JOURNAL OF VIROLOGY, Oct. 1991, p. 5289–5296
0022-538X/91/105289-08$02.00/0

Vol. 65, No. 10

Characterization of a Potent Varicella-Zoster Virus-Encoded *trans*-Repressor

SUNIL NAGPAL† AND JEFFREY M. OSTROVE*

Medical Virology Section, Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892

Received 30 November 1990/Accepted 2 July 1991

Using a transient expression assay in Vero cells, we have shown that the protein product from gene 61 of varicella-zoster virus (VZV) can repress the function of the VZV encoded *trans*-activators on putative viral immediate-early, early, and late gene promoters. The repression is exerted at the transcriptional level and requires functional gene 61 protein. This *trans*-repressor is the herpes simplex type 1 ICP0 (a *trans*-activator) homolog, as defined by gene location, the sharing of a cysteine-rich putative zinc-binding finger in the amino-terminal region, and limited amino acid homology. Open reading frame 61 (ORF61)-mediated *trans*-repression appears to be specific for VZV-encoded *trans*-activators in that it has no effect on simian virus 40 and Rous sarcoma virus promoters. Moreover, it does not inhibit *trans*-activation of the human T-lymphotropic virus type I and human immunodeficiency virus long terminal repeats by *tax* and *tat* genes, respectively. We constructed plasmids with mutations in ORF61 and tested them for their ability to inhibit *trans*-activator (VZV genes 4 and 62)-mediated activation of the viral thymidine kinase promoter-chloramphenicol acetyltransferase construct. Mutants containing interruptions in ORF61 lost their *trans*-repressing ability, as demonstrated at both the protein and steady-state RNA levels. These results suggest that the ORF61 protein product can mediate down-regulation of VZV gene expression.

The control of gene transcription is brought about either by the direct interaction of *trans*-acting proteins with *cis*-acting promoter elements (20) or by the interaction of *trans*-acting proteins with cellular factors. The newly formed complexes, in turn, bind to the *cis*-acting DNA elements (13, 28, 31, 36). A number of viral *trans*-acting proteins have been shown to be important for the expression of different classes of viral genes. Simian virus 40 (SV40) large T antigen has both repressor and activator functions. It negatively regulates SV40 early gene transcription and positively regulates late gene expression (16, 21, 29, 38, 45). Adenovirus codes for a regulatory gene, E1a, whose products stimulate the adenovirus early promoters (1, 24, 39). Adenovirus E1a products also have repressor activity, since they repress the activity of SV40, polyomavirus, and adenovirus type 2 E1a enhancers (2, 46). Herpesviruses offer more complexity in regulation because they harbor a bigger genome, with herpes simplex virus type 1 (HSV-1) coding for at least three immediate-early regulatory proteins, ICP0, ICP4, and ICP27. Among these, ICP0 and ICP4 have been shown to *trans*-activate various viral promoters (5, 8, 9, 11, 27, 32, 33, 37, 42). ICP27 has been shown to be a *trans*-repressor or *trans*-activator in combination with HSV-1 *trans*-inducing genes ICP4 and ICP0 (41). Synergistic *trans*-activation of HSV early gene expression by ICP4 and ICP0 has been demonstrated (8, 9, 11, 27, 32, 37), suggesting a possible interaction between ICP4 and ICP0. Therefore, HSV immediate-early gene products may interact with each other in order to perform their functions. A similar type of interaction has been envisioned for the products of ICP27 and those of ICP4 and ICP0 (41), resulting in either transcriptional activation or repression, depending on the target promoter.

In our laboratory, we have mapped putative immediate-early *trans*-activating genes of varicella-zoster virus (VZV) to products of gene 4 and gene 62 (17). Open reading frame 4 (ORF4) and HSV-1 ICP27 share amino acid homology (4), but functionally it is not clear how they are related. ORF62 is the ICP4 homolog, as shown at the amino acid level as well as functionally (4, 10). Both of these VZV regulatory genes act at the level of transcription and synergistically activate the expression of putative immediate-early and late gene promoters (17).

We present here the identification of a VZV *trans*-repressor gene to ORF61, whose protein product can down-regulate the stimulation of viral putative immediate-early, early, and late promoters by the VZV *trans*-activator genes 4 and 62. Our results suggest that the ORF61 protein product interacts with ORF4 and ORF62, thus resulting in ORF61-mediated *trans*-repression. This *trans*-repression occurs at the level of RNA. Additionally, the ORF61 product represses, in *trans*, the activation of the human immunodeficiency virus (HIV) long terminal repeat (LTR) by ORF4 and ORF62. Deletion and insertion mutants of ORF61 no longer possess the *trans*-repressing ability. ORF61 alone had no effect on the expression of these target genes.

MATERIALS AND METHODS

Cells and viruses. Vero cells (American Type Culture Collection, Rockville, Md.) were maintained in minimal essential medium and medium 199 (1:1) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, penicillin G (100 U/ml), and streptomycin (100 μg/ml). Stocks of VZV Ellen strain were propagated, and titers were determined in Flow 5000 cells as previously described (44).

Transfections and CAT assays. Transfection of plasmid DNAs was carried out in 60-mm tissue culture dishes. Cells were plated the day before transfection at 60 to 70% confluency (approximately 5×10^5 cells per dish). Vero cells were

* Corresponding author.
† Present address: Laboratory of Molecular Genetics of Eukaryotes, Strasbourg, France.

transiently transfected by the calcium phosphate coprecipitation method of Graham and van der Eb (15). At 4 h posttransfection, cells were shocked with 15% glycerol in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered saline for 1 min, washed, and incubated in growth medium. Lysates from transfected cells were prepared 40 to 44 h after transfection by freeze-thawing the harvested cells three times in 100 μl of 0.25 M Tris HCl (pH 7.8) and centrifuging them for 10 min at 4°C. The extracts were incubated for 5 min at 65°C to inactivate an endogenous deacetylase activity. Chloramphenicol acetyltransferase (CAT) assays were performed by the procedure of Gorman et al. (16) with 50 μg of protein for each CAT assay. Protein concentrations were determined by using Bio-Rad protein estimation reagent. The percentage of chloramphenicol that was acetylated was quantitated by cutting out regions containing unacetylated [^{14}C]chloramphenicol (New England Nuclear) and acetylated forms from thin-layer chromatograms after autoradiography and counting them in a liquid scintillation counter.

Plasmid constructions and gene disruptions. The construction of recombinant plasmids pltk-CAT, p68-CAT, pGi26 (ORF62), p29, and pGORF4 has been described previously (17). pORF61 was constructed by *Hin*dIII digestion of the VZV *Eco*-A fragment (17) and insertion of a 4.5-kb fragment into the *Hin*dIII site of plasmid pGEM-3Z (Promega, Madison, Wis.). The resulting plasmid contained a single copy of the VZV ORF (ORF61) with 2,256 bp upstream of the AUG codon and 973 bp downstream of the protein stop codon (Fig. 1). According to the DNA sequence, ORF61 is 1.4 kb and codes for 467 amino acid residues (4).

The construction of pGORF4-CAT is shown in Fig. 1. A 1.1-kb *Pst*I-*Sst*I fragment from pORF4 (17) was subcloned into the *Pst*I-*Sst*I sites of plasmid pUC19 (Bethesda Research Laboratories) that had been previously digested with *Pst*I and *Sst*I. The latter plasmid contained CAT gene block (Pharmacia, Piscataway, N.J.) in the *Hin*dIII site. Thus, the resulting plasmid contains ORF4 promoter sequences from bp −895 to +192 relative to the AUG codon upstream of the CAT gene.

Deletion mutants of pORF61 were constructed in the following ways. pORF61-Δ1 was constructed by digesting pORF61 with *Xho*I and religating the resulting plasmid. Thus, it lacked bp −2063 to +493 with respect to the AUG codon. pORF61-Δ2 was constructed by *Nco*I digestion of pORF61 and religating the resulting plasmid. Thus, it had a deletion from bp +3 to +690 within the protein-coding frame. pORF61 insertion mutant pORF61-i3 was prepared by *Bgl*II digestion of ORF61, fill-in, and religation of the plasmid. The fill-in reaction inserted 4 bp, resulting in a frameshift in the protein-coding frame and insertion of a stop codon at amino acid 143 (Fig. 1). This mutant plasmid produced a truncated protein which shared the first 140 amino acids with the parent wild-type gene 61 protein. Plasmid p29-Δ61 was constructed by digestion of the parent plasmid p29 (17) with *Nco*I such that gene 61 within p29 carries an internal deletion of 229 amino acids, leaving ORF60 intact. Construction of plasmids LTR-CAT and pSV2tat has been described elsewhere (12); *tax* and human T-lymphotropic virus type I (HTLV-I)-LTR-CAT constructs were a generous gift from K.-T. Jeang (19, 30, 43). pSV2CAT and RSV-CAT were kindly provided by B. Howard (National Institutes of Health) and U. Siebenlist (National Institutes of Health), respectively.

RNA analysis. Preparation of total RNA from VZV-infected and uninfected MRC-5 cells and Northern (RNA) blot

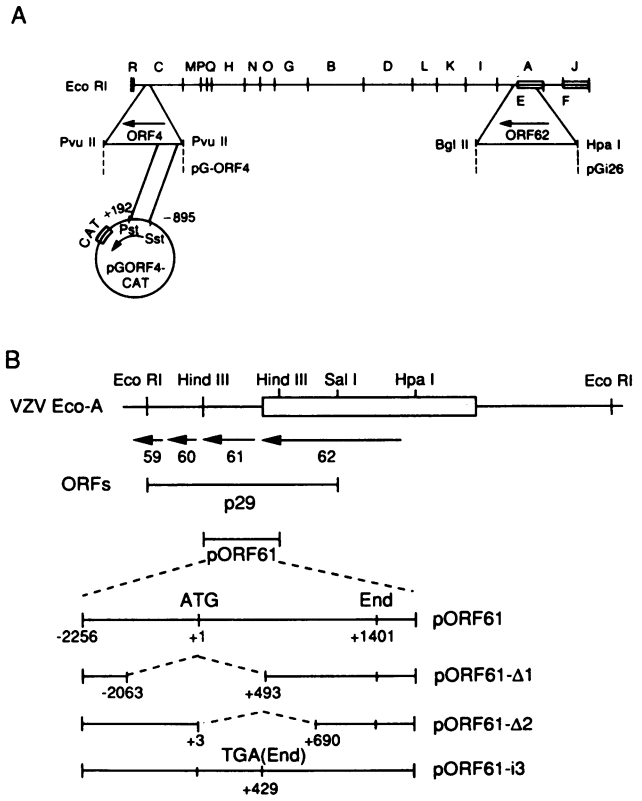

FIG. 1. (A) Construction of plasmid pGORF4-CAT. The diagram represents the *Eco*RI restriction endonuclease map of the VZV genome and positions of the two immediate-early *trans*-activating genes, ORF4 and ORF62. The relative position of the *Pst*I-*Sst*I fragment containing the promoter and regulatory sequences of ORF4 is also shown. Construction of the CAT plasmid is described in Materials and Methods. (B) ORF61 and its deletion and insertion mutants. The diagram represents the restriction map of the VZV *Eco*-A fragment. p29, pORF61, and various mutants of pORF61 are shown. ORFs present in p29 are indicated. Coordinate numbers in pORF61 and its mutants are presented with respect to the ATG codon. Deletions within pORF61 are indicated by broken lines. For details of the construction of pORF61 and its mutants, see Materials and Methods.

analysis have been described previously (35). RNA molecular weight markers (Bethesda Research Laboratories) used in each experiment were visualized by ethidium bromide staining. For slot blot, Vero cells were seeded in 150-mm dishes and transfected as described. Forty-eight hours posttransfection, total cellular RNA was extracted by the guanidinium isothiocyanate-cesium chloride method (3). Slot blots were performed as described earlier (17).

Primer extension analysis. Primer extension analysis of ORF61 RNA was conducted by using a 20-base synthetic primer (5'-TCCATGGTAACAACTGGCTG-3') complementary to nucleotides +5 to −15 relative to the AUG of ORF61. One-half nanogram of labeled primer was hybridized to 30 μg of total cellular RNA from either uninfected or VZV-infected MRC-5 cells. Hybridization and subsequent reverse transcription were carried out as described previously (40), and reaction products were resolved by electrophoresis in 8% polyacrylamide gels containing 8 M urea.

In vitro transcription and translation of ORF61. Plasmid DNAs (10 μg each) carrying ORF61 and ORF61-i3 (purified

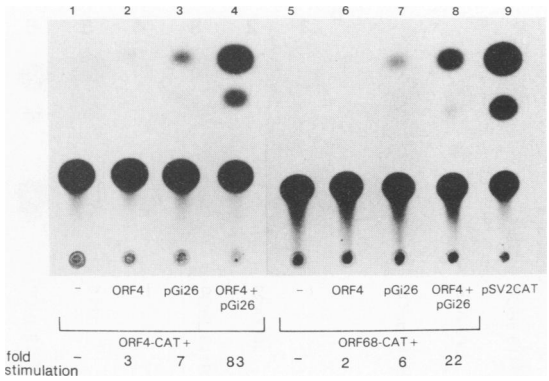

FIG. 2. *trans*-activation of VZV putative immediate-early and late gene promoter-CAT constructs by pGORF4 and pORF62 (pGi26). Vero cells were transfected with pORF4-CAT (5 μg; lanes 1 to 4) or pORF68-CAT (5 μg; lanes 5 to 8) along with either pGORF4 (5 μg; lanes 2 and 6) or pORF62 (5 μg; lanes 3 and 7) or a combination of pGORF4 and pORF62 (5 μg each; lanes 4 and 8). Lane 9 represents a positive control in which Vero cells were transfected with pSV2CAT DNA (2 μg) alone. Fold stimulation produced by ORF4 (pGORF4) and ORF62 (pGi26) plasmids is shown at the bottom.

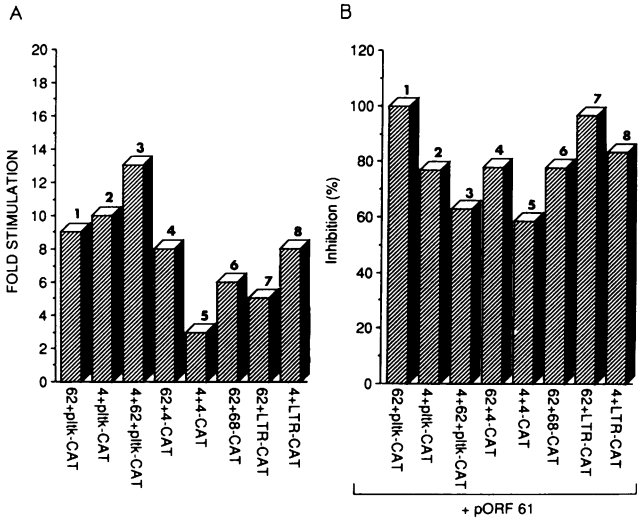

FIG. 3. Effects of ORF61 on transactivation of VZV and HIV promoters. The data shown are from a number of transfection experiments. Vero cells were transfected with pltk-CAT (1 μg; columns 1 to 3), pORF4-CAT (5 μg; columns 4 and 5), pORF68-CAT (5 μg; column 6), or HIV-LTR-CAT (2 μg; columns 7 and 8) along with pGORF4 (5 μg; columns 2, 5, and 8), pORF62 (5 μg; columns 1, 6, and 7), or a combination of pGORF4 and pORF62 (5 μg each; column 3). All transfections were performed with or without pORF61 (5 μg). Plasmids used for the experiments are indicated at the bottom.

by equilibrium centrifugation in cesium chloride) were digested with *Stu*I (923 bp downstream of the 3′ stop codon), treated with proteinase K (1 mg/ml solution), phenol-chloroform extracted, and ethanol precipitated. To synthesize 5′ capped RNA, the linearized DNA template was transcribed in vitro by using an mRNA capping kit (Stratagene Cloning Systems, La Jolla, Calif.). RNA was purified by phenol-chloroform extraction and ethanol precipitation and resuspended in 10 μl of RNase-free TE buffer (10 mM Tris HCl [pH 7.5], 1 mM EDTA). One-fourth of the in vitro-transcribed RNA was translated in vitro, using a rabbit reticulocyte lysate with 50 μCi of [^{35}S]methionine. Labeled translated proteins were analyzed by 4 to 20% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (22). A prestained protein mixture (Sigma) was used for molecular weight standards. After electrophoresis, the gel was fixed with 10% acetic acid–30% methanol, treated with Amplify (a fluorographic reagent; Amersham), dried, and autoradiographed for 4 h.

RESULTS

***trans*-activation of VZV putative immediate-early, early, and late promoters by ORF4 and ORF62.** Two previously mapped VZV *trans*-activating genes, 4 and 62 (17), were tested for their ability to stimulate the expression of different classes of putative VZV promoters: immediate-early (pORF4-CAT), early (pltk-CAT), and late (pORF68-CAT) genes. Vero cells were cotransfected with pORF4-CAT (IE4-CAT), pltk-CAT (thymidine kinase [*tk*]-CAT), or pORF68-CAT (glycoprotein I [gpI]-CAT) along with gene 4 and/or gene 62. The level of CAT activity observed 40 h posttransfection is shown in Fig. 2. Both *trans*-activators were able to induce the expression of all three types of promoter-CAT constructs. ORF4 and ORF62 stimulated the expression of pORF4-CAT 3- and 7-fold, respectively (Fig. 2, lanes 2 and 3), that of pORF68-CAT 2- and 6-fold, respectively (Fig. 2, lanes 6 and 7), and that of pltk-CAT 17- and 10-fold (17; data not shown), respectively. A synergistic transactivation of pORF4-CAT and pORF68-CAT was observed when pGORF4 and pGi26 (gene 62) were used together in cotransfection assays. Cotransfection of pGORF4 and pGi26 (ORF62) along with either pORF4-CAT or pORF68-CAT resulted in 83- and 22-fold increases in the CAT activity, respectively (Fig. 2, lanes 4 and 8).

Gene 61 inhibits the *trans*-activation of all classes of VZV promoters by ORF4 and ORF62. We have previously reported that clone p29 contains a *trans*-repressing activity because of its ability to down-regulate the activation of pltk-CAT by ORF4 and ORF62 (17). Nucleotide sequence analysis of the virus indicates that plasmid p29 contains two ORFs, ORF60 and ORF61. To delineate the *trans*-repressor activity, we subcloned gene 61 as a single copy on a plasmid and used that plasmid in transient cotransfection assays to test its ability to down-regulate ORF4- and ORF62-mediated *trans*-activation of VZV putative immediate-early, early, and late promoter constructs. pORF62, pGORF4, and a combination of pGORF4 and pORF62 stimulated the expression of pltk-CAT by 9-, 10-, and 13-fold, respectively (Fig. 3A). Similarly, pGORF4 and pORF62 induced the expression of pORF4-CAT by three- and eight-fold, respectively. A six-fold stimulation of pORF68-CAT expression was observed with pORF62. Cotransfection of cells with pORF62 and pGORF4 induced the expression of HIV-LTR-CAT by five- and eight-fold, respectively (Fig. 3A). We first tested the effect of pORF61 on pGORF4- and pORF62-induced *trans*-activation of pltk-CAT. Vero cells were cotransfected with pORF61 along with pltk-CAT and either pGORF4 or pORF62. ORF61 inhibited the *trans*-activation of pltk-CAT by pORF62 and pGORF4 by 100 and 77%, respectively (Fig. 3B, columns 1 and 2). pORF61 was able to down-regulate the activation of pltk-CAT by VZV putative immediate-early genes but had no effect on the endogenous activity of pltk-CAT. Cotransfection with ORF61 resulted in CAT

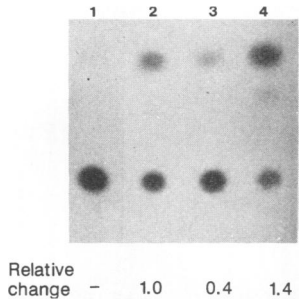

FIG. 4. Evidence that ORF61 encodes p29-associated *trans*-repressive activity. Vero cells were transfected with pltk-CAT alone (1 μg; lanes 1 to 4), pltk-CAT plus pGORF4 (5 μg; lanes 2 to 4), pltk-CAT plus pGORF4 plus p29 (5 μg; lane 3), and pltk-CAT plus pGORF4 plus p29-Δ61 (5 μg; lane 4). The relative change in CAT expression produced by p29 and p29-Δ61 CAT expression is shown at the bottom.

activity levels which were almost identical to the basal level of expression of the target gene in the absence of effector plasmids, i.e., pGORF4 and pORF62. ORF61 also inhibited the synergistic *trans*-activation of pltk-CAT by pGORF4 and pORF62 by 63% (Fig. 3B, column 3). Similarly, we tested the effect of pORF61 on VZV immediate-early and late promoter-CAT constructs. pORF61 inhibited pORF62, and pGORF4 induced *trans*-activation of pORF4-CAT by 78 and 59%, respectively (Fig. 3B, columns 4 and 6). Similarly, pORF61 inhibited pORF62-mediated *trans*-activation of pORF68-CAT by 78% (Fig. 3B, column 6). The *trans*-repressing effect of ORF61 on pGORF4-mediated *trans*-activation of pORF68-CAT could not be assessed because pGORF4 stimulated the expression of pORF68-CAT by only twofold.

It has been previously reported that VZV can stimulate the expression of the (HIV LTR) (18). To observe the effect of pORF61 on *trans*-activation of a non-VZV target, cotransfection assays were performed in the presence of pORF61, HIV-LTR-CAT, and pGORF4 or pORF62. pORF61 down-regulated the pORF62- and pGORF4-mediated expression of HIV-LTR-CAT by 97 and 84%, respectively (Fig. 3B, columns 7 and 8). In addition, plasmid p29-Δ61, which contains a deletion in its ORF61 gene, thus leaving a functional ORF60 gene, was not able to repress pGORF4-mediated *trans*-activation of pltk-CAT. pGORF4 stimulated the *trans*-activation of pltk-CAT (Fig. 4; compare lanes 1 and 2), and p29 down-regulated this increased expression of pltk-CAT (lanes 2 and 3). As shown in lane 4, cotransfection of Vero cells with p29-Δ61 did not down-regulate pGORF4-mediated activation of pltk-CAT, thus proving that ORF61, and not ORF60, is associated with *trans*-repressive activity.

Effect of ORF61 on expression of heterologous promoters. To eliminate the possibility that pORF61 acts in a nonspecific fashion, Vero cells were cotransfected with either pSV2CAT (CAT construct driven by the SV40 promoter and enhancer sequences) or RSV-CAT (CAT cassette driven by the Rous sarcoma virus LTR). In these assays, ORF61 failed to repress the expression of both pSV2CAT (Fig. 5A, lanes 1 and 2) and RSV-CAT (Fig. 5A, lanes 3 and 4). We also tested the effect of pORF61 on *trans*-activation of the HTLV-I and HIV LTRs by *tax* and *tat* genes, respectively. pSV2tat (carrying the HIV *tat* gene) activated the expression of the HIV-LTR-CAT construct (Fig. 5B, lanes 1 and 2). Similarly, a plasmid carrying the HTLV-I *tax* gene activated the expression of the HTLV-I-LTR-CAT construct (lanes 4

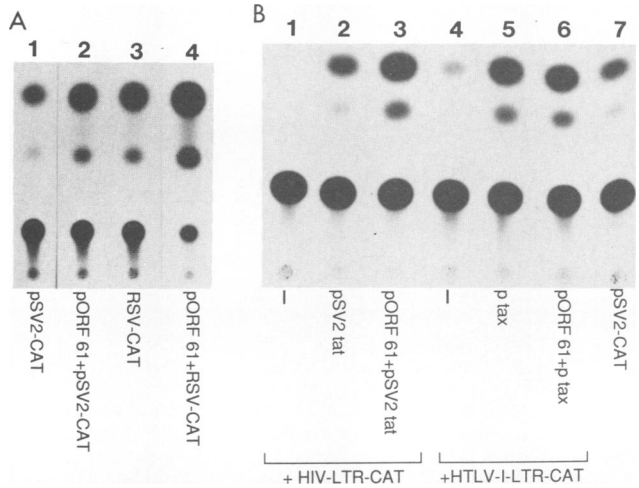

FIG. 5. Effects of ORF61 on the expression of heterologous promoters. (A) Vero cells were cotransfected with either pSV2CAT or RSV-CAT with or without pORF61. The plasmid DNA used in each transfection experiment is indicated at the bottom. (B) Plasmids HIV-LTR-CAT (2 μg; lanes 1 to 3), HTLV-I-LTR-CAT (1 μg; lanes 4 to 6), pSV2tat (5 μg; lanes 2 and 3), ptax (1 μg; lanes 5 and 6), and pORF61 (5 μg; lanes 3 and 6) were transfected into Vero cells. Lane 7 is the positive control in which Vero cells were transfected with pSV2CAT (5 μg).

and 5), whereas pORF61 failed to down-regulate the *tat*- as well as *tax*-mediated expression of the HIV-LTR-CAT and HTLV-I-LTR-CAT constructs, respectively (lanes 3 and 6). Lane 7 is a positive control in which Vero cells were transfected with pSV2CAT alone.

Deletion and insertion mutants of ORF61 do not act as *trans*-repressors. We next wanted to verify whether the down-regulation seen in the presence of pORF61 was, in fact, due to the action of the protein product of ORF61 gene and not due to some other nonspecific effects such as competition for transcription factors by ORF61 promoter elements. To address this question, we engineered mutations within ORF61 and constructed two deletion (pORF61-Δ1 and pORF61-Δ2) mutants and one insertion (pORF61-i3) mutant. Insertion mutant pORF61-i3 was checked for the production of truncated ORF61 protein by in vitro transcription and translation. As shown in Fig. 6, pORF61-i3 made a truncated protein product of 19 kDa, compared with the full-length ORF61 protein product of 56 kDa. A 143-amino-acid product of pORF61-i3 is expected to have a molecular

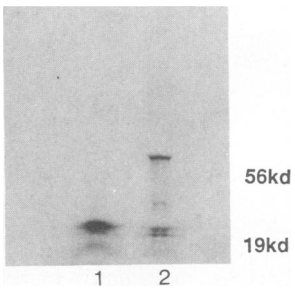

FIG. 6. In vitro translation of ORF61-i3 (lane 1) and ORF63 (lane 2). The genes were transcribed and translated as described in Materials and Methods. In vitro-translated products were resolved by 4 to 20% gradient SDS-PAGE.

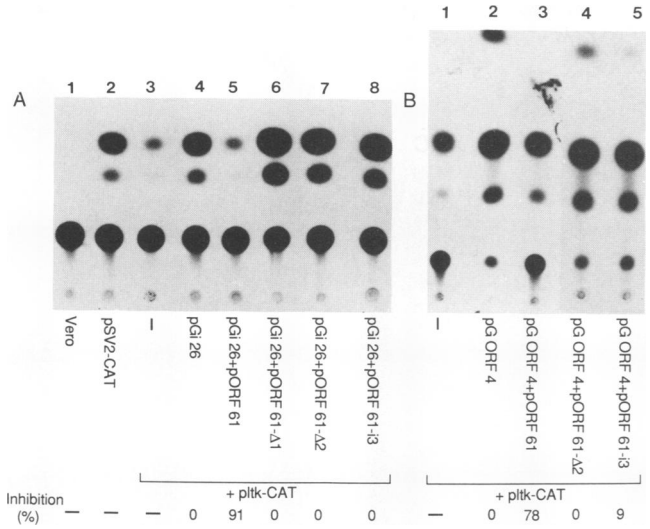

FIG. 7. (A) Effects of ORF61 mutants on *trans*-activation of the VZV *tk* promoter by pORF62 in transient cotransfections. Vero cells were cotransfected with pltk-CAT (1 μg; lanes 3 to 8), pORF62 (pGi26, 5 μg; lanes 4 to 8), pORF61 (5 μg; lane 5), pORF61-Δ1 (5 μg; lane 6), pORF61-Δ2 (5 μg; lane 7), and pORF61-i3 (5 μg; lane 8). Lane 1 represents a negative control in which untransfected Vero cells were used, and lane 2 represents a positive control in which Vero cells were transfected with pSV2CAT (5 μg) alone. (B) Effects of ORF61 and its mutants on transactivation of the VZV *tk* promoter by pGORF4. Plasmids pltk-CAT (1 μg; lanes 1 to 5), pGORF4 (5 μg; lanes 2 to 5), pORF61 (5 μg; lane 3), pORF61-Δ2 (5 μg; lane 4), and pORF61-i3 (5 μg; lane 5) were transfected into Vero cells as described in Materials and Methods. Percentage inhibition of pGi26 (pORF62)- and pGORF4-mediated pltk-CAT expression produced by cotransfection with wild-type or mutant pORF61 is shown at the bottom.

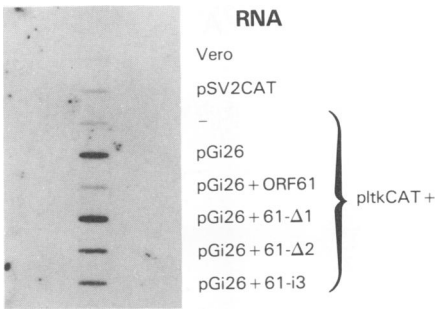

FIG. 8. Evidence that ORF61 inhibits transactivation at the RNA level. Total cellular RNA was extracted from Vero cells transfected with pltk-CAT (1 μg), pORF62 (5 μg), and either pORF61 (5 μg) or one of its deletion or insertion mutants (5 μg). Five μg of RNA was applied to the nitrocellulose through the slot blot manifold. Plasmid DNA used in individual transfection experiments for the isolation of total cellular RNA is shown beside each slot. CAT mRNA level in untransfected Vero cells was used as a negative control. A positive control showing the CAT mRNA level in pSV2CAT (2 μg)-transfected Vero cells is also shown.

mass of 19 kDa. To prove that the pORF61 protein product is responsible for *trans*-repressor activity, we performed cotransfection assays in Vero cells, using pORF62 (pGi26) and pltk-CAT along with pORF61 or one of its deletion or insertion mutants. pORF62 enhanced the expression of pltk-CAT (Fig. 7A, lanes 3 and 4), whereas cotransfection with pORF61 repressed the pORF62-enhanced expression of pltk-CAT (compare lanes 4 and 5). In contrast, all mutants of pORF61 lost their ability to down-regulate the pORF62-enhanced expression of pltk-CAT (lanes 6 to 8). Lane 1 shows the background CAT activity of mock-transfected cells, and lane 2 shows the CAT activity obtained when Vero cells were transfected with pSV2CAT as a positive control. Similarly, pGORF4 activated the expression of pltk-CAT (Fig. 7B, lanes 1 and 2). Cotransfection with pORF61 down-regulated the pGORF4-induced expression of pltk-CAT (lanes 2 and 3), whereas both deletion (pORF61-Δ2) and insertion (pORF61-i3) mutants of ORF61 were unable to repress the activation of pltk-CAT by pGORF4 (lane 4 and 5).

ORF61 inhibits transcription at the RNA level. To determine whether the *trans*-repression of CAT activity by pORF61 occurs at the RNA level, CAT mRNA levels were quantitated by slot blot analysis. pltk-CAT showed a low basal level of CAT mRNA (Fig. 8), but upon cotransfection with pORF62 (pGi26), an increased level of CAT mRNA could be detected. The addition of pORF61 in this cotransfection assay along with pltk-CAT and pORF62 (pGi26) resulted in down-regulation of the CAT mRNA level which almost matched the basal level obtained in a transfection assay with pltk-CAT alone. In contrast, the addition of pORF61 deletion or insertion mutants instead of pORF61 did not decrease pORF62-induced expression of CAT mRNA, thus demonstrating the role of ORF61 protein in *trans*-repressing at the level of RNA. Therefore, repression occurred either at the level of transcription or at the level of mRNA stability.

RNA analysis of gene 61. The published sequence of VZV (4) reveals that ORF61 spans 1.4 kb from the start codon to the stop codon and has a putative polyadenylation signal 80 bp downstream of the stop codon. Northern hybridization analysis using a ^{32}P-labeled 687-bp internal fragment of ORF61 as a probe and total RNA from VZV-infected MRC-5 cells revealed a single band of 1.8 kb (Fig. 9A). To map the start site of the ORF61 gene, primer extension analysis was performed, using total RNA from VZV-infected MRC-5 cells and a synthetic oligonucleotide primer whose sequence and location are described in Materials and Methods. Primer extension analysis positioned the mRNA start site at 69 bp upstream of the AUG codon (Fig. 9B). According to the VZV DNA sequence, there are two putative TATA boxes at 25 and 56 bp upstream of the mRNA start site. The putative CAAT box was found at 114 bp upstream of mRNA start site.

DISCUSSION

We have demonstrated that the VZV-encoded protein product of gene 61 *trans*-represses the activation of the promoters from an immediate-early gene, ORF4, an early gene, the *tk* gene, and a late viral gene encoding gpI by VZV *trans*-activating genes. These *trans*-activating genes are the products of ORF4 and ORF62 (17). To characterize this VZV-encoded *trans*-repressor, we constructed plasmids carrying the putative promoter and regulatory regions of the VZV *tk*, ORF4, and gpI genes. The reporter plasmids were used in cotransfection assays with putative immediate-early *trans*-acting genes pGORF4 and pORF62. ORF61 reproducibly down-regulated the ability of these *trans*-activators to function. To prove the specificity of ORF61-mediated *trans*-repression, we used three criteria. First, pORF61 did not inhibit the expression of pSV2CAT and RSV-CAT (Fig. 5A).

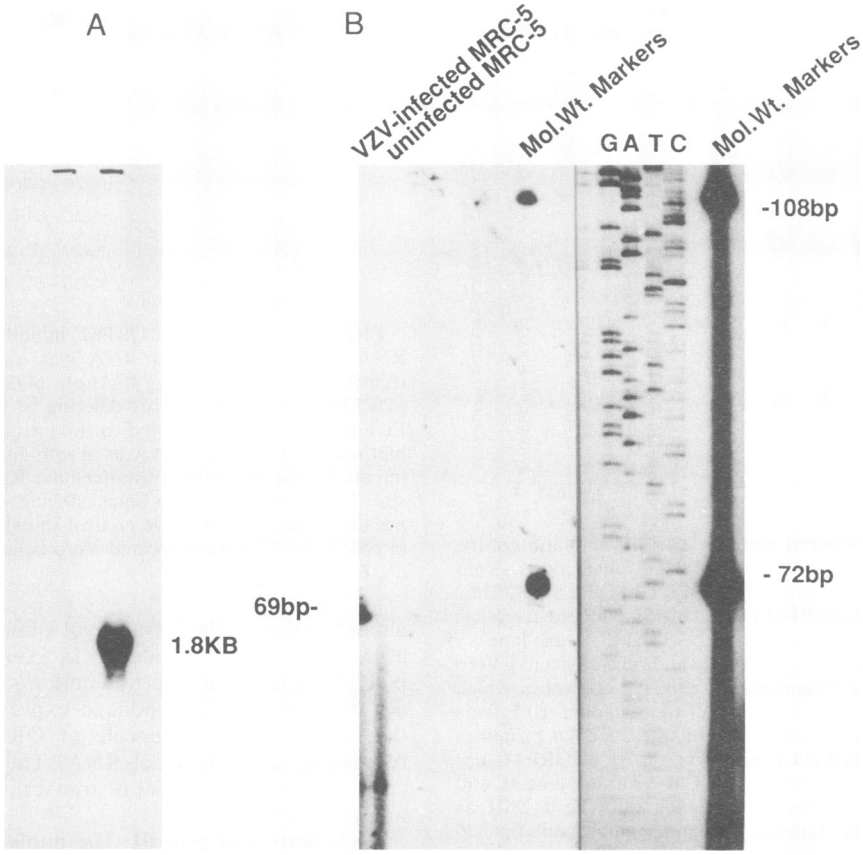

FIG. 9. Northern hybridization and primer extension analysis of gene 61. (A) Autoradiogram of Northern blot analysis of ORF61 mRNA. Total RNA (10 μg) from VZV-infected MRC-5 cells was fractionated by electrophoresis, blotted, and hybridized with a ^{32}P-labeled ORF61 DNA probe as described in Materials and Methods. (B) Determination of the transcriptional start site of the ORF61 gene through primer extension analysis. The transcription start site, indicated with an arrow, was determined by running DNA size markers, whose light and dark exposures are shown alongside the gel.

Second, it had no *trans*-repressing effect on the activation of the HTLV-I and HIV-1 LTRs by *tax* and *tat* genes, respectively (Fig. 5B). Third, both deletion and insertion mutants of gene 61 lost their ability to *trans*-repress (Fig. 7). By coupled in vitro transcription and translation, we have provided evidence that the insertion mutant of ORF61, in fact, makes a truncated protein. These results suggest that *trans*-repression requires the expression of the ORF61 gene and VZV putative immediate-early *trans*-activating proteins. We do not know whether the observed *trans*-repression of viral promoters is a direct or an indirect effect brought about by the ORF61 product. Our results indicate that the products of ORF4 and ORF62 may interact with the product of ORF61 to negatively regulate gene expression, since the latter has no direct effect on the basal level of promoter activity but, in contrast, acts to counteract the stimulatory effects of pGORF4 and pORF62. Interestingly, ORF61 is the HSV-1 ICP0 homolog, as defined by gene location, sharing of a cysteine-rich putative zinc-binding finger in the amino-terminal region, and limited amino acid homology (4). Functionally, HSV-1 ICP0 has been found to be a potent *trans*-activator of many promoters (8, 9, 11, 27, 32, 34, 37, 42). The mechanisms of action of ORF61 and ICP0 have yet to be elucidated. Further, no *trans*-repressing activity has been associated with HSV-1 ICP0. To our knowledge, this is the first report regarding the identification of a viral gene which negatively regulates the expression of all sets of viral genes. It has been shown that the HSV-1 immediate-early gene ICP27 protein product can *trans*-repress or *trans*-activate, depending on the presence of two other immediate-early *trans*-activating proteins, ICP4 and ICP0 (41). ICP27 significantly repressed the induction of HSV-1 *tk*, glycoprotein B, glycoprotein D, and glycoprotein C promoter-CAT constructs by ICP0 and ICP4 (41). Cotransfection of cells with ICP27 reduced the expression of each of these HSV-1 targets to the basal level seen in the absence of ICP0 or ICP4. Therefore, VZV ORF61 seems to be functionally analogous to HSV-1 ICP27, except that repression with ORF61 in in vitro assays is more general and pronounced. HSV ICP4 has been shown to negatively autoregulate expression from its own promoter (reference 39a and references therein). Such a down-regulation differs from that of ORF61-mediated expression in that the ICP4 protein directly binds to its own promoter. A similar autorepression has been observed in the case of VSV putative immediate-early gene 62 (5a). Furthermore, mutational analysis of HSV-1 ICP4, HSV-1 ICP27, and adenovirus E1a genes has shown that the activator and repressor activities reside in different domains of these proteins.

There are three possible mechanisms to explain the *trans*-repressive activity of the ORF61 gene product. The first is that the ORF61 protein directly binds to the promoter regions of target genes. The NH_2-terminal region of the ORF61 protein product contains a number of cysteine resi-

dues which resemble metal-binding domains (4). A putative zinc-binding finger similar to the predicted zinc fingers of the steroid/thyroid hormone receptor DNA-binding domain (7) can be seen in its amino-terminal region. All of these cysteines are conserved between HSV-1 ICP0 and VZV ORF61. Since HSV-1 ICP0 has not been shown to be a sequence-specific DNA-binding protein, a similar analogy can be extrapolated to ORF61. Therefore, it may be possible that ORF61, like HSV-1 ICP0, does not interact with DNA. Adenovirus E1a also has a zinc-binding domain but does not bind to DNA in a sequence-specific manner on its own (25). Instead, E1a requires a cellular protein, ATF-2, for binding to cyclic AMP-responsive sequences in the promoter region of the effector viral as well as cellular genes (26). Similarly, HSV-1 ICP0 also acts through ATF sequences to activate the expression of heterologous viral and cellular promoters (22a). Therefore, a second possibility is that the ORF61 protein product interacts with a cellular transcription factor or with a so-called adaptor or intermediary protein which interacts both with viral immediate-early genes and with the RNA polymerase II transcription machinery. A final possibility is that the ORF61 protein interacts with the ORF4 and ORF62 gene products. Interaction of the ORF61 product with ORF4 and ORF62 proteins or with the adaptor molecule is suggested by the fact that the ORF61 product has little or no ability on its own to *trans*-repress in cotransfection experiments but invariably requires the presence of pGORF4 and pORF62. Another suggestion supporting the model of protein-protein interaction comes from analysis of the predicted amino acid sequences of ORF4 and ORF62. Both contain putative leucine zipper motifs which have been shown to be involved in protein-protein interaction. In the carboxy-terminal region of ORF4, a putative leucine zipper (L-L-C-L-L) in which leucines are repeated seven amino acids apart is seen. A similar leucine zipper region (with three consecutive leucine repeats) is also represented in the gene 62 protein. Although a typical leucine zipper consists of four to six leucine residues placed seven amino acids apart (23), it has been shown that in the cyclic AMP response element-binding protein, the first three leucine residues are necessary for efficient protein-protein interaction (6). If ORF61 acts by virtue of protein-protein interaction, it may involve domains that are structurally homologous rather than domains that show significant amino acid sequence homology, since the predicted protein products of ORF4 and ORF62 do not have significant amino acid homology.

The encoding by VZV of a potent *trans*-repressor raises several interesting questions about the link between ORF61-mediated repression and latency of VZV. Is the mechanism of *trans*-repression of immediate-early, early, and late gene promoters related to latent infection with VZV? At what stage of the viral life cycle is ORF61 protein expressed, and is it carried along with the virus during infection? Is this negative regulation of gene expression related to the low frequency of recurrent VZV infection and its difficult growth in cultured cells? Establishing such a link could help in determining the mechanisms that control gene expression during viral replication and latency.

ACKNOWLEDGMENTS

We thank Stephen E. Straus for continuous support and helpful suggestions. We thank K.-T. Jeang for HTLV-I LTR-CAT and HTLV-I *tax* plasmids, Holly Smith for help in tissue culture, and the members of the Medical Virology Section for useful discussions.

S.N. was the recipient of a Fogarty International Visiting Fellowship.

REFERENCES

1. **Berk, A. J., F. Lee, T. Harrison, J. Williams, and P. Sharp.** 1979. Pre-early adenovirus 5 gene product regulates synthesis of early viral messenger RNAs. Cell **17**:935–944.
2. **Borelli, E., R. Hen, and P. Chambon.** 1984. Adenovirus-2 E1a products repress enhancer-induced stimulation of transcription. Nature (London) **312**:608–612.
3. **Chirgwin, J. M., A. E. Przybyla, R. M. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
4. **Davison, A. J., and J. E. Scott.** 1986. The complete sequence of varicella-zoster virus. J. Gen. Virol. **67**:1759–1816.
5. **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 ICP4. Mol. Cell. Biol. **5**:1997–2008.

5a. **Disney, G. H., T. A. McKee, C. M. Preston, and R. D. Everett.** 1990. The product of varicella-zoster virus gene 62 autoregulates its own promoter. J. Gen. Virol. **71**:2999–3003.

6. **Dwarki, V. J., M. Montminy, and I. M. Verma.** 1990. Both the basic region and the leucine zipper domain of the cyclic AMP response element binding (CREB) protein are essential for transcriptional activation. EMBO J. **9**:225–232.
7. **Evans, R. M.** 1988. The steroid and thyroid hormone receptor superfamily. Science **240**:889–895.
8. **Everett, R. D.** 1984. Transactivation of transcription by herpes virus product: requirements for two HSV-1 immediate-early polypeptides for maximum activity. EMBO J. **3**:3135–3141.
9. **Everett, R. D.** 1986. A detailed mutational analysis of Vmw 110, a *trans*-acting transcriptional activator encoded by herpes simplex virus type 1. EMBO J. **6**:2069–2076.
10. **Felser, J. M., P. R. Kinchington, G. Inchauspe, S. E. Straus, and J. M. Ostrove.** 1988. Cell lines containing varicella-zoster virus open reading frame 62 and expressing the IE175 protein complement ICP4 mutants of herpes simplex virus type 1. J. Virol. **62**:2076–2082.
11. **Gelman, I. H., and S. Silverstein.** 1985. Identification of immediate early genes from herpes simplex virus that transactivate the virus thymidine kinase gene. Proc. Natl. Acad. Sci. USA **82**:5265–5269.
12. **Gendelman, H. E., W. Phelps, L. Feigenbaum, J. M. Ostrove, A. Adachi, P. M. Howley, G. Khoury, H. S. Ginsberg, and M. A. Martin.** 1986. *Trans*-activation of human immunodeficiency virus long terminal repeat sequence by DNA viruses. Proc. Natl. Acad. Sci. USA **83**:9759–9763.
13. **Gerster, T., and R. G. Roeder.** 1988. A herpesvirus *trans*-activating protein interacts with transcription factor OTF-1 and other cellular proteins. Proc. Natl. Acad. Sci. USA **85**:6347–6351.
14. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
15. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
16. **Hansen, U., D. G. Tenen, D. M. Livingston, and P. A. Sharp.** 1981. T antigen repression of SV40 early transcription from two promoters. Cell **27**:603–612.
17. **Inchauspe, G., S. Nagpal, and J. M. Ostrove.** 1989. Mapping of two varicella-zoster virus-encoded genes that activate the expression of viral early and late genes. Virology **173**:700–709.
18. **Inchauspe, G., and J. M. Ostrove.** 1989. Differential regulation by varicella-zoster virus (VZV) and herpes simplex virus-1 *trans*-activating genes. Virology **173**:710–714.
19. **Jeang, K.-T., S. G. Widen, O. J. Semmes IV, and S. H. Wilson.** 1990. HTLV-I *trans*-activator protein, tax, is a *trans*-repressor of the human β-polymerase gene. Science **247**:1082–1084.
20. **Jones, N. C., P. W. J. Rigby, and E. B. Ziff.** 1988. *Trans*-acting protein factors and the regulation of eukaryotic transcription: lessons from studies on DNA tumor viruses. Genes Dev. **2**:267–281.
21. **Khoury, G., and E. May.** 1977. Regulation of early and late

simian virus 40 transcription: overproduction of early viral RNA in the absence of a functional T antigen. J. Virol. **23**:167–176.

22. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.

22a. **Nagpal, S., and J. Ostrove.** Submitted for publication.

23. **Landschulz, W. M., P. F. Johnson, and S. L. McKnight.** 1988. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science **240**:1759–1764.

24. **Leff, T., R. Elkaim, C. T. Goding, P. Jalinot, P. Sassone-Corsi, M. Perricaudet, C. Kedinger, and P. Chambon.** 1984. Individual products of the adenovirus 12S and 13S mRNAs E1a stimulate viral EIIa and EIII expression at the transcriptional level. Proc. Natl. Acad. Sci. USA **81**:4381–4385.

25. **Lillie, J. W., and M. R. Green.** 1989. Transcription activation by the adenovirus E1a protein. Nature (London) **338**:39–44.

26. **Liu, F., and M. R. Green.** 1990. A specific member of the ATF transcription factor family can mediate transcription activation by the adenovirus E1a protein. Cell **61**:1217–1224.

27. **Mavromara-Nazos, P., S. Silver, J. Hubenthal-Voss, J. C. McKnight, and B. Roizman.** 1986. Regulation of herpes simplex virus type 1 genes. α gene sequence requirements for transient induction of indicator genes regulated by β or late (γ_2) promoters. Virology **149**:152–164.

28. **McKnight, J. L. C., 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.

29. **Myers, R. M., D. C. Rio, A. K. Robbins, and T. Tjian.** 1981. SV40 gene expression is modulated by the cooperative binding of T antigen to DNA. Cell **25**:373–384.

30. **Nerenberg, M., S. H. Minrichs, R. K. Reynolds, G. Khoury, and G. Jay.** 1987. The *tat* gene of human T-lymphotropic virus type I induces mesenchymal tumors in transgenic mice. Science **237**:1324–1329.

31. **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**:435–442.

32. **O'Hare, P., and G. S. Hayward.** 1985. Evidence for direct role of both the 175K and 110K immediate-early proteins of herpes simplex virus in the transactivation of delayed-early promoters. J. Virol. **53**:751–760.

33. **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.

34. **Ostrove, J. M., J. Leonard, K. E. Weck, A. B. Rabson, and H. E. Gendelman.** 1987. Activation of the human immunodeficiency virus by herpes simplex virus type 1. J. Virol. **61**:3726–3732.

35. **Ostrove, J. M., W. Reinhold, C. M. Fan, S. Zorn, J. Hay, and S. E. Straus.** 1985. Transcription mapping of the varicella-zoster virus genome. J. Virol. **56**:600–606.

36. **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.

37. **Quinlan, M., and D. Knipe.** 1985. Stimulation of expression of a herpes simplex virus DNA-binding protein by two viral functions. Mol. Cell. Biol. **5**:957–963.

38. **Reed, S. I., G. R. Stark, and J. C. Alwine.** 1976. Autoregulation of simian virus 40 gene A by T antigen. Proc. Natl. Acad. Sci. USA **73**:3083–3087.

39. **Ricciardi, R., R. Jones, C. Cepko, P. Sharp, and B. Roberts.** 1981. Expression of early adenovirus genes requires a viral encoded acidic polypeptide. Proc. Natl. Acad. Sci. USA **78**: 6121–6125.

39a. **Robers, M. S., A. Boundy, P. O'Hare, M. C. Pizzorno, D. M. Cinfo, and G. S. Hayward.** 1988. Direct correlation between a negative autoregulatory response element at the cap site of the herpes simplex virus type 1 IE175 (α4) promoter and a specific binding site for the IE175 (ICP4) protein. J. Virol. **62**:4307–4320.

40. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

41. **Sekulovich, R. E., K. Leary, and R. M. Sandri-Goldin.** 1988. The herpes simplex virus type 1 α protein ICP27 can act as a *trans*-repressor or a *trans*-activator in combination with ICP4 and ICP0. J. Virol. **62**:4510–4522.

42. **Shapira, M., F. L. Homa, J. C. Glorioso, and M. Levine.** 1987. Regulation of the herpes simplex virus type 1 late (γ_2) glycoprotein C gene: sequences between base pairs −34 to +29 control transient expression and responsiveness to transactivation by the products of the IE4 and 0 genes. Nucleic Acids Res. **15**:3097–3111.

43. **Sodroski, J. G., C. A. Rosen, and W. A. Haseltine.** 1984. *Trans*-acting transcriptional activation of the long terminal repeat of human T-lymphotropic viruses in infected cells. Science **225**:381–385.

44. **Straus, S. E., H. S. Aulakh, W. T. Ruyechan, J. Hay, T. A. Casey, G. F. Vande Wonde, J. Owens, and H. A. Smith.** 1981. Structure of varicella-zoster virus DNA. J. Virol. **40**:516–525.

45. **Tjian, R.** 1981. T antigen binding and control of SV40 gene expression. Cell **26**:1–2.

46. **Velcich, A., and E. Ziff.** 1985. Adenovirus E1a proteins repress transcription from the SV40 early promoter. Cell **40**:705–716.