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Construction and Characterization of a Herpes Simplex Virus Type 1 Mutant Unable To Transinduce Immediate-Early Gene Expression

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A herpes simplex virus mutant, *in*1814, possessing a 12-base-pair insertion in the gene encoding the transinducing factor Vmw65 has been constructed. The insertion abolished the ability of Vmw65 to transinduce immediate-early (IE) gene expression and to form a protein-DNA complex with cell proteins and the IE-specific regulatory element TAATGAGAT. Accumulation of IE RNA 1 and 2 was reduced four- to fivefold in *in*1814-infected cells, but the level of IE RNA 4 was reduced only by twofold, and IE RNA 3 was unaffected. Mutant *in*1814 had a high particle/PFU ratio, but many of the particles, although unable to form plaques, were capable of normal participation in the early stages of infection at high multiplicity of infection. The defect of *in*1814 was overcome partially by transfection of a plasmid encoding the IE protein Vmw110 into cells prior to titration and by prior infection with ultraviolet light-inactivated herpes simplex virus. Mutant *in*1814 was essentially avirulent when injected into mice. The results demonstrate that transinduction of IE transcription by Vmw65 is important at low multiplicity of infection and in vivo but that at high multiplicity of infection the function is redundant.

Herpes simplex virus type 1 (HSV-1) encodes 70 predicted genes which are expressed as three temporally regulated classes (35, 70). The five immediate-early (IE or α) genes are the first to be transcribed after infection, and their expression does not require de novo protein synthesis, whereas early (β) and late (γ) gene expression is dependent on the prior synthesis of IE polypeptides (8, 23). The products of IE gene 1 (polypeptide Vmw110 or ICP0) and IE gene 3 (Vmw175 or ICP4) are potent transactivators of early- and late-gene promoters in transient expression assays (12, 18, 40, 53). The IE gene 2 product (Vmw63 or ICP27) has also been implicated in the regulation of viral promoters (13, 54, 60). Analysis of temperature-sensitive mutants indicates that both Vmw175 and Vmw63 are essential for productive infection; Vmw175 is required for early- and late-gene expression (11, 47, 71), whereas Vmw63 appears to be required after the onset of early-gene expression and DNA replication (56). Viable mutants with deletions in Vmw110 exhibit restricted growth in certain cell types at low multiplicity of infection (MOI) but are apparently normal at high MOI (57, 65). Deletion mutations in IE gene 4 (which specifies Vmw68 or ICP22) also confer a host range phenotype to the virus (45, 59). The IE gene 5 product (Vmw12 or ICP47) appears to be unimportant for virus replication in tissue culture cells since deletions within the gene have little effect on growth of HSV (4, 29, 69).

A distinguishing feature of IE genes is the presence of the *cis*-acting element TAATGARAT (where R is a purine residue) in their 5′ regulatory regions. This element responds to the HSV-1 virion polypeptide Vmw65 (otherwise designated VP16 or αTIF), resulting in a stimulation of transcription from IE promoters (2, 5, 7, 9, 17, 25, 31–33, 41, 43, 44, 48). Although Vmw65 does not itself bind to DNA (34), the evidence currently available suggests that the polypeptide mediates transinduction of IE genes by associating with cellular proteins, including nuclear factor III, to form an IE complex (IEC) which is able to bind specifically to DNA sequences that contain TAATGARAT (1, 19, 38, 39, 49). Mutation analysis of cloned DNA fragments encoding Vmw65 suggests that the polypeptide contains at least two separable regions, both of which are necessary for transinduction of IE transcription. The amino-terminal 411 amino acids are sufficient for binding to the cellular factor (1; T. A. McKee, C. I. Ace, and C. M. Preston, manuscript in preparation), and the acidic carboxy-terminal domain defined by amino acids 411 to 490 (the "acid tail," a feature common to many other eucaryotic and procaryotic transactivators [3, 20, 30, 52, 58, 66, 67]) is required for stimulating transcription and may interact with fundamental transcription components, for example, the TATA binding factor TFIID or RNA polymerase, or both (24).

Because Vmw65 regulates the set of genes expressed at the earliest stages of infection, it is important to determine the role of the polypeptide during HSV growth. All information to date regarding the properties of Vmw65 has been obtained by transfection, using either stably transformed cell lines or transient expression assays. Such systems are clearly artificial, and the crucial biological question concerns the phenotype of virus mutants which lack the transinducing activity of Vmw65. A difficulty inherent in attempts to construct such mutants is that, apart from its role in transinducing IE genes, Vmw65 is also a major structural component which is required for virion assembly (1). To address this problem, functional domains of Vmw65 required for virion assembly and for transinduction were identified by insertion mutagenesis of a cloned gene fragment (1). The construction and characterization of a viable HSV-1 mutant that contains an insertion which affects only the transinducing activity of Vmw65 are described here. The results indicate that IE gene transinduction by Vmw65 is not essential for virus growth at high MOI but plays a critical role in determining whether infection is lytic or nonproductive at low MOI. Vmw65 is also important for the virulence of HSV-1 in mice.

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MATERIALS AND METHODS

Cells and viruses. BHK cells (clone 13) were grown in Eagle medium with 10% newborn calf serum, 10% tryptose phosphate broth, and 100 U of penicillin and 100 μg of streptomycin per ml. Human fetal lung (HFL) cells (Flow Laboratories) were grown in Eagle medium with 10% fetal calf serum and 100 U of penicillin and 100 μg of streptomycin per ml. The wild-type (wt) HSV-1 virus used in these studies was Glasgow strain 17 syn^+. Virus particle concentrations were determined by comparison of virus stocks with bead preparations of known concentration.

Plasmids. Plasmid pMC1, which contains the coding sequences for Vmw65, has been described previously (7). The construction of pMC1.in14, which contains a 12-base-pair (bp) *Bam*HI oligonucleotide linker inserted in the Vmw65 gene, has also been described (1). Plasmid pIE3CAT contains the HSV-1 IE gene 3 promoter and regulatory sequences linked to the chloramphenicol acetyltransferase coding region (64), and p111 expresses wt HSV-1 Vmw110 (14).

Isolation of *in*1814 and Southern blot analysis. A BHK cell monolayer in a 35-mm-diameter petri dish was cotransfected with 0.5 μg of intact HSV-1 DNA, 0.5 μg of an *Eco*RI-cleaved plasmid with the mutation of pMC1.in14 in the larger plasmid pGX158 (which contains *Bam*HI *f* [7]), and 2.0 μg of calf thymus carrier DNA by the calcium phosphate precipitation method (51). After incubation for 3 days at 31°C, the progeny viruses were harvested and titrated on BHK cells. Single plaques were picked and used to infect BHK cells in multiwell plates containing 15-mm-diameter wells. After 2 days at 37°C, total DNA was prepared from infected cells (65) and the medium was retained as a viral stock. DNA samples were screened for the presence of viral genomes containing a *Bam*HI linker insertion within the Vmw65 gene. DNA was cleaved with *Bam*HI, and viral DNA analyzed by agarose gel electrophoresis and Southern blotting (61). Plasmid pMC17, which contains the Vmw65 coding sequences cloned in pUC9 (1), was radiolabeled with ^{32}P by nick translation (55) and used as a probe. Hybridization, membrane washing, and autoradiography conditions were as described previously (42). Progeny from a sample which contained viral DNA with a linker insertion was plaque purified and screened by hybridization twice more, and a working stock of virus was prepared from BHK cells. The virus was named *in*1814.

Marker rescue of *in*1814. A BHK cell monolayer was cotransfected with 0.1 μg of intact *in*1814 DNA, 0.5 μg of pMC1 cleaved with *Eco*RI, and 2.0 μg of calf thymus DNA, as described above. After 5 days at 31°C, progeny were harvested and used to infect a BHK cell monolayer on a 90-mm-diameter petri dish at 0.0001 PFU per cell. After 3 days, the progeny were harvested and titrated on BHK cells. Single plaques were picked and used to infect BHK cells on multiwell plates. Virus stocks were prepared from wells, and their genomes were analyzed as described above. Progeny from a sample that exhibited a wt HSV-1 DNA structure was plaque purified, and a working stock of virus was prepared in BHK cells. The virus was named 1814R.

Quantitation of viral DNA in nuclei. BHK cell monolayers in 90-mm-diameter petri dishes were infected in the presence of 200 μg of cycloheximide (CH) per ml. After incubation for 3 h at 38.5°C, cell nuclei were isolated (46) and DNA was extracted. Virus DNA was quantitated by Southern blotting as described above but by using ^{32}P-labeled pTK1, which contains the cloned HSV-1 *Bam*HI *p* fragment, in the hybridization procedure.

Radiolabeling of viral polypeptides. For IE polypeptides, BHK cell monolayers were infected in the presence of 200 μg of CH per ml. After 4 h at 38.5°C, CH was washed from the plates and proteins were radiolabeled for 1 h with [^{35}S]methionine in the presence of 1 μg of actinomycin D per ml and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (46). For early and late polypeptides, BHK cell monolayers were infected and incubated at 37°C for 8 h, and proteins were radiolabeled for 1 h with [^{35}S]methionine and analyzed by SDS-PAGE.

Gel retardation analysis. Virion extracts were prepared (49), and proteins were analyzed by SDS-PAGE. The gel was stained with Coomassie brilliant blue. Virion extract was added to a mixture containing HeLa cell nuclear extract and a ^{32}P-labeled 74-bp DNA fragment containing HSV-1 IE gene 4/5 regulatory sequences (49). Reaction conditions for complex formation and analysis of protein-DNA complexes were as described previously (49).

Transinduction assay. BHK cell monolayers in 35-mm-diameter petri dishes were transfected with 3 μg of pIE3CAT by the calcium phosphate precipitation method (7), except that the dimethyl sulfoxide boost was performed 1 h after the medium overlay. The cells were incubated at 38.5°C for 1 h and then superinfected. After incubation for a further 3 h at 38.5°C, extracts were made from the cells and chloramphenicol acetyltransferase assays were performed (21).

Quantitation of IE RNA. BHK cells were infected in the presence of 200 μg of CH per ml. After incubation for 4 h at 38.5°C, cytoplasmic RNA was extracted and quantitated by dot blot analysis (72) by using DNA probes radiolabeled with ^{32}P by primer extension (15). Gene-specific probes were prepared from DNA fragments that correspond to IE genes 1 (a 1,367-bp *Sal*I-*Nru*I fragment from pJR3 [12]), 2 (a 2,760-bp *Mlu*I-*Bam*HI fragment from *Bam*HI *b* [35]), 3 (a 3,210-bp *Hin*cII fragment from *Xho*I *c* [36]), and 4 (a 2,200-bp *Nru*I-*Mlu*I fragment from *Bam*HI *n* hybridizing predominantly to IE RNA 4 [37]).

TK assay. BHK cells were infected in the presence of 200 μg of phosphonoacetic acid per ml. After incubation for 15 h at 38.5°C, cytoplasmic extracts were made and thymidine kinase (TK) assays were performed (9).

Complementation assay. BHK cell monolayers in 35-mm-diameter petri dishes were transfected with 3 μg of p111 or pUC9 by the calcium phosphate precipitation method (7) and treated with dimethyl sulfoxide 1 h later. After a further 1 h at 37°C, virus was titrated on the monolayers. Alternatively, monolayers were treated with the HSV-1 mutant *ts*K, which had been UV irradiated to reduce its titer by 5×10^5 (48). The MOI of UV-irradiated *ts*K corresponded to 0.1 PFU of unirradiated virus per cell. Wt HSV-1 or *in*1814 was titrated on the UV-irradiated-*ts*K-pretreated cells. After 2 days at 37°C, plates were stained and plaques were counted.

Virulence assay. Female Charles River mice, each weighing approximately 15 g, were inoculated either intracranially (ic) with 20 μl or intraperitoneally (ip) with 200 μl of 10-fold dilutions of virus stocks, as described previously (6). Ten mice were inoculated for each virus dilution, and the number of survivors after 21 days was recorded. The mean 50% lethal dose values from two experiments were calculated.

RESULTS

Isolation of a mutant containing an insertion within the Vmw65 gene. The gene encoding Vmw65 lies between map

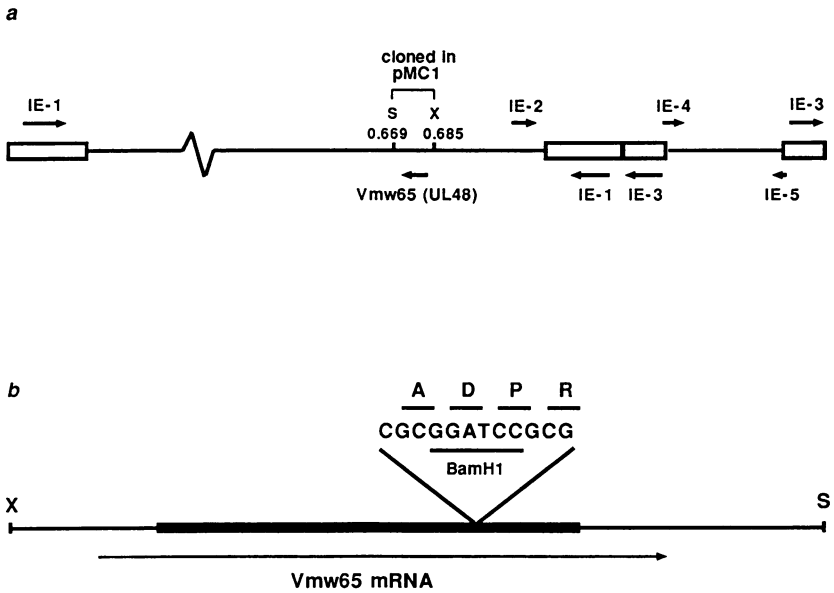

FIG. 1. (a) Structure of the HSV-1 genome showing the positions of the five IE genes (without introns) and Vmw65. The open boxes represent repeated sequences. (b) Structures of the insertion mutation in Vmw65 encoded on a *Sal*I(S)-*Xho*I(X) fragment in plasmid pMC1.in14.

coordinates 0.669 and 0.685 in the U_L region of the prototype HSV-1 genome and is contained within plasmid pMC1 (Fig. 1A; 7, 10). The isolation of a number of plasmids with in-frame *Bam*HI linker insertion mutations within the gene encoding Vmw65 has been described previously (1). In particular, a four-amino-acid insertion at codon 397, specified on plasmid pMC1.in14 (Fig. 1B), abolished the transinducing activity of the polypeptide in transfection assays. The mutation disabled the binding of Vmw65 to the host-cell factors and thus defined a region of the polypeptide involved in this interaction. The essential role of the polypeptide during virion assembly was not affected by the mutation, as inferred from its ability to rescue an HSV-2 mutant with a temperature-sensitive mutation in Vmw65 (1), suggesting that a viable virus could be constructed that contained the transinducing mutation specified by pMC1.in14. To construct such a mutant virus, a plasmid consisting of *Bam*HI *f* containing the pMC1.in14 mutation was cotransfected with intact wt HSV-1 DNA into BHK cells, and the structure of progeny virus DNA was examined by restriction enzyme analysis. One plaque from a total of 84 screened was identified as a recombinant that contained the *Bam*HI linker insertion. This mutant isolate, *in*1814, was plaque purified twice more, and a large-scale stock of virus was prepared. To rule out the possibility of a second site mutation in *in*1814 that might affect the phenotype of the mutant virus, a rescued virus was constructed by recombining *in*1814 DNA with pMC1. If, as desired, the phenotype of *in*1814 depended on the insertion mutation, then a rescued virus should behave as wt HSV-1. Initial observation of the properties of *in*1814 suggested that it grew poorly in comparison with wt HSV-1; thus, it was expected that rescued recombinants would outgrow *in*1814 during successive passages of a mixed population. This turned out to be the case, since after a single passage of the progeny virus from the initial cotransfection of *in*1814 DNA and pMC1, 75% of the plaques screened had the wt DNA structure. These viruses were unlikely to result from spontaneous reversion of *in*1814, since no reversion was detected at any stage during the passaging and propagation of mutant virus. A stock of rescued virus, 1814R, was prepared after plaque purification. Figure 2 shows a Southern blot of wt HSV-1, *in*1814, and 1814R DNA which was digested with *Bam*HI and probed with pMC17, a plasmid containing the Vmw65 coding sequences. The *Bam*HI *f* fragment of 8 kilobase pairs was seen in both wt HSV-1 (lane 1) and 1814R (lane 3), whereas in *in*1814 (lane 2), this fragment was replaced by two fragments of the sizes (5 and 3 kilobase pairs) expected from the presence of the *Bam*HI linker insertion. Overexposure of the autoradiograph revealed no detectable *Bam*HI *f* fragment in

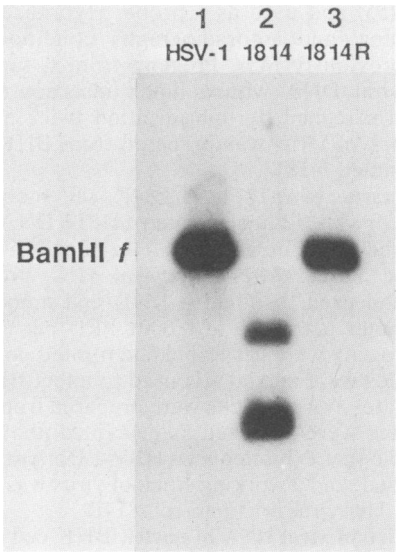

FIG. 2. Structure of the *in*1814 genome. Wt HSV-1 (lane 1), *in*1814 (lane 2), or 1814R (lane 3) DNA was cleaved with *Bam*HI, and the fragments were separated on a 1.5% agarose gel, transferred to nitrocellulose, and hybridized to ^{32}P-labeled pMC17. The position of HSV-1 *Bam*HI *f* is indicated.

TABLE 1. Titration of wt HSV-1, *in*1814, and 1814R on BHK and HFL cells

Virus	Particles/ml	BHK titer (PFU/ml)	HFL titer (PFU/ml)
wt HSV-1	1.9×10^{11}	5.0×10^{9}	1.7×10^{10}
*in*1814	1.2×10^{11}	1.3×10^{7}	7.0×10^{5}
1814R	4.6×10^{10}	4.0×10^{9}	ND[a]

[a] ND, Not determined.

*in*1814 and therefore that the stock of mutant virus was essentially pure.

The efficiency of plaque formation by *in*1814 is markedly reduced and dependent on cell type. The successful isolation and propagation of *in*1814 confirms that the insertion mutation is compatible with virus growth in BHK cells. When *in*1814 was titrated on BHK cells, however, a low titer was obtained, and therefore virus particle concentrations were determined (Table 1). It was found that the particle concentrations of wt HSV-1, *in*1814, and 1814R stocks were comparable but that the particle/PFU ratio was approximately 100 times greater for *in*1814 than for wt HSV-1 and 1814R. The apparent titer of a given preparation of *in*1814 on BHK cells varied by as much as 10-fold on different batches of cells, whereas the titers of wt HSV-1 and 1814R were much more consistent, suggesting that the cellular metabolic state affects the efficiency of plaque formation by *in*1814. When titrations were performed on HFL cells, an even higher particle/PFU ratio, 1.7×10^{5}, was observed for *in*1814. In view of the variation in titer of *in*1814 when expressed in terms of PFU, cell monolayers were infected with equal numbers of particles of wt HSV-1, *in*1814, or 1814R in subsequent experiments.

DNA migration to the nucleus. The early stages of infection by *in*1814 were examined, since it was possible that the insertion mutation affected virus adsorption, penetration, or uncoating. In initial experiments, the rate of adsorption of wt HSV-1 or *in*1814 preparations, radiolabeled by incubation with [^{3}H]thymidine during virus propagation, to BHK cell monolayers was investigated. The adsorption rates of wt HSV-1 and *in*1814 particles were indistinguishable (results not shown). The efficiency of DNA migration to the cell nucleus was also determined. BHK cell monolayers were infected in the presence of CH with 1,000, 100, or 10 particles of wt HSV-1, *in*1814, or 1814R per cell, nuclei were prepared at 3 h postinfection, and nuclear DNA was analyzed by Southern blot hybridization (Fig. 3). No significant differences were detected in the levels of HSV DNA, showing that the nuclear migration of *in*1814 DNA is not impaired at either high or low MOI.

This result underlines the requirement to use particles rather than PFU as a basis for the design of experiments with *in*1814; 1,000 particles of wt HSV-1 and *in*1814 represent 26 and 0.1 PFU, respectively.

***in*1814 does not exhibit virion-mediated transinduction of IE genes.** The ability of Vmw65, encoded by *in*1814, to form the protein-DNA complex IEC and to transinduce expression from transfected IE promoters was investigated, since both of these properties were disrupted in pMC1.in14 (1).

Extracts of wt HSV-1, *in*1814, and 1814R virions were prepared and analyzed by SDS-PAGE (Fig. 4). The levels of Vmw65 in these extracts were very similar, and the slightly increased molecular weight of the mutant polypeptide due to the four-amino-acid insertion was apparent (lane 2). The virion extracts were incubated with HeLa cell nuclear extract and a 74-bp DNA fragment containing the TAAT GAGAT sequence motif of IE gene 4/5. As shown in Fig. 5, the IEC was readily detected with extracts of wt HSV-1 (lane 2) and 1814R (lane 4) virions but not with extracts of *in*1814 (lane 3) or when no virion extract was present (lane 1). This result demonstrates that Vmw65 specified by *in*1814 is not capable of binding the cellular proteins required for IEC formation because of the mutation in the viral polypeptide.

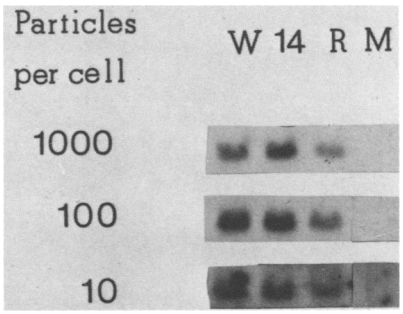

FIG. 3. DNA migration to the nucleus. DNA isolated from nuclei of cells infected with wt HSV-1 (W), *in*1814 (14), or 1814R (R) or mock infected (M), in the presence of CH, was cleaved with *Bam*HI, and the fragments were separated on an agarose gel, transferred to GeneScreen Plus membrane, and probed with radiolabeled pTK1. The portion of the blot representing *Bam*HI *p* is presented. Exposure times were 0.4 h (1,000 particles per cell), 4 h (100 particles per cell), or 40 h (10 particles per cell).

The ability of *in*1814 to transinduce IE gene expression was investigated by comparing the level of activation from a transfected IE promoter in the presence or absence of superinfecting virus (Fig. 6). BHK cells were transfected with pIE3CAT and infected with 1,000 particles of wt HSV-1, *in*1814, or 1814R per cell. An increase of approximately sixfold in chloramphenicol acetyltransferase activity was observed when cells were superinfected with wt HSV-1 (lane 2) or 1814R (lane 4), but infection with *in*1814 (lane 3)

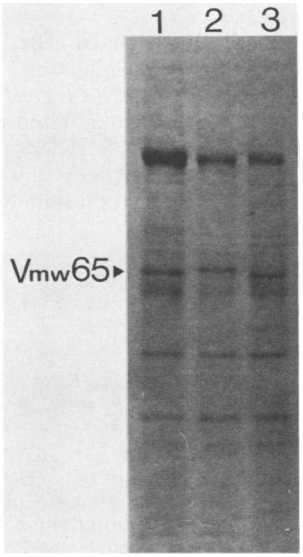

FIG. 4. Proteins extracted from virions of wt HSV-1 (lane 1), *in*1814 (lane 2), and 1814R (lane 3) and used as a source of Vmw65 for gel retardation analysis. The gel was stained with Coomassie brilliant blue.

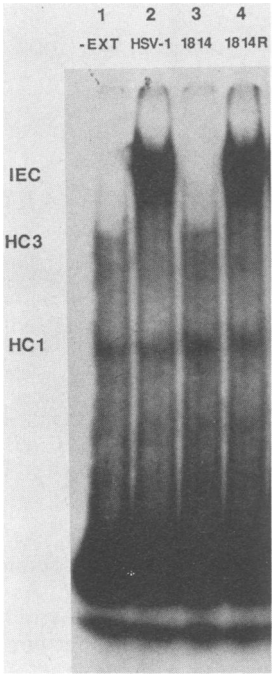

FIG. 5. IEC formation by Vmw65. A 74-bp DNA fragment containing the IE gene 4/5 TAATGAGAT sequence was incubated with HeLa cell nuclear extract alone (lane 1) or with virion extract (EXT) from wt HSV-1 (lane 2), *in*1814 (lane 3), or 1814R (lane 4), and was analyzed by gel electrophoresis. The positions of IEC and the cell-specific complexes HC1 and HC3 (49) are indicated.

gave no stimulation over the level in mock-infected cells (lane 1).

Taken together, these results confirm that the properties of the mutant Vmw65 polypeptide in the viral context reflect the observations and expectations implicit in the initial characterization of the mutation in cloned copies of the gene; that is, the mutation in *in*1814 disables the ability of the virus to direct the formation of IEC and consequently abolishes its transducing activity.

Gene expression in *in*1814-infected cells. It would be expected that the abolition of transinduction by Vmw65 would affect the expression of viral genes, especially IE genes. The accumulation of IE RNA was quantitated by hybridization by using IE gene-specific probes. BHK cells were infected in the presence of CH with 1,000 particles of wt HSV-1, *in*1814, or 1814R per cell for 4 h, and cytoplasmic RNA was applied

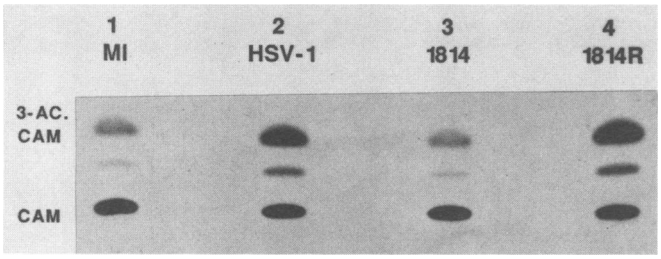

FIG. 6. Transinduction of IE transcription. Cells were transfected with pIE3CAT and mock infected (lane 1) or infected with wt HSV-1 (lane 2), *in*1814 (lane 3), or 1814R (lane 4). Chloramphenicol acetyltransferase assays were carried out on cytoplasmic cell extracts. The positions of chloramphenicol (CAM) and 3-acetyl chloramphenicol (3-AC. CAM) are shown.

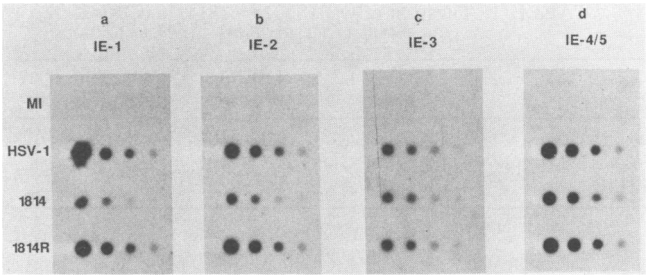

FIG. 7. Production of IE RNA. Cells were mock infected (MI; row 1) or infected with wt HSV-1 (row 2), *in*1814 (row 3), or 1814R (row 4) in the presence of CH. RNA was prepared after 4 h and applied to nitrocellulose filters in four sequential dilutions (3 μg, 1 μg, 0.3 μg, and 0.1 μg). Filters were separately hybridized with ^{32}P-labeled DNA probes corresponding to IE genes 1, 2, 3, and 4/5 (panels a, b, c, and d, respectively).

to nitrocellulose and separately hybridized with radiolabeled DNA fragments corresponding to the IE-1, IE-2, IE-3, or IE-4 genes. The levels of IE-1- and IE-2-specific RNA, as determined by densitometric analysis, were reduced four- to fivefold in *in*1814-infected cells compared with wt HSV-1- and 1814R-infected cells (Fig. 7, a and b), whereas the reduction in IE-4/5-specific RNA was only twofold (Fig. 7d), and no significant effect on IE-3-specific RNA was detected (Fig. 7c).

The expression of IE polypeptides was also investigated. BHK cells were infected as described above, but after 4 h CH was washed from the cells and polypeptides were radiolabeled in the presence of actinomycin D and separated by SDS-PAGE (Fig. 8). Densitometric analysis was used to

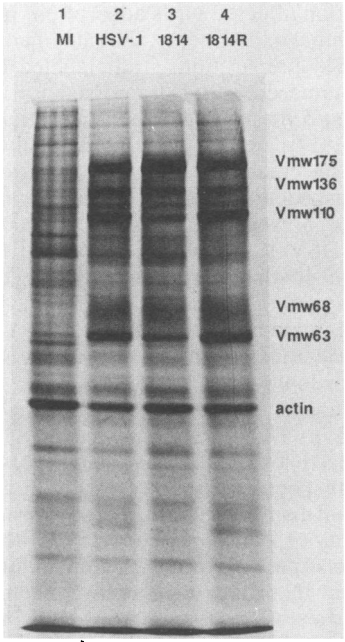

FIG. 8. IE polypeptide synthesis. Cells were mock infected (MI; lane 1) or infected with wt HSV-1 (lane 2), *in*1814 (lane 3), or 1814R (lane 4) in the presence of CH. Proteins were labeled with [^{35}S]methionine after removal of CH by washing at 4 h postinfection. The positions of viral IE polypeptides and cellular actin are indicated.

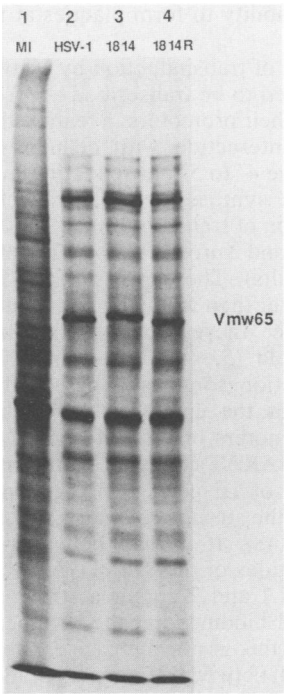

FIG. 9. Late polypeptide synthesis. Cells were mock infected (MI; lane 1) or infected with wt HSV-1 (lane 2), *in*1814 (lane 3), or 1814R (lane 4), and proteins were labeled with [^{35}S]methionine at 8 h postinfection. The position of Vmw65 is indicated.

determine the relative rates of synthesis of individual IE polypeptides, and the values were normalized to that of actin. The rates of synthesis of Vmw110 and Vmw63, the products of IE genes 1 and 2, respectively, were reduced four- to fivefold in *in*1814-infected cells, whereas the rates of synthesis of Vmw175, the product of IE gene 3, were equivalent for the three viruses. It was not possible to measure accurately the rate of synthesis of Vmw68, the product of IE gene 4, since this polypeptide ran as a diffuse band. It is clear, however, that the data on IE RNA levels and IE protein synthesis rates are in good agreement and show that the expression of IE genes 1 and 2 is significantly reduced in *in*1814-infected cells but that the expression of IE gene 3 is essentially unaffected.

Protein synthesis at 8 h postinfection, a time when early and, especially, late polypeptides are synthesized, was also examined (Fig. 9). The profiles of wt HSV-1, *in*1814-, and 1814R-infected cells were very similar, the increased molecular weight of Vmw65 specified by *in*1814 being the only major difference.

Thus, upon infection of BHK cells with 1,000 particles of *in*1814 per cell in the presence of CH, the level of expression of IE genes 1, 2, 4, and presumably 5 is reduced, but under normal conditions infection procedes to the late stage, suggesting that there is no overall consequence of reduced IE gene expression. From the high particle/PFU ratio, however, it appears that the growth of *in*1814 is inefficient when cells are infected with 1 virus particle per cell. To investigate whether the incapacity of *in*1814 at low MOI is reflected in reduced gene expression, the synthesis of TK, an early enzyme that can be detected with high sensitivity, was examined. BHK cells were infected with 1,000, 100, 10, or 1 particle of wt HSV-1, *in*1814, or 1814R per cell, and incubation was continued for 15 h in the presence of phosphonoacetic acid to prevent the secondary spread of virus. Table 2 shows the results of TK assays performed on the cell extracts. The level of TK after infection with 1,000 or 100 particles per cell was indistinguishable for wt HSV-1, *in*1814, and 1814R, reemphasizing that *in*1814 is not detectably impaired at high MOI. At 10 particles per cell, the TK level in *in*1814-infected cells relative to that in wt HSV-1-infected cells was reduced by 6-fold, and at 1 particle per cell the decrease was 33-fold. Therefore, the expression of TK (and presumably of other early and late genes) is more strictly dependent on MOI for *in*1814 than for wt HSV-1 or 1814R, and it is likely that the observed reduction in expression is large enough to account for the inefficiency of plaque formation by the mutant.

TABLE 2. TK production by wt HSV-1, *in*1814, and 1814R at high and low MOI[a]

MOI (particles/cell)	Viral TK activity (cpm/min of assay per μg of protein)			Ratio of wt HSV-1/*in*1814
	wt HSV-1	*in*1814	1814R	
1,000	2,693	2,285	2,789	1.2
100	1,988	2,549	2,478	0.8
10	935	161	792	6.0
1	100	3	63	33.3

[a] BHK cells were infected at the multiplicities indicated. Cytoplasmic extracts were diluted as necessary to ensure that TK determinations were within the linear response range of the assay. A background of 3 cpm per min of assay per μg of protein has been subtracted from all values.

Complementation of *in*1814 by Vmw110 and Vmw65. If *in*1814 fails to form plaques at low MOI because of the reduction in IE gene expression, then complementation of this state should increase the efficiency of plaque formation and consequently the apparent titer of the mutant virus. In contrast, a compensating increase in IE gene expression would not complement *in*1814 growth if the mutant phenotype resulted from a defect at a stage before the onset of IE transcription. Two experiments were carried out to test these possibilities.

BHK cells were transfected with p111 (a plasmid encoding the HSV-1 transactivator Vmw110) or pUC9 and then used separately for titration of wt HSV-1, *in*1814, or 1814R (Table 3). Although the titers of wt HSV-1 and 1814R were constant in both cell samples, the apparent titer of *in*1814 increased approximately 10-fold on cells transfected with p111. Since only a proportion of cells (normally between 5 and 50%) in a BHK monolayer express Vmw110 after transfection of p111, it is likely that a higher level of complementation could potentially be obtained. Therefore, raising the level of Vmw110 can, at least partially, rectify the defect of *in*1814 in BHK cells.

Complementation of *in*1814 in HFL cells was achieved by infecting monolayers with UV-irradiated *ts*K, which supplied functional Vmw65 in *trans* (48), prior to titration of wt HSV-1 or *in*1814. The apparent titer of *in*1814 increased from 5.7×10^6 to 1.5×10^9 PFU/ml, whereas the titer of wt HSV-1

TABLE 3. Titration of wt HSV-1, *in*1814, and 1814R on BHK cells transfected with pUC9 or p111 (encoding Vmw110)

Plasmid	PFU/ml		
	wt HSV-1	*in*1814	1814R
+pUC9	1.2×10^{10}	4.0×10^6	1.7×10^9
+p111	9.5×10^9	4.2×10^7	2.2×10^9

TABLE 4. Virulence of wt HSV-1, *in*1814, and 1814R in mice

Virus	50% Lethal dose (PFU)[a]	
	ip injection	ic injection
wt HSV-1	9.7×10^2 (4.0×10^4)	3.1 (1.3×10^2)
*in*1814	$>7.4 \times 10^4$ ($>2.4 \times 10^8$)	$>7.4 \times 10^3$ ($>2.4 \times 10^7$)
1814R	3.0×10^3 (9.2×10^4)	19.1 (5.8×10^2)

[a] 50% Lethal dose in terms of particles per mouse is shown in parentheses.

was 6.0×10^{10} on both cell monolayers. No plaques were observed on monolayers treated only with UV-irradiated *ts*K. The titer of *in*1814 on UV-irradiated-*ts*K-treated cells represented a particle/PFU ratio of 74, and after account is taken of the fact that the MOI for UV-irradiated *ts*K was only 0.1 PFU per cell, it is clear that the efficiency of plaque formation was similar to that of wt HSV-1. Thus, the observed phenotype of *in*1814 on HFL cells was reversed by provision of Vmw65 in *trans*, arguing against a *cis*-acting defect, for example, inhibition of uncoating by the mutant protein.

***in*1814 has reduced virulence in mice.** An assessment of the in vivo properties of *in*1814 was made by studying virulence after inoculation of mice either ic or ip. The results (Table 4) show that *in*1814 was much less virulent than wt HSV-1 or 1814R, regardless of the method of inoculation. In fact, all mice challenged with *in*1814 survived, with the exception of three mice injected ic with undiluted virus. In these cases, death was atypically rapid, occurring within 12 h as opposed to the usual 3 to 5 days, and it is suspected that the effect was due to the large number of virus particles injected. The 50% lethal dose values in terms of particles per mouse, the more relevant value, show that virulence of *in*1814 was reduced by a factor of at least 3×10^3 for ip or 2.5×10^4 for ic inoculation, compared with wt HSV-1 or 1814R.

DISCUSSION

The isolation of a mutant defective in transinduction of IE transcription is a crucial step in determining the biological role of Vmw65. The 12-bp insertion mutation in *in*1814 appears to be stable, since no revertants have been detected during passage and growth of virus stocks; reversion to the phenotype of wt HSV-1 would readily be detected, as shown by the ease with which 1814R was isolated. Two features of *in*1814 are particularly noteworthy. At MOI of 100 or more particles per cell, no significant effect was observed on the overall pattern of virus gene expression, whereas at low MOI, the efficiency of plaque formation was severely reduced in a cell-dependent manner. The phenotype is similar to that exhibited by deletion mutants which do not express Vmw110 (57, 65).

Although the results presented here suggest that transinduction by Vmw65 is not essential for HSV gene expression at high MOI, this interpretation must be taken cautiously, as the assays available are of limited sensitivity. The degree of impairment of transinduction is difficult to assess because the stimulation of expression from a transfected IE promoter is only 5- to 10-fold, and thus, as argued previously (1), it is possible to state only that *in*1814 is reduced by at least 90% in its ability to stimulate IE transcription. Analysis of the ability to form IEC, as shown in Fig. 5, is more sensitive, and by this criterion *in*1814 is disabled by 99% or more. Nevertheless, each HSV particle contains approximately 1,000 molecules of Vmw65 (22), and therefore a cumulative effect of a low residual activity might be sufficient to endow *in*1814 with the ability to form plaques at the observed low efficiency.

In the absence of transinduction by Vmw65, the IE genes would be expected to be transcribed according to the inherent strengths of their promoters, a feature that is presumably determined by interaction with cellular proteins. For IE genes 1 and 2, the 4- to 5-fold reduction in RNA accumulation and protein synthesis correlates well with the 5- to 10-fold stimulation of transcription in BHK cells from transfected IE gene 1 and 2 promoters by Vmw65 (C. M. Preston, unpublished results). The expression of IE genes 3 and 4, however, is greater than would be anticipated from transfection studies, since these promoters are also activated by more than fivefold (5, 48), and it is difficult to offer an obvious explanation for this apparent discrepancy. One possibility is that the enhancer-like sequence which lies between the promoters of IE genes 3 and 4 (28, 50), rather than the TAATGARAT elements, is the major requirement for transcription of IE gene 3 in the context of the viral genome and that the strong proximal promoter suffices for IE genes 4 and 5 (48). It is also noteworthy that the four upstream nucleotides of the TAATGARAT elements which control IE genes 1 and 2 confer a strong homology to the nuclear factor III binding site, the octamer element ATGC AAAT, whereas this is not the case for the TAATGARAT elements located between IE genes 3 and 4/5. A further consideration is that the topology of the DNA template and the stoichiometric relationships between DNA and protein factors may vary considerably between transfected and infected cells. Clearly, the findings with virus-infected cells are the more relevant.

Even though *in*1814 lacks transinducing activity, the major polypeptides synthesized under IE conditions are the IE proteins. Activation by Vmw65 is therefore not a definitive characteristic of IE genes, and other features must distinguish them from early and late genes. It may be that the presence of strong promoters and enhancer-like sequences determines the relatively high efficiency of IE gene transcription in the absence of IE proteins, but equally, the TAATG ARAT or other IE-specific elements might be responsible. It is known that cellular proteins bind to various sequences in IE gene upstream regions (26, 27, 39, 49, 68), and these factors might increase the availability of IE promoters to transcription components in the absence of Vmw65. Thus, IE-specific DNA sequences, rather than Vmw65, may be the primary determinants of an IE gene.

Transinduction by Vmw65 is important for infection only at low MOI. At a superficial level, it is straightforward to view this property as a reasonable adaptation, since the initial interaction of HSV with an organism is likely to involve a small number of virus particles. The inability to replicate at low MOI appears to result from the failure to produce IE proteins at levels sufficient to initiate infection, and it is probable that the reductions in Vmw110 and Vmw63 are crucial, since these polypeptides are required for gene expression (56, 57, 65). Thus, it seems that threshold levels of IE polypeptides must be attained, and the role of Vmw65 is to ensure that such levels are reached, especially at low MOI. It is not clear whether the few cells in which infection with *in*1814 results in the formation of a plaque represent a subpopulation in a particular metabolic state or simply random variation in response to infection. Furthermore, the basis for the difference in behavior of *in*1814 in BHK and HFL cells remains undefined. It may be that IE transcription in the absence of Vmw65 is less efficient in HFL cells at low MOI or that HFL cells are less able to compensate for low

levels of IE proteins. The fact that deletion mutants in Vmw110 are also impaired for growth at low MOI and show a relatively greater reduction in HFL cells than BHK cells (65; R. D. Everett, J. Gen. Virol., in press) supports the latter proposal, but further work is needed to clarify this important point.

Recently, Friedman et al. have shown that a transformed cell line which expresses the protein-binding portion of Vmw65 supports virus growth poorly, presumably because the expressed protein sequesters the cell factors required to mediate transinduction (16). In essence, transinduction by Vmw65 is thought to be abrogated in the transformed cell line. The experiments dealt only with infection at low MOI (0.1 or 0.3 PFU per cell), but the results are similar to those found with *in*1814, namely, a significant reduction in the efficiency of plaque formation, inefficient virus growth, and a decrease (by 12-fold) in accumulation of IE RNA 1. From the results reported here, it is predicted that virus replication in the transformed cells should not be as severely affected at high MOI.

The avirulence of *in*1814 in mice, even after injection of high doses, demonstrates that transinduction is important for infection in vivo and emphasizes the importance of host-cell factors in the replication of *in*1814. Furthermore, Vmw65 may be a good target for the design of new antiviral agents.

It is interesting to speculate on the role of Vmw65 in HSV latency in the light of the phenotype of *in*1814, since the majority of genes, including IE genes, are silent during latency (62, 63), suggesting that an early transcriptional block may operate. One hypothesis is that Vmw65 may be lost or rendered inactive during transport of the HSV nucleocapsid from the neuronal cell surface to the nucleus (27). From the analysis of *in*1814 presented here it is possible to predict that under such circumstances, at low MOI, virus replication would not ensue, and thus latency might be established. Support for this view comes from our recent observation that noninfectious particles of *in*1814 can be retained by tissue culture cells after infection at low MOI and can subsequently be reactivated to form plaques (C. Ace and C. M. Preston, unpublished results), as found in studies with a mutant lacking Vmw110 (N. D. Stow and E. C. Stow, J. Gen. Virol., in press). Therefore, the failure to transinduce IE transcription by interference with Vmw65 function is worthy of serious consideration as a basic precondition for latency.

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