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Deletion Mutants in the Gene Encoding the Herpes Simplex Virus Type 1 Immediate-Early Protein ICP0 Exhibit Impaired Growth in Cell Culture

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We report the construction and characterization of deletion mutants in the herpes simplex virus type 1 gene encoding the immediate-early protein ICP0. In the event that ICP0 proved to play an essential role in virus replication, ICP0-transformed Vero cells were generated to serve as permissive hosts for such mutants. Two mutants, *dl*X0.7 and *dl*X3.1, were isolated in these cells by a marker rescue-transfer procedure involving the rescue of an ICP4 deletion mutant and the simultaneous insertion of a linked deletion in the ICP0 gene. Mutant *dl*X0.7 contained a 700-base-pair deletion in both copies of ICP0. The deletion lay entirely within the transcript specified by the gene. *dl*X0.7 induced the synthesis of an ICP0-specific mRNA that was ~0.7 kilobases smaller than the corresponding mRNA specified by wild-type virus. The 3.1-kilobase deletion in both copies of the ICP0 gene in mutant *dl*X3.1 removed the majority of the transcriptional-regulatory signals and coding sequences, retaining only sequences at the 3′ end of the gene. As expected, no ICP0-specific mRNA was detected in *dl*X3.1-infected Nero cells (G418-resistant Vero cells). Both mutants grew in all cells tested, although their burst sizes were 10- to 100-fold lower than that of wild-type virus. Although the plaque sizes of *dl*X0.7 and *dl*X3.1 were equally small on Nero and ICP0-transformed cells, the plating efficiency of the mutants was 15- to 50-fold greater on ICP0-transformed cells than on Nero cells. The mutants exhibited modest interference with the growth of wild-type virus in mixed infections, an effect that was abolished by UV irradiation of the mutants, implying that interference required viral gene expression. Polypeptide profiles generated by the mutants in Nero cells were qualitatively similar to that of wild-type virus. Quantitatively, only slight reductions in the levels of certain late viral polypeptides were observed, a phenomenon also borne out by analysis of viral glycoproteins. Both mutants induced the synthesis of significant, although reduced, levels of viral DNA relative to wild-type virus. Taken together, the results demonstrate that ICP0 is not essential for productive infection in cell culture but that this protein plays a significant role in viral growth, as indicated by the impaired abilities of the mutants to replicate.

Of the three major kinetic classes of genes of herpes simplex virus type 1 (HSV-1), the immediate-early (α) genes are those that are expressed first in the replicative cycle of the virus and are functionally defined by their ability to be expressed in the absence of prior viral protein synthesis (6, 17, 46). Their expression is required for the onset of expression of the early (β) and late (γ) classes of viral polypeptides (18). The immediate-early class of viral genes is composed of five members whose products are designated ICP0, -4, -22, -27, and -47. The locations of the transcripts encoding these proteins are indicated in Fig. 1 (top line).

Efforts to elucidate the functions of each of the immediate-early gene products in the viral replicative cycle have focused on ICP4, because temperature-sensitive mutants in the gene for this protein have long been available. Analyses of these mutants and, more recently, of deletion mutants in this gene have demonstrated that ICP4 plays an essential role in the life cycle of the virus, is involved in negative regulation of immediate-early genes, and is required throughout the viral replicative cycle for the expression of early and late genes at the level of transcription (8, 10, 35, 45). Although far less is known about the role of ICP27, recent studies of temperature-sensitive mutants in the gene for this protein have shown that it, too, performs an essential regulatory function and is involved in some manner in regulation of late gene expression (38). The roles of ICP22 and ICP47 are obscure in that deletion mutants in the genes for these proteins are viable in cells normally used to host the virus in culture (22, 34, 41), suggesting a nonessential role for these two proteins, at least in cell culture.

The function of ICP0 remains unknown owing to the absence of mutants in this gene. Like ICP4, the gene encoding ICP0 is located in reiterated sequences of the viral genome and is therefore present in two copies (1, 46). Setting it apart from ICP4, -22, -27, and -47 is the fact that the level of ICP0 declines earlier than the levels of other immediate-early proteins (48). Like ICP4, -22, and -27, ICP0 is phosphorylated and accumulates in the nuclei of infected cells (32, 48). In transient expression experiments in which cloned immediate-early genes were cotransfected with appropriate indicator genes, ICP0, like ICP4, was shown to *trans*-activate expression from all three major classes of HSV promoters (12, 27, 30, 31, 36). *trans*-inducing activity has yet to be demonstrated for the other three immediate-early genes (ICP22, -27, and -47).

The fact that ICP0 alone is able to *trans*-activate early and late promoters in transient assays in vitro presents a paradox when the results of studies of ICP4 deletion and temperature-sensitive mutants are considered (8). These mutants contain wild-type copies of ICP0 yet fail to express early and

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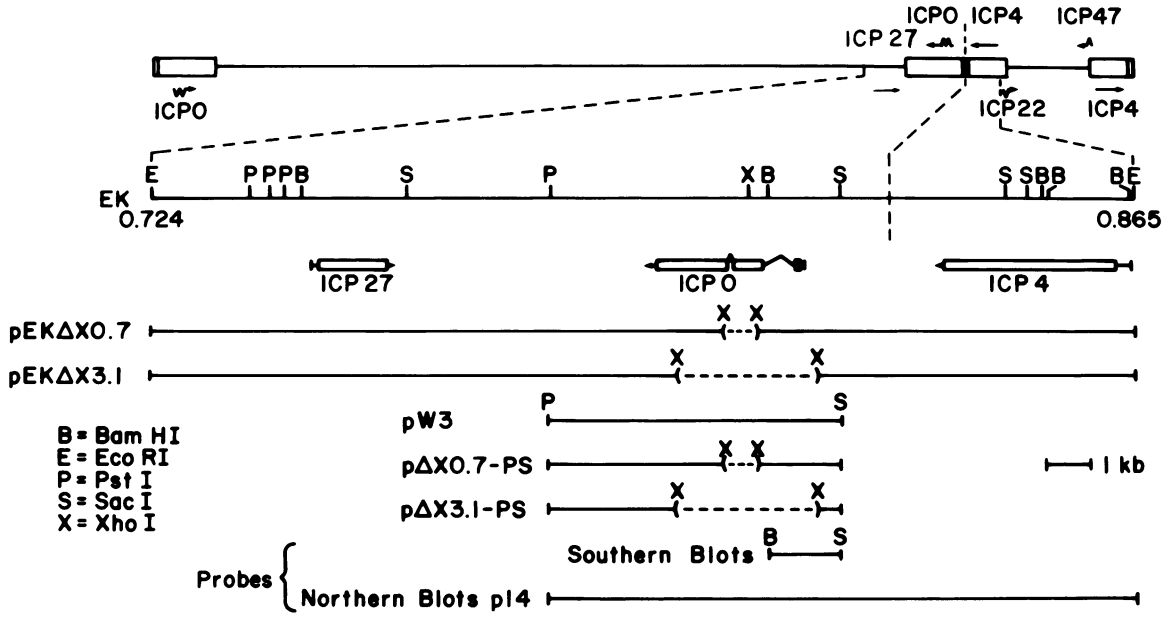

FIG. 1. Map locations of the transcripts specifying ICP27, -0, and -4 and the viral inserts in plasmids used in this study. Beneath the representation of the prototype arrangement of the HSV-1 genome (top line), showing the locations of transcripts specifying the five immediate-early proteins, is an expansion of the *Eco*RI joint fragment EK (pSG28; line 2) showing the locations of relevant restriction sites. The locations and structures of immediate-early transcripts within EK are shown in line 3 (24, 28, 32a, 37). Open boxes represent exons, and solid horizontal lines represent untranslated regions. The remaining lines show the HSV-1 inserts in plasmids used in this study. The locations of deleted sequences are indicated by dashed lines; deletion endpoints (parentheses) were determined by restriction enzyme analysis and are therefore approximations. × indicates the presence of *Xho*I linkers. The last two lines show the viral DNA fragments used as probes in Southern and Northern blot analysis.

late genes. The failure of ICP0 to activate early and late genes in the context of the viral genome suggests that additional levels of gene regulation involving ICP0 exist.

To begin to address the question of the role of ICP0 in the context of the viral genome, we isolated deletion mutants in this gene. To propagate such mutants, in the event that the gene proved to perform an essential function, we first constructed cell lines containing intact copies of the wild-type gene in a manner similar to that used in the study of mutants of the adenovirus E1A region (20) and ICP4 deletion mutants of HSV-1 (8). It is clear from the results obtained in this study that ICP0 plays an important, although not absolutely essential, role during productive infection in cell culture.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney (Vero and CV-1) cells, human embryonic lung (HEL) cells, and human epidermoid carcinoma 2 (HEp-2) cells were propagated as previously described (38). Nero cells, as described previously (8), were derived by pooling the population of G418-resistant cells generated by transformation of Vero cells with pSV2neo. The specific use of each cell type is indicated below.

Procedures for the propagation and assay of the KOS strain of HSV-1 have been previously described (40). Since stocks of the KOS mutants used in this study yielded low titers when prepared in the standard way, concentrated stocks were prepared by reducing the contents of roller bottles of infected-cell material to a total of 2 to 4 ml and adding 10% glycerol. *d*202, provided by N. DeLuca, is an ICP4 deletion mutant (8). 85gC is unable to induce the synthesis of detectable levels of glycoprotein C (gC) (B. Pancake, unpublished results).

Plasmids. The structures and genomic locations of HSV-1 sequences in the plasmids used in this study are shown in Fig. 1. pSG28, containing the *Eco*RI EK fragment in pBR325 (39; Fig. 1, line 2) was provided by R. Sandri-Goldin and propagated in strain DH-1, a derivative of *Escherichia coli* K-12 1100. pEKΔX0.7 and pEKΔX3.1 were derived from pSG28 by linearizing the plasmid with *Xho*I, carrying out limited BAL 31 digestion, filling in the ends with the Klenow fragment of DNA polymerase I, blunt-end ligating *Xho*I linkers to the plasmid, cleaving again with *Xho*I, and religating the molecules with T4 DNA ligase. pW3 was derived from pSG28 by cleavage with *Pst*I and *Sac*I and insertion of the 6.5-kilobase (kb) ICP0-containing fragment into pUC13. pΔX0.7-PS and pΔX3.1-PS were derived from pEKΔX0.7 and pEKΔX3.1, respectively, in an identical manner. p14, provided by N. DeLuca, was generated by cleavage of pSG28 with *Pst*I, followed by religation. pSV2neo contains the procaryotic gene conferring neomycin resistance under the control of the simian virus 40 early promoter (43). ptkCAT, in which the chloramphenicol acetyl transferase (CAT) gene is under the control of the HSV-1 thymidine kinase (TK) promoter (7), was also provided by N. DeLuca. All of the enzymes and *Xho*I linkers used in the construction of the plasmids were obtained from New England BioLabs, Inc., Beverly, Mass.

Nucleic acid isolation. HSV-1 DNA was purified as described by DeLuca et al. (7) except that (i) ICP0-transformed or Nero cells were used for virus propagation and (ii) the final step was ethanol precipitation rather than dialysis. Cellular DNAs for genomic blots were isolated in the same manner from cell monolayers in 85-mm dishes except that

they were extracted three times with phenol-chloroform-isoamyl alcohol (25:24:1). The viral DNA (see blot described in the legend to Fig. 4) was purified from whole-cell extracts of several roller bottles of infected cells and banded in CsCl.

Cytoplasmic RNA from infected ICP0-containing 0-28 cells and from Nero cells was isolated as previously described (25).

Southern and Northern blot analyses. Restriction enzyme-cleaved DNAs separated by agarose gel electrophoresis were transferred to nitrocellulose by the method of Southern (25, 42). The probe used for Southern blots (Fig. 1) was generated by the digestion of either pKBK (containing *Bam*HI joint fragment K in pBR325) or pW3 with *Bam*HI and *Sac*I. The 1.6-kb fragment shown in Fig. 1 was electroeluted after separation by preparative polyacrylamide gel electrophoresis (25) and purified over an Elutip-d column (Schleicher & Schuell, Inc., Keene, N.H.).

Formaldehyde gel electrophoresis of infected-cell RNAs and Northern blot hybridization were performed as described (8) except that the final washes were for 1 h at room temperature and 1 h at 68°C.

Probes for both Southern and Northern blot analyses were nick translated with ^{32}P-labeled dCTP and dGTP (Amersham Corp., Arlington Heights, Ill.) as previously described (25).

Transformation and transfection. Transformation of Vero cells with a derivative of pW3 that also contained the neomycin resistance gene from pSV2neo (pW3neo) was performed as described by DeLuca et al. (8) except that 2 μg of the plasmid was coprecipitated with 38 μg of salmon testis DNA in a total volume of 2 ml.

Transfection of 0-2 and 0-28 cells was performed by using 1 μg of *d*202 DNA and ~2 μg of linearized pEKΔX0.7 or pEKΔX3.1, respectively, in the marker rescue procedures described previously (38) but with the addition of a 2-min glycerol (15%) shock at 4 h posttransfection.

CAT assays. Cotransfection of ICP0-containing plasmids with tkCAT and the assay of CAT activity were performed by using the modifications of the methods of Gorman et al. (14) described previously (9).

Analysis of infected-cell polypeptides. Lysates of radioactively labeled infected cells were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (21) as modified by Manservigi et al. (26). For this purpose, cells were infected with 10 PFU of virus per cell and incubated at 37°C with [^{35}S]methionine, [^{3}H]mannose (New England Nuclear Corp., Boston, Mass.), or $^{32}P_i$ (Amersham) in the quantities and at the times indicated below.

Viral DNA phenotypes. Viral DNA phenotypes of mutants were determined in Nero cells infected at a multiplicity of infection (MOI) of 10 PFU per cell at 37°C by using the method of Aron et al. (2).

RESULTS

ICP0-transformed cell lines. To ensure that ICP0 mutants could be propagated in the event that deletions in the gene proved lethal, cell lines containing the wild-type ICP0 gene were constructed as permissive hosts. For this purpose, the neomycin resistance gene under the control of the simian virus 40 early promoter (pSV2neo) was cloned into the ICP0-containing plasmid pW3 (Fig. 1). The resulting hybrid plasmid (pW3neo) was used to transform Vero cells, giving rise to G418-resistant colonies that were picked and amplified. The lines were screened for the presence of ICP0 sequences by Southern blot analysis. The probe used for this analysis, as well as for all other Southern blots in this study, was the 1.6-kb *Bam*HI-*Sac*I fragment shown in Fig. 1. Of 28 lines screened, 4 were found to contain intact copies of the 6.5-kb ICP0-containing *Pst*I-*Sac*I fragment (Fig. 2). The standards on the right indicate that the sequences were present in all four cell lines in approximately one to three copies per haploid genome equivalent. Evidence will be presented below to indicate that the sequences were, in fact, expressed. Bands, both larger and smaller than the 6.5-kb *Pst*I-*Sac*I fragment, which hybridized to the probe are also evident (Fig. 2). Contamination of the gel-purified probe with nonviral vector sequences cannot explain the observed hybridization patterns, since no hybridization was detected in Nero cells which contained pSV2neo sequences. These bands must, therefore, represent rearranged copies of the *Pst*I-*Sac*I viral DNA fragment. Their origin and structure were not examined further.

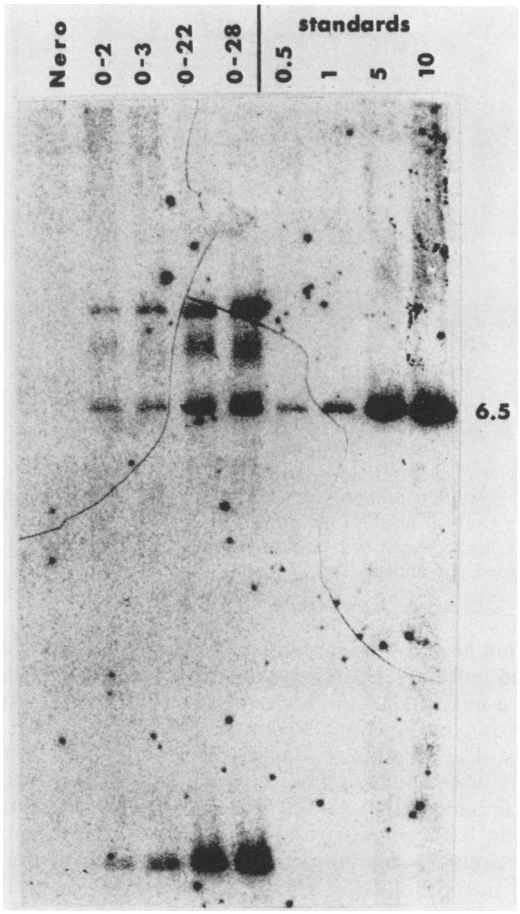

FIG. 2. Southern blot analysis of ICP0-specific sequences in G418-resistant cell lines. DNA (10 μg) from the indicated cell line was digested with *Pst*I and *Sac*I, electrophoresed on a 0.7% agarose gel, Southern blotted, and probed with the ^{32}P-labeled 1.6-kb *Bam*I-*Sac*I fragment shown in Fig. 1. The standards, representing 0.5, 1, 5, and 10 copies of the 6.5-kb ICP0-containing fragment per 3×10^9 bp, are 22, 43, 220, and 430 pg, respectively, of pW3neo (12.9 kb) cut with *Pst*I and *Sac*I.

Of the four ICP0-containing cell lines, lines 0-2 and 0-28 were initially used as the permissive hosts for the propagation of ICP0 deletion mutants. Line 0-28 was later found to be a somewhat more efficient host for the mutants than were the other three lines.

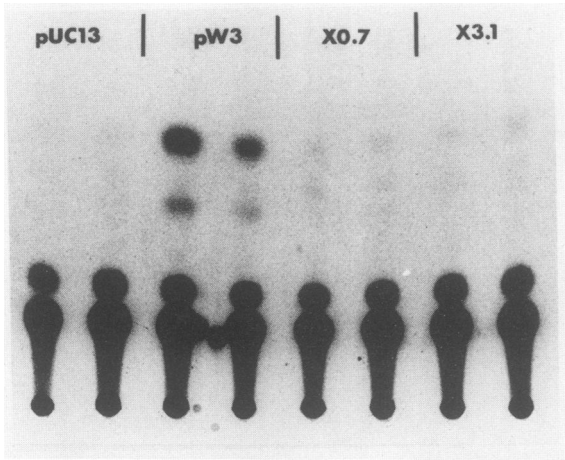

FIG. 3. Effect of deletions in ICP0 on *trans*-induction of ptkCAT activity. Duplicate monolayers of CV-1 cells were cotransfected with 2 μg each of ptkCAT and the indicated plasmid. Transfected cells were harvested at 40 h posttransfection, and cell extracts were assayed for CAT activity.

Isolation of plasmids containing deletions in ICP0. Having identified cell lines containing intact copies of the gene for ICP0, we next undertook construction of plasmids containing deletions in the gene as described in Materials and Methods. The location and approximate endpoints of these deletions are shown in Fig. 1 in the plasmids designated pEKΔX0.7 and pEKΔX3.1. The 0.7-kb deletion lay entirely within the ICP0 transcript, although the reading frame and the exact relationship between the endpoints of the deletion and the introns of ICP0 (32a) have not been determined. Restriction mapping of the 3.1-kb deletion indicated that its right-hand endpoint lay approximately 500 base pairs (bp) from the *Sac*I site at −784 bp with respect to the ICP0 mRNA start site. On the basis of previously reported data, this would remove the mRNA start site, promoter, and part of the regulatory domain of ICP0 (24) but would leave intact the recently identified late gene whose transcript begins in the *a* sequence and whose predicted coding sequence extends into the *b* sequences of IR_L and TR_L (5). This deletion plasmid, therefore, retained only approximately 900 bp of the 3′-terminal portion of the gene (37).

To evaluate the effect of these deletions on ICP0 *trans*-activating activity before their introduction into the background of the viral genome, we compared plasmids containing deleted forms of ICP0 with a plasmid containing wild-type ICP0 in cotransfection-induction experiments. For this purpose, pΔX0.7-PS, pΔX3.1-PS, or pW3 (Fig. 1) was cotransfected with ptkCAT. CAT activity in cell extracts was assayed in duplicate, and the results are shown in Fig. 3. Whereas pW3 stimulated expression of ptkCAT to a level 12-fold higher than that obtained with pUC13, both deletion plasmids failed to induce CAT activity above pUC13 levels, indicating that both deletions abolished this particular activity of ICP0.

Introduction of ICP0 deletions into the viral genome. The plasmids pEKΔX0.7 and pEKΔX3.1 contained wild-type copies of ICP4 and therefore could be used to rescue viruses with mutations in that gene. This, in effect, provided a means of selecting recombinants between viral DNA and the deletion plasmids. ICP0-containing cells were, therefore, cotransfected with linearized forms of the plasmids and intact viral DNA from the ICP4 deletion mutant *d*202. Since ICP4 is an essential protein, the only viable viruses generated in ICP0-transformed cells would be those in which recombination had occurred between *d*202 DNA and the wild-type ICP4 gene in plasmid sequences. Such recombinants were plaque purified and amplified. Plaque isolates were then screened for their ability to grow in ICP0-containing cells relative to Nero cells and were found to fall into two phenotypic classes, i.e., those that grew well in both cell types and those that grew poorly in both cell types. When the DNAs of isolates were analyzed, it became evident that the presence of wild-type or deleted copies of ICP0 correlated perfectly with the phenotypic properties of the isolates, i.e., isolates possessing wild-type copies of ICP0 grew well in both cell types, whereas isolates with deletions in ICP0 grew poorly. Deletion mutants were ultimately plaque purified three times and two, *dl*X0.7 and *dl*X3.1, were arbitrarily chosen for further study.

The Southern blot shown in Fig. 4 compares the DNAs of both deletion mutants, the plasmids used to generate them, and the wild-type virus KOS. The left half of the figure, in which the DNAs were cleaved with *Pst*I and *Sac*I, demonstrates that *dl*X0.7 possessed two copies of the ICP0-

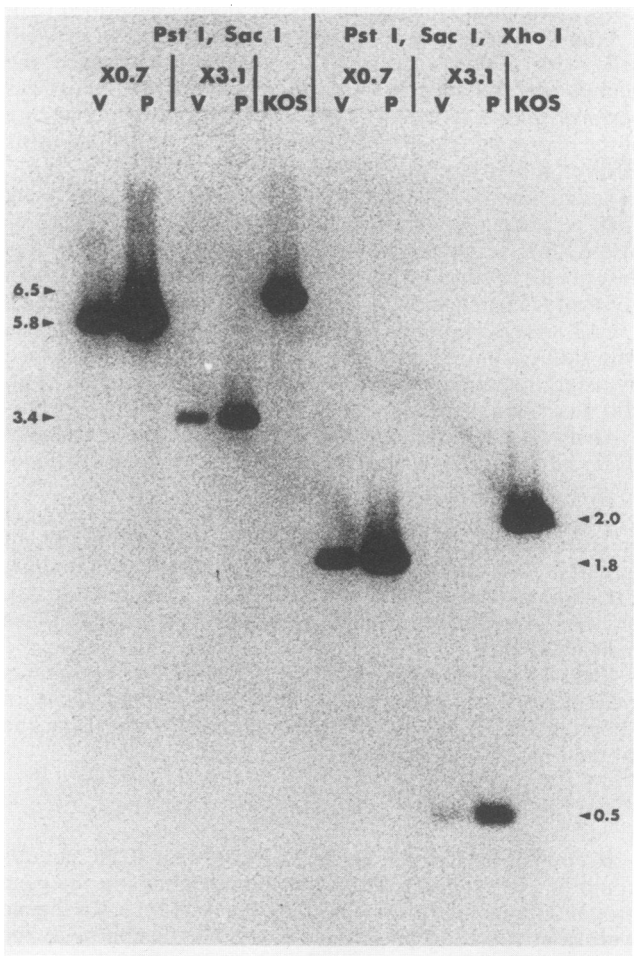

FIG. 4. Comparison of ICP0 sequences in deletion mutants and in deleted plasmids. Viral (V) and plasmid (P) DNAs containing either 0.7- or 3.1-kb deletions, as well as KOS DNA, were cleaved either with *Pst*I and *Sac*I (left-hand lanes) or *Pst*I, *Sac*I, and *Xho*I (right-hand lanes). The electrophoretically separated DNAs were then Southern blotted as described in the legend to Fig. 2. Sizes of fragments (in kilobases) are indicated.

TABLE 1. Titers of ICP0 deletion mutant stocks generated in Nero cells[a]

Virus stock	Titer (PFU/ml) (ratio of KOS per mutant)		Efficiency of plating (0-28/Nero)
	0-28	Nero	
Standard			
KOS	6×10^8	6×10^8	1
*dl*X0.7	3×10^7 (20)	2×10^6 (300)	15
*dl*X3.1	7×10^6 (86)	4×10^5 (1,500)	18
Concentrated			
*dl*X0.7	4×10^8	2×10^7	20
*dl*X3.1	3×10^8	6×10^6	50

[a] Stocks of the indicated viruses were generated in Nero cells, and processed as described in Materials and Methods; titers were determined on the indicated cell line.

containing *Pst*I-*Sac*I fragment that were indistinguishable from that of pEKΔX0.7, being 0.7 kb smaller than the wild-type 6.5-kb fragments of KOS. Likewise, the *Pst*I-*Sac*I fragments of *dl*X3.1 were 3.1 kb smaller than those of KOS and identical to that of pEKΔX3.1. The right half of the figure, in which DNA was cleaved with *Xho*I as well as *Pst*I and *Sac*I, demonstrates that the ICP0-containing *Pst*I-*Sac*I fragments of all five DNAs could be cleaved with *Xho*I. This analysis demonstrates that both mutant viruses contained deletions that were indistinguishable from those in the parental plasmids, with regard to both size and the presence of *Xho*I linkers.

Phenotypic analysis of ICP0 deletion mutants. (i) Growth properties. As mentioned above, the two deletion mutants were capable of growing, albeit poorly, on both ICP0-transformed and Nero cells. One manifestation of this fact is that both mutants gave rise to extremely small plaques on both cell types. Notably, these plaques often contained a focuslike clump of rounded cells in their centers in contrast to the large clear plaques generated by KOS.

The titers of stocks of the mutants on ICP0-transformed and Nero cells (Table 1) reflect another manifestation of their impaired ability to replicate. Whereas a standard stock of KOS yielded approximately 10^8 PFU/ml, the titers of similarly prepared stocks of the mutants were 10- to 1,000-fold lower. A further point evident in Table 1 is that although plaque sizes of both mutants were equally small on both cell types, both plated reproducibly 15- to 50-fold more efficiently on 0-28 cells than on Nero cells. In contrast, KOS plated equally well on both cell types.

The results of the yield experiment shown in Table 2 demonstrate that (i) in addition to Nero and 0-28 cells, both deletion mutants replicated in a variety of other cell types and (ii) the yields and burst sizes of both mutants were approximately 10- to 100-fold lower than those of KOS, in accordance with their small plaque sizes.

(ii) Interference. Having demonstrated the restricted abilities of the deletion mutants to replicate in culture, we next sought to determine whether the mutants could interfere with replication of the wild-type virus. For this purpose, mixed infections were performed in Nero cells. In these tests, the MOI of KOS was held constant at 3 PFU per cell and the MOI of the mutants was varied from 0 to 10 PFU per cell. The ICP4 deletion mutant *d*202, which has little effect on the growth of KOS in mixed infections (N. DeLuca, unpublished results), was included for comparison. Infected cells were harvested at 24 h postinfection, and virus was assayed on 0-28 cells. KOS plaques were readily distinguishable from mutant plaques on these cells, and the results (Fig. 5) show the yields of KOS obtained.

As expected, even at 10 PFU per cell, *d*202 effected only a threefold reduction in the yield of KOS. In contrast, infections with *dl*X0.7 and *dl*X3.1 led to similar reductions in yield at an MOI of 1 PFU per cell and 10-fold reductions at an MOI of 10 PFU per cell. This level of interference is not as profound as that exhibited by a mutant such as *ts*B32, however, whose temperature-sensitive ICP4 possesses growth-inhibitory activities (8, 9) and consequently leads to a 100-fold reduction in the yield of KOS under the same conditions (N. DeLuca, unpublished results). In the case of *dl*X0.7, inhibition was probably not due to the activity of a nonfunctional, truncated form of ICP0, since the effect was more severe in the null mutant *dl*X3.1.

Because concentrated stocks of the mutants were needed for phenotypic characterization, a concern throughout the course of this study was whether the stocks contained high proportions of noninfectious particles that interfered with the normal course of infection. To assess this possibility, we UV-irradiated preparations of the mutant viruses corresponding to an MOI of 3 PFU per cell and coinfected with KOS as before. The results (Fig. 5, open circles) show that UV irradiation abolished the interfering effects observed with all three mutant viruses, demonstrating that interference is a consequence of the expression of viral genetic information by the mutants and not a consequence of the presence of inert particles or virion components.

(iii) Synthesis of ICP0-specific mRNA. Based on the sizes and locations of the deletions in the two mutants, it seemed likely that *dl*X0.7 would express a truncated form of ICP0 mRNA, whereas *dl*X3.1, lacking critical transcriptional signals, would fail to express the message. To test this prediction, Nero and 0-28 cells were infected with mutant or wild-type viruses or mock infected in the presence of anisomycin. Under these conditions, immediate-early mRNAs are the predominant transcription products (23). At 6 h postinfection, total cytoplasmic RNA was isolated and subjected to Northern blot analysis, using p14, the plasmid containing the *Pst*I-*Eco*RI fragment shown in Fig. 1, as a probe. This fragment contained both ICP0- and ICP4-specific sequences.

The results shown in Fig. 6 demonstrate that both mutants induced the synthesis of ICP4 mRNAs of wild-type size (4.3

TABLE 2. Growth of ICP0 deletion mutants in various cell lines

Virus	Virus yield (PFU/dish) (burst size)[a]				
	Nero	0-28	Vero	HEp-2	HEL
KOS	5×10^8 (700)	2×10^8 (300)	5×10^8 (1,000)	1×10^8 (100)	5×10^8 (500)
*dl*X0.7	1×10^7 (10)	2×10^7 (30)	1×10^7 (20)	6×10^6 (6)	4×10^6 (4)
*dl*X3.1	5×10^6 (7)	8×10^6 (10)	4×10^6 (8)	6×10^6 (6)	8×10^6 (8)

[a] Confluent 35-mm dishes of cells were infected at an MOI of 2.5 PFU per cell at 37°C and harvested at 24 h postinfection; the progeny were assayed on 0-28 cells. Burst size was calculated on the basis of 7.5×10^5 Nero and 0-28 cells, 5×10^5 Vero cells, and 1×10^6 HEp-2 and HEL cells per dish.

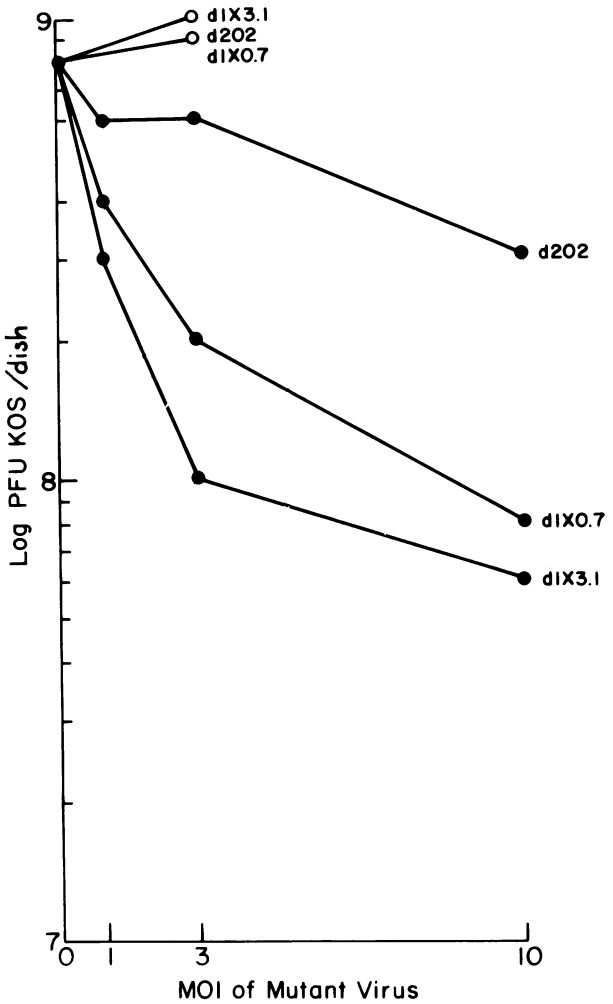

FIG. 5. Interference of ICP0 deletion mutants with the growth of KOS. Confluent monolayers of Nero cells were coinfected with a constant MOI (3 PFU per cell) of KOS and various MOIs (0, 1, 3, and 10 PFU per cell) of deletion mutants. After incubation at 37°C for 24 h, infected cells were harvested and the titers of the progeny were determined on 0-28 cells. Symbols: ●, yields of KOS (which were readily distinguishable from mutant plaques); ○, coinfection of 3 PFU per cell of KOS with preparations of mutants that would have corresponded to an MOI of 3 PFU per cell had they not been UV-irradiated under conditions known to reduce the titer of KOS by 10^{-6} (9).

kb) in both cell types as expected. As predicted, *dl*X0.7 induced the synthesis an ICP0 mRNA that was approximately 0.7 kb smaller (2.1 kb) than that of the wild-type virus (2.8 kb), whereas cells infected with *dl*X3.1 synthesized no detectable truncated ICP0 message, since an RNA 900 bp or larger would be readily visible on the gel. The tests also indicate that 0-28 cells expressed low levels of wild-type ICP0 mRNA that were detectable only after superinfection (Fig. 6, compare lanes 3, 5, and 7). This is presumably due to the induction of resident ICP0 genes by a virion component (3, 33).

(iv) Immediate-early protein synthesis. To evaluate the ability of the deletion mutants to induce the synthesis of immediate-early polypeptides, 0-28 and Nero cells were infected in the presence of cycloheximide. Cycloheximide-containing medium was removed at 6 h postinfection and replaced with medium containing actinomycin D and either [^{35}S]methionine or $^{32}P_i$. After a further 3 h of incubation, samples were harvested and analyzed by SDS-PAGE. The results shown in Fig. 7 represent profiles of infected Nero cell samples and were indistinguishable from those generated in infected 0-28 cells (not shown). The ICP4 deletion mutant *d*202 was included for comparison. With the exception of ICP0, it is clear that both ICP0 mutants, like wild-type virus, were able to induce the synthesis of immediate-early polypeptides (ICP4, -22, and -27) and the early protein ICP6. Moreover, phosphorylation of the polypeptides (32, 48) also appeared to be normal, indicating that phosphorylation of these polypeptides did not require functional ICP0 (nor did it require functional ICP4, as demonstrated by the *d*202 profile shown in Fig. 7).

Since *dl*X0.7 specifies a truncated ICP0 mRNA, we anticipated that it would induce the synthesis of a truncated polypeptide. Unfortunately, no such polypeptide was evident on the gels, under either set of labeling conditions. Notably, the truncated form of ICP4 expressed in *d*202-infected cells was also undetectable under these conditions since it comigrates with other infected-cell proteins, most

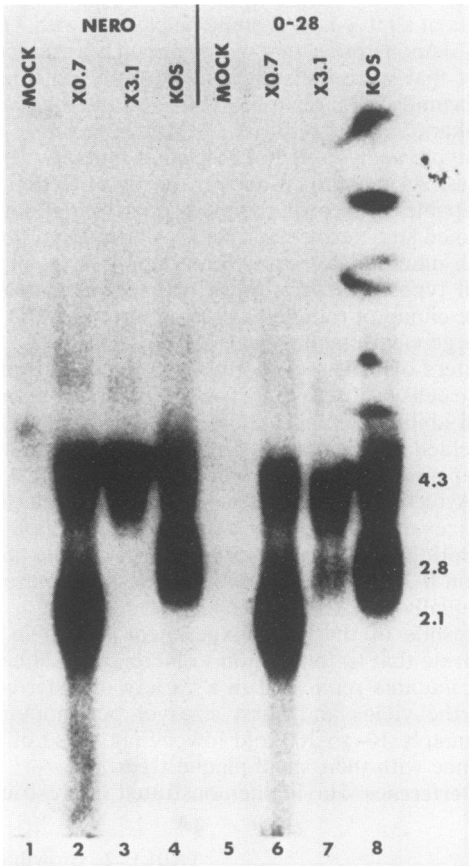

FIG. 6. Northern blot analysis of mRNAs encoding ICP4 and ICP0 in cells infected with ICP0 deletion mutants. Monolayers of Nero or 0-28 cells were incubated for 1 h at 37°C in the presence of 80 μM anisomycin before infection with 5 PFU of the indicated virus per cell (in the presence of the same concentration of the drug). At 6 h postinfection, cells were harvested. Cytoplasmic RNAs were isolated as described in the text and analyzed by Northern blot analysis using ^{32}P-labeled p14 (Fig. 1) as a probe. The approximate sizes of the mRNAs (in kilobases) are indicated on the right.

likely ICP6 or ICP0 (8). A similar situation may also exist for the ICP0 of *dl*X0.7.

(v) Polypeptide synthesis. Given the restricted abilities of both ICP0 deletion mutants to replicate, the patterns of polypeptides expressed by the mutants were of interest. Infected Nero cell monolayers were therefore labeled from 4.5 to 24 h postinfection with [^{35}S]methionine, and infected-cell extracts were analyzed by SDS-PAGE (Fig. 8). As expected from their replication competence, both *dl*X0.7 and *dl*X3.1 generated protein profiles that were qualitatively similar to that of wild-type virus. Quantitative differences in polypeptide synthesis were, however, evident. *dl*X0.7 induced slightly reduced levels of certain polypeptides, particularly those of the late class (i.e., ICP5, -19, -20, and -25), and *dl*X3.1 induced even lower levels of these species. The results of a yield experiment performed on aliquots of the samples used to generate the protein profiles shown in Fig. 8 demonstrate again the reduced yields characteristic of the mutants relative to KOS. In this experiment, the yield of KOS was 3×10^8 PFU/ml, while the yields of *dl*X0.7 and *dl*X3.1 were 4×10^7 and 1×10^7, respectively. Similar experiments performed in 0-28 cells gave rise to gel profiles

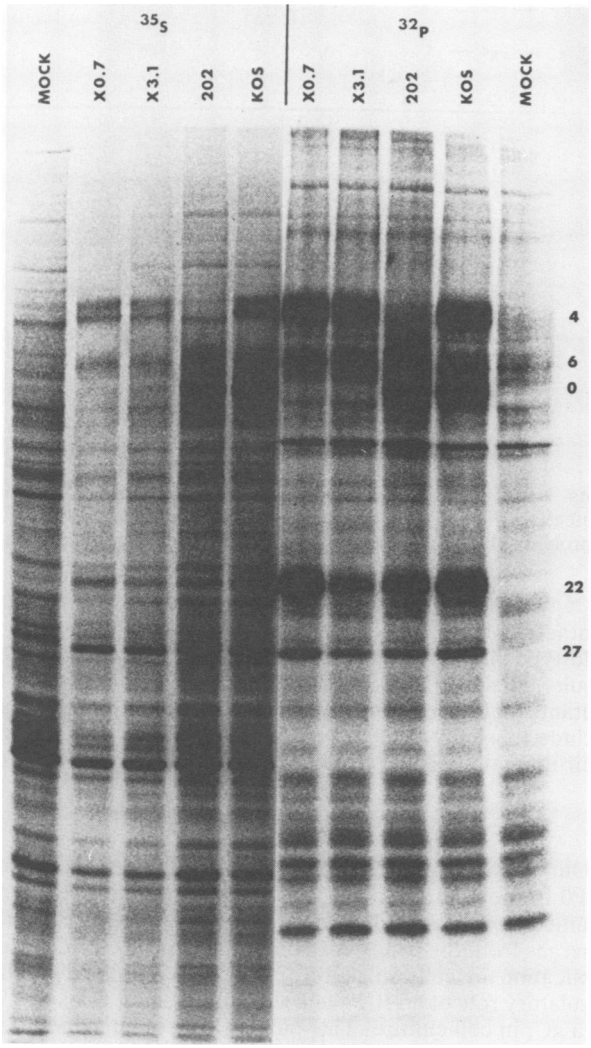

FIG. 7. Synthesis of immediate-early polypeptides in cells infected with ICP0 deletion mutants. Cells were preincubated for 1 h at 37°C in the presence of 100 μg of cycloheximide per ml. They were then infected with 10 PFU of the indicated viruses per cell in the presence of the same concentration of drug. At 6 h postinfection, infected monolayers were washed and overlaid with medium containing 10 μg of actinomycin D and 100 μCi of either [^{35}S]methionine or $^{32}P_i$ per ml. After a further 3 h of incubation, monolayers were harvested as described and subjected to SDS-PAGE on a 9% gel cross-linked with *N,N'*-dicyclohexylcarbodiimide. The ^{35}S half of the gel represents an ~52-h exposure, and the ^{32}P half represents an ~18-h exposure. The positions of the four immediate-early polypeptides visible on this gel (ICP4, -0, -22 and -27) and of one early polypeptide (ICP6) are indicated on the right.

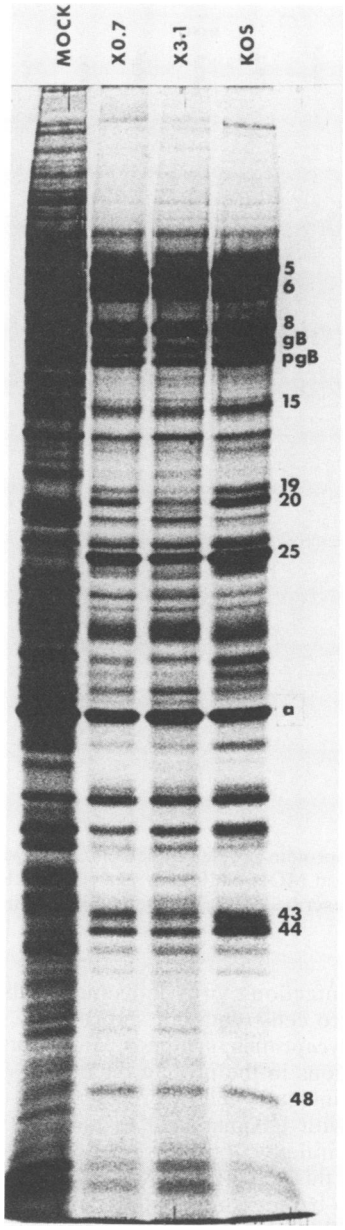

FIG. 8. Polypeptide profiles of ICP0 deletion-mutant-infected cells. Nero cells were infected with 10 PFU of the indicated viruses per cell at 37°C and labeled with 2.5 μCi of [^{35}S]methionine per ml from 4.5 to 24 h postinfection. Lysates were then prepared and electrophoresed as described in the legend to Fig. 7. The positions of several early polypeptides (ICP6, ICP8, and gB) and late polypeptides (ICP5, -15, -19, -20, -25, -43, -44, and -48) are indicated.

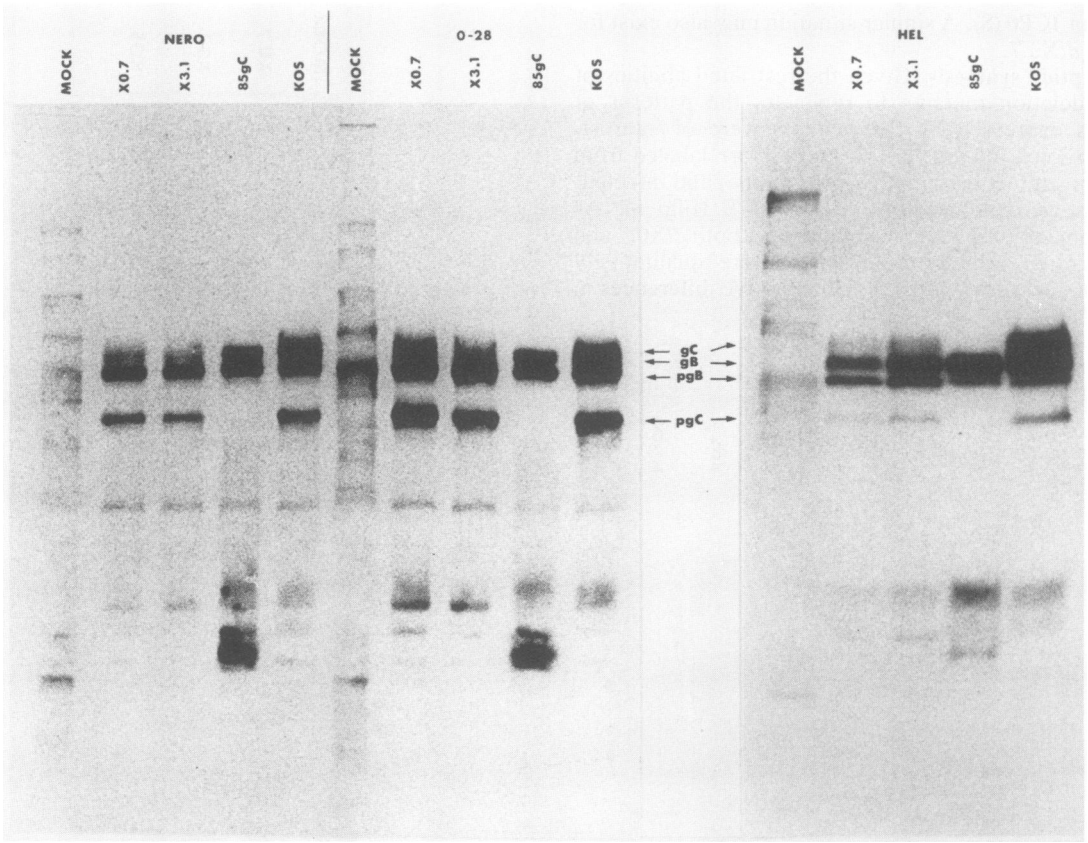

FIG. 9. Glycoprotein profiles of cells infected with ICP0 deletion mutants. Nero, 0-28, and HEL cells were infected in parallel with the indicated viruses at MOIs of 10 PFU per cell, labeled from 5 to 17 h postinfection with 100 μCi of [^{3}H]mannose per ml, and processed for SDS-PAGE as described in the legend to Fig. 7. The positions of the glycoproteins gB and gC, as well as of precursor forms, are indicated.

and yields of infectious virus indistinguishable from those obtained in Nero cells (data not shown).

(vi) Viral glycoprotein synthesis. In a further effort to identify alterations in the protein phenotypes of ICP0 deletion mutants, infected cells were labeled from 5 to 17 h postinfection with [^{3}H]mannose to monitor synthesis and processing of viral glycoproteins (Fig. 9). Also included for comparison in this experiment was 85gC, a mutant previously shown to be nonconditionally deficient in the expression of gC (B. Pancake, unpublished results). In addition to 0-28 and Nero cells, glycoproteins were also visualized in infected HEL cells.

When the quantities of both precursor and mature forms of gB and gC induced by the ICP0 deletion mutants are considered, a number of trends are suggested. Regardless of the cell type infected, the total quantity of the early polypeptide gB (i.e., pgB + gB) induced by all of the viruses appeared similar. In *dl*X0.7- and *dl*X3.1-infected Nero or HEL cells, however, the total quantity of the late polypeptide gC (i.e., pgC + gC) seemed greatly reduced, as did the proportions of the mature forms of both gB and gC relative to their precursor forms. 0-28 cells appeared more permissive for the deletion mutants for both synthesis and processing of these glycoproteins.

(vii) Viral DNA synthesis. We next analyzed the abilities of *dl*X0.7 and *dl*X3.1 to induce viral DNA synthesis in Nero cells; we did this by labeling with [^{3}H]thymidine from 4 to 24 h postinfection. The CsCl gradient profiles shown in Fig. 10 demonstrate that both mutants were able to induce the synthesis of viral DNA, although to levels below that of wild-type virus (*dl*X0.7, 38% of wild type; *dl*X3.1, 24%). The results are qualitatively consistent with the abilities of the mutants to induce the synthesis of early polypeptides, which include those involved in viral DNA synthesis (2), and with their ultimate abilities to produce viable progeny.

DISCUSSION

Our approach to addressing the question of the role of ICP0 in the context of the virus has been to construct and characterize deletion mutants in the gene. The picture that emerges from this study is that of a gene whose role in virus replication in cells in culture falls between the essential, regulatory role of an ICP4 and the nonessential roles of a TK or a gC, in cell culture. This picture is also indicative of the complex manner in which HSV-1 modulates expression of its genes.

Isolation of ICP0 deletion mutants. The two plasmids pEKΔX0.7 and pEKΔX3.1, shown by transient-expression assays to have lost ptkCAT inducing activity, were introduced into the viral genome by cotransfection of ICP0-transformed cells with viral DNA from the ICP4 deletion mutant *d*202. It is notable that despite the fact that ICP0 is diploid, we had no difficulty obtaining mutants with deletions in both copies of the gene. In fact, after one plaque purification of mixed populations of viruses carrying wild-type or deleted copies of the gene or both, all isolates examined contained two identical copies of either allele, suggesting

that in the absence of other types of selective pressure, homozygosity is favored over heterozygosity. This was also found to be true in the generation of deletion mutants of ICP4 (8).

Phenotypic properties of ICP0 deletion mutants. The most striking characteristic of *dl*X0.7 and *dl*X3.1 was that they replicated in cell culture. Their replicative abilities were significantly restricted relative to that of the wild-type virus, however, as reflected by their extremely small plaque sizes and by titers that were 10- to 1,000-fold lower than that of wild-type virus. Consistent with their small plaque sizes, their burst sizes were 10- to 100-fold lower than that of KOS, irrespective of the cell type used. The reduced burst sizes exhibited in this growth experiment did not reflect a delay in the production of infectious virus by the mutants, since no further increase in yields was observed when the experiment was extended to 48 h postinfection (data not shown).

It is notable that in contrast to the situation observed for deletion mutants of ICP22 (41), no particular cell type (such as HEL) was more or less permissive for the ICP0 deletion mutants in a growth experiment. Furthermore, of the growth properties considered, the only manner in which ICP0-transformed cells were observed to be more permissive for the mutants was in plating efficiency (Table 1). This peculiar observation, i.e., that the mutants plated 15 to 50 times more efficiently on 0-28 cells than on Nero cells, whereas plaque and burst sizes were similar on both cell types, suggests that there is a restriction in the ability of the mutants to initiate plaque formation in Nero cells and that this restriction is complemented in 0-28 cells. Once a plaque was initiated, however, the mutants replicated equally well in either cell type.

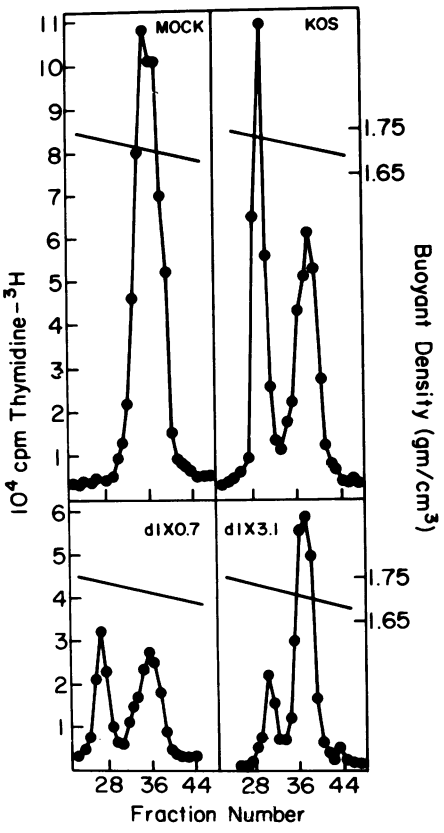

FIG. 10. Viral DNA phenotypes of ICP0 deletion mutants. Nero cell monolayers were infected with 10 PFU of the viruses indicated per cell at 37°C and labeled with 12.5 μCi of [^{3}H]thymidine per ml from 4 to 24 h postinfection. CsCl gradient profiles were obtained by using the method of Aron et al. (2). In these experiments, viral DNA peaked at a density of ~1.735 g/cm^3 and cellular DNA peaked at ~1.712 g/cm^3.

Viral polypeptide and DNA phenotypes. Our preliminary efforts to discern specific restrictions in the replicative cycles of the two ICP0 deletion mutants revealed generalized quantitative reductions in the abilities of the mutants to induce later viral polypeptides and viral DNA but no absolute blocks. Long-term labeling of mutant-infected cells generated polypeptide profiles in which the quantities of viral proteins synthesized were somewhat reduced, particularly those of the late class (e.g., ICP5, -19, -20, and -25), relative to the levels induced by KOS. Further corroboration of this phenomenon comes from the analysis of viral glycoprotein synthesis in which levels of the late protein gC induced by the mutants were reduced. Furthermore, the proportions of mature to precursor forms of both of these glycoproteins appeared greatly reduced in mutant-infected cells (especially in non-ICP0-transformed cells). This likely reflects reduced levels of a late viral gene product(s) involved in glycoprotein processing in mutant-infected cells.

Analysis of the abilities of the deletion mutants to induce viral DNA synthesis generated results similar to those obtained from analysis of viral polypeptide synthesis, i.e., no specific block, but rather a reduction in the abilities of the mutants to induce viral DNA synthesis, was observed relative to wild-type virus. This reduced ability may be sufficient to explain the reduced levels of late polypeptides noted above.

With regard to the overall phenotype exhibited by the mutants, several further observations can be made. It was not unexpected that the deletion mutants exhibited no discernible qualitative phenotypic differences from KOS, given their abilities to generate viable progeny in all cell types tested. However, the slight quantitative differences noted in the abilities of the mutants to induce viral polypeptide and DNA synthesis relative to KOS did not appear severe enough to explain the much larger reductions in burst sizes noted. This observation, i.e., that levels of viral polypeptide and DNA synthesis were reduced by 2- to 4-fold, whereas in the same experiment, burst sizes were reduced up to 30-fold, indicates that even a smaller proportion than normal of the products of viral replication constituted viable progeny. It indicates that in addition to the deficiencies already noted, other undetected effects of the absence of ICP0 must also contribute to the low yields generated by the mutants. It is suggestive of the existence of a large population of mutant particles, only a small portion of which complete the replicative cycle successfully. The interference of the mutants with KOS may, therefore, reflect competition for limiting factors in a manner analogous to the effects of defective interfering particles.

Finally, although we have as yet no direct evidence for the existence of a truncated form of ICP0 with residual activity induced by *dl*X0.7, the existence of such a polypeptide cannot be ruled out. Moreover, the fact that the phenotypes exhibited by *dl*X0.7 were consistently and reproducibly somewhat less severe than those of *dl*X3.1 is suggestive of just such residual activity.

The role of ICP0. Our characterization of mutants lacking functional ICP0 has not yet identified a specific role for the protein in the life cycle of the virus in culture. Despite the ability of the isolated ICP0 gene to *trans*-activate all three classes of HSV promoter in cotransfection experiments, our

studies indicate that, at least in cell culture, there was no absolute requirement for ICP0 *trans*-inducing activity in the viral replicative cycle. Most reports of ICP0 *trans*-activation have, however, noted synergism between the activities exhibited by ICP0 and ICP4, suggesting the possibility that some of the pleiotropic defects exhibited by the ICP0 deletion mutants may be due to the absence of ICP0 *trans*-activation. Further experimentation will be required to determine, for example, whether the reduced levels of late gene products are due simply to the reduced levels of viral DNA observed or due more directly to the absence of ICP0 and its associated *trans*-activating ability. The finding that deletion mutants of ICP0 were replication competent is, however, consistent with the observation that, despite the apparent redundancy of the activities of ICP0 and ICP4 demonstrated in vitro, the wild-type copies of ICP0 present in ICP4 mutants do not suffice to facilitate the normal course of viral gene expression (8).

It is clear from this study that, although ICP0 was not absolutely essential for replication of the virus in culture, it nevertheless carried out a function(s) of some importance. The role of the gene cannot be categorized as nonessential, as those of TK and gC are. Mutations in either of these genes give rise to viruses whose growth properties in culture are indistinguishable from that of wild-type virus (11, 15, 19, 26). On the other hand, the abilities of the ICP0 deletion mutants to replicate in culture under all conditions tested was greatly impaired. In this regard, the effects of deletions of ICP0 on the growth of HSV-1 are somewhat reminiscent of the effects of deletions in the E1A region of adenovirus. Although deletion of E1A has profound effects on early gene expression and viral replication, the effects are not absolute, in that early gene expression and virus production ultimately occur, but they occur much later than normal (29). It is therefore notable that (i) levels of ICP0 decline earlier than do those of the other immediate-early gene products (48); (ii) the effects of deleting ICP0 appear global, being detectable by the onset of viral DNA synthesis; and (iii) the onset of early and late protein synthesis induced by the deletion mutants was, in fact, delayed (manuscript in preparation). It is, therefore, possible that ICP0 plays a fine-tuning role in gene regulation during productive infection of HSV-1 in culture. Our further efforts to better define the role of the gene will include attempts to identify more specifically the earliest time after the onset of infection when the effect of the absence of ICP0 is observed and to determine what the direct consequence of its absence is.

In the past several years an increasing number of HSV genes have been identified as being more or less dispensable for the growth of the virus in culture. In addition to TK and gC, these include ICP22 and ICP47, US 10 and US 11 (22, 34, 41), and now ICP0. It is highly likely that these dispensable functions play roles in aspects of the normal life cycle of the virus left unaddressed by studies of productive infection in cell culture, namely, replication and latency in the normal host. In the case of the five immediate-early genes, it is intriguing that those identified as essential (ICP4 and ICP27) are unspliced, whereas those that appear to be nonessential (ICP0, -22, and -47) are spliced. In the Epstein-Barr virus system, in which latency is more amenable to study than it is in the HSV system, of the genes that have been mapped and characterized thus far, genes expressed during latency, such as Epstein-Barr virus nuclear antigens I and II, are spliced (16, 44, 47), whereas those that have been identified as lytic genes, such as ribonucleotide reductase and certain diffuse early antigens, are unspliced (4, 13). A gene such as ICP0, which exhibits many of the qualities of a regulatory protein, would seem a likely candidate for involvement in the switch from latent to lytic modes of infection. On the basis of their finding that ICP0 stimulates immediate-early promoters, whereas ICP4 tends to inhibit their activity in cotransfection experiments, O'Hare and Hayward (31) have proposed that the two gene products mediate the transition between the two states. We find this model an attractive one.

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