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Varicella-Zoster Virus Open Reading Frame 4 Encodes an Immediate-Early Protein with Posttranscriptional Regulatory Properties

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Varicella-zoster virus (VZV) encodes four putative immediate-early proteins based on sequence homology with herpes simplex virus type 1: the products of ORF4, -61, -62, and -63. Until now, only two VZV proteins have been described as being truly expressed with immediate-early kinetics (IE62 and IE63). The ORF4-encoded protein can stimulate gene expression either alone or in synergy with the major regulatory protein IE62. Making use of a sequential combination of transcription and protein synthesis inhibitors (actinomycin D and cycloheximide, respectively), we demonstrated the immediate-early nature of the ORF4 gene product, which can thus be named IE4. The fact that IE4 is expressed with kinetics similar to that of IE62 further underlines the possible cooperation between these two VZV proteins in gene expression. Analysis of the IE4-mediated autologous or heterologous viral gene expression at the mRNA levels clearly indicated that IE4 may have several mechanisms of action. Activation of the two VZV genes tested could occur partly by a posttranscrip-tional mechanism, since increases in chloramphenicol acetyltransferase (CAT) mRNA levels do not account for the stimulation of CAT activity. On the other hand, stimulation of the human immunodeficiency virus type 1 long terminal repeat- or the cytomegalovirus promoter-associated CAT activity is correlated with an increase in the corresponding CAT mRNA.

Varicella-zoster virus (VZV) is a neutrophic alphaherpesvi-rus which is responsible for two common, well-defined diseases in humans: chicken pox, upon primary infection, and shingles, after reactivation of latent virus from the dorsal root ganglia. Studies of VZV biology have greatly suffered from the inability to produce high-titer infectious cell-free virus. Consequently, part of a researcher's knowledge about VZV is derived from the determination of the nucleotide sequence of the entire VZV genome (8), which has enabled structural and functional comparisons with herpes simplex virus type 1 (HSV-1), another intensively studied alphaherpesvirus (7, 14).

The HSV-1 infectious cycle is characterized by the regulation of gene expression ordered in a cascade (20). The viral genes are subdivided into three classes according to their expression kinetics. The immediate-early genes (IE, **α**) are transcribed first, following penetration of the virus in the absence of de novo protein synthesis (3, 5). The IE proteins play an important role in viral gene regulation (reviewed in references 13 and 43). Early (E, **β**) gene expression occurs next and provides most of the proteins necessary for viral DNA replication. After DNA synthesis has occurred, late (L or **7**) genes are expressed. These genes mainly encode virion structural proteins.

Insight into the functions of several VZV proteins has been gained by transient-cell-transfection assays with plasmids carrying corresponding open reading frames (ORFs). These experiments have shown that the ORF4-, ORF61-, ORF62-, and ORF63-encoded proteins can regulate gene expression (4, 10, 19, 21–23, 33, 36, 39). On the basis of amino acid sequences, these four VZV regulatory proteins were defined as homologs to HSV-1 IE proteins ICP27, -0, -4, and -22, respectively (7, 8). Until now, the true nature of IE proteins has been demonstrated only for the ORF62- and ORF63-encoded proteins (9, 15).

The ORF4 gene product (51 kDa) is a transactivator of gene expression (10, 21, 35, 39, 40) which presents sequence similarity, especially in the carboxyl-terminal region, with ICP27 of HSV-1, an important multifunctional viral regulatory protein (30, 42, 44, 46). It has been shown that ICP27 and the ORF4 gene product are not functionally homologous (34). However, the amino-terminal region of the ORF4 gene product can efficiently replace that of ICP27 (35). Unlike ICP27, the ORF4 gene product does not exhibit any *trans*-repressing activities (10, 21, 39) but is capable of stimulating gene expression either alone or in synergy with the major VZV regulatory protein IE62 (10, 21, 36, 39).

The aim of this report was to address the kinetic class of the ORF4-encoded protein. The IE nature of this protein confirms its importance in the very early stages of viral gene expression. Analysis of IE4-

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mediated transactivation in transient transfec-tion assays at the RNA level also suggested that IE4 can stimulate heterologous and autologous gene expression through distinct mechanisms, one of which could involve posttranscrip-tional regulation.

The ORF4-encoded protein is expressed as an immediate-early protein during the VZV infectious cycle.

To analyze the expression and localization of the ORF4-encoded protein during the viral infectious cycle, cell-free virus was produced by shaking infected cells with 0.5-mm-diameter glass beads. Infected Vero cells (at 80% cytopathic effect) were scraped into sucrose-phosphate-glutamic acid medium (218 mM sucrose, 3.8 mM KH_2PO_4,7.2 mM K_2HPO_4,4.9 mM sodium glutamate, 1% bovine serum albumin, and 10% fetal calf serum) (45). Infected cells and glass beads were shaken for 10 s in a Mini Bead-Beater (Biospec Products, Bartlesville, Okla.) and then centrifuged twice at 3,000 rpm at 20°C in a 5415C centrifuge (Eppendorf, Hamburg, Germany). Supernatants were used to infect confluent MRC5 cells (grown on glass coverslips) with a multiplicity of infection of 5 **X** 10^{-4} (i.e., 1 PFU per 2,000 cells). This multiplicity of infection was chosen because it is impossible to produce high-titer infectious cell-free VZV. The inoculum probably included a significant number of noninfectious particles which were not quantified. Unadsorbed viruses were washed after 1 h, and infected MRC5 cells were fixed in acetone at various times postinfection (p.i.) (3, 6, 9, 12, 16, 20, and 24 h). The cells were studied by immunolabeling with an antiserum raised against a branched peptide covering amino acids 169 to 181 of the ORF4 gene product (11, 35). One or two coverslips were analyzed per condition, and the experiment was repeated at least five times. Uninfected cells were subjected to the same treatment and tested with the antibody as a negative control. Infectivity of the virus was determined in parallel in control wells. Briefly, foci were counted 3 days after infection under identical conditions. Plaques were detected with primary antibodies directed against either IE63 (an IE protein) or glycoprotein E (gE, a late glycoprotein) and were revealed with secondary antibodies coupled with horseradish peroxidase or fluorescein. No major differences in the number of plaques or in the number of IE4-specific fluorescent cells were detected.
Immunofluorescence associated with the ORF4 gene product could be detected as early as 9 h p.i. The immunolabeling was mainly localized in the cytoplasm of infected cells, although a less intense fluorescence could also be observed in the nucleus (Fig. 1A). Twelve hours p.i., labeling in the nucleus decreased to only a few dots, while cytoplasmic staining remained intense (Fig. 1B). These observations suggest that the ORF4 gene product could be present in nuclei at very early stages of infection. In cells fixed from 12 to 24 h p.i., labeling is predominantly cytoplasmic (Fig. 1C, D, and E). The presence of the ORF4 gene product late in infection could also reflect its incorporation into the viral tegument (26). gE, a late protein, was detected with the monoclonal antibody VL8 (9) at 20 h and 24 h p.i. Staining was localized in the endoplasmic reticulum or the trans-Golgi network or else in a punctate cytoplasmic distribution (data not shown). Detection of the ORF4-encoded protein at 9 h p.i. suggests its involvement in the very early stages of the viral cycle, since it is expressed very shortly after IE62 and IE63, which are two clearly defined immediate-early proteins (9, 15).
To demonstrate the true immediate-early nature of the ORF4-encoded protein, MRC5 cells infected with cell-free VZV as described above were treated with metabolic inhibitors (15). First, cells were treated with cycloheximide (CHX), which blocks protein synthesis, and then with actinomycin D (Act D), which prevents transcription. Briefly, after adsorption of the virus for 1 h in the presence of CHX (100 μg/ml), the cells were washed three times and incubated with CHX (100 μg/ml) at 37°C for 5 h. After three washes, Act D-containing medium (10 μg/ml) was added to the cells for 18 h to block transcription. From 6 to 24 h p.i., cells were fixed and subjected to immunofluorescence analysis with antibodies directed against the ORF4 gene product, IE62 (Viro-Research Inc., Rockford, Ill.), IE63, and gE (9). Two coverslips were analyzed in each condition, and the experiment was repeated at least four times. No labeling could be detected with these antibodies in infected cells treated with CHX at 6 h p.i. (data not shown).
Fluorescence associated with the ORF4 gene product could be detected at 12 h p.i. (6 h after removal of CHX) both in cytoplasm and nuclei (Fig. 2B). Under the same experimental conditions, IE63 could be detected as early as 9 h p.i. (3 h after the removal of CHX) (Fig. 2A), whereas IE62 was observed at 12 h p.i. (6 h after the removal of CHX) (Fig. 2C) as described before (9, 15). Similar numbers of IE4-, IE62-, and IE63-positive cells were counted in each case (9). When the cells were tested with the anti-gE, no signal was detected in the infected cells treated with the metabolic inhibitors (data not shown). The sequential combination of metabolic inhibitors (CHX and Act D) (9, 20) allowed us to demonstrate the true immediate-early nature of the ORF4 gene product. CHX blocked protein synthesis for a 6-h

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period; under these conditions, only the immediate-early transcripts could be synthesized without any viral translation (in the absence of de novo synthesis). After CHX removal, Act D was added, which prevented any new transcription, and only pre-existing transcripts could be translated into proteins. The lack of gE synthesis confirmed the efficiency of the block and reversal experiments. In these experiments, the ORF4-encoded protein could be detected 6 h after the removal of CHX. Its expression kinetic is similar to that of IE62. Since synthesis of the ORF4 gene product was not prevented in the presence of these metabolic inhibitors, it can be concluded that the ORF4-encoded protein is an immediate-early protein which can accurately be named IE4.
Confirmation of the IE nature of the ORF4-encoded protein stresses the importance of this viral regulator in the regulation of VZV gene expression. IE4 can act in synergy with IE62 to stimulate gene expression in transfection assays (21, 36, 39); moreover, IE62 influences the intracellular localization of IE4 (11). Our present results suggest that these activities could occur in the very early stages of viral infection and contribute to the precise control of the expression cascade. Interestingly, the ORF10 gene product, the VZV homolog of HSV-1 VP16 triggering factor, is a tegument protein (24) that only transac-tivates the expression of IE62 but has no effect on IE63 and IE4 promoters (31, 32). Therefore, another mechanism must exist to trigger IE gene expression. It is known that IE62 as well as IE4 and IE63 is a tegument protein (24, 26). Consequently, the initiation of transcription could be dependent upon IE62, either alone or in combination with IE4.

IE4 can stimulate heterologous and autologous gene expression by different mechanisms.

We and others have previously shown that IE4 can transactivate the expression of autologous and heterologous viral promoters (10, 21, 39, 40). Some evidence has indicated that IE4 could act transcriptionally and/or posttranscriptionally (10, 40). We wanted to assess whether autologous and heterologous IE4-mediated activation followed identical mechanisms. We analyzed the ability of IE4 to increase the steady-state levels of mRNA encoded from two VZV promoters, the cytomegalovirus IE promoter and the human immunodeficiency virus type 1 (HIV-1) long terminal repeat. The IE4 expression vector, pGi4 (21), was cotrans-fected in Vero cells cultivated in 9-cm-diameter petri dishes by using the lipofection technique (Gibco-BRL) either with 4 **µ**g of pHIV-chloramphenicol acetyltransferase (CAT) (which carries the HIV-1 long terminal repeat) (12, 17), 6 **µ**g of pCMV-CAT (which carries the major IE promoter of cytomegalovirus) (10, 37), 6 **µ**g of p62-CAT (which carries the VZV IE62 gene promoter) (10, 28), or 6 **µ**g of ptk-CAT (which carries the VZV ORF36 promoter, a putative early gene) (10). Figure 3 illustrates the structure of the various CAT constructs used in this study. CAT transcription and expression were analyzed in a dose-dependent fashion with increasing amounts of pGi4.

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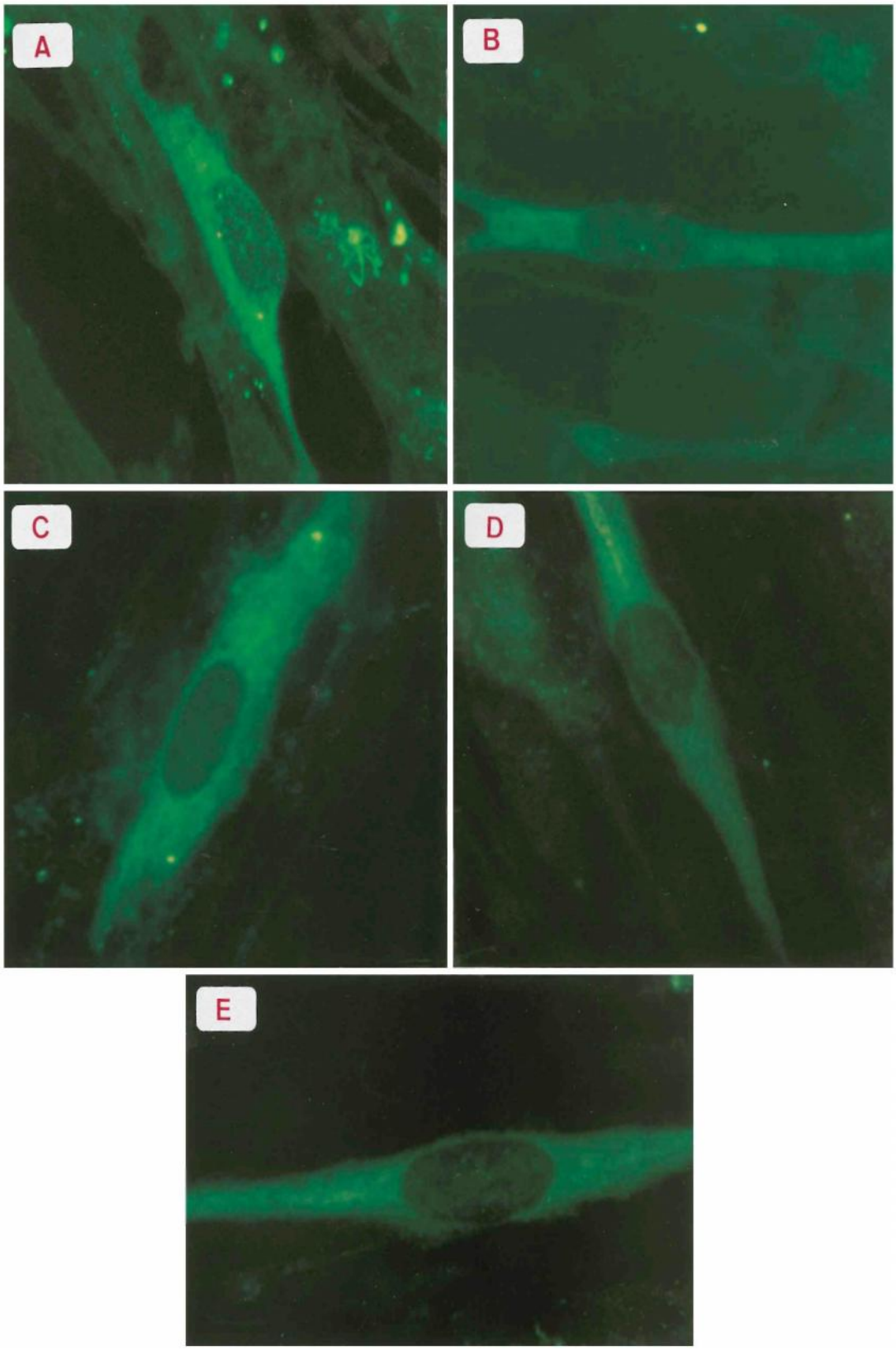

FIG. 1. Immunofluorescence of MRC5 cells infected with cell-free VZV. Infected cells were fixed at 9 h (A), 12 h (B), 16 h (C), 20 h (D), and 24 h (E) p.i. and revealed with an antiserum directed against the ORF4 gene product as the primary antibody. Incubation was pursued with fluorescein isothiocyanate-conjugated swine anti-rabbit immunoglobulin G as secondary antibodies (DAKO).

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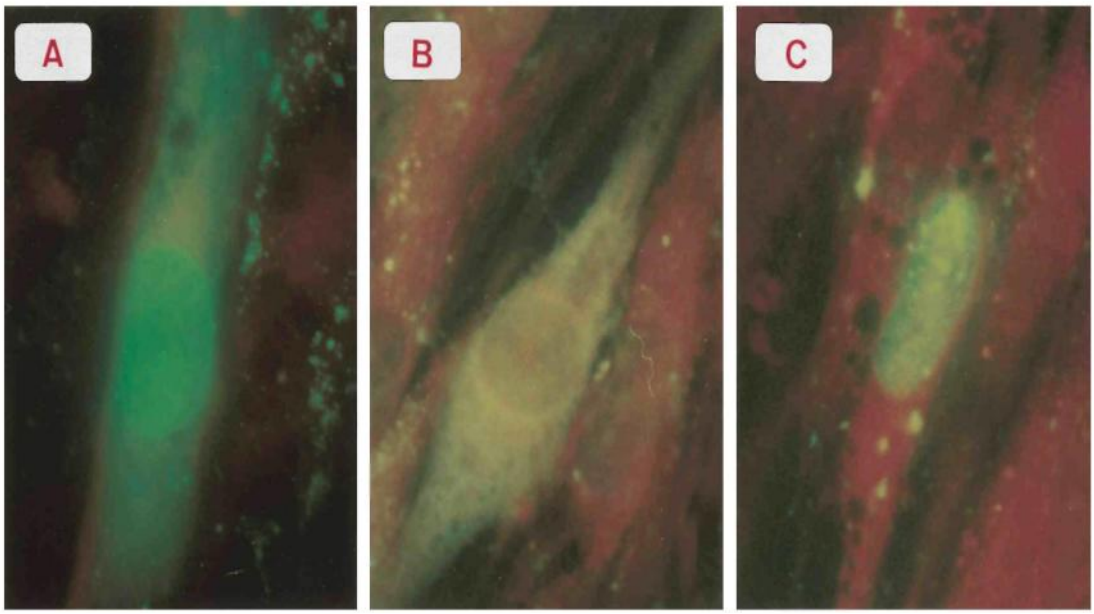

FIG. 2. Immunofluorescence of MRC5 cells infected with cell-free VZV in the presence of a combination of metabolic inhibitors. Cells were sequentially treated with cycloheximide (100 µg/ml) and Act D (10 µg/ml); then cells were fixed 9 h (A) or 12 h (B and C) p.i. Following fixation, cells were revealed with anti-IE63 (A), anti-ORF4 gene product (B), and anti-IE62 antibodies before being processed as described in the legend for Fig. 1.

Forty-eight hours posttransfection, cells were split for CAT activity analysis (10) and for total cellular RNA extraction by the guanidinium isothyocyanate-cesium chloride method (6). CAT mRNA was quantified after DNase I treatment by the ribonuclease protection assay (RPA), a highly sensitive technique which allows the specific detection of mRNA with an antisense RNA probe (2). Briefly, 30 **µ**g of total cellular RNA was hybridized in solution to 5 **X** 10^5 cpm of a single-stranded RNA probe (420 nucleotides [nt]) antisense to the CAT mRNA. This probe spans the CAT gene from an internal *Eco*RI site to the ATG codon and onwards into the promoter of the HSV-1 thymidine kinase gene (in plasmid pSpt18-tk). Therefore, this probe does not allow the determination of the transcript initiation sites. Following hybridization, samples were digested with ribonucleases (H and T1), and protected fragments (255 nt) were run on a 5% acrylamide-7 M urea denaturing gel along with radiolabeled molecular-weight (MW) markers and a ^{35}S sequence ladder of M13 template to precisely define fragment size. The protected fragments correspond to the CAT mRNA region running from the initiation codon to the internal *Eco*RI site.

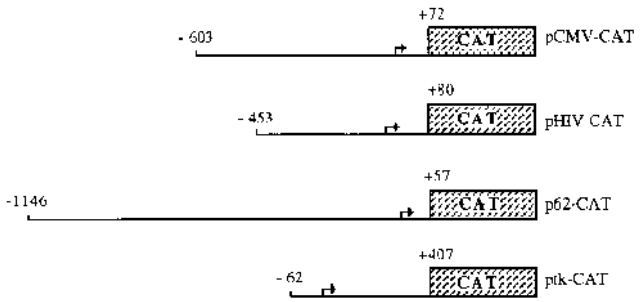

FIG. 3. Schematic structures of the promoter/upstream regulatory elements of CAT constructs. Positions are indicated relative to the CAP site (small arrow). The large box represents the CAT coding sequence. pHIV-CAT, pCMV-CAT, and ptk-CAT carry simian virus 40 early poly(A) signals, whereas p62-CAT carries HSV-2 IE5 gene poly(A) signals (28).

Total RNA amounts were standardized by quantification of actin mRNA in each sample of harvested RNA; this evaluation was used to normalize CAT mRNA levels. Quantitative analysis was performed

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with a PhosphorImager scanner with ImageQuant software (Molecular Dynamics, Sunnyvale, Calif.). The fold stimulation in CAT activity was compared with the fold stimulation in CAT mRNA levels for each condition.

Figure 4 illustrates the effect of IE4 on the level of pHIV-CAT activity versus the increase in pHIV-CAT mRNA. IE4-mediated activation was associated with an important increase in the amount of the 255-nt protected fragment (Fig. 4A). This increase in CAT mRNA was correlated with the stimulation of CAT activity (Fig. 4B). In the case of pCMV-CAT, the degree of mRNA stimulation observed can also account for the levels of CAT activity observed (Fig. 4C). These results indicate that IE4-mediated activation could be associated with augmentation in the rate of transcription, efficient stabilization of the messenger or more efficient mRNA processing, such as poly-adenylation, which could also lead to enhanced RNA levels. These results suggest that heterologous transactivation by IE4 is not predominantly associated with an effect on translation efficiency or CAT protein stability. Very interestingly, when autologous transactivation was investigated by using the same approach, a clear discrepancy was observed between CAT mRNA stimulation and CAT activity induction (Fig. 5). In the case of ptk-CAT, the small increase in CAT mRNA levels, determined by RPA analysis, accounted for only 17 to 20% of the increase in CAT activity over the range of the pGi4 concentrations analyzed (Fig. 5B). IE4 could function partly at the level of ptk-CAT mRNA transcription or stabilization, since some increase in mRNA levels was observed (a two- to threefold increase). Our observations in the case of the p62-CAT construct were similar (Fig. 5C). There was little correlation between the CAT mRNA increase and the induction of CAT activity. The absolute mRNA levels for p62-CAT were also always very low, but despite the strong transactivation by IE4 in CAT assays, no mRNA increase was ever determined. The

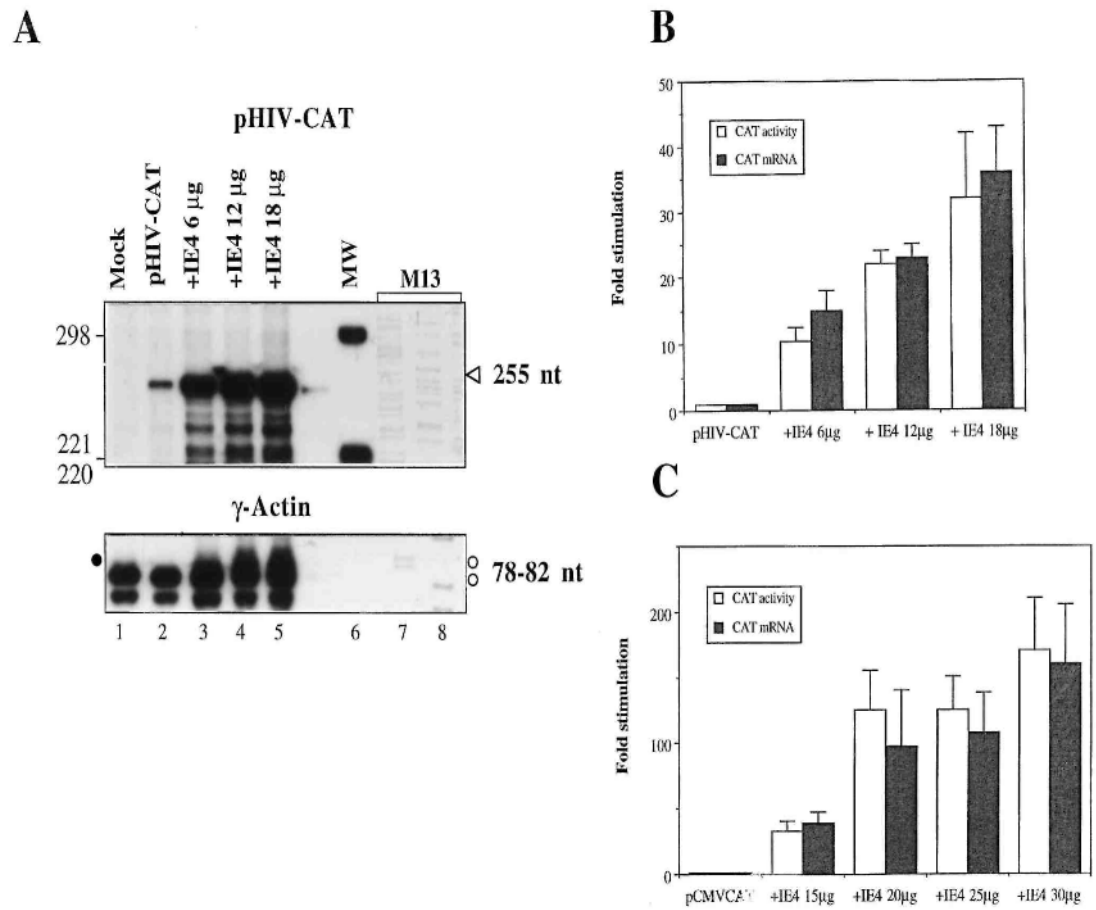

FIG. 4. Stimulation of CAT activity versus increase in CAT mRNA levels by the IE4 protein in heterologous transactivation. (A) RPA with pHIV-CAT. Thirty micrograms of total RNA isolated from Vero cells mock transfected (lane 1) or cotransfected with 4 µg of pHIV-CAT and IE4-expressing vector (pGi4) (lanes 2 to 5), as indicated above the autoradiogram, was hybridized to 5 X 10^5 cpm of the single-stranded RNA probe (420 nt) antisense to the CAT mRNA and to 5 X 10^4 cpm of the 7-actin probe (145 nt). After digestion with RNases, the protected CAT fragments (255 nt) were detected (arrowhead) along with some smaller fragments which result from the highly labile structure of CAT

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mRNA. The 7-actin fragments (78 to 82 nt) are labeled with open circles; we labeled a smaller CAT-specific fragment (86 nt) with a filled circle, since it overlaps with the 7-actin upper band. The MW markers are end-labeled HinfI restriction fragments of pBR322 (lane 6) and a ^{35}S sequencing reaction with M13 template DNA (lanes 7 and 8). (B) Comparison of CAT activity with CAT mRNA stimulation with pHIV-CAT. Band intensities on RPA autoradiograms were quantified with a PhosphorImager scanner in each cotransfection condition. Simultaneously, CAT activities were determined as described previously (11). Fold inductions in CAT mRNA levels and CAT activities were then determined relative to values obtained with pHIV-CAT (4 μg) in the absence of IE4, arbitrarily set at 1. (C) Comparison of CAT activity with CAT mRNA stimulation in IE4-mediated transactivation of pCMV-CAT (6 μg). Band intensities on RPA autoradiograms and CAT activities were quantified simultaneously. Fold inductions in CAT mRNA levels and CAT activities were then determined relative to values obtained in the absence of IE4, arbitrarily set at 1. All experiments were repeated independently three times. Data are presented as mean values with standard errors.

increase in CAT protein expression out of proportion to the mRNA level evokes additional mechanisms, such as mRNA processing and transport and the translational efficiency of ptk-CAT and p62-CAT mRNA. It should be taken into consideration that CAT mRNAs carry a different 5' untranslated leader region. In p62-CAT and ptk-CAT, computer-assisted analysis has allowed the prediction of secondary structures. A hairpin with a stability (free energy = -27 kcal [1 cal = 4.184 J]/mol) close to that of the TAR region present in pHIV-CAT can be found in p62-CAT. However, despite this similarity in the short 5' leader region, p62-CAT and pHIV-CAT responded differently to IE4 in our assay. Also interesting is the potentially complicated structure of the 5' leader in ptk-CAT. This long region (400 bp) can presumably form hairpin structures *(AG* =-64.6 kcal/mol). Such secondary structures can be important in the translatability of mRNAs (27). Hence, in the EBV DNA polymerase gene, the structures present in the 5' noncoding region could influence translation efficiency (16). Predicted hairpins of similar stability introduced in the leader portion of the HSV-1 thymidine kinase gene have been shown to impair translation (38). One hypothesis is that IE4 enhances the translational efficiency of ptk-CAT and possibly p62-CAT through the destabilization of inhibitory secondary structures. We find it very unlikely that the discrepancy between the CAT mRNA levels and CAT activity for the two VZV promoters that were tested is linked to the intrinsic CAT mRNA instability in the system, since CAT mRNA levels associated with both heterologous constructs were shown to increase under identical experimental conditions. It must be noted that since we do not precisely map the initiation sites of our transcripts, the steady-state mRNA levels we measure may include incorrectly initiated transcripts. Spurious initiation could influence mRNA turnover rates; however, we feel that random initiation would not lead to a reproducible and clear discrepancy like the one we observed with p62-CAT and ptk-CAT.

These results confirmed our previous observations demonstrating the possibility of a posttranscriptional effect of IE4 in VZV promoter activation (10) and established that IE4 may have more than one transactivation mechanism.

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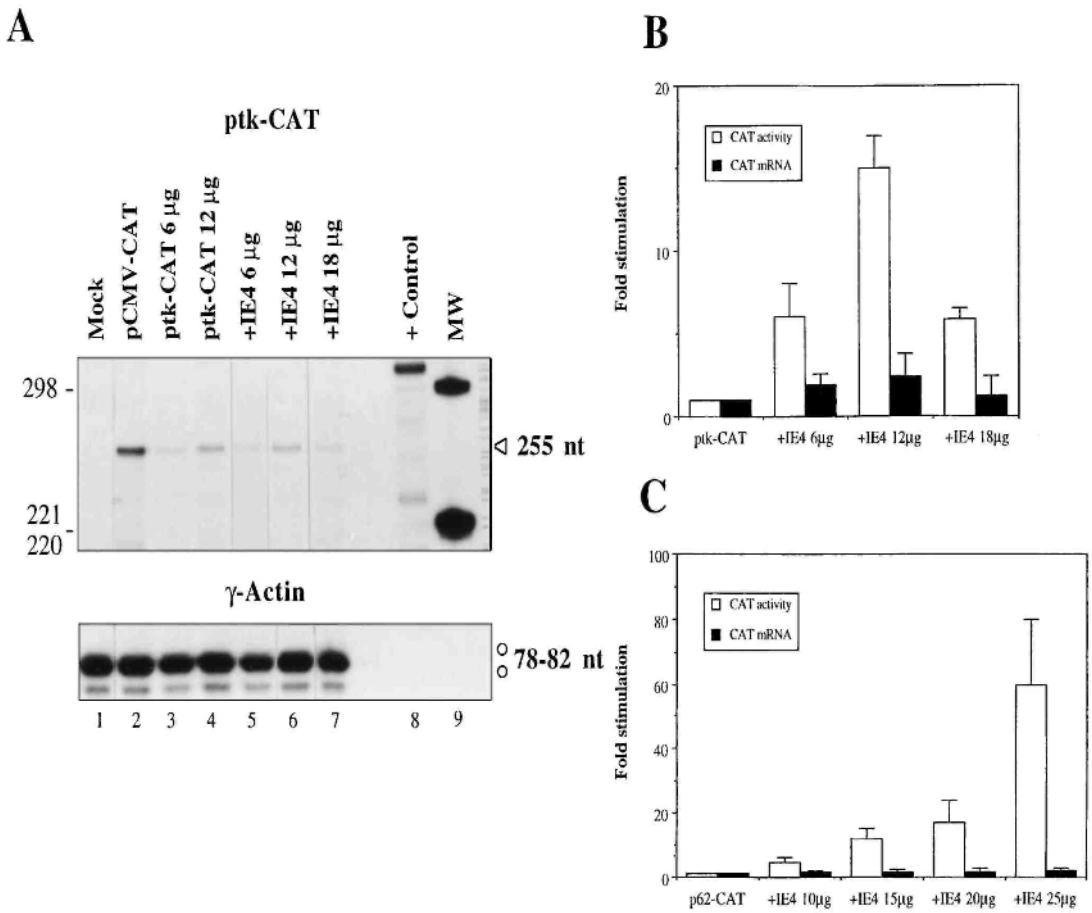

FIG. 5. Comparison of CAT activity with CAT mRNA stimulation in IE4-mediated autologous transactivation. (A) RPA with ptk-CAT. Thirty micrograms of total RNA was isolated from Vero cells mock transfected (lane 1), transfected with pCMV-CAT (6 µg) as a positive control (lane 2), or cotransfected with combinations of the different plasmids (lanes 3 to 7) as indicated above the autoradiogram (6 µg of ptk-CAT was used in the cotransfections with pGi4). Sample RNAs were processed for RPA as described in the legend for Fig. 1. The protected CAT fragments (255 nt) are identified by an arrowhead. The 7-actin fragments (78 to 82 nt) are labeled with open circles. Lane 8 shows the positive RPA control (316 nt) of CAT mRNA synthesized in vitro. The MW markers are end-labeled HinfI restriction fragments of pBR322 (lane 9). (B) Band intensities on RPA autoradiograms and CAT activities were quantified in each cotransfection condition with 6 µg of ptk-CAT, as described in the legend for Fig. 3. (C) Comparison of CAT activity with CAT mRNA stimulation in IE4-mediated transactivation of p62-CAT (6 µg). Band intensities on RPA autoradiograms and CAT activities were quantified simultaneously. Fold inductions in CAT mRNA levels and CAT activities were then determined relative to values obtained in the absence of IE4, arbitrarily set at 1. All experiments were repeated independently three times. Data are presented as mean values with standard errors.

Indeed, the stimulation of the heterologous promoters tested was associated with a strong increase in mRNA levels, in contrast to the situation observed with the autologous promoters. Therefore, this effect of IE4 on heterologous viral gene expression could be associated with an effect on transcription. Indeed, some data indicate that IE4 requires some critical *cis* elements in the HIV and simian virus 40 promoters to mediate its transactiva-tion (12, 40). Although ICP27 and IE4 display distinct properties, i.e., IE4 does not complement an ICP27-defective HSV-1 mutant (34), ICP27 is nuclear, and IE4 is predominantly cytoplasmic (1, 11, 28), ICP27 possesses transrepressing functions (42); strikingly, IE4 appears to be a multifunctional protein as is ICP27. Both proteins could be involved in tran-scriptional and posttranscriptional regulation. Hence, ICP27, which has been identified as a potential posttranscriptional regulator which affects mRNA processing (29, 46, 47), would also be capable of interacting with the transcription process, although there is no direct evidence of this yet (41).
Our work demonstrates that, unlike equine herpesvirus 1, VZV encodes at least three polypeptides which are expressed as immediate-early proteins: IE62, IE63, and IE4 (9, 15, 18). Moreover, IE4

expression kinetics are similar to those of IE62; this observation further enhances the importance of the cooperation between these two proteins in the control of VZV gene expression. Despite differences in their promoter structures and in their functions, the three IE genes are expressed during the very early stages of the viral cycle (19, 25). Our results favor the existence of mechanisms of controlling gene expression in VZV which could involve the IE proteins brought into the cells in large quantities by the virus.

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