Characterization of Regulatory Functions of the Varicella-Zoster Virus Gene 63-Encoded Protein

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Abstract

Varicella-zoster virus (VZV) gene 63 encodes a protein (IE63) with a predicted molecular mass of 30.5 kDa which has amino acid similarities to the immediate-early (IE) protein 22 (ICP22) of herpes simplex virus type 1. ICP22 is a polypeptide synthesized in herpes simplex virus type 1-infected cells, and as is the case for its VZV counterpart, its regulatory functions are unknown. On the basis of the VZV DNA sequence, it has been shown that IE63 exhibits hydrophilic and acidic properties, suggesting that this protein could play a regulatory role during the infectious cycle. We report in this article cotransfection experiments which demonstrate that the VZV gene 63 protein strongly represses, in a dose-dependent manner, the expression of VZV gene 62. On the other hand, transient expression of the VZV gene 63 protein can promote activation of the thymidine kinase gene but cannot affect the expression of the genes encoding glycoproteins I and II. The results of transient expression experiments strongly suggest that the VZV gene 63 protein could play a pivotal role in the repression of IE gene expression as well as in the activation of early gene expression.

Varicella-zoster virus (VZV), a member of the neurotropic alphaherpesvirus subfamily, is the causative agent of two human diseases. Chicken pox, which most people contract as children, is the result of generalized primary infection, while shingles is the consequence of reactivation of virus that has remained latent. Despite strong interest in studying this medically important virus, our knowledge of VZV is still rudimentary in comparison with our knowledge of the other herpesviruses which infect humans. The major reason for this is the poor growth of VZV in the available tissue culture systems, which produce predominantly cell-associated virus with low-titer stocks and a high degree of instability. Consequently, the identification and mapping of specific VZV functions have been particularly difficult. These limitations have been somewhat overcome since Davison and Scott (4) determined the complete VZV DNA sequence and identified 71 major open reading frames (ORFs), allowing similarity comparisons between VZV and herpes simplex virus type 1 (HSV-1) and assignment of potential functions to some of the VZV ORFs by using transient expression in eukaryotic cells (7, 15, 16).

A number of viral *trans*-acting proteins have been shown to be important for the expression of different classes of viral genes during HSV-1 infection (for a review, see reference 9). At least three viral proteins expressed during the immediate-early (IE) phase of infection exhibit regulatory properties. ICP0 *trans* activates various promoters (1), ICP4 represses the expression of all five IE genes and activates the expression of early (E) and late (L) genes (8, 18), and ICP27 has been shown to be a *trans* repressor or *trans* activator in combination with ICP4 and ICP0 (19, 27). Thus, it is envisioned that IE gene products interact in order to perform their functions. The key role played by these polypeptides in mediating control of viral gene expression has been confirmed by analysis of viruses with temperature-sensitive mutations. Indeed, ICP4 and ICP27 have been shown to be essential for growth in tissue culture (5), while ICP0 is not required for growth in cell culture because mutant viruses in which both copies of the diploid ICP0 gene have been deleted are replication competent (24). As for the other IE proteins, it has been known for a long time that ICP22 is not required for virus replication in HEp-2 and Vero cells but that ICP22-deficient virus grows poorly in a rabbit skin cell line (23, 26). The observed disparity in the growth properties of HSV-1 ICP22-deficient mutants in various cell lines and in at least one human cell strain (HEL) demonstrates that a host factor can substitute for ICP22 with different degrees of efficiency. The complementation of ICP22 function by a host cell factor has been shown to occur to a greater extent in HEp-2 and Vero cells than in confluent, resting HEL cells. Analysis of properties of growth in HEL cells suggests that the lack of growth can be attributed to underproduction of late viral gene products (26). The VZV gene exhibiting the highest degree of similarity to the HSV-1 gene encoding ICP22 is VZV ORF 63 (4). Although the sizes of the two proteins are rather different, i.e., the VZV gene 63 product has a theoretical molecular mass of 30.5 kDa whereas its HSV-1 homolog has a molecular mass of 68 kDa, and although the total degree of amino acid conservation is low, they have one rather conserved domain with a similarity degree ranging from 25 to 49% (3). Thus, it is quite possible that these two proteins are functionally equivalent, but it

remains to be demonstrated whether they fulfill a similar function.

Because VZV grows poorly in the available tissue culture systems and the production of site-specifically mutated VZV is difficult, it is of interest to determine whether the VZV protein encoded by ORF 63 exhibits regulatory functions toward VZV promoters controlling the expression of genes thought to belong to the three classes, i.e., the IE62 gene (7), the E36 gene (which encodes thymidine kinase [TK] [4]), and L68 and L31 (which encode the gpl and gpII proteins, respectively [4]). The use of transient expression of the VZV gene 63 product in eukaryotic cells turns out to be the most appropriate way to characterize the potential regulatory function of this protein. Therefore, plasmid Tdn IE63, in which the coding sequence of VZV gene 63 is driven by the Rous sarcoma virus long terminal repeat and followed by the bovine growth hormone polyadenylation site, was constructed (Fig. 1A). Placing VZV gene 63 under the control of a heterologous promoter, such as the Rous sarcoma virus long terminal repeat, instead of its own promoter reduces difficulties in the interpretation of the results which would arise from promoter competition effects and a possible regulatory effect of the VZV gene 63 product on its own regulatory sequence. The coding sequence of VZV gene 63 was obtained by cutting the VZV *Eco*RI A fragment (20) with restriction enzymes and ligating subfragments to generate an 834-bp fragment containing the ATG codon, to which a partially double-stranded oligonucleotide was added by ligation to restore the 3' end. Target plasmids contain the chloramphenicol acetyltransferase (CAT) gene under the control of various VZV promoter regions (Fig. 1B): (i) the gene 62 control sequences (p62-CAT [7]), (ii) the sequence situated upstream of gene 36 controlling the TK gene (pTK-CAT [15]), (iii) the regulatory region of gene 68 encoding gpI (pgpI-CAT [15]), and (iv) the gene 31 control sequences (pgpII-CAT).
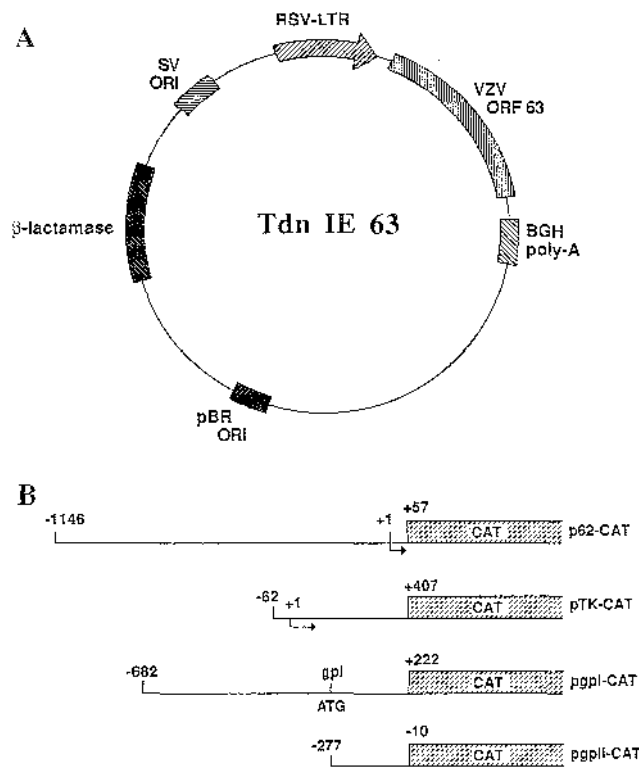

FIG. 1. (A) Structure of plasmid Tdn IE63. An 834-bp fragment representing the entire gene 63 coding sequence from nucleotide 110581 to 111415 (4) was isolated from the VZV *Eco*RI A fragment (20) and inserted at the EcoRV site of Tdn 8066 after blunt ending. VZV gene 63 is driven by the Rous sarcoma virus long terminal repeat (RSV-LTR) and followed by the bovine growth hormone polyadenylation (BGH poly-A) site. SV, simian virus 40; pBR, pBR322; ORI, origin of replication. (B) Structures of the target plasmids used. p62-CAT (7) contains the gene 62 control sequence from nucleotide -1146 to +57 respective to the gene 62 transcription start (+1) upstream from the CAT coding sequence. pTK-CAT (15) contains, upstream of the CAT coding sequence, the gene 36 control sequence from nucleotide -62 to +407 respective to the gene 36 transcription start. pgpI-CAT (15) contains the gene 68 control sequence from nucleotide -682 to +222 relative to the ATG codon (4) upstream of the CAT coding sequence. pgpII-CAT contains the gene 31 control region extending from nucleotide -277 to -10 relative to the ATG codon (4).

Vero cells, at a density of 0.8 x 10^6 cells per 10-cm^2 dish, were transfected by lipofection using cationic lipid vesicles of DOTAP (*N*-[l-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylam-moniummethylsulfate) (Boehringer, Mannheim, Germany) (11). Mixtures of Tdn IE63 and target plasmids were prepared in 100 µl of Hanks balanced salt solution. Next, 5 µg of DOTAP per ml was added, and the mixtures were kept at room temperature for 10 min before being added to the cells and incubated for 24 h. The total amount of plasmid DNA in each transfection mixture was equalized by addition of sonicated herring sperm DNA. CAT assay extracts were prepared after 48 h of cell cultivation, and CAT activity was determined as described by Gorman et al. (14). The CAT activities were expressed as the percentage of conversion of [^{14}C]chloramphenicol to acetylated forms by cutting out the spots revealed on thin-layer plates and determining their radioactivity in a liquid scintillation counter. CAT activities were normalized with respect to the protein amount determined by the Micro BCA protein assay reagent (Pierce, Rockford, III.). A typical experiment involved cotransfec-tion of various molar ratios of Tdn IE63 and target plasmids together with both positive and negative controls consisting of plasmids pCMV-CAT and pSVO-CAT, respectively. RNA analysis by slot blotting was carried out 48 h posttransfec-tion. Total RNA was extracted by the guanidinium isothio-cyanate-cesium chloride method (2), and three twofold dilutions of the RNA preparation were slot blotted on nylon membranes as previously described (17) before being hybridized with a CAT probe. Autoradiograms were scanned with a laser photodensitometer (Ultroscan XL; LKB-Pharmacia).

To elucidate the regulatory role of the VZV gene 63 product in the expression of an IE gene, we chose as a target sequence the regulatory region of VZV gene 62 encoding a 175-kDa protein that has several functional similarities to ICP4 of HSV-1 (6, 12). Vero cells were cotransfected with 1.5 µg of p62-CAT and Tdn IE63 in amounts of up to 8.5 µg. The amount of transfected p62-CAT had been previously determined to reach a level of chloramphenicol acetylation of about 50%, allowing detection of both *trans*-activating and *trans*-repressing effects of the VZV gene 63 product on the gene 62 regulatory sequence. As shown in Fig. 2A, transfection of 1.5 µg of p62-CAT alone resulted in 49.5% chloramphenicol acetylation with 45 µg of protein, and this value dropped progressively to 3% when the transfection was carried out in the presence of 8.5 µg of Tdn IE63. These results are depicted graphically in Fig. 2B, where it can be seen that the relative CAT activity was strongly repressed by the presence of the VZV gene 63 product in a dose-dependent manner. The results presented above clearly demonstrate that cotransfected Tdn IE63 repressed the gene 62 promoter and that relative CAT activities as low as 6% of the initial CAT value could be reached in the presence of 8.5 µg of Tdn IE63. To determine whether the *trans* repression of CAT activity by Tdn IE63 occurs at the RNA level, CAT mRNAs were quantitated by slot blot analysis. p62-CAT showed a rather high CAT mRNA concentration (Fig. 2C, lane 1), but upon cotransfection with Tdn IE63, a decreased CAT mRNA level could be detected (Fig. 2C, lanes 2 to 4). Photodensitometric scanning showed that the CAT mRNA concentration matched the drop in CAT activity, demonstrating that *trans* repression of VZV gene 62 expression by the VZV gene 63 product occurs either at the level of transcription or by decreasing the stability of the mRNA. Experiments were also carried out to determine whether the VZV gene 63 product can regulate the expression of VZV genes encoding proteins expressed during the E and L phases of the infectious cycle. Cotransfection of Vero cells with 0.6 µg of pTK-CAT, which carries the VZV gene 36 upstream control sequences, with increasing amounts of Tdn IE63 demonstrated that the VZV gene 63 protein could stimulate the CAT activity associated with the TK promoter (Fig. 3A). In the absence of Tdn IE63, the basal CAT activity measured in the presence of 0.6 µg of pTK-CAT led to 3.41% conversion of substrate in the presence of 45 µg of protein. This CAT activity was stimulated up to 28.8-fold in the presence of 2.8 µg of Tdn IE63. It should be noted that a further addition of Tdn IE63 (from 3.0 to 3.4 µg) clearly decreased this optimal stimulation to a lower CAT activity value of about 85% conversion of substrate in the presence of 45 µg of protein. These results show that the VZV gene 63 protein can stimulate the expression of the gene encoding TK, which is probably required for DNA replication (22). This stimulation seemed to be maximum for a pTK-CAT/ Tdn IE63 molar ratio of 2.45; for higher values, this effect decreased slightly without giving rise to repression of the basal CAT activity. RNA analysis carried out on Vero cells transfected with pTK-CAT and increasing amounts of Tdn IE63 unambiguously showed that the *trans* activation mediated by the VZV gene 63 product was induced by an increase in the level of CAT mRNA (Fig. 3B).
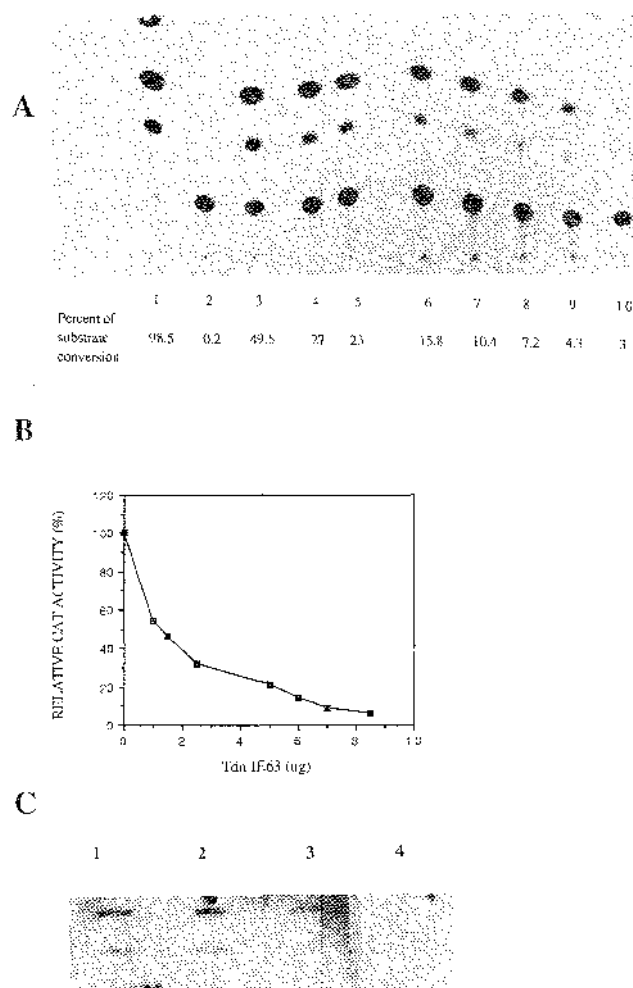
FIG. 2. (A) Effects of plasmid Tdn IE63, which expresses the product of VZV gene 63, on CAT activity controlled by the VZV gene 62 promoter-leader region (p62-CAT). Vero cells were trans-fected with 1.5 µg of p62-CAT alone (lane 3) and with the following amounts of Tdn IE63: 1 µg (lane 4), 1.5 µg (lane 5), 2.5 µg (lane 6), 5 µg (lane 7), 6 µg (lane 8), 7 µg (lane 9), and 8.5 µg (lane 10). The total amount of DNA was brought to 10 µg by addition of sonicated herring sperm DNA. The percentage of substrate converted in the presence of 45 µg of protein is shown at the bottom. The positive control consisted of 1.5 µg of pCMV-CAT (lane 1), and the negative control consisted of 1.5 µg of pSVO-CAT (lane 2). (B) Repression of expression of VZV gene 62 promoter-leader sequences (p62-CAT) by VZV gene 63 product expressed in Vero cells after transfection of various amounts of Tdn IE63. The relative CAT activity is plotted against the amount of Tdn IE63 plasmid. (C) Evidence that the VZV gene 63 product *trans* represses gene 62 expression at the RNA level. Total cellular RNA (2.4 µg) was extracted from Vero cells transfected with 1.5 µg of p62-CAT (lane 1), 1.5 µg of p62-CAT and 1 µg of Tdn IE63 (lane 2), 1.5 µg of p62-CAT and 6 µg of Tdn IE63 (lane 3), or 1.5 µg of p62-CAT and 8.5 µg of Tdn IE63 (lane 4). Hybridization was carried out with a ^{32}P-labeled CAT probe.
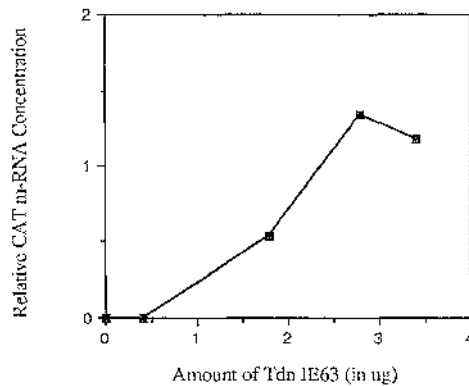
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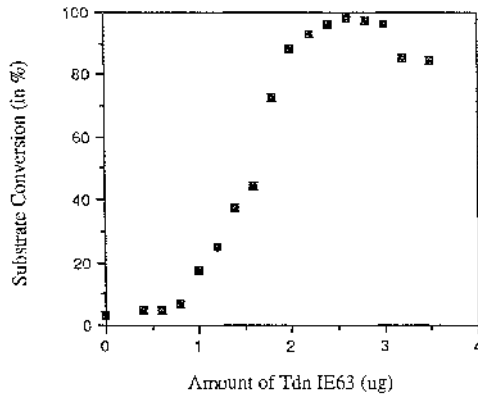
B.

FIG. 3. (A) Activation of expression of VZV gene 36 (encoding TK) promoter-leader sequences (pTK-CAT) by the VZV gene 63 product expressed in Vero cells after transfection of various amounts of Tdn IE63. pTK-CAT (0.6 µg) was transfected with increasing amounts of Tdn IE63 (from 0 to 4.4 µg). The total amount of DNA was brought to 5 µg with sonicated herring sperm DNA. The percentage of substrate converted in the presence of 45 µg of protein is plotted against increasing amounts of Tdn IE63. (B) Stimulation of the level of CAT rnRNA, whose expression is controlled by the VZV gene 36 regulatory region. Transfections were carried out as described for panel A.

Because the VZV gene 63 protein can regulate IE and E gene expression, it is worthwhile to determine whether this protein can regulate L genes. To estimate the basal CAT activity driven by both the gene 68 (gpl) and gene 31 (gpII) control regions, Vero cells were transfected with either pgpI-CAT (7 µg) or pgpII-CAT (7 µg). In both cases, the CAT activity detected 48 h after transfection was low and could be slightly increased when larger amounts of these plasmids were used for transfection. A maximal detected CAT activity of 0.5% substrate conversion with 40 µg of protein could be reached after transfection with 10 µg of pgpII-CAT. Cotransfections of Vero cells by either pgpl-CAT or pgpII-CAT in the presence of increasing amounts of Tdn IE63 were carried out to search for a regulatory activity of the gene 63 protein on the promoters of these L genes. Regardless of the concentrations of the reporter plasmid and Tdn IE63, no significant stimulation or repression of the CAT activity could be observed, demonstrating that the VZV gene 63 protein cannot affect expression of these L genes.

In this study, we have shown for the first time that the protein encoded by VZV gene 63 exhibits important regulatory functions such as repressing the expression of VZV gene 62 and activating the expression of VZV gene 36, at least in vitro. Because these two genes encode proteins important for the virus replication cycle, it can be inferred that the VZV gene 63 product could be essential to terminate the IE phase and to initiate the E phase. Indeed, VZV gene 62 encodes a 175-kDa protein having functional and sequential similarities to ICP4 of HSV-1 (6, 10, 13). Because of these considerable similarities and the fact that the 175-kDa protein largely complements the growth defect that results from the loss of ICP4 function in HSV-1 (6), it is now generally believed that VZV gene 62 encodes an IE protein. The repression of major IE62 protein expression by the VZV gene 63 product is thus a very important result which should lead to a better understanding of how the IE phase

of VZV infection is terminated. Moreover, the observation that the VZV gene 63 product can activate the expression of the TK gene, which encodes a typical E protein required for DNA replication, leads us to think that the VZV gene 63 product could play a pivotal role in the VZV replication cycle by both repressing the expression of the major IE protein and activating an important E protein. To determine whether the VZV gene 63 product is a major repressor of the IE phase, it would be worthwhile to investigate its regulatory effect on other potential IE genes such as VZV genes 4, 61, and 62. In the same manner, activation of other E genes such as those encoding DNA polymerase or ribonucleotide reductase would indicate that this protein is a factor in triggering the E phase and DNA replication. An interesting result also reported in this article concerns the absence of VZV gene 63 product activity on promoters controlling L gene expression.

Neither the gpI nor the gpII promoter can be regulated by this protein. The absence of regulatory effects on pgpI-CAT is not due to the plasmid construct, since Inchauspe et al. have shown that it can be *trans* activated by the VZV gene 4 and 62 products (16). A study of the regulation of pgpII-CAT by VZV gene products 4 and 62 is under way. The VZV gene 63 product might not regulate L gene expression by itself, perhaps needing synergy with either other IE or E proteins or cellular factors.

Because these regulatory functions are very important, it is worthwhile to determine which domains of the protein possess either DNA-binding capacities or regulatory functions. Linker insertion mutagenesis in the gene 63 coding sequence, which has been done for other HSV-1 IE genes (9, 18), should lead to the localization of these functional domains. Investigations of the DNA-binding properties could be done by using nuclear extracts from cells transfected with Tdn IE63 in gel retardation with probes encompassing the entire gene 62 promoter region in p62-CAT. Furthermore, the identification of the target sequence to which the VZV gene 63 product binds can be carried out by footprinting using this protein expressed in prokaryotic cells and probes made of the gene 62 promoter region. Because no DNA-binding activity has been shown to be elicited by ICP22 from HSV-1, it is impossible to deduce from sequence alignments the amino acids involved in DNA binding of the VZV gene 63 product. Moreover, a computer search for a DNA-binding domain in the VZV gene 63 product reveals none of the major motifs associated with this function, such as a helix-turn-helix, zinc finger, or leucine zipper. On the other hand, the regulatory functions also need a mutagenesis approach in order to be detected in the sequence. Although this protein is rather hydrophilic, negatively charged amino acids such as glutamic acid and aspartic acid are very abundant between residues 155 and 168 and between residues 172 and 220, possibly pointing out these two regions as potential activating domains as has been shown for GAL4, GCN4, and VP16 (see reference 21 for a review). It is obvious that mutation analysis should first be carried out with these two regions to characterize activator domains. For the domain involved in the repressing functions, random mutagenesis seems to be required because, on the basis of a computer search, no domain can be suspected to exert this function. All of the information collected by these biochemical studies will help to elucidate the biological properties of this protein and the reason why VZV gene 63 is heavily transcribed in rat neurons persistently infected by VZV (20, 25).

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