JOURNAL OF VIROLOGY, Mar. 1990, p. 984–991
0022-538X/90/030984-08$02.00/0

Vol. 64, No. 3

Enhanced Infectivity of Herpes Simplex Virus Type 1 Viral DNA in a Cell Line Expressing the *trans*-Inducing Factor Vmw65

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Received 3 August 1989/Accepted 6 November 1989

Vmw65 is a structural component of herpes simplex virus (HSV) which is involved in transactivating the expression of the viral immediate-early (IE) genes. To gain further insight into the function of this protein, a cell line, BSV65, was established which expresses biologically active Vmw65 under control of the Moloney leukemia virus long terminal repeat. This cell line was shown to specifically activate IE genes as demonstrated by transient transfection assays with reporter genes linked to HSV IE or delayed-early promoter-regulatory regions. Furthermore, by using mobility shift assays, cell extracts were shown to be capable of forming a Vmw65-containing complex with oligonucleotides that contained a TAATGARAT motif, a conserved *cis*-acting IE regulatory element which is required for Vmw65-mediated *trans* induction. BSV65 cells were able to complement HSV type 1 *in*1814, a mutant which is unable to *trans*-induce IE gene expression and whose growth is impaired at low multiplicities of infection. Transfection of purified HSV type 1 viral DNA into BSV65 cells resulted in an approximately 200-fold increase in virus production compared with the parental cell line. In addition, in comparison to wild-type cells, infectious virus production occurred sooner and efficiency of plaque formation was higher in BSV65 cells following transfection of viral DNA but not following infection with virus. Northern (RNA) dot blot analysis of cells transfected with viral DNA showed that transcription of the IE gene Vmw175 was approximately 10-fold greater in BSV65 cells compared with wild-type cells. These results indicate that, in the presence of functional Vmw65, there is a greater probability that transfected viral DNA will lead to a productive infection.

The genes of herpes simplex virus (HSV) fall into three categories depending on the order in which they are expressed during the course of an infection. Members of each class are coordinately regulated in a positive and negative manner through the actions of both viral and cellular factors (7, 21, 22, 51). The immediate-early (IE) genes are the first to be expressed following infection, and their products are required for the activation of both early and late genes (11, 12, 37, 38, 40, 41). Transcription of the IE genes does not require de novo protein synthesis; however, their expression is strongly stimulated in *trans* by Vmw65 (also called VP16 or αTIF), a virally encoded structural polypeptide present in the virus (3, 5, 9, 39). *trans* induction mediated by Vmw65 involves recognition of conserved *cis*-acting sequence elements of the type TAATGARAT (R = purine) which are present in the upstream regulatory regions of all IE genes (4, 8, 15, 27, 30, 42). Whereas Vmw65 has no intrinsic DNA-binding properties (31), recent studies have demonstrated that Vmw65 is able to interact with TAATGARAT elements as part of a multicomponent complex which includes essential host factors, one of which has been identified as the ubiquitous octamer-binding protein oct-1 (16, 28, 32, 35, 36, 43, 50). Transactivation by Vmw65 is not restricted to HSV type 1 (HSV-1) viral IE genes, and recent evidence has demonstrated that certain cellular genes can also be activated by this protein (23, 34). Mutational studies of Vmw65 have shown that it contains two separable and modular domains: a highly acidic COOH-terminal region which is required for transactivation and a region closer to the amino terminus which is involved in protein-protein interactions (1, 19, 44, 49, 53).

That purified HSV-1 viral DNA, devoid of Vmw65, is infectious when introduced into cells by transfection suggests that transactivation of IE promoters by Vmw65 is not absolutely essential for initiation of productive infection by viral DNA. It is possible, however, that in the presence of Vmw65, virus production following transfection would be more efficient because of enhanced levels of IE gene expression. To address this question and to understand the role of Vmw65 in the activation of viral as well as cellular genes further, we have established a cell line which constitutively expresses biologically active Vmw65. Using this cell line, we demonstrate that the efficiency of virus production following transfection of HSV-1 viral DNA is markedly increased compared with wild-type cells.

MATERIALS AND METHODS

Cells and virus. BSC40 and Vero cells were maintained in Dulbecco modified essential medium plus 10% calf serum. HSV-1 strain 17 (Syn^+) was used throughout and was obtained from D. Johnson, McMaster University. Virus stocks were prepared and titrated by plaque assay on Vero cells. HSV-1 *in*1814 was obtained from Chris Preston, MRC Virology Unit, Glasgow, Scotland. A detergent extract from HSV-1 was prepared by treating HSV-1 virions, purified from infected Vero cells, with 0.03% Nonidet P-40 as described previously (31). The final concentration in the viral extract was 8 mg/ml; it contained 10 mM Tris hydrochloride (pH 7.5), 50 mM NaCl, 1 mM EDTA, and 0.03% Nonidet P-40.

Plasmids. p175*cat* contains the *Escherichia coli* chloramphenicol acetyltransferase (CAT) gene (*cat*) under control of the promoter-regulatory region from the HSV-1 IE gene Vmw175 (also called ICP4 or α4) (53). This construct is responsive to *trans* induction by Vmw65. p38K*cat*, obtained from G. Hayward, contains the *cat* gene under control of the promoter-regulatory domain of the gene for the small subunit of ribonucleotide reductase from HSV-2, a delayed-early

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gene which is nonresponsive to Vmw65 (37). pMC1, obtained from C. Preston, contains the entire gene of Vmw65 from HSV-1 strain 17 (9). pMC1-B contains a dodecameric *Bam*HI linker inserted 260 nucleotides downstream from the Vmw65 TAG termination codon and was obtained from a linker insertion library of pMC1 (53). pZip65 contains the Vmw65-coding region driven from the Moloney murine leukemia virus long terminal repeat. To construct pZip65, pMC1-B was digested at the unique *Eco*RV site 28 nucleotides upstream from the initiator ATG of Vmw65 and ligated to the *Bam*HI linker 5′-CCCGGATCCGGG. After complete digestion with *Bam*HI, the 1,761-nucleotide-long fragment containing the gene was isolated and cloned into the *Bam*HI site of pZipNeoSV(X)1 (6). pSV2*neo* contains the bacterial neomycin phosphotransferase (*neo*) gene under control of the simian virus early promoter (48), and pRSV*cat* contains the *cat* gene under control of the Rous sarcoma virus long terminal repeat (17).

Isolation of cell lines expressing Vmw65. BSC40 cells were seeded at a density of 3×10^6 cells per 10-cm dish. The following day, cells were cotransfected by the calcium phosphate method with 10 μg of pZip65, 0.5 μg of pSV2*neo*, and sonicated salmon sperm DNA up to 20 μg (18). Cells were shocked for 2 min with 10% dimethyl sulfoxide in phosphate-buffered saline 6 h later and incubated at 37°C. Cells were trypsinized 24 h later and split 1:4. The following day, cells were washed and incubated with media containing 600 μg of G418 per ml. Individual G418^r colonies were picked 14 days later, expanded, and tested for activity by transient transfection assays, using p175*cat* and p38K*cat*. One such cell line, designated BSV65, displayed significant and specific transactivation of p175*cat* and was chosen for further study.

DNA isolation. HSV-1 viral DNA was isolated from infected-cell nucleocapsids and purified as described previously (52). DNA was suspended in 10 mM Tris hydrochloride (pH 7.8)–100 mM NaCl–1 mM EDTA. Cellular genomic DNA for Southern blot analysis was isolated from monolayer cultures as described before (10).

Transfection and CAT assays. Plasmid transfections and CAT assays were performed as described previously (17, 53). For transfection of HSV-1 viral DNA, cells were seeded at a density of 5×10^5 cells per 60-mm dish 24 h prior to transfection. Cells were transfected with various amounts of purified viral DNA by the $CaPO_4$ method, using a dimethyl sulfoxide shock as described above. Total DNA was kept constant at 20 μg per dish with sonicated salmon sperm DNA.

Southern and Northern blot analysis. Southern blot analysis was carried out as described previously (47). The probe used was the 1.2-kilobase *Sal*I fragment contained entirely within the coding region of Vmw65 from pMC1 and was labeled by using random priming and [α-^{32}P]dATP (13). For Northern (RNA) analysis, cells were plated in 10-cm dishes at 80% confluency and incubated in the presence of 50 μg of cycloheximide per ml for 30 min prior to transfection with 10 μg of HSV-1 DNA. Following transfection, cultures were incubated in the presence of cycloheximide for 4 h and cellular RNA was isolated by the guanidinium hydrochloride method (10). Samples were spotted onto nitrocellulose, using a slot blot apparatus, hybridized with the probe, and processed as described before (54). The probe used for Northern analysis consisted of the 1.8-kilobase *Bam*HI fragment contained within the coding region of the HSV-1 Vmw175 gene which was isolated from plasmid pRHP6 and labeled with random primers (40). Duplicate blots were also probed with [γ-^{32}P]ATP 5′-end-labeled oligo(dT) (Pharmacia) to normalize for the amount of RNA spotted in each case (20).

Mobility shift analysis. Nuclear extract from BSC40 or BSV65 cells was prepared from monolayer cultures, and gel retardation experiments were carried out as described previously (29, 43, 46). Reaction mixtures contained, in a final volume of 20 μl, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9), 60 mM KCl, 2 mM EDTA, 4% (vol/vol) glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 2 μg of poly(dI-dC):poly(dI-dC), 4 μg of nuclear extract, and 0.2 ng of 3′-end-labeled probe. Oligonucleotide 5′ GATCCCGTGCATGCTAATGA TATTCTTT and its complement, 5′ CTAGAAAGAATATC ATTAGCATGCACGG, were synthesized on an Applied Biosystems DNA synthesizer, annealed, and labeled at the 3′ end with Klenow polymerase and [α-^{32}P]dATP. The labeled duplex was purified by gel electrophoresis prior to use in the binding reactions. Reactions were preincubated for 4 min at room temperature prior to addition of probe. After incubation at 30°C for 20 min, reaction mixtures were electrophoresed at 4°C on a 3.5% polyacrylamide gel (30:1, acrylamide–*N*,*N*′-bisacrylamide), using 45 mM Tris borate–1 mM EDTA as running buffer.

RESULTS

Establishment of permanent cell lines expressing Vmw65. Plasmid pZip65 contains the Vmw65 gene from HSV-1 strain 17 expressed from the Moloney murine leukemia virus long terminal repeat and was constructed as outlined in Fig. 1. This plasmid also contains the bacterial neomycin phosphotransferase (*neo*) gene which specifies resistance to the antibiotic G418. The biological activity of Vmw65 in this construct was assessed through transient cotransfection assays in BSC40 cells in which pZip65 was shown to stimulate expression of CAT activity approximately 40-fold over basal levels of p175*cat*, a reporter plasmid which contains the promoter-regulatory region of the HSV-1 Vmw175 gene (data not presented).

To generate cell lines expressing Vmw65, BSC40 cells were cotransfected with pZip65 and pSV2*neo*. The latter plasmid was included in the transfections because it was found that pZip65 on its own produced very few G418^r colonies, presumably because insertion of the Vmw65 gene interferes with expression of the downstream *neo* gene present in pZip65, as has been demonstrated in other studies that used pZipNeoSV(X)1 for expression of foreign genes (26).

Forty independent G418^r colonies were isolated and expanded into cell lines. Functional expression of Vmw65 in individual cell lines was assessed by measuring CAT activity following transfection with p175*cat*. Four cell lines were found that strongly stimulated CAT activity compared with levels obtained in a parallel transfection of wild-type BSC40 cells. One such cell line, herein designated BSV65, was chosen for further investigation.

CAT assays on this cell line are shown in Fig. 2. As can be seen, CAT activity is substantially elevated in BSV65 cells transfected with p175*cat* as compared with transfection of the parental cell line BSC40 (lanes a and b). To demonstrate that this transactivation was specific for IE promoters, cells were also transfected with p38K*cat*, a plasmid in which the *cat* gene is expressed from the promoter of the gene for the small subunit of the HSV ribonucleotide reductase gene and which is not responsive to Vmw65 *trans* induction. The

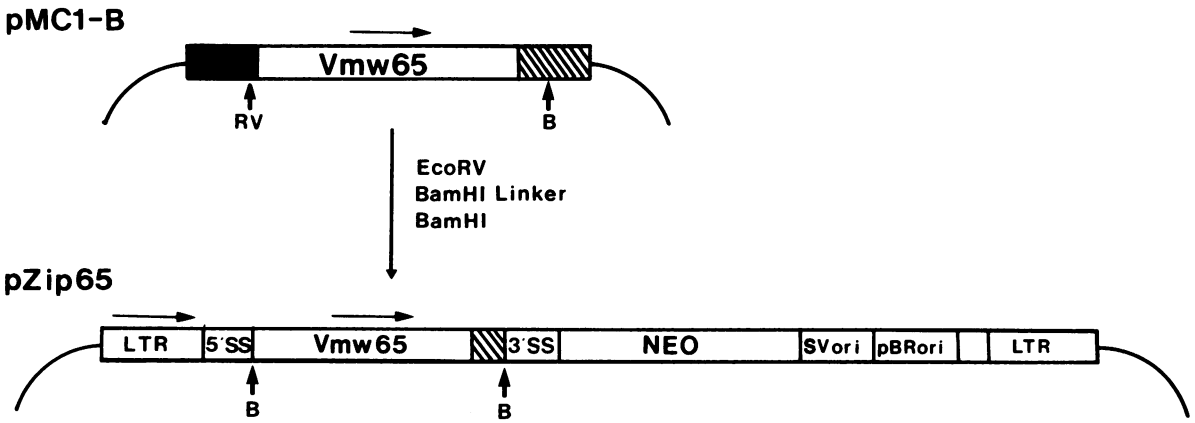

FIG. 1. Construction of pZip65. The coding region of Vmw65 from pMC1-B was cloned into the *Bam*HI site of the retroviral shuttle vector pZipNeoSV(X)1 (6) as described in Materials and Methods. The retroviral vector was used to leave open a future option of using infection to introduce the Vmw65 gene into cells. The solid and hatched areas in pMC1-B represent the 5′ and 3′ noncoding sequences, respectively. B, *Bam*HI; RV, *Eco*RV. LTR, Long terminal repeat.

levels of CAT activity obtained after transfection of BSV65 cells with this plasmid were indistinguishable from the levels obtained after transfection of wild-type cells (Fig. 2, lanes c and d).

Attempts at directly detecting Vmw65 protein in BSV65 cells, or in other cell lines capable of transactivating p175*cat*, by immunoprecipitation of [^{35}S]methionine-labeled cell extracts or by Western blotting (immunoblotting) with Vmw65-specific LP1 monoclonal antibody (31, 33) were unsuccessful. This suggests that BSV65 cells are making amounts of Vmw65 below the detection limit of this antibody or that the protein is highly unstable.

Presence of the Vmw65 gene in BSV65 cells. The results of Southern blot analysis to establish the presence of and determine the copy number of the Vmw65 gene in BSV65 cells are presented in Fig. 3. Genomic DNA from BSV65 cells and BSC40 cells was prepared, digested with *Bam*HI or *Cla*I, fractionated on an agarose gel, and blotted onto nitrocellulose. The blot was probed with a ^{32}P-labeled 1.2-kilobase *Sal*I fragment from pMC1, which is contained entirely within the Vmw65 gene. Included in the blot were different amounts of *Bam*HI-digested pZip65 corresponding to 1, 5, and 10 genomic equivalents and which served as copy number controls (lanes a to c). BSV65, but not BSC40, cells contain a band specifically hybridizing to the probe which corresponds to the *Bam*HI fragment from pZip65 (lanes d and e). Comparison to the copy number controls indicates that BSV65 cells contain approximately five copies of the intact Vmw65 gene. Digestion of genomic DNA with

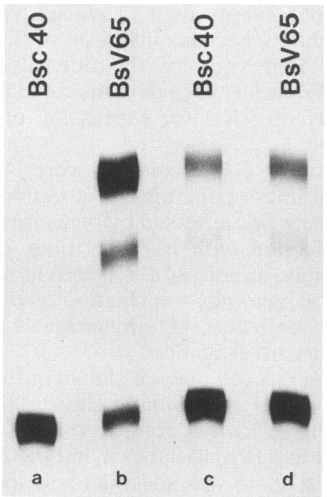

FIG. 2. Activation of the Vmw175 IE promoter in BSV65 cells. BSC40 or BSV65 cells, as indicated, were transfected with p175*cat* (lanes a and b) or p38K*cat* (lanes c and d). Cells were harvested 40 h after transfection, and CAT assays were performed.

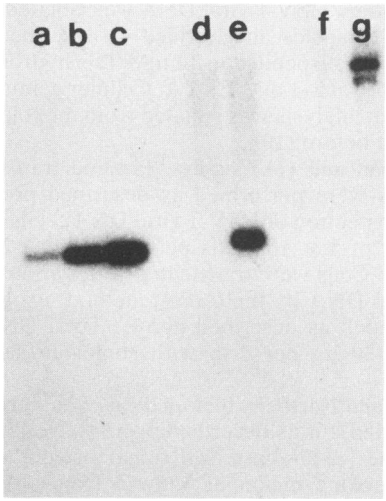

FIG. 3. Southern blot hybridization analysis of BSV65 cells. Total DNA was extracted from BSC40 (lanes d and f) or BSV65 (lanes e and g) cells and blotted onto nitrocellulose after digestion with *Bam*HI (lanes d and e) or *Cla*I (lanes f and g) and electrophoresis on a 1% agarose gel. The blot was hybridized with a gel-purified, Vmw65-specific *Sal*I restriction fragment from pMC1. Lanes a to c contain *Bam*HI-digested pZip65 corresponding to 1, 5, and 10 genome copy equivalents, respectively. Digestion of BSV65 DNA with *Bam*HI (lane e) generated a band with mobility corresponding to the *Bam*HI fragment of pZip65, which in comparison with the copy number controls is present at approximately five copies of the intact Vmw65 gene per cell. Digestion with *Cla*I (lane g), which cuts plasmid pZip65 once, produced a major band with a mobility of unit-length plasmid, indicating that most of the DNA is integrated in intact head-to-tail concatamers.

*Cla*I, which cuts only once in pZip65, demonstrates that the Vmw65 gene copies are mostly integrated in a head-to-tail array (lane g).

Protein-DNA complex formation in extracts of BSV65 cells. Several recent studies have demonstrated that the transactivating function of Vmw65 is correlated with its ability to interact with host factors and assembly into a protein complex which binds specifically to *cis*-acting TAATGARAT motifs present in the promoter-regulatory regions of IE genes (16, 32, 35, 43).

To determine whether BSV65 cells are capable of forming a Vmw65-containing complex on TAATGARAT elements, mobility shift experiments were undertaken. Complementary oligonucleotides, containing the promoter-proximal TAATGARAT element from the HSV-1 ICP0 gene, were synthesized and used in band shift assays. This oligonucleotide contains an overlapping octamer motif which is involved in sequence-specific interaction with the host transcription factor oct-1 (16, 36). Incubation of nuclear extract from wild-type BSC40 cells resulted in the formation of a major protein-DNA complex which is referred to as TRF (for TAATGARAT recognition factor) (Fig. 4, lane a), as shown previously by O'Hare and Goding (35). Others have referred to this complex as OTF, α-H1, or HC3, and it most likely represents the interaction of the host transcription factor oct-1 with the oligonucleotide (16, 32, 43). The mobility of this complex is identical whether HeLa cell or BSC40 nuclear cell extracts are used (unpublished observations). Addition of Vmw65, obtained from a detergent extract of purified HSV-1 virions, to the binding reactions results in the formation of a novel complex designated VIC (for Vmw65-induced complex), as previously shown by others (16) (lane b). That Vmw65 is a component of VIC is demonstrated in lane c, where LP1 antibody, directed against Vmw65, was included in the reaction and which caused a decrease in the mobility of VIC. Vmw65, in the absence of cellular extract, was unable to interact with the probe (lane d).

When extract from BSV65 cells was incubated with the probe, the major protein-DNA complex observed was TRF (lane e); however, when higher amounts of extract were used, a faint additional band which comigrated with VIC was also observed (lane f). Vmw65 was shown to be a component of this complex by the demonstration that LP1 antisera but not control antisera resulted in a decrease in the electrophoretic mobility of the complex (lanes g and h, respectively). VIC was not observed in extracts from wild-type BSC40 cells when an amount of extract equivalent to that for BSV65 was used (lane i). VIC formation in BSV65 cells was specific since it could be inhibited with unlabeled homologous but not heterologous oligonucleotides, and it could not form when labeled oligonucleotides containing mutations which were previously shown to prevent VIC, but not TRF, formation were used in the reactions (data not presented) (16).

The observation that BSV65 cells can form a specific Vmw65-containing complex on TAATGARAT elements is consistent with the ability of these cells to transactivate IE promoters specifically and directly demonstrates that these cells are synthesizing Vmw65.

Complementation of *in*1814 in BSV65 cells. Ace et al. recently isolated an HSV-1 mutant, *in*1814, which contains a defined linker insertion mutation in Vmw65 (2). This virus is defective in *trans* induction of IE gene transcription, presumably because the mutation in Vmw65 renders it unable to assemble into a protein-DNA complex with TAATGARAT elements. *in*1814 behaves like wild-type virus when cells are

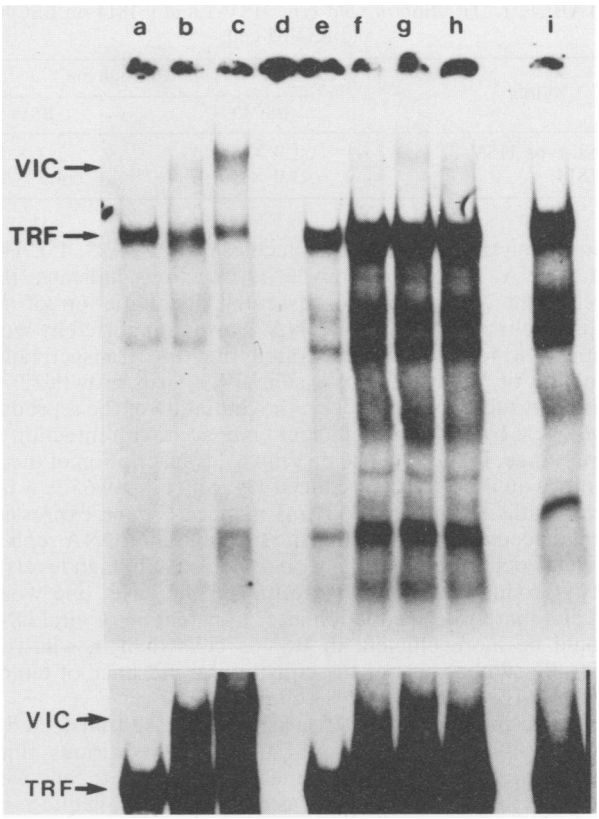

FIG. 4. Analysis of protein-DNA complex formation in extracts of BSV65 cells. Nuclear extracts from BSC40 (4 μg, lanes a to c; 8 μg, lane i) or BSV65 (4 μg, lane e; 8 μg, lanes f to h) cells were incubated with labeled oligonucleotide probe containing a TAATGA RAT element and analyzed by electrophoresis as described in Materials and Methods. Lanes b and c also contained 0.1 μg of a Nonidet P-40 detergent extract from purified HSV-1. Reactions in lanes c and g contained 0.1 μl of LP1 antibody, while the reaction in lane h contained normal mouse serum. TRF corresponds to the specific complex formed with cellular factors, while VIC (for Vmw65-induced complex) indicates a new complex seen only after addition of Vmw65. VIC was shifted in mobility upon inclusion of Vmw65-specific monoclonal antibody LP1 (lanes c and g), indicating that Vmw65 is part of this complex. Lane d is a control in which HSV-1 extract was incubated with probe in the absence of cellular extract, showing that Vmw65 on its own cannot interact with the probe. Extract from BSV65 cells shows a major TRF band and a faint band corresponding to VIC when higher amounts of extract were used (lanes e to h). Addition of LP1 but not normal mouse serum caused a shift in the mobility of VIC (lanes g and h, respectively). Increasing the amount of BSV40 extract in the reaction did not generate VIC (lane i). The figure at the bottom is a longer exposure of the relevant region of the above gel which more clearly demonstrates the formation of VIC in BSV65 cells.

infected at a high multiplicity of infection (MOI); however, virus production and plating efficiency are severely reduced when a low MOI is used. To ascertain whether BSV65 cells are capable of complementing this growth defect, *in*1814 was titrated on these cells and the efficiency of plaque formation was determined. Efficiency of plating of *in*1814 was 2 orders of magnitude greater in BSV65 cells compared with the parental cell line (Table 1). Thus, BSV65 cells are making sufficient functional Vmw65 to complement the *trans*-induction defect of *in*1814.

Enhanced infectivity of HSV-1 viral DNA in BSV65 cells. Highly purified deproteinized HSV-1 DNA has a specific

TABLE 1. Titration of wild-type HSV-1 and *in*1814 on BSC40 and BSV65 cells

Virus	Titer (PFU/ml) on:	
	BSC40	BSV65
Wild-type HSV-1	1.9×10^8	1.2×10^8
*in*1814	3.0×10^5	4.3×10^7

activity on the order of 10^6 molecules per PFU (25, 45). That viral DNA, devoid of Vmw65, is infectious indicates that preformed Vmw65 is not essential for initiation of the reproductive cycle by viral DNA. This is supported by work with *in*1814 which demonstrated that the transactivation function of Vmw65 is not essential for virus growth (2). It has been suggested, however, that initiation of the reproductive cycle by DNA is inefficient compared with infection by virus since, in the absence of Vmw65, transcription of the IE genes would be greatly reduced (3). Since Vmw65 is a late gene, sufficient protein to *trans* induce IE gene expression would accumulate only after the first round of DNA replication has occurred, and this would then sustain high levels of virus production. If this were indeed the case, one would predict that virus production after transfection of viral DNA would be more efficient in BSV65 cells than in wild-type BSC40 cells because of the constitutive presence of biologically active Vmw65 in this cell line.

To test this, HSV-1 DNA was purified and transfected in parallel into BSV65 and BSC40 cells. At various times posttransfection, the growth media and infected cells were collected and the virus yield was determined by plaque assay on Vero cells. Virus was detected from BSV65-transfected cells as early as 24 h posttransfection and continued to accumulate for 6 days posttransfection (Fig. 5). In contrast, virus was first detected from BSC40-transfected cells at 48 h posttransfection. Between 1 and 4 days posttransfection, virus production was 100 to 200 times greater in BSV65 than in BSC40 cells. Thus, virus production following transfection of viral DNA is markedly increased in BSV65 cells.

The possibility that the above results could be explained by a generalized increase in transfection efficiency of BSV65 cells compared with BSC40 cells was addressed by using pRSV*cat* to measure transfection efficiency. Numerous transfection experiments indicated that there was no significant difference in the ability of BSV65 cells to take up and express foreign DNA compared with the parental BSC40 cell line (data not presented). This is also demonstrated in Fig. 2, lanes c and d, which shows no difference in CAT activity when cells were transfected with p38K*cat*.

To examine the ability of the cell line to support plaque formation following transfection, BSV65 and BSC40 cells were transfected in duplicate with various amounts of viral DNA and plaque numbers were determined. The results are shown in Table 2. On average, the number of PFUs per microgram of viral DNA was approximately 1.5 to 2.5 times higher in BSV65 cells compared with wild-type cells. These results indicate that the ability of transfected viral DNA to support plaque formation is slightly higher in BSV65 cells. In addition, plaques were observable approximately 24 h sooner on BSV65 cells compared with BSC40 cells. While the efficiency of plaque formation cannot account completely for the 200-fold increase in virus yield following transfection, the finding that plaques appear sooner in BSV65 cells suggests that the first round of replication is more efficient in these cells.

We next investigated the infectivity of virus in BSV65

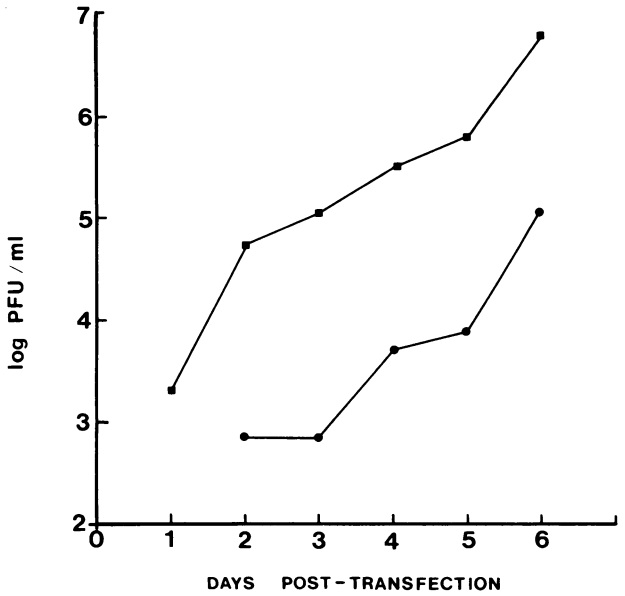

FIG. 5. Comparison of virus production after transfection of HSV-1 viral DNA in BSV65 and BSC40 cells. BSC40 (●) or BSV65 (■) cells were seeded at 10^6 cells per 60-mm culture dish in duplicate for each time point and transfected with 3 μg of HSV-1 viral DNA. After the dimethyl sulfoxide shock treatment, cells were washed with Dulbecco modified Eagle medium and incubated with 5 ml of growth medium. Media and cells from individual plates were collected at 24-h intervals and, after sonication, virus titers were determined by plaque assay on Vero cell monolayers. Shown are the average titers obtained from duplicate transfections.

cells by examining the efficiency of plating and by measuring virus production during the course of an infection. Interestingly, the efficiency of plating was approximately 50 to 60% lower in BSV65 cells as compared with wild-type cells; in addition, the plaques which formed on BSV65 cells appeared somewhat smaller. The virus growth curves shown in Fig. 6 support this finding. In this experiment, cells were infected with virus at an MOI of 0.1 and virus was collected and assayed at 12-h intervals. Virus production in BSV65 cells was approximately 10-fold lower compared with wild-type cells. The possibility exists that constitutive expression of Vmw65, even at the low levels generated in BSV65 cells, may impair virus production somewhat.

Enhanced transcription of viral IE genes in BSV65 cells. Since the products of the viral IE genes are required for subsequent events leading to DNA replication and virus production, the enhanced infectivity of viral DNA in BSV65 cells is likely due to increased levels of IE gene transcription, as a result of *trans* activation of these genes by endogenous Vmw65.

TABLE 2. Plaque formation following transfection of viral DNA

μg of HSV-1 DNA transfected	No. of plaques ± SD[a]	
	BSC40	BSV65
0.5	1.0	4 ± 0.7
1	4 ± 1.4	10 ± 3.5
2	23 ± 2.0	28 ± 0.7
2.5	63 ± 3.5	144 ± 32
5	400 ± 28.3	584 ± 31

[a] Values are the average of two separate determinations.

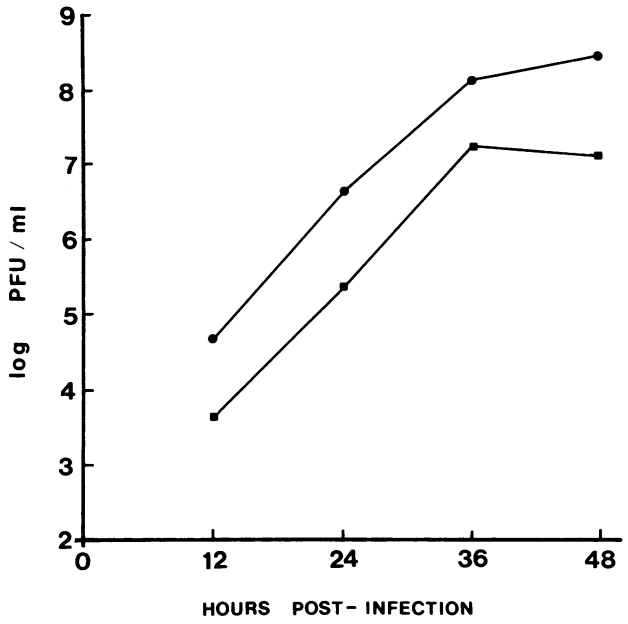

FIG. 6. Comparison of virus growth after infection of BSV65 and BSC40 cells. BSC40 (●) or BSV65 (■) cells were seeded in duplicate into 60-mm dishes as described in the legend to Fig. 5. Cells were infected at an MOI of 0.1 with HSV-1. After viral absorption, cells were washed and incubated with 5 ml of fresh medium. Total media and cells were harvested from individual plates at 12-h intervals and sonicated, and virus titers were determined by plaque assay. Values shown were averaged from duplicate infections.

To test this directly, cells were transfected with viral DNA in the presence of cycloheximide and the level of transcription of the IE gene coding for Vmw175 was measured by RNA dot blot analysis. Cycloheximide was included to block translation of IE gene products since some are able to autoregulate their own expression. At 4 h posttransfection, total RNA was isolated and examined by slot blot analysis, using a probe corresponding to part of the transcribed portion of the IE gene Vmw175. BSV65-transfected cells produce much more Vmw175-specific RNA than BSC40-transfected cells or mock-transfected controls (Fig. 7). The levels of expression were quantitated by densitometric scanning of the autoradiogram and normalized to duplicate filters probed with oligo(dT), which served to control for differences in amounts of RNA spotted. This analysis indicated that BSV65 cells accumulated approximately 10-fold more Vmw175-specific RNA than wild-type cells at 4 h posttransfection. Thus, the more efficient and earlier appearance of virus in BSV65 cells following transfection of HSV-1 DNA correlate with enhanced transcription of the IE gene for Vmw175.

DISCUSSION

The availability of stable cell lines constitutively expressing the HSV IE gene transactivator Vmw65 provides a useful tool for investigating some of the properties of this important transcription factor. Kmetz et al. recently reported on the isolation of a mouse cell line expressing Vmw65 from its own promoter, although this cell line also contained two other HSV-1 genes (24). This cell line was also able to specifically *trans* activate the Vmw175 promoter.

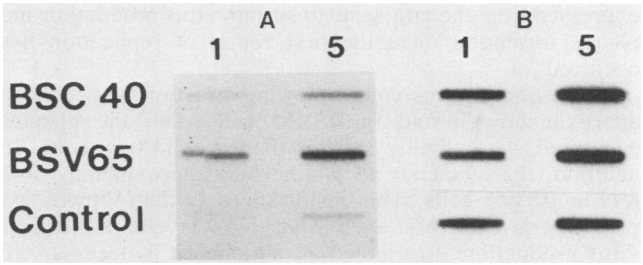

FIG. 7. Enhanced transcription of Vmw175 after transfection of viral DNA in BSV65 cells. BSC40 or BSV65 cells were incubated in the presence of 50 μg of cycloheximide per ml for 30 min prior to transfection with 10 μg of HSV-1 viral DNA. After transfection, cells were maintained in cycloheximide for 4 h, after which total RNA was isolated and spotted onto nitrocellulose (numbers above each lane correspond to micrograms of RNA). Blots were hybridized with a purified restriction fragment contained within the transcribed portion of the IE gene Vmw175. The control represents mock-transfected cells. A duplicate filter shown on the right was probed with labeled oligo(dT) to normalize for the amount of RNA spotted.

The presence of biologically active Vmw65 in BSV65 cells was confirmed by using transient assays which demonstrated strong and specific activation of the IE promoter from Vmw175, through mobility shift experiments which showed that extracts from these cells are capable of forming a Vmw65-containing protein complex which specifically binds to TAATGARAT containing oligonucleotides, and by complementation of *in*1814. Our inability to detect Vmw65 protein in this cell line by immunoprecipitation of metabolically labeled proteins or by Western blot analysis indicates that very little protein is made or that it is highly unstable. Given the degree of transactivation observed (40-fold with p175*cat*), the results suggest that very small amounts of Vmw65 are sufficient for substantial levels of activation of IE promoters, at least in transient assays. Since it is believed that Vmw65 functions through modulation of or interaction with preexisting cellular transcription-activation pathways, it may be difficult to establish permanent cell lines which constitutively express large amounts of this protein since this may compromise normal cellular processes. In support of this, transfection experiments have indicated that the transactivation function of Vmw65 is biphasic in that high levels of protein result in decreased activity, possibly because of sequestration of essential transcription factors (19, 23).

Virus production is substantially greater (2 orders of magnitude) and infectious virus is produced sooner after transfection of purified viral DNA in BSV65 cells compared with wild-type cells. This is consistent with the idea that the first round of replication of viral DNA following transfection is accelerated in BSV65 cells, presumably because of enhanced levels of IE gene transcription mediated by endogenous Vmw65. Plaque formation following transfection was found to be approximately 1.5- to 2.5-fold higher in BSV65 cells over a range of DNA concentrations compared with wild-type cells. Since the transfection efficiency in this cell line is similar to that in wild-type cells, it would appear that, on average, transfected viral molecules have a slightly higher probability of entering a lytic cycle in the presence of functional Vmw65. In wild-type cells, on the other hand, a threshold level of IE gene products must accumulate before efficient activation of early genes and DNA replication can occur. Since Vmw65 is a late gene, sufficient Vmw65 is

expressed from the viral gene to sustain virus production and plaque formation once the first round of replication has occurred.

Interestingly, virus yield following infection was reduced approximately 10-fold in BSV65 cells, and the plaques generated were slightly smaller than in wild-type cells. In addition, the efficiency of plating was approximately 50% less in BSV65 cells. This result suggests that Vmw65 expressed constitutively, even at very low levels, may impair virus production after infection, although it is necessary to test a number of independent cell lines to rule out the possibility of clonal variation. Vmw65 is a major viral structural protein which, in addition to its transactivating properties, has been postulated to play a critical role in virus assembly (1, 9). It is possible that the endogenous Vmw65 present in BSV65 cells may disrupt a critical stoichiometric balance needed for proper and efficient viral assembly. This seems unlikely, given the low amounts of Vmw65 produced in these cells. Alternatively, the continuous expression of Vmw65, which is expressed from a non-HSV promoter, may disrupt some important regulatory event. Recently, Freidman et al., using a cell line expressing a truncated variant of Vmw65 defective in transactivation, showed that virus production following infection with HSV was greatly reduced (14). It was suggested that this was the result of the defective Vmw65 interfering with transactivation of IE gene expression by wild-type Vmw65, thereby impeding progression of the lytic cycle.

Ace et al., using *in*1814, clearly demonstrated that Vmw65-mediated *trans* induction of IE gene transcription is not essential for viral growth at high MOIs; however, it is important for efficient viral growth when a low MOI is used (2). They determined that reduced virus production of *in*1814 at low MOI correlated with reduced levels of expression of some but not all of the IE genes. It is reasonable to speculate that a similar situation may occur in viral DNA-transfected cells when DNA uptake is relatively low and the expression of the IE genes may be at a level which is in most cases suboptimal to promote efficient progression of the first reproductive cycle. Presumably, higher levels of DNA uptake per cell, corresponding to a higher MOI, would overcome the need for Vmw65 since sufficient IE gene products would accumulate to allow efficient early gene expression and DNA replication to occur.

The availability of cell lines expressing functional Vmw65, in the absence of any other viral gene, should prove to be of value for further investigations concerning the role of Vmw65 in viral gene expression as well as the effects of Vmw65 on cellular gene expression.

ACKNOWLEDGMENTS

We thank G. Hayward and S. Bacchetti for plasmids p38K*cat* and pRHP6, respectively. We are especially grateful to Chris Preston for providing *in*1814.

This work was supported by grants from the National Cancer Institute (NCI) of Canada. G.W. is the recipient of a National Cancer Institute studentship, and J.P.C. is a Research Scholar of the National Cancer Institute.

LITERATURE CITED

1. **Ace, C. I., M. A. Dalrymple, F. H. Ramsay, V. G. Preston, and C. M. Preston.** 1988. Mutational analysis of the herpes simplex virus type 1 *trans*-inducing factor Vmw65. J. Gen. Virol. **69**:2595–2605.
2. **Ace, C. I., T. A. McKee, J. M. Ryan, J. M. Cameron, and C. M. Preston.** 1989. Construction and characterization of a herpes simplex virus type 1 mutant unable to transinduce immediate-early gene expression. J. Virol. **63**:2260–2269.
3. **Batterson, W., and B. Roizman.** 1983. Characterization of the herpes simplex virion-associated factor responsible for the induction of α genes. J. Virol. **46**:371–377.
4. **Bzik, D. J., and C. M. Preston.** 1986. Analysis of sequences which regulate the transcription of herpes simplex virus immediate-early gene 3: DNA sequences required for enhancer-like activity and response to transactivation by a virion polypeptide. Nucleic Acids Res. **14**:929–943.
5. **Campbell, M. E. M., J. W. Palfreyman, and C. W. Preston.** 1984. Identification of herpes simplex virus DNA sequences which encode a *trans*-acting polypeptide responsible for stimulation of immediate-early transcription. J. Mol. Biol. **180**:1–19.
6. **Cepko, C. L., B. E. Roberts, and R. C. Mulligan.** 1984. Construction of a highly transmissible murine retrovirus shuttle vector. Cell **37**:1053–1062.
7. **Clements, J. B., R. J. Watson, and N. M. Wilkie.** 1977. Temporal regulation of herpes simplex virus type 1 transcription: location of transcripts on the viral genome. Cell **12**:275–285.
8. **Cordingley, M. G., M. E. M. Campbell, and C. M. Preston.** 1983. Functional analysis of a herpes simplex virus type 1 promoter: identification of far-upstream regulatory sequences. Nucleic Acids Res. **11**:2347–2365.
9. **Dalrymple, M. A., D. J. McGeoch, A. J. Davison, and C. M. Preston.** 1985. DNA sequence of the herpes simplex virus type 1 gene whose product is responsible for transcriptional activation of immediate early promoters. Nucleic Acids Res. **13**: 7865–7879.
10. **Davis, L. G., M. D. Dibner, and J. F. Battey (ed.).** 1986. Basic methods in molecular biology. Elsevier Science Publishing Inc., New York.
11. **DeLuca, N. A., and P. A. Schaffer.** 1985. Activation of immediate-early, early, and late promoters by temperature-sensitive and wild-type forms of herpes simplex virus type 1 protein IPC4. Mol. Cell. Biol. **5**:1997–2008.
12. **Everett, R. D.** 1984. Transactivation of transcription by herpes virus products: requirement for two HSV-1 immediate-early polypeptides for maximum activity. EMBO J. **3**:3135–3141.
13. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
14. **Friedman, A. D., S. J. Triezenberg, and S. L. McKnight.** 1988. Expression of a truncated viral trans-activator selectively impedes lytic infection by its cognate virus. Nature (London) **335**:452–454.
15. **Gaffney, D. F., J. Mclauchlan, J. L. Whitton, and J. B. Clements.** 1985. A modular system for the assay of transcription regulatory signals: the sequence TAATGARAT is required for herpes simplex virus immediate early gene activation. Nucleic Acids Res. **13**:7874–7882.
16. **Gerster, T., and R. G. Roeder.** 1988. A herpesvirus transactivating protein interacts with transcription factor OTF-1 and other cellular proteins. Proc. Natl. Acad. Sci. USA **85**:6347–6351.
17. **Gorman, C. M., L. F. Moffatt, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
18. **Graham, F. L., and A. Van der Eb.** 1973. New technique for assay of infectivity of adenovirus 5 DNA. J. Virol. **59**:456–467.
19. **Greaves, R., and P. O'Hare.** 1989. Separation of requirements for protein-DNA complex assembly from those for functional activity in the herpes simplex virus regulatory protein Vmw65. J. Virol. **63**:1641–1650.
20. **Harley, C. B.** 1987. Hybridization of oligo(dT) to RNA on nitrocellulose. Gene Anal. Technol. **4**:17–22.
21. **Honess, R. W., and B. Roizman.** 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J. Virol. **14**:8–19.
22. **Honess, R. W., and B. Roizman.** 1975. Regulation of herpesvirus macromolecular synthesis: sequential transition of polypeptide synthesis requires functional viral polypeptides. Proc. Natl. Acad. Sci. USA **72**:1276–1280.
23. **Kemp, L. M., and D. S. Latchman.** 1988. Differential regulation

of octamer-containing cellular genes by the herpes simplex virus virion protein Vmw65 is mediated by sequence differences in the octamer element. EMBO J. 7:4239–4244.
24. **Kmetz, M., M. Ostrander, J. Schwartz, and K. G. Draper.** 1988. MTX5: a cell line expressing biologically active HSV-1 Vmw65 protein. Nucleic Acids Res. **16**:4735.
25. **Knipe, D. M., W. T. Ruyechan, and B. Roizman.** 1979. Molecular genetics of herpes simplex virus. III. Fine mapping of a genetic locus determining resistance to phosphonoacetate by two methods of marker transfer. J. Virol. **29**:698–704.
26. **Korman, A. J., J. D. Frantz, J. L. Strominger, and R. C. Mulligan.** 1987. Expression of human class II major histocompatibility complex antigens using retrovirus vectors. Proc. Natl. Acad. Sci. USA **84**:2150–2154.
27. **Kristie, T. M., and B. Roizman.** 1984. Separation of sequences defining basal expression from those conferring α gene recognition within the regulatory domains of herpes simplex virus 1 α genes. Proc. Natl. Acad. Sci. USA **81**:4065–4069.
28. **Kristie, T. M., and B. Roizman.** 1987. Host cell proteins bind to the cis-acting site required for virion-mediated induction of herpes simplex virus 1 α genes. Proc. Natl. Acad. Sci. USA **84**:71–75.
29. **Lee, K. A., A. Bindereif, and M. R. Green.** 1988. A small scale procedure for preparation of nuclear extracts that support efficient transcription and pre-mRNA splicing. Gene Anal. Technol. **5**:22–31.
30. **Mackem, S., and B. Roizman.** 1982. Structural features of the herpes simplex virus α gene 4, 0, and 27 promoter-regulatory sequences which confer α regulation on chimeric thymidine kinase genes. J. Virol. **44**:939–949.
31. **Marsden, H. S., M. E. M. Campbell, L. Haarr, M. C. Frame, D. S. Parris, M. Murphy, R. G. Hope, M. T. Muller, and C. M. Preston.** 1987. The 65,000 M_r DNA-binding and virion *trans*-inducing proteins of herpes simplex virus type 1. J. Virol. **61**:2428–2437.
32. **McKnight, J. L., T. M. Kristie, and B. Roizman.** 1987. Binding of the virion protein mediating α gene induction in herpes simplex virus 1-infected cells to its cis site requires cellular proteins. Proc. Natl. Acad. Sci. USA **84**:7061–7065.
33. **McLean, C., A. Buckmaster, D. Hancock, A. Buchan, A. Fuller, and T. Minson.** 1982. Monoclonal antibodies to three nonglycosylated antigens of herpes simplex virus type 2. J. Gen. Virol. **63**:297–305.
34. **Offord, E. A., R. E. Leake, and J. C. M. MacNab.** 1989. Stimulation of estrogen receptor mRNA levels in MCF-7 cells by herpes simplex virus infection. J. Virol. **63**:2388–2391.
35. **O'Hare, P., and C. R. Goding.** 1988. Herpes simplex virus regulatory elements and the immunoglobulin octamer domain bind a common factor and are both targets for virion transactivation. Cell **52**:435–445.
36. **O'Hare, P., C. R. Goding, and A. Haigh.** 1988. Direct combinatorial interaction between a herpes simplex virus regulatory protein and a cellular octamer-binding factor mediates specific induction of virus immediate-early gene expression. EMBO J. **7**:4231–4238.
37. **O'Hare, P., and G. S. Hayward.** 1985. Three *trans*-acting regulatory proteins of herpes simplex virus modulate immediate-early gene expression in a pathway involving positive and negative feedback regulation. J. Virol. **56**:723–733.
38. **O'Hare, P., and G. S. Hayward.** 1985. Evidence for a direct role for both the 175,000- and 110,000-molecular-weight immediate-early proteins of herpes simplex virus in the transactivation of delayed-early promoters. J. Virol. **53**:751–760.
39. **Pellet, P. E., J. L. C. McKnight, F. J. Jenkins, and B. Roizman.** 1985. Nucleotide sequence and predicted amino acid sequence of a protein encoded in a small herpes simplex virus DNA fragment capable of trans-inducing α genes. Proc. Natl. Acad. Sci. USA **82**:5870–5874.
40. **Persson, R. H., S. Bacchetti, and J. R. Smiley.** 1985. Cells that constitutively express the herpes simplex virus immediate-early protein ICP4 allow efficient activation of viral delayed-early genes in *trans*. J. Virol. **54**:414–421.
41. **Preston, C. M.** 1979. Control of herpes simplex virus type 1 mRNA synthesis in cells infected with wild-type virus or the temperature-sensitive mutant *ts*K. J. Virol. **29**:275–284.
42. **Preston, C. M., M. G. Cordingley, and N. D. Stow.** 1984. Analysis of DNA sequences which regulate the transcription of a herpes simplex virus immediate early gene. J. Virol. **50**: 708–716.
43. **Preston, C. M., M. C. Frame, and M. E. M. Campbell.** 1988. A complex formed between cell components and an HSV structural polypeptide binds to a viral immediate early gene regulatory DNA sequence. Cell **52**:425–434.
44. **Sadowski, I., J. Ma, S. Triezenberg, and M. Ptashne.** 1988. GAL4-VP16 is an unusually potent transcriptional activator. Nature (London) **335**:563–564.
45. **Sheldrick, P., M. Laithier, D. Lando, and M. L. Ryhiner.** 1973. Infectious DNA from herpes simplex virus: infectivity of double-stranded and single-stranded molecules. Proc. Natl. Acad. Sci. USA **70**:3621–3625.
46. **Singh, H., R. Sen, D. Baltimore, and P. A. Sharp.** 1986. A nuclear factor that binds to a conserved sequence motif in transcriptional control elements of immunoglobulin genes. Nature (London) **319**:154–158.
47. **Southern, K. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
48. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–341.
49. **Triezenberg, S. J., R. C. Kingsbury, and S. L. McKnight.** 1988. Functional dissection of VP16, the transactivator of herpes simplex virus immediate early gene expression. Genes Dev. **2**:718–729.
50. **Triezenberg, S. J., K. L. LaMarco, and S. L. McKnight.** 1988. Evidence of DNA:protein interactions that mediate HSV-1 immediate early gene activation by VP16. Genes Dev. **2**:730–742.
51. **Wagner, E. K.** 1985. Individual HSV transcripts: characterization of specific genes, p. 45–104. *In* B. Roizman (ed.), The herpesviruses, vol. 3. Plenum Publishing Corp., New York.
52. **Wagner, M. J., and W. C. Summers.** 1978. Structure of the joint region and the termini of the DNA of herpes simplex virus. J. Virol. **27**:374–378.
53. **Werstuck, G., and J. P. Capone.** 1989. Mutational analysis of the herpes simplex virus *trans*-inducing factor Vmw65. Gene **75**: 213–224.
54. **White, B. A., and F. C. Bancroft.** 1982. Cytoplasmic dot hybridization. Simple analysis of relative mRNA levels in multiple small cell or tissue samples. J. Biol. Chem. **257**: 8569–8572.