always contained 1 to 5% ([titer of Vero cells/titer of 2-6 cells] × 100) wild-type recombinants which produced plaques in Vero cells. Such recombinants, when isolated and plated in Vero and 2-6 cells, always yielded plating efficiencies of 1.0. When individual plaque isolates of d120 and d202 were propagated in 1-2, 1-5, or 4-2 cells, wild-type recombinants were rarely detected. Occasionally, however, when d120 or d202 was amplified through several rounds of growth in ori$^-$ ICP4$^+$ cell lines, wild-type recombinants were generated at a frequency approaching 10^{-5}. The growth characteristics of d120 and d202 on control Nero (G418-resistant Vero cells [see Materials and Methods]), 1-2, 4-2, 1-5, and 2-6 cells are shown in Table 2. In this experiment, wild-type virus was not present in lysates obtained from ori$^-$ cells. Recombinant-free stocks of d120 and d202 grown in 4-2 cells were used for further analysis.

Characterization of deletions in d120 and d202. d120 and d202 are the progeny of cotransfection of pKX2-Δ*Sac*I and KOS DNA. To determine whether these isolates contained the deletion in pKX2-Δ*Sac*I, we analyzed viral DNA from d120 and d202 by restriction enzyme digestion. Electrophoretically separated restriction fragments of KOS, d120, and d202 were transferred to nitrocellulose paper and probed with the indicated ^{32}P-labeled fragment (Fig. 5).

The isolate d202 contained the expected 0.5-kb *Sac*I deletion in both copies of ICP4. The 3.2-kb *Hin*cII fragment of pKX2 comigrated with that of KOS, whereas the *Hin*cII fragment of pKX2-Δ*Sac*I comigrated with that of d202. Furthermore, the *Sac*I fragments of d202 homologous to the 3.2-kb *Hin*cII probe were identical to those of KOS except for the absence of the 0.5-kb deletion in pKX2-Δ*Sac*I.

The *Hin*cII and *Sac*I digests of d120 DNA indicated a

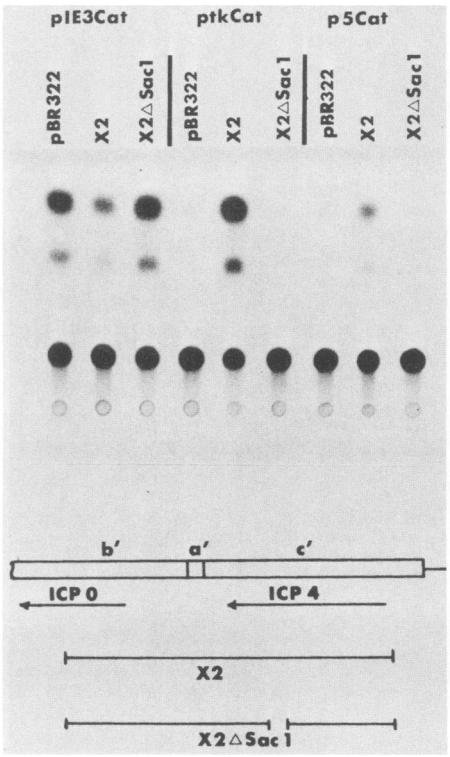

FIG. 4. *trans*-Inducing activities of wild-type and deleted forms of ICP4. CV-1 cells were cotransfected with 2 μg each of the indicated CAT plasmid and the indicated test plasmid. At 40 h posttransfection, cell extracts were prepared and analyzed for CAT activity in vitro as previously described (15). Shown are the autoradiographic images of the chromatographically separated input, unacetylated chloramphenicol (lower spot), and the acetylated products (upper spots). Also diagrammed are the test plasmids used in the cotransfection with respect to the genes for the infected cell polypeptides ICP4 and ICP0. b', a', and c' represent the elements of the HSV-1 joint region (17).

TABLE 2. Growth of ICP4 mutants on transformed cells

Virus	Virus grown on[a]				
	Nero cells	4-2 Cells	2-6 Cells	1-5 Cells	1-2 Cells
KOS	1.9×10^{8}[b] 1.8×10^{8}[c]	2.2×10^{8} 1.5×10^{8}	1.4×10^{8} 1.1×10^{8}	2.0×10^{8} 1.7×10^{8}	9.0×10^{7} 1.0×10^{8}
d120	2.5×10^{4}[b] <5[c]	4.0×10^{7} <10^{2}	4.9×10^{7} 1.7×10^{5}	5.2×10^{7} <10^{2}	4.2×10^{7} <10^{2}
d202	7.2×10^{4}[b] <5[c]	6.5×10^{7} <10^{2}	3.7×10^{7} 2.2×10^{5}	6.5×10^{7} <10^{2}	3.3×10^{7} <10^{2}
*ts*B32	6.2×10^{2}[d] <5[e]	1.0×10^{4} <5	3.0×10^{4} 2.0×10^{2}	2.0×10^{3} <5	2.0×10^{3} <5

[a] Nero, 4-2, 2-6, 1-5, and 1-2 cells were infected with the appropriate virus under conditions identical to those stated in Fig. 9.
[b] Infected cultures were harvested at 18 h postinfection and assayed at 34°C on 1-2 cells.
[c] Same culture as that described in footnote *b* assayed simultaneously at 34°C on Nero cells.
[d] Infected cultures were harvested at 18 h postinfection and assayed at 34°C on Nero cells.
[e] Same culture as that described in footnote *d* assayed simultaneously at 39°C on Nero cells.

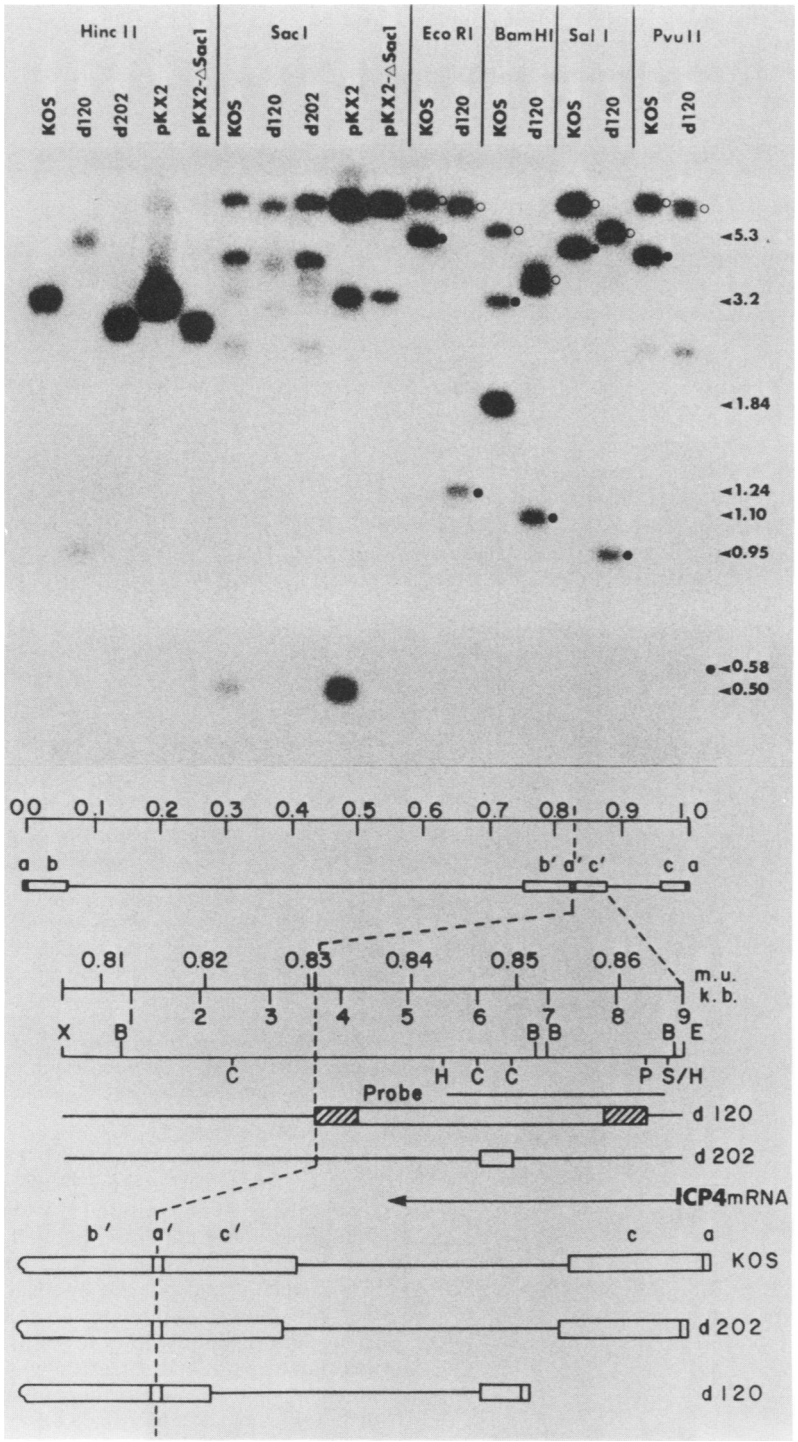

FIG. 5. Genome structures of d120 and d202. Viral (KOS, d120, and d202) and plasmid (pKX2 and pKX2-Δ*Sac*I) DNAs were digested with the indicated restriction enzymes and electrophoretically separated on a 1.2% agarose gel. Separated DNA fragments were analyzed as described in the legend to Fig. 2 with the indicated 3.2-kb *Hin*cII fragment as probe. For comparison of KOS and d120 fragments (right half of the gel), terminal (○) and joint (●) fragments are indicated. Shown below the autoradiogram in descending order are the KOS genome, an expansion of the joint region of the genome between 0.806 and 0.867 map units (m.u.), the locations of relevant restriction sites, the location of the fragment used as probe, and the deduced structures of d120 and d202. The open and shaded boxes in the structures of d120 and d202 indicate deleted sequences and regions of uncertainty, respectively. The boundaries between deleted sequences and regions of uncertainty in d120 DNA were deduced from the following observations. (i) d120 contains a 4.1-kb deletion. (ii) The *Pvu*II site is present at m.u. 0.863. (iii) The functional a sequences are present at the joint, as demonstrated by evidence of inversion of L and S. (iv) The restriction sites spanned by the open box in d202 are absent in d120. The right portions of the KOS, d120, and d202 genomes are diagrammed in the lower portion of the figure to illustrate the effects of deletions in both of the inverted repeats (C and C') on the sizes of the short components of the genomes. A dashed line is drawn to indicate the L-S junction. The relevant restriction sites are the same as in Fig. 1, with the addition of *Hin*cII (H), *Pvu*II (P), and *Sal*I (S).

more substantial deletion. The structure of d120 was analyzed further by Southern blot hybridization. The right half of Fig. 5 is an example of one such experiment. The 5.3-kb terminal *Eco*RI K fragment of the KOS short component was absent in d120 DNA, and a new 1.24-kb band was present. The deletion in d120 spanned the two *Bam*HI sites located near 0.853 map units (Fig. 5) in KOS, and a new 1.1-kb band was present in d120. The 5.1-kb terminal *Sal*I fragment of KOS was replaced by a 0.95-kb fragment in d120. Likewise, the 4.7-kb terminal *Pvu*II fragment of KOS was replaced by a 0.58-kb fragment in d120. The size of the novel terminal fragments in d120 was consistent with the separation between, and order of, the *Eco*RI, *Bam*HI, *Sal*I, and *Pvu*II sites near the 5′ terminus of the ICP4 gene in wild-type HSV-1 (Fig. 5). The sizes of the reductions in the above mentioned terminal fragments (Fig. 5) and in the joint fragments (Fig. 5; data not shown) are consistent with the existence of a 4.1-kb deletion in both copies of ICP4.

Based on other Southern blots (data not shown), restriction sites in the long component of the genome did not appear to be affected in either d120 or d202, and no sequences homologous to pBR322 were detectable in the DNA of either virus. In addition, the expected patterns generated by genome isomerization were reflected in *Eco*RI and *Pvu*II digests (Fig. 5; data not shown). Thus, because the presence of a sequences at both the joint and molecular termini are required for isomerization (33), their presence in d120 in a functional configuration is implied.

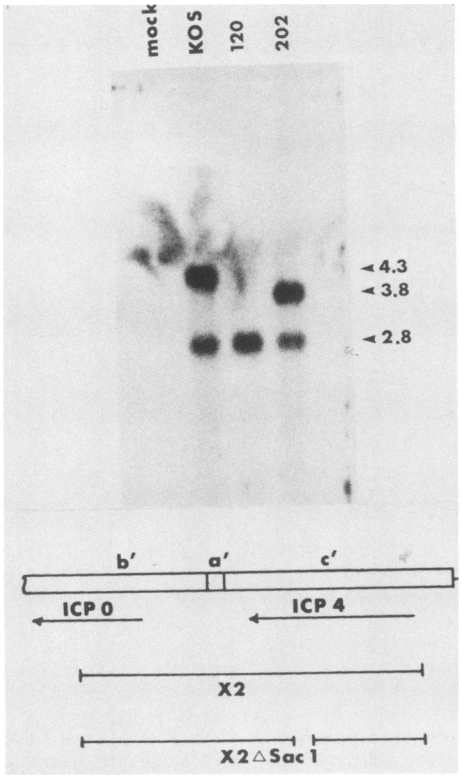

FIG. 6. ICP4 mRNA synthesized in d120 and d202. Vero cell monolayers were preincubated in the presence of 75 μg of cycloheximide per ml for 1 h at 37°C. Monolayers were then infected with 10 PFU of KOS, d120, or d202 per cell in the presence of 75 μg of cycloheximide per ml. After 6 h of incubation at 37°C, the cultures were harvested and cytoplasmic RNA was analyzed as described in the text. The locations of the ICP0 and ICP4 mRNAs and of the deletion in pKX2Δ*Sac*I used to generate d202 are shown beneath the autoradiogram. The numbers on the right indicate size in kilobases. b′, a′, and c′ are described in the legend to Fig. 4.

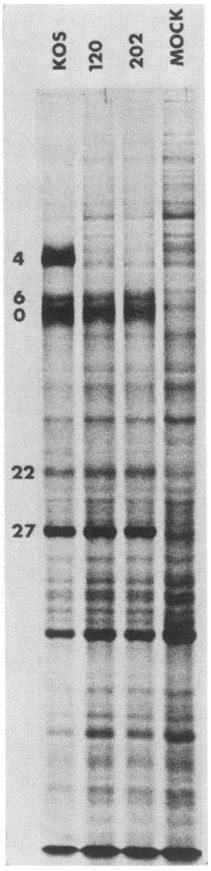

FIG. 7. Immediate-early polypeptides synthesized in d120- and d202-infected Vero cells. Monolayers of Vero cells were treated and infected as described in the legend to Fig. 6. At 6 h postinfection, cycloheximide was washed out with medium containing 10 μg of actinomycin D per ml. Incubation was continued for an additional 3 h in the presence of 10 μg of actinomycin D and 50 μCi of [^{35}S]methionine per ml. Cell lysates were then prepared and analyzed by SDS-PAGE as described in the legend to Fig. 3. Numbers on the left are described in the legend to Fig. 3.

ICP4 mRNA synthesis by d202 and d120. d202 contains the 0.5-kb deletion introduced by pKX2-Δ*Sac*I. Therefore, d202 was expected to synthesize an ICP4 mRNA 0.5 kb smaller than that of the wild-type virus. d120 contains a large deletion (4.1 kb) and therefore was expected to synthesize a much smaller message or none at all. Northern blot analysis of cytoplasmic RNA from KOS-, d120-, and d202-infected Vero cells verified that this was the case (Fig. 6). Infected Vero cells were incubated in the presence of cycloheximide for 6 h. Cytoplasmic RNA was then isolated, electrophoretically separated, transferred to nitrocellulose, and probed with nick-translated pKX2 DNA. pKX2 contains sequences homologous to the immediate-early mRNA for ICP0 and ICP4. KOS induced the synthesis of a 4.3-kb mRNA specifying ICP4 and a 2.8-kb mRNA specifying ICP0 (50) (Fig. 6). d120 synthesized only the 2.8-kb message, whereas d202 synthesized a 3.8- and a 2.8-kb message. Therefore, although ICP0 mRNA was synthesized in cells infected with the deletion mutants, as it was in KOS-infected cells, ICP4

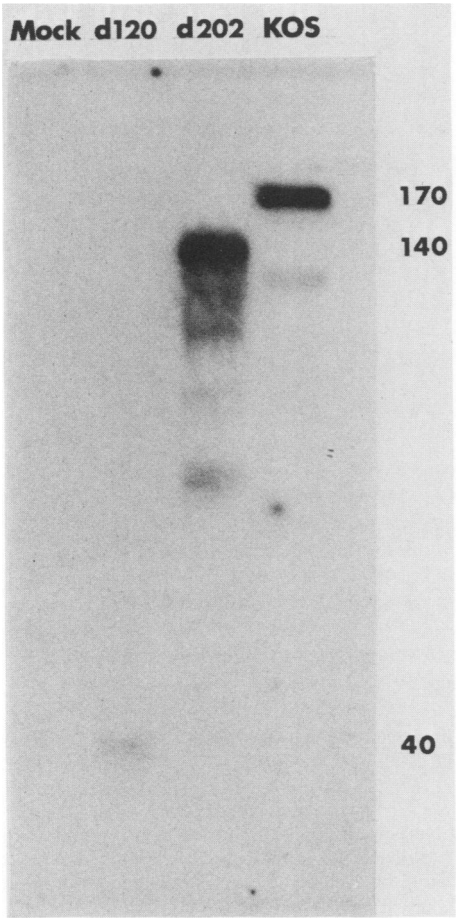

FIG. 8. Western blot analysis of d120- and d202-infected Vero cells. Non-ICP4-containing cells were infected with the indicated virus at a multiplicity of 10 PFU per cell. At 5 h postinfection, SDS lysates were prepared and run on a 9% SDS-polyacrylamide gel. The SDS peptides were electrophoretically transferred to nitrocellulose and analyzed as described in Materials and Methods. Numbers on the right indicate apparent molecular weight.

mRNA was 0.5 kb smaller in d202-infected cells and not detectable by this procedure in d120-infected cells.

Immediate-early polypeptide synthesis in d120- and d202-infected Vero cells. To determine what immediate-early polypeptides where synthesized in d120- and d202-infected Vero cells, we performed an experiment similar to that shown in Fig. 6, except that at 6 h postinfection, cycloheximide was washed out and incubation was continued for 3 h in the presence of actinomycin D and [^{35}S]methionine. Immediate-early polypeptides ICP0, ICP22, and ICP27 were clearly visible in extracts of d120-, d202-, and KOS-infected cells on SDS-polyacrylamide gels (Fig. 7). As in other cycloheximide reversal experiments, small quantities of ICP6 were also observed. The ICP4 polypeptide, however, was detectable only in extracts of KOS-infected cells. Whereas one might not expect an ICP4 polypeptide to be synthesized in d120-infected cells, it is possible that a deleted form of ICP4 was synthesized in d202-infected cells. Although this is not evident in Fig. 7, the possibility of an ICP4-like peptide comigrating with a peptide of a different electrophoretic mobility than ICP4 exists. This possibility was investigated further by Western blot analysis. Electrophoretically separated SDS peptides prepared from infected and uninfected Nero cells were transferred to nitrocellulose, probed with anti-ICP4 rabbit serum, and visualized by staining with ^{125}I-labeled protein A. The anti-ICP4 serum used was generated from rabbits inoculated with a gel-eluted SDS ICP4 polypeptide (6). Such polyclonal antibody would be expected to react efficiently with truncated SDS ICP4 peptides. Cells infected with d202 contained a polypeptide shorter than KOS by about 30 kb (Fig. 8). This polypeptide probably comigrated with ICP6 or ICP0 (Fig. 7). The d202 form of ICP4 appeared relatively unstable with respect to the wild-type form of ICP4, as reflected by the descending smear and several specific lower-molecular-weight bands. The origin of the lower-molecular-weight band in KOS is unclear and may represent a degradation product of the wild-type ICP4 polypeptide. It is not, in all likelihood, a cellular band or another HSV-1 immediate-early peptide, since it was absent from mock-infected and d120-infected cell profiles. d120 appears to specify a peptide of approximately 40 kilodaltons, which is reactive with ICP4 antiserum. This peptide represents the amino terminus of ICP4, since only the 5′ end of the ICP4 gene was retained in d120 (Fig. 5).

Other phenotypic properties of d120 and d202. The growth properties and polypeptide phenotypes of d120, d202, *ts*B32, and KOS were compared in Nero and ICP4-transformed cells. Nero cells and cells of four ICP4-transformed lines were infected at a multiplicity of 10 PFU per cell and labeled with [^{35}S]methionine from 5 to 16 h postinfection. All infected cultures were incubated at 34°C, except those infected with *ts*B32, which we incubated at 39°C to visualize the mutant phenotype. Duplicate cultures were harvested for simultaneous infectivity determinations.

The polypeptide profiles of d120 and d202 differed drastically from that of KOS in Nero cells (Fig. 9). Qualitatively, the profiles of d120- and d202-infected cells were quite similar to each other and to that of *ts*B32-infected cells, with the exception of ICP4, which was evident only in *ts*B32-infected cells. The predominant viral polypeptides detected in Nero cells infected with the deletion mutants were ICP6, ICP0, and ICP27. The truncated forms of ICP4 are not evident in Fig. 9. In our hands, ICP22 was discernible only in cycloheximide reversal experiments. Both ICP0 and ICP27 are immediate-early polypeptides (28), whereas ICP6 is generally not regarded as a member of this class of HSV-1 genes. It is clear, however, that the expression of ICP6 does not require functional ICP4 (Fig. 9).

The polypeptide profiles of d120 and d202 in ICP4-transformed cells resembled that of the wild-type virus, indicating efficient complementation by resident, wild-type ICP4. Enhanced expression of early polypeptides in *ts*B32-infected ICP4-transformed cells relative to *ts*B32-infected Nero cells (i.e., ICP8, ICP11, and ICP36) was also evident. Aspects of the mutant phenotype characteristic of *ts*B32, however, were evident in all ICP4-transformed cell lines at this multiplicity. This observation is consistent with the results shown in Fig. 3, in which the *ts* mutant was inefficiently complemented in ICP4-transformed cells at high multiplicity of infection. The fact that early and late polypeptides were expressed relatively inefficiently in ICP4-transformed cells infected with the *ts* mutant, whereas those of the deletion mutants were efficiently expressed, indicates that the *ts* mutant form of the ICP4 polypeptide exhibits an interfering effect on gene expression, thus inhibiting complementation.

The growth characteristics of KOS, d120, d202, and *ts*B32 are summarized in Table 2. The following statements concerning these viruses can be made. (i) The growth of d120,

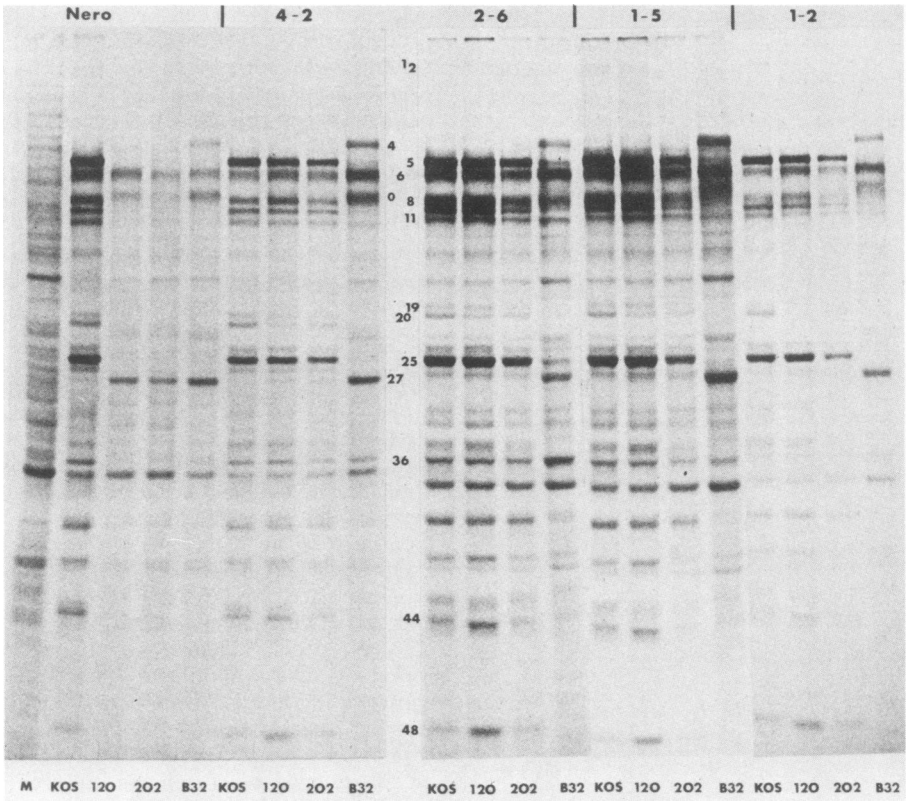

FIG. 9. Viral polypeptides synthesized in KOS-, d120-, d202-, and *ts*B32-infected Nero and ICP4-transformed cells. Confluent monolayers of each cell type were infected as described in the text and analyzed by SDS-PAGE as described in the legend to Fig. 3. All infections were performed at 34°C, except those with *ts*B32, which were performed at 39°C. An uninfected Nero cell culture was maintained for visualization of cellular bands (M). The uninfected cell polypeptide profiles of Nero, 4-2, 2-6, 1-5, and 1-2 cells were identical (data not shown). Numbers in the center are described in the legend to Fig. 3.

d202, and *ts*B32 (at 39°C) in Nero cells was severely restricted. (ii) All ICP4-transformed cell lines complemented d120 and d202 efficiently. (iii) ICP4-transformed cell lines complemented the growth of *ts*B32 poorly at a multiplicity of infection of 10 PFU per cell. Only in 2-6 and 4-2 cells were significant levels of complementation demonstrated. (iv) Wild-type recombinants were observed only when ICP4 deletion or *ts* mutants were propagated in 2-6 cells. Thus, the yields of mutant and wild-type viruses in cell lines expressing ICP4 support the results of polypeptide analysis.

The experimental results shown in Fig. 9 illustrate a qualitative comparison of polypeptide synthesis by d120, d202, and *ts*B32 (at 39°C) in Nero and ICP4-transformed cells. When all of the incubations were carried out at 39°C, the same pattern of polypeptide synthesis was observed in ICP4-transformed cell lines. The polypeptide profiles of d120- and d202-infected 4-2 cells were similar to that of KOS, whereas the profile of *ts*B32-infected 4-2 cells was more restricted, as shown by the relative underproduction of ICP5 and ICP25 (Fig. 10). Also clear is the fact that, in Nero cells, d120 and d202 synthesized reduced levels of immediate-early polypeptides ICP0 and ICP27 relative to *ts*B32. ICP6 was also reduced in d120- and d202-infected cells relative to *ts*B32-infected cells. To determine more specifically whether the rates of synthesis of ICP0, ICP27, and ICP6 in d120- and d202-infected cells differ from that in *ts*B32-infected cells and to examine the possible effects of protein turnover on polypeptide phenotype, we labeled Nero cells infected with these mutants for 0.5-h intervals at selected times from 0 to 8 h postinfection at 39°C. The rates of synthesis of ICP0, ICP27, and ICP6 in cells infected with the deletion mutants were slower than that seen in *ts*B32-infected cells (Fig. 11). This difference may be attributed to a direct or indirect effect of the *ts*B32 form of ICP4. In this case, it would be postulated that the presence of the *ts* mutant form of the ICP4 polypeptide resulted in exaggerated expression of immediate-early polypeptides.

DISCUSSION

The genome of HVS-1 is capable of coding for approximately 100 polypeptides. Despite the large size of its genome, HSV-1 is highly conducive to genetic analysis, thus permitting the identification of specific HSV-1-encoded functions. *ts* mutants, in particular, have proven invaluable in this regard (1, 42) in that, to date, complementation groups representing approximately 30 essential replicative genes have been localized on the HSV-1 genome. In addition to *ts* mutants, mutants resistant to a variety of anti-herpes drugs (4), mutants which render cells resistant to immune cytolysis (31), and mutants resistant to neutralization with monoclonal antibodies (18) have proven useful in identifying and characterizing the genomic locations and functions of individual HSV-1 gene products.

Using cloned HSV-1 genes and selections for both the thymidine kinase-negative (tk^-) and -positive (tk^+) phenotypes, Post and Roizman (34) have developed a method to delete nonessential genes of HSV-1. This approach led to the isolation of viable deletion mutants in the immediate-early

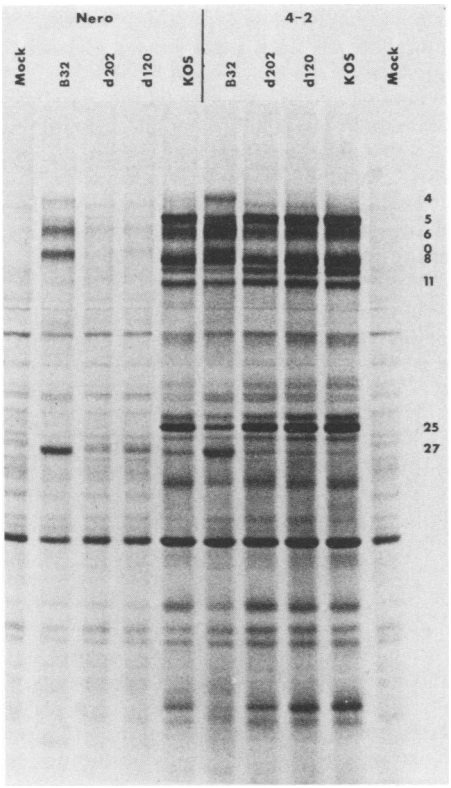

FIG. 10. Viral polypeptides synthesized in KOS-, d120-, d202-, and *ts*B32-infected Nero and 4-2 cells at 39°C. Nero and 4-2 cells were infected, labeled, and analyzed as described in the legend to Fig. 8, except that all incubations were carried out at 39°C. Numbers on the right are described in the legend to Fig. 3.

(α) gene for ICP22, indicating the nonessentiality of this gene for virus replication in the cell lines tested. Moreover, a viable deletion mutant has recently been isolated in a nonessential region of ICP4 (44). In this study, we generated cell lines transformed with the gene for the HSV-1 immediate-early gene encoding ICP4. ICP4-containing cells were used to isolate and propagate two HSV-1 deletion mutants in the gene for ICP4. The phenotypes of the two deletion mutants, d120 and d202, were then analyzed on cells which do not contain the ICP4 gene.

Isolation of ICP4 deletion mutants. ICP4-transformed cells were generated by cotransformation of Vero cells with pKXC and pSV2neo. ICP4-containing 2-6 cells were used to screen for ICP4 deletion mutants. Two ICP4 deletion mutants were isolated in this manner. Consistent with the findings of Davidson and Stow (7), passage of ICP4 *ts* or deletion mutants on 2-6 cells resulted in the generation of wild-type (ts^+ for *ts* mutants; host cell independent for deletion mutants) virus in the progeny. This presumably occurs in the presence of helper functions provided by the superinfecting virus by replication, mobilization, and propagation of wild-type sequences containing ICP4 in 2-6 cells. Amplified sequences are packaged as defective genomes (7) which are then able to recombine with the superinfecting mutant helper virus to produce wild-type virus. Cells generated by transformation with pKX2-P4 lack ori_S and did not result in the generation of wild-type virus after infection with *ts* or deletion mutants (Table 2).

ICP4 deletion mutants d120 and d202 were isolated from the progeny of a cotransfection of pKX2-Δ*Sac*I (Fig. 1) and intact KOS (wild-type) DNA. pKX2-Δ*Sac*I contains a 0.5-kb deletion in the coding sequence for ICP4. In the absence of sequence information in this region of the genome, it was of interest to determine if the ICP4 gene in pKX2-Δ*Sac*I retained any ICP4 activity. The transient expression assay shown in Fig. 4 indicates that pKX2-Δ*Sac*I does not specify ICP4-like activity. This experiment enabled us to ascertain what the consequences of such a deletion would be before introducing the deleted gene into wild-type virus. As noted above, a deletion mutant in ICP4 has been isolated recently which does not inactivate the gene product (44).

d202 contains the expected 0.5-kb deletion in both copies of ICP4. Consistent with the presence of a 0.5-kb deletion internal to the ICP4-coding sequences was the synthesis of an mRNA which was smaller than its wild-type counterpart by 0.5 kb and the synthesis of a correspondingly truncated form of ICP4. The structure of d120, on the other hand, was quite unexpected. This mutant contains a 4.1-kb deletion in both copies of ICP4 and failed to express detectable cytoplasmic ICP4 mRNA. A severely foreshortened form of ICP4 was, however, detected by Western blot analysis and

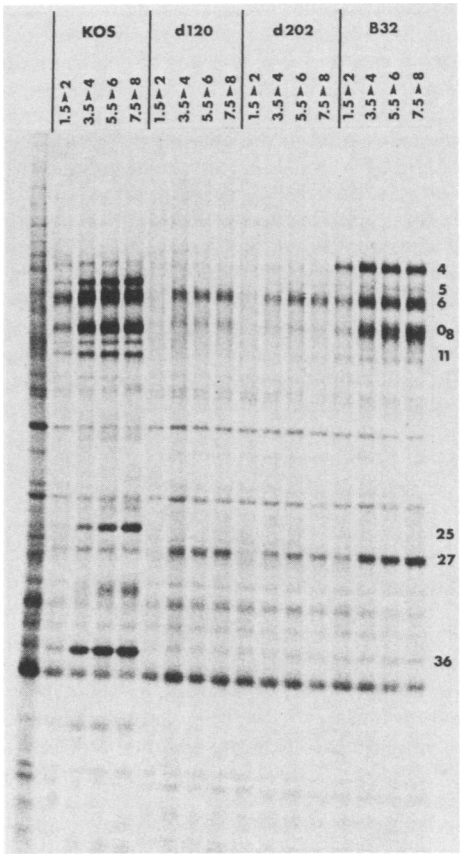

FIG. 11. Rates of synthesis of viral polypeptides at 39°C in KOS-, d120-, d202-, and *ts*B32-infected Nero cells. Nero cells were infected with the indicated viruses and incubated at 39°C as described in the legend to Fig. 8. [^{35}S]methionine (100 μCi) was added during the indicated time intervals. At the end of the labeling period, monolayers were washed and cell lysates were prepared and analyzed by SDS-PAGE as described in the legend to Fig. 3. Numbers on the right are described in the legend to Fig. 3.

may represent expression of the amino terminus of ICP4. One would predict that an α mRNA would initiate from the ICP4 mRNA start site in d120, since this region of the ICP4 gene is intact. Repeated efforts to detect an ICP4-specific mRNA in d120-infected cells have failed, however. As anticipated, the quantity and mobility of the ICP0 mRNA were identical to the analogous mRNA synthesized in KOS- and d202-infected, cycloheximide-treated cells.

Despite differences in the size and structure of the ICP4 gene and the size of the ICP4 mRNA synthesized in d120- and d202-infected cells, the immediate-early polypeptides expressed in cells infected with the two mutants were identical in a cycloheximide reversal experiment (Fig. 7). In fact, with the exception of ICP4, which was not detected by standard polyacrylamide gel electrophoresis (PAGE) analysis in extracts of deletion mutant-infected cells, the quantities and mobilities of ICP6, ICP0, ICP22, and ICP27 were identical to those observed in cells infected with KOS. The experiments of Fig. 7 and 8 established two points. (i) Full-length ICP4 polypeptides are not expressed in d120- and d202-infected Nero cells. Independent of whether the deletion in d202 is in frame, a peptide antigenically reactive with ICP4 antiserum with an apparent molecular weight reduction of 30,000 with respect to the wild-type protein was synthesized. An antigenically reactive peptide of approximately 40 kilodaltons was synthesized in d120-infected cells. The sizes of the shortened peptides were consistent with the sizes of the deletions in d120 and d202 (Fig. 5). (ii) Expression of the other immediate-early polypeptides in cells infected with d120 and d202 was identical to that seen in wild-type virus-infected cells in a cycloheximide reversal experiment.

Viral gene expression in the absence of ICP4. The absence of functional ICP4 in d120- and d202-infected cells had profound effects on the expression of other viral genes relative to that seen in wild-type infection. Qualitatively, with the exception of ICP4, the peptides synthesized in d120- and d202-infected Nero cells were the same species as those expressed in *ts*B32-infected cells at 39°C. ICP0 and ICP27 were produced in moderate quantities in d120- and d202-infected Nero cells (Fig. 9, 10, and 11). Interestingly, ICP6 was the most abundantly expressed polypeptide in cells infected with the deletion mutants. Honess and Roizman (19) have previously shown that ICP6 is inefficiently expressed in the absence of prior viral protein synthesis, placing ICP6 in the early (β) class of HSV-1 genes. They have also shown that ICP6 is synthesized to some degree in the presence of the amino acid analog canavanine (20). In our hands, reduced quantities of ICP6 relative to ICP0 were synthesized in a cycloheximide reversal experiment (Fig. 7). This ICP6-ICP0 ratio was reversed in d120- and d202-infected Nero cells (Fig. 11). The synthesis of ICP6 in this genetic background indicates that ICP4 is not required for efficient expression of the ICP6 gene. This observation and the minimal expression of ICP6 in a cycloheximide reversal experiment may well reflect the involvement of an α gene other than ICP4 (i.e., ICP0, ICP22, ICP27, or ICP47) in ICP6 gene expression.

Recently it has been shown by a number of investigators that the immediate-early gene product ICP0 can act in *trans* in transient expression systems to enhance expression of early HSV-1 genes (30, 39). With the exception of ICP6, early genes were underexpressed to the same or a greater extent in deletion mutant-infected cells than in cells infected with an ICP4 *ts* mutant at 39°C. Therefore, the lack of expression of early genes in ICP4 *ts* mutant-infected cells at 39°C cannot be due solely to inhibitory effects manifested by the *ts* form of the ICP4 polypeptide. Studies of the ICP4 deletion mutants reflect the insufficiency of ICP0 to act alone to stimulate early gene expression.

The *ts* form of ICP4 affects gene expression. The *ts* form of ICP4 in *ts*B32-infected cells was apparently inhibitory to the activity of the wild-type form of ICP4 in all of the transformed cells tested. Inhibition was more prominent at higher multiplicities (Fig. 3). At a multiplicity of 10 PFU per cell, all of the cell lines complemented d120 and d202 efficiently, yet *ts*B32 was complemented relatively inefficiently at 39°C (Fig. 9 and 10; Table 2). In fact, only 2-6 and 4-2 cells significantly enhanced the yield of *ts*B32 at 39°C above the yield in Nero cells. Possible explanations for this inhibition include the following. (i) The *ts* form of ICP4 binds tightly to sites bound by wild-type ICP4, inhibiting the activity of the latter by competition. (ii) The *ts* form of ICP4 forms a stable complex with the wild-type form of the protein in the cytoplasm, thereby inhibiting either transport of the latter to the nucleus or its activity once in the nucleus. (iii) The *ts* form of ICP4 alters cellular metabolism in such a way as to render the wild-type form of ICP4 less effective.

Another property of the *ts* form of ICP4, as indicated by comparison with the deletion mutants, is its ability to induce overproduction of immediate-early polypeptides. This was evident from continuous labeling experiments and by pulse-labeling at 39°C (Fig. 9, 10, and 11). The rates of synthesis and accumulation of ICP0 and ICP27 were decreased in d120- and d202-infected Nero cells relative to *ts*B32-infected cells. Consistent with these findings is the observation that an ICP4-containing clone derived from *ts*B32 can induce CAT activity at 39°C when CAT gene expression is under the control of an immediate-early promoter (10). A possible explanation for this finding is that the *ts* form of ICP4 retains some of the activity associated with that of the wild-type polypeptide.

Analysis of ICP4 deletion mutants has both confirmed and extended the findings previously obtained with ICP4 *ts* mutants (11, 36). ICP4 is required for the expression of early and late genes. In the absence of functional ICP4, the remaining immediate-early genes are either insufficient or insufficiently expressed to induce significant levels of early genes, with the possible exception of ICP6. The phenotypes of d120 and d202 also indicate that the exaggerated levels of immediate-early polypeptides induced by ICP4 *ts* mutants are due, in part, to activities associated with the *ts* ICP4 polypeptides themselves.

Many of the contentions put forth in this study rest in part on the assumption that the deleted forms of ICP4 have little or no effect on gene expression or on the activity of the wild-type ICP4 in any of the transformed cell lines tested. The 40-kilodalton peptide synthesized in d120-infected cells has no effect on the restoration of the wild-type polypeptide phenotype in d120-infected ICP4-containing cells. On the other hand, the slightly reduced intensities of some late polypeptides on gel profiles of d202-infected ICP4-containing cells (Fig. 9 and 10) suggest a small inhibitory effect. Although we cannot formally rule out an activity associated with the d202 ICP4-related polypeptide, it should be noted that the plasmid clone used to generate d202 had no activity in a transient assay (Fig. 4). It remains of interest to determine the molecular basis for the apparent inhibitory effect of the *ts* ICP4 polypeptide and possibly that of the d202 peptide. An attractive hypothesis is that the mutant ICP4 polypeptides (*ts*B32 and possibly d202) are, for the most part, nonfunctional and yet may still bind to the normal site of action, thereby competing with any wild-type ICP4

present. Further studies are under way to test this hypothesis.

Several other observations concerning the growth of d120 and d202 in ICP4-negative cells suggest interesting avenues for further study. Unlike the *ts* mutants in ICP4 (29), d120 and d202 do not induce certain cellular stress proteins on infection of HEL cells (data not shown), nor are cytopathic effects evident after 24 h at a multiplicity of 10 PFU per cell, suggesting additional differences between the deletion mutants and the *ts* mutants. Also of interest is the genesis of d120. Efforts are in progress to determine whether the deletion in d120 was induced as a result of marker transfer or whether deletions such as that found in d120 arise spontaneously.

ACKNOWLEDGMENTS

We thank Maryellen Polvino-Bodnar for expert assistance with Western blot experiments. We also thank Don Coen for useful comments on the manuscript and Madelon Datz for manuscript preparation.

This investigation was supported by Public Health Service grant CA20260 from the National Cancer Institute. N.A.D. is the recipient of Public Health Service postdoctoral fellowship AI06790 from the National Institute of Allergy and Infectious Diseases.

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