Published in : Nucleic acids research (1995), vol. 23, iss. 8, pp. 1341-1349
Status : Postprint (Author's version)

Mutational analysis of varicella-zoster virus major immediate-early protein IE62

Baudoux L, Defechereux P, Schoonbroodt S, Merville M-P, Rentier B, Piette J

Laboratory of Fundamental Virology, Department of Microbiology and laboratory of Clinical Chemistry, Institute of Pathology B23, University of Liege, B-4000 Liege, Belgium

Abstract

The varicella-zoster virus (VZV) open reading frame 62 encodes an immediate-early protein (IE62) that trans-activates expression of various VZV promoters and autoregulates its own expression in transient expression assays. In Vero cells, IE62 was shown to transacti-vate the expression of all putative immediate-early (IE) and early (E) genes of VZV with an up-regulating effect at low intracellular concentrations. To define the functional domains involved in the regulatory properties of IE62, a large number of in-frame insertions and deletions were introduced into a plasmid-borne copy of the gene encoding IE62. Studies of the regulatory activities of the resultant mutant polypeptides in transient expression assays allowed to delineate protein regions important for repression of its own promoter and for transactivation of a VZV putative immediate-early gene (ORF61) promoter and an early gene (ORF29) promoter. This mutational analysis resulted in the identification of a new functional domain situated at the border between regions 4 and 5 which plays a crucial role in the IE62 regulatory functions. This domain turned out to be very well conserved amongst homologous alphaherpesvirus regulatory proteins and appeared to be rich in bulky hydrophobic and proline residues, similar to the proline-rich region of the CAAT box binding protein CTF-1. By immunofluorescence, a nuclear localization signal has been mapped in region 3.

Introduction

Varicella-zoster virus (VZV) is a neurotropic alphaherpesvirus which is responsible for two common, well-defined diseases: chickenpox, upon primary infection, and shingles, after reactivation of latent virus from the dorsal root ganglia. Classic studies of VZV biology have been severely hindered by the inability to produce high-titer cell-free virus with a good infectivity ratio (for reviews see 1,2). Determination of the entire nucleotide sequence of the VZV genome (3), however, has enabled structural and functional comparisons with herpes simplex virus type 1 (HSV-1), a more intensively studied alphaherpesvirus.
In HSV-1 infection, regulation of gene expression is ordered in a cascade fashion (4), implicating highly complex interactions between viral and cellular proteins. The immediate-early (IE, α) genes are transcribed first following penetration of the virus in the absence of *de novo* protein synthesis (5,6). A virion tegument protein, Vmw65 (VP-16, α-Tif), interacts with cellular factors, including the ubiquitous octamer binding protein Oct-1, and forms protein-DNA complexes with the consensus sequence TAATGARAT located upstream in the 5' promoter/regulatory regions of the five IE genes (7-10). The net consequence is stimulation of IE gene transcription by the host RNA polymerase II. Functional IE gene products are required for expression of the other classes of genes (11). Early (E, β) genes, encoding proteins necessary for DNA synthesis, are expressed next. Transcription of the leaky-late (γl) genes starts before viral DNA replication and peaks after DNA synthesis, whereas true-late (γ2) genes are transcribed after DNA synthesis only. Late genes encode virion structural proteins. Insight into the functions of four of the five IE gene products of HSV-1 (i.e. ICPO, ICP4, ICP22, ICP27 and ICP47) has been gained by studies of numerous viral mutants and findings from transient assay experiments (for a review see 12). Four potent VZV IE genes have been proposed as homologs of HSV-1 IE genes [i.e. open reading frame 4 (ORF4), ORF61, ORF62 and ORF63] (2,3,13). VZV genes 62 and 71 (the identical gene is present in both copies of the short repeat regions) encode an immediate-early protein (IE62) which is a relatively large phosphoprotein containing 1310 amino acid residues with a predicted molecular mass of 140 kDa (VZV 140k). However, the actual size of the protein appears to be -175 kDa, as determined by polyacrylamide gel electrophoresis (13-16). IE62 protein is present in the virion tegument (16) and can be found in the nucleus of VZV-infected cells

Published in : Nucleic acids research (1995), vol. 23, iss. 8, pp. 1341-1349
Status : Postprint (Author's version)

(15). In transfection assays, at least, IE62 functions as a powerful transcriptional activator of viral and selected cellular gene expression (17-21) and autoregulates negatively and positively its own promoter (22,23).
IE62 shares considerable predicted amino acid similarity with HSV-1 ICP4 (Vmwl75) and is divided into five regions (1-5) on the basis of sequence homology (3,24). For several reasons IE62 is believed to be the functional counterpart of ICP4: (i) HSV-1 ICP4 mutants can be complemented either by transfected plasmids or by transformed cell lines expressing VZV IE62 (14,25); (ii) a recombinant HSV-1 virus with both copies of the ICP4 coding sequences replaced by the homologous ORF62 gene is viable in tissue culture (26). ICP4 is essential for virus growth in cell culture (27,28) and is required for transcriptional activation of early and late genes and also for repression of IE genes (29). ICP4 acts via a common mechanism which involves binding on DN A and interactions with the cellular proteins TFIIB and TBP (30). Although at present there is no definitive proof for an essential role for IE62 in VZV biology, its ability to regulate expression of VZV genes of all three putative kinetic classes, as well as its functional similarity with ICP4, certainly argues for an important role in the VZV replicative cycle.
Comparison of the predicted primary sequence of VZV IE62 with those of the related proteins expressed by HSV-1 (24), pseudorabies virus (PRV) (31,32) and equine herpesvirus (EHV) type 1 (33) reveals two highly conserved regions (region 2 and 4) interspersed with three other regions (1,3 and 5) that have lesser similarities. Extensive mutagenic studies have revealed that regions 2 and 4 contain sequences that are essential for ICP4 functions in early gene activation and IE gene repression (34-39). Mutations in region 2 can also affect the ability of ICP4 to bind *in vitro* to DNA fragments which encompass the consensus recognition sequence ATCGTnnnnnYSG (34,38-42). Binding to such a consensus sequence at the cap site of the HSV-1 IE-3 and IE-1 promoters can be directly implicated in the ability of ICP4 to repress IE gene transcription (34,43,44), while binding to a similar site upstream of the gD promoter of HS V-1 contributes to promoter activation (45,46). A nuclear localization signal has been mapped within region 3 (34,37,47).
Little is known about the functional domains of the IE62 protein. Recently it has been described that region 2 (amino acids 472-633) is able to bind DNA (42,48) and that the 90 N-terminal residues constitute a potent activator domain rich in acidic amino acids (49,50). This paper describes a mutational analysis of the IE62 protein to further characterize regions of this regulatory protein which are important for both transactivation and autore-gulation. A large number of small in-frame, insertion and deletion mutations have been introduced into the coding region of an expression plasmid bearing the IE62 gene. Short-term transfec-tion assays coupled with immunofluorescence staining have been used to determine the ability of these mutated IE62 proteins to transactivate both the ORF61 and ORF29 promoters and to repress ORF62 promoter activity. This mutational analysis provides information for the definition of a new functional domain located at the border between regions 4 and 5 which plays a crucial role in IE62 regulatory properties.

Materials and methods

Plasmid construction

Reporter plasmids containing the chloramphenicol acetyltrans-ferase (CAT) gene under the control of various VZV regulatory/ promoter regions have been described previously: p4CAT, p61CAT, p63CAT, pMDBPCAT, pPolCAT, pTKCAT, pgpIICAT (51); pgpICAT (19) and p62CAT (52). The IE62 expression plasmid (pSV62) contains the ORF62 gene under the control of the SV40 early promoter (22). Plasmid pMC1 includes the HS V-1 gene encoding Vmw65 under its cognate promoter (6).
Nonsense and in-frame insertions were introduced into the IE62 coding region in the following manner. Plasmid pSV62 (10 µg) was digested with *NlalV* (1 U) in the presence of 50 µg/ml ethidium bromide in order to produce a maximum of singly cut linear molecules with blunt ends in a final volume of 300 µl of the appropriate buffer. Linearized molecules generated by this reaction were eluted from agarose gel (0.8%). The oligonucleotide linkers (5'-GGCTAGTTAACTAGC-3' and 5'-CCCGTTA: ACGGG-3'), containing an *Hpal* restriction site which does not exist in pSV62, were ligated to the linearized plasmid, then digested with an excess *of Hpal* and finally recircularized. After transformation of *Escherichia coli* DH5α, plasmids from individual transformants were analyzed for the presence of the *Hpal* linker. Location of the introduced *Hpal* site was done by digestion with *Sacll.* The first oligonucleotide encodes termination codons in all three reading frames and the second introduces an in-frame insertion.

Published in : Nucleic acids research (1995), vol. 23, iss. 8, pp. 1341-1349
Status : Postprint (Author's version)

The deletion mutants were derived from the insertion mutants which contain a unique *Hpal* site, to generate in-frame deletions. Deletion plasmids were constructed by standard procedures (53). Briefly, to construct pΔ90-124 and pΔ90-570, insertion mutants pHN124 and pHN570 (which contain an *Hpal* insertion site after codons 124 and 570) were digested with *Hpal* and *SnaBl* and then recircularized. To create pΔ612-865, pΔ865-971 and pΔ865-1097, insertion mutants pSN612, pSN971 and pSN1097 (containing a stop codon after codons 612,971 or 1097) were cut with *Hpal* and *NruI* and recircularized. pΔ636-733 was constructed by digesting pHN636 with *Hpal* and partially with *BamHl.* After removing 3' overhangs with T4 DNA polymerase, the plasmid was recircularized. To obtain pΔ90-416, pSV62 was cut with *SnaBI* and *ClaI,* blunted by filling in with Klenow enzyme and religated. Verification of deletions was done by digestion with appropriate restriction enzymes.

DNA transection

Vero cells at a density of 0.8 x 10^6 cells in 35 mm diameter six-well cluster dishes were transfected by the lipofection technique, using cationic vesicules of DOTAP (Boehringer Mannheim, Germany). Various amounts of target constructs were prepared in 100 µl Hank's buffered salt solution. To avoid any promoter competition effects, an equivalent molar amount of SV40 promoter was kept constant by addition of pSVL (which contains the SV40 promoter region present in pSV62 but lacks ORF62 coding sequences); the total amount of DNA in each experiment was kept constant by the addition of sonicated herring sperm DNA. A solution of N-[1-(2,3-dioleoyloxy)propyl)-N,V,N-trimethylammonium methylsulfate] was then added to a final concentration of 10 µg/ml and the mixtures were kept at room temperature for 10 min before being layered on the cells. The cells were washed after 24 h and placed in fresh medium for another 22 h before total protein extracts were made.
CAT assays
CAT assay extracts were prepared 46 h after transfection and CAT activities were determined essentially as described previously (54), based on the use of [^{3}H]acetyl coenzyme A and chloramphenicol as substrates. Briefly, cells were washed once with phosphate-buffered saline (PBS), resuspended in 50 µl 0.1 M
Tris-HCl (pH 7.8) and disrupted by three freeze-thaw cycles. CAT activity was assayed using the whole protein extract. All experiments were repeated at least four times independently.
Immunofluorescence study Vero cells seeded into 10 mm dishes were transfected with 2 µg pSV62 and its derivatives as described above, then washed and refed after 24 h. After a further 24 h, the cells were fixed in acetone/methanol (v/v) and stained. A rabbit antiserum directed against a synthetic peptide corresponding to the C-terminus of the IE62 protein (iepl278; kindly supplied by Dr P. Jacobs, ULB, Nivelles, Belgium) was used at a 1/250 dilution in PBS to detect IE62. Cells were then stained with fluorescein-conju-gated swine anti-rabbit Ig before examination by fluorescence microscopy.

Results

Modulation of VZ V gene promoters by IE62 protein

IE62 could activate the different classes of VZV gene promoters (ORF4, ORF61, ORF31, ORF67 and ORF68) (17,19-21), but could also repress or activate its own promoter in BHK cells (22) or in human T and rat neuronal cells (23) respectively. The activation effect, however, depended strongly upon the amount of IE62 expressed, with an obvious diminution in effect at high IE62 concentrations in T lymphocytes (23). To determine whether the IE62 concentration influenced its regulatory activities in a cell line where VZV could undergo a productive infectious cycle, titration experiments were performed in Vero cells using a unique concentration of target plasmid (1 µg) transfected together with increasing amounts of pSV62 DNA (0-4 µg). The use of pSV62 as expressing plasmid was based on the low SV40 promoter/enhancer responsiveness to IE62 protein (data not shown).
As shown in Figure 1 A, IE62 protein is able to activate all the putative IE gene promoters in Vero cells in a dose-dependent fashion, maximal stimulation (64- and 102-fold) being observed with 500 ng pSV62 on the ORF61 and ORF4 gene promoter. Lower transcriptional activations were detected on the ORF63 (8.3-fold) and ORF62 (4.4-fold) promoters with 10 ng and 2 µg pSV62 added respectively. Above these concentrations, the stimulation diminished. Identical dose-response curves were observed on the early gene promoters (Fig. IB); maximal stimulations of 13-, 42- and 7-fold on pTKCAT, pMDBPCAT and pPolCAT respectively were reached with 500 ng pSV62. No regulatory effect was seen when pgpICAT or pgpIICAT were transfected with increasing amounts of pSV62 (Fig. 1C).

Published in : Nucleic acids research (1995), vol. 23, iss. 8, pp. 1341-1349
Status : Postprint (Author's version)

To fully characterize the regulatory properties of IE62 on its own promoter, Vero cells were transfected with p62CAT (4 µg) and increasing amounts of pSV62 (0-6 µg). The ORF62 promoter was very effective at driving CAT gene expression in Vero cells (51), in contrast to the situation recorded by others in baby hamster kidney cells (22). Using these experimental conditions, where the intrinsic activity of the ORF62 promoter was high enough to detect either a repressing or a transactivating effect, we showed (Fig. ID) that the IE62 protein was able to transactivate its own promoter at concentrations below 1 µg transfected pSV62. Above this concentration, transactivating effects disappeared and the ORF62 promoter was repressed by IE62 protein, as described previously (22). Some authors have earlier reported that gene 62 promoter activity is increased ~ 15-fold by co-transfection with a plasmid expressing Vmw65, the HSV-1 virion-associated IE promoter transactivator (52).

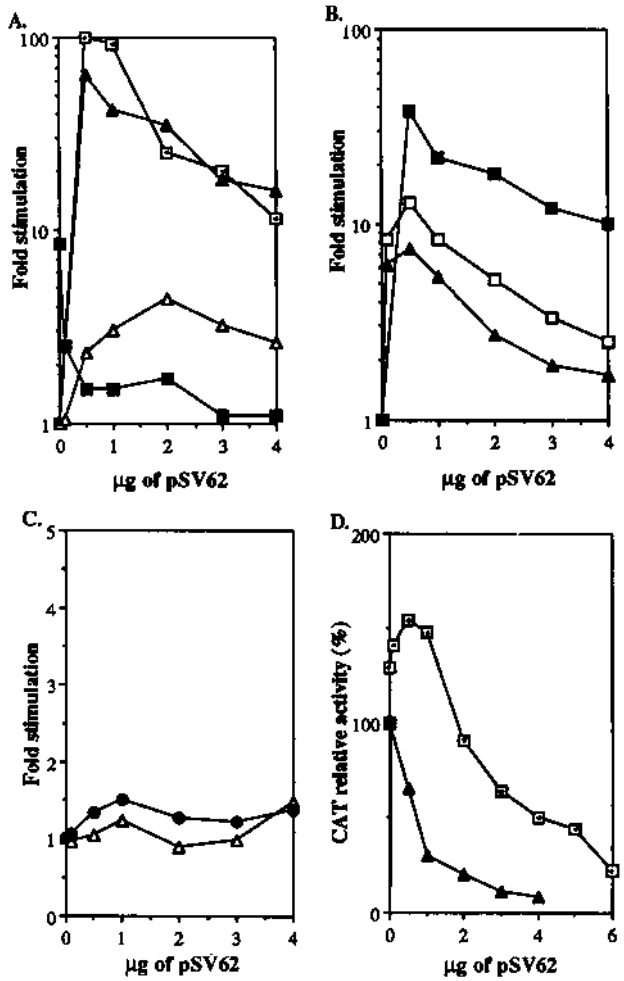


Figure 1. Regulation of the VZV promoters by IE62. (A) Dose-dependent activation of the putative IE gene promoters. Vero cells were co-transfected with 1 µg p4CAT (□), p61CAT (A), p62CAT (Δ), p63CAT (■) and increasing amounts of pSV62 by lipofection. (B) Effect on the putative E gene promoters. Vero cells were transfected by 1 µg pTKCAT (□), pPolCAT (A) or pMDBPCAT (■) and pSV62 in the indicated amounts. (C) Effect on the L gene promoters. Vero cells were co-transfected with 1 µg pgpICAT (Δ) or pgpHCAT (•) and increasing amounts of pSV62. Cells were harvested 46 h after transfection and CAT activities were assayed using total protein extract under each condition. Initial CAT rate activities were calculated from kinetics and data are presented as fold induction of CAT activity relative to the uninduced values, obtained with the reporter plasmid alone and arbitrarily set to 1.0. Mean values from at least three independent experiments are presented. (D) Regulation of the VZV gene 62 promoter by IE62. Vero cells were transfected with 4 µg p62CAT and increasing amounts of pSV62 without (B) or with (A) pMC1 plasmid (2 µg) expressing HSV-1 Vmw65. Initial CAT reaction rates were calculated from kinetics and data are presented as percentage of CAT activity of the value obtained with p62CAT without effector plasmid, arbitrarily set to 100%. These experiments have been repeated at least four times.

Published in : Nucleic acids research (1995), vol. 23, iss. 8, pp. 1341-1349
Status : Postprint (Author's version)

When a plasmid expressing Vmw65 (pMC1, 2 µg) was transfected together with p62CAT and increasing amounts of pSV62 (0-4 µg), no transactivating effect of the IE62 protein on its own promoter could be detected, because the resulting CAT activity was too high to measure any putative stimulation. Instead, Vmw65-stimulated levels of CAT activity from p62CAT were repressed up to 50-fold under these experimental conditions (Fig. ID), as described by others (22).

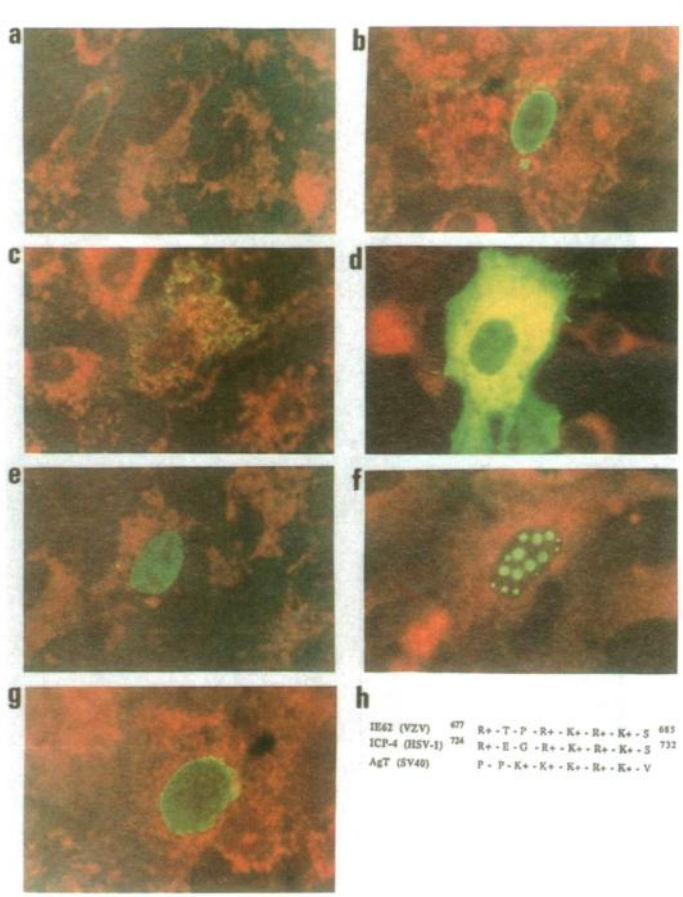


Figure 2. Expression of wild-type and deleted IE62 protein in Vero cells. Cells were transfected with (a) irrelevant plasmid, (b) pSV62, (c) pΔ636-733, (d) pΔ612-865, (e) pΔ90-416, (f) pΔ90-570 or (g) pΔ865-1097 and reacted with polyclonal antibody (iep1278) before immunofluorescence detection, (h) The nuclear localization signals of SV40 large T antigen and HSV-1 ICP4 are presented for comparison with the putative nuclear localization signal of IE62.

Linker scanning mutagenesis

As shown earlier, the IE62 protein could positively transactivate putative IE and E promoters in short-term transfection experiments. In order to determine which IE62 regions were involved in these activities, a panel of 14 nonsense, 18 in-frame insertions and seven deletions were introduced into IE62 encoding sequences inserted in pSV62. Nonsense and in-frame mutations were introduced by insertion of oligonucleotides containing a new *Hpal* site. Functional analysis of the truncated IE62 mutants created by inserting translation^ stop codons in the DE62 gene constituted a good approach for the potential regulatory domains situated at the C-terminus, but not in the N-terminal regions. Indeed, such mutations produced larger truncations for which the biological relevance was questionable. In-frame insertions along the IE62 gene are better suited to study the N-terminal part of the protein and they are essential to delineate functional domains precisely. Several deletion mutants were built up to confirm the results obtained with truncated and insertion mutants and were constructed by excision of specific segments within the IE62 gene.
In order to visualize the production and localization of the mutated proteins and to verify the stability of the mutants, indirect immunofluorescence studies were performed on Vero cells transfected with

Published in : Nucleic acids research (1995), vol. 23, iss. 8, pp. 1341-1349
Status : Postprint (Author's version)

wild-type or mutated IE62-expressing plasmids. We used a rabbit antiserum directed against a synthetic oligopeptide corresponding to the C-terminus of IE62. However, a few nonsense mutants lacking the epitope could not be recognized and were not tested. The cells transfected with pSV62 (Fig. 2b) exhibited a strong nuclear fluorescence, revealing the accumulation of IE62 protein in the nucleus, as in VZV-infected cells (15). All in-frame mutated IE62 proteins and the deletion proteins expressed by pΔ90-124 and pΔ865-971 were localized in the nucleus, as did the wild-type protein (data not shown). Some deletions gave rise to disperse cytoplasmic immunofluorescence, especially mutants encoded by plasmids pΔ636-733 and pΔ612-865 (Fig. 2c and d). These results indicated that a signal required for proper nuclear localization lay between amino acids 636 and 733 in region 3 of the protein. In this region there exists an amino acid sequence (Fig. 2h) similar in composition and charge character to the nuclear localization signal of SV40 large T antigen (55) and HS V-1ICP4 (47). The deletion mutant lacking residues 90-570 exhibited a granular nuclear localization (Fig. 2f), whereas the deletion mutant pΔ90-416 showed nuclear staining (Fig. 2e), like that observed with pSV62. A signal was thus required for correct intranuclear localization and it lies between amino acids 416 and 570. The limits of this signal were not more precisely defined in this study and we cannot exclude the possibility that intranuclear localization of the wild-type protein could be due to cooperation between several amino acid stretches. IE62 protein deleted between residues 865 and 1097 (region 4) exhibited a nuclear localization in transfected cells (Fig. 2g). All the mutated proteins exhibited an immunostaining similar in intensity to the wild-type protein, demonstrating that the mutations did not affect the level of expression and the stability of the mutated proteins.

Mapping of IE62 transactivating domains involved in ORF61 promoter stimulation

To detect sequences within IE62 which could be involved in transactivation of a potent immediate-early gene promoter, transactivating phenotypes of truncated, insertion and deletion mutants were examined by transient transfection experiments. In a first step, transfections were performed with both p61CAT (1 μg) and some mutated IE62 expression plasmid (0.5,1 and 2 μg) and these experimental conditions gave similar results (data not shown). In order to be near the optimum transactivation level of pSV62 on the ORF61 promoter and to minimize CAT activity fluctuations due to variations in DNA quantities, Vero cells were co-transfected with 1 μg p61CAT and 1 μg expression plasmid, in either wild-type or mutated form, as reported above. In each transfection experiment, two positive control assays (with pSV62) were included to determine precisely the relative activities of the IE62 mutants. Transactivating activities of the various mutants are given in Table 1, together with standard errors of the mean of individual values, and are plotted in Figure 3.
As shown in Table 1, the truncated peptide expressing only the first 1263 residues (pSN1263) was able to transactivate the ORF61 promoter to the same extent as the full-length protein, whereas polypeptides expressed by plasmids pSN1042, pSN1097, pSN 1115 and pSN 1137 lost ~ 80% of the transactivat-ing activity exhibited by the controls. Although their transactivat-ing properties were strongly reduced, they could still stimulate the ORF61 promoter 4-fold. On the other hand, shorter truncated IE62 proteins, like those expressed by pSN971, pSN960 and pSN859, were not able to significantly stimulate ORF61 promoter activity. This indicated that an important functional domain is situated at the border between regions 4 and 5, with its C-terminus positioned at around residue 1137. Indeed, the deletion mutant pΔ865-1097 completely lost its transactivating properties, whereas pΔ865-971 retained partial stimulating activity on the ORF61 promoter (Fig. 3D), demonstrating unambiguously the functional relevance of this particular domain located in region 4. The results obtained with insertion mutants confirmed the importance of this region, because insertions of five amino acids after residue 1137 strongly reduced the transactivating properties of IE62 (Table 1).
Other deletion mutants were also investigated (Fig. 3D). Mutations in region 1 of the protein did not seem to have important consequences for IE62 transactivating properties, proteins where residues 90-124 and 90-416 had been deleted fully transactivating the ORF61 promoter. On the other hand, stimulating activity was lost when deletions removed regions 2 or 3 (pΔ90-570, pΔ636-733 and pΔ612-865). The fact that these deleted proteins, pΔ636-733 and pΔ612-865, were localized in the cytoplasm instead of the nucleus (Fig. 2c and d) could explain why both lost their stimulating effect on the ORF-61 promoter. Analysis of the transactivating phenotype of the insertion mutants confirmed these results. While most of the insertion mutants preserved an induction similar to that of the wild-type protein, proteins with an insertion located in region 2 (pHN458,472 and 570) partially or completely lost their transactivating properties, confirming the crucial role of the DNA binding activity of region 2 (42,48) in IE62 regulatory functions.
Table 1. Summary of positions and activities of the insertion mutants

Published in : Nucleic acids research (1995), vol. 23, iss. 8, pp. 1341-1349
Status : Postprint (Author's version)

Mutant	Inserted AA	Site[a]	Transactivating activity on IE61[b]	Transactivating activity on MDBP[b]	Repressing activity[c]
pIE1-124	124-GPVNG	372	98 (19)	96 (14)	82(16)
pIE1-255	255-VPLTG	764	97 (17)	99(21)	87 (14)
pIE1-435	435-DPLTG	1304	98 (17)	82 (14)	80(15)
pIE1-459	459-GPLTG	1376	49(10)	67 (12)	90(15)
pIE1-473	473-APLTG	1418	34(9)	48 (10)	98 (25)
pIE1-510	510-DPLTG	1529	68 (12)	87 (15)	96(18)
pIE1-570	570-GPVNG	1710	5(4)	26(6)	1(1)
pIE1-636	636-GPVNG	1908	97 (15)	90(15)	88(16)

Published in : Nucleic acids research (1995), vol. 23, iss. 8, pp. 1341-1349
Status : Postprint (Author's version)

657-APLTG	1970	100 (25)	98(18)	85 (14)
747-APLTG	2240	71 (12)	100 (23)	95 (23)
764-VPLTG	2291	60(11)	77(11)	83(13)
859-GPVNG	2577	95 (26)	100(15)	80(15)
870-GPLTG	2609	74(11)	100(19)	73(13)
960-GPVNG	2880	98(16)	85 (15)	83(12)
1115-GPVNG	3345	68(10)	60(11)	68(13)
1138-DPLTG	3413	21(6)	4(3)	61 (13)

Published in : Nucleic acids research (1995), vol. 23, iss. 8, pp. 1341-1349
Status : Postprint (Author's version)

1182-GPVNG	3546	62(9)	84 (16)	66(12)
1264-APLTG	3791	76 (12)	55(9)	83(8)
544-PRstop	1630	3(2)	4(1)	0
613-ARstop	1837	4(2)	10(3)	0
656-AASstop	1970	10(5)	9(4)	1(1)
733-EASstop	2201	12(5)	12(5)	3(2)
758-GLVNstop	2964	10(6)	7(3)	2(2)
859-GGSstop	2577	23(8)	11(1)	40(6)
960-GGSstop	2880	15(6)	6(2)	47(13)

Published in : Nucleic acids research (1995), vol. 23, iss. 8, pp. 1341-1349
Status : Postprint (Author's version)

972-ARstop	1837	14(5)	7(2)	50 (10)
1042-GGSstop	3129	23(7)	10(2)	48(8)
1097-ARstop	3292	25(7)	14(5)	50(8)
1115-GGSstop	3345	24(9)	10(6)	52(12)
1137-EASstop	3413	23(9)	7(4)	46(11)
1263-AASstop	3791	96 (10)	98 (25)	66(12)
1303-RLVNstop	3910	101 (9)	100 (24)	100(5)

Published in : Nucleic acids research (1995), vol. 23, iss. 8, pp. 1341-1349
Status : Postprint (Author's version)

[a]*The coordinate given is the position in the recognition sequence of the restriction enzyme (NlaIV) site at which insertion occurs, numbering from the first base of the reading frame.*

[b]*Activation of the IE61 or MDBP gene promoters (using p61CAT or pMDBPCAT) is given as a percentage of that obtained in parallel experiments with pSV62. The mean of at least four independent determinations is given, with the standard errors of the mean in parentheses.*

[c]*The quantitative ability to repress the IE62 promoter on p62CAT is given as a percentage of pSV62 (100%) and the standard errors of the mean of at least four independent transfections is shown in parentheses.*

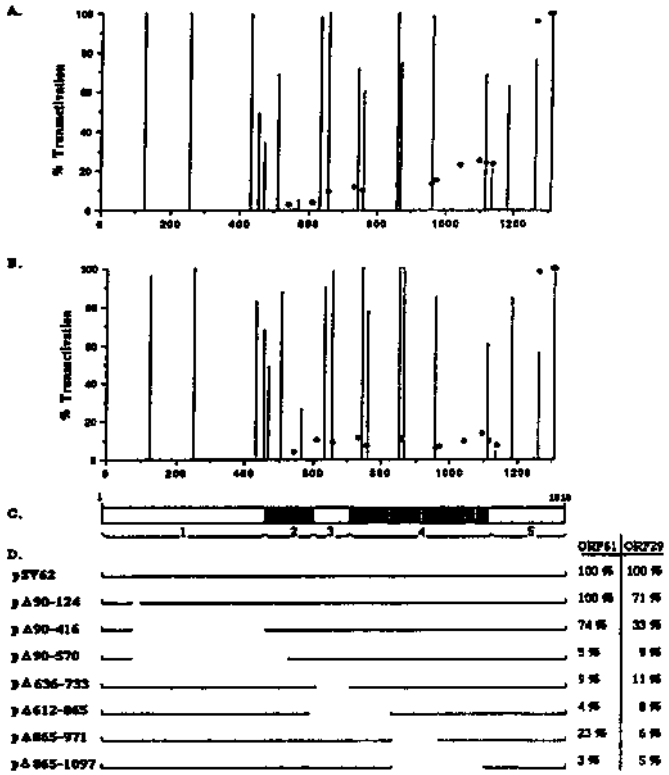


Figure 3. Relative transactivating activities of IE62 mutated proteins. (A) Transactivation values promoted by each insertion mutant on the ORF61 promoter are plotted above insertion site in the coding region. Insertion mutants are represented by bars and nonsense insertion mutants by black dots. Percentages of transactivation are given in Table 1. (B) Transactivation values on the ORF29 promoter exhibited by each insertion mutant as described above. (C) Structure of the IE62 protein. JE62 was divided into five regions (1-5) on the basis of sequence homology with HSV-1 ICP4 (24). Amino acid residues are numbered from 1 to 1310 from N- to C-terminus. Homologous sections are shown as boxes; white, hatched and black boxes correspond respectively to regions with low, medium and high homologies. (D) Deletion mutants used in this study and their relative stimulating activities on the ORF61 promoter or the ORF29 promoter. Transfections were performed in Vero cells with 1 µg p61CAT and 1 µg pSV62 or its derivatives.

Mapping of the EE62 transactivation domains involved in MDBP promoter activation

To detect IE62 sequences involved in transactivation of a putative early gene promoter, we have examined the transactivating phenotype of the truncated, insertion and deletion mutants on the ORF29 promoter regulating the gene encoding the major DNA binding protein. Transfections were performed as described above except for use of the pMDBP-CAT plasmid (1 µg). The global results are presented in Table 1 and in Figure 3.

The truncated peptide expressing the first 1263 residues (pSN1263) was able to stimulate the ORF29 promoter like the full-length protein, whereas the polypeptide expressed by pSN1137 lost all its transactivating activity, while preserving partial stimulating activity on the ORF61 promoter (Table 1). This indicated that the amino acid sequence lying between residues 1137 and 1263 could be essential for IE62 activity on putative early gene promoters.

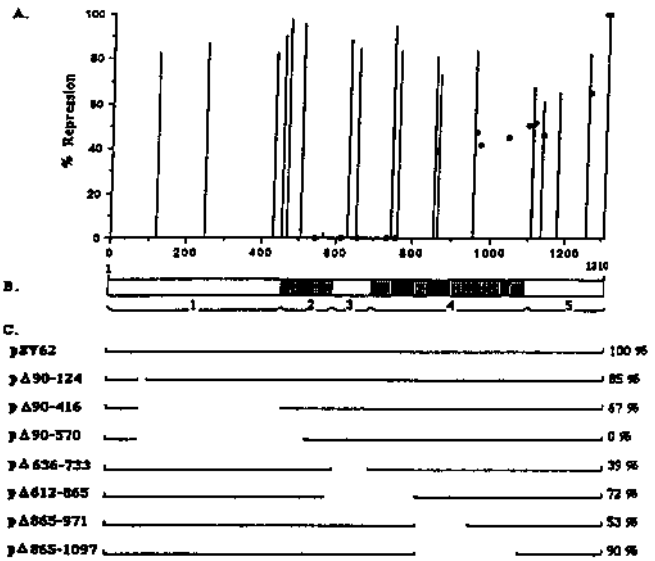
Deletion of sequence 90-124, in region 1 of the protein, had little effect on the transactivating

Published in : Nucleic acids research (1995), vol. 23, iss. 8, pp. 1341-1349
Status : Postprint (Author's version)

properties, whereas the deletion mutant pΔ90-416, still active on the ORF61 promoter, lost part of its activity on the ORF29 promoter (Fig. 3D). All stimulating activities were extinguished when deletions removed regions 2 or 3 (pΔ90-570, pΔ636-733 and pΔ612-865), but these mutants exhibited incorrect cellular localization, as shown previously (Fig. 2c, d and f)- Again, region 4 turned out to be very important for stimulation of the ORF29 promoter, because the deletion mutations expressed by pΔ865-971 and pΔ865-1097 were unable to transactivate this promoter. Moreover, amino acids 865-971 seemed to be needed more for transactivation of the MDBP gene promoter than of the ORF61 promoter. Indeed, mutant pΔ865-971 completely lost its capacity to stimulate the ORF29 promoter, while keeping 36% of full transactivating activity on the ORF61 promoter (Fig. 3D).
Analysis of the transactivating phenotype of the insertion mutants confirmed these results (Fig. 3B, Table 1). While most insertion mutants exhibited induction similar to the wild-type protein, some mutations located in regions 2 (pHN 458,472 and 570) or 4 (pHN 1137) partially or completely lost their transactivating properties on the ORF29 promoter. These results suggest an important role for the DNA binding activity of region 2 (42,48) in transactivation of the ORF29 promoter. As reported for the ORF61 promoter, region 4/5 is also essential for the transactivating activities of IE62.

Mapping of IE62 domains involved in transrepressing activity

It has been shown that the IE62 protein could repress its own gene transcription (22). To determine which regions of the protein are involved in this activity, the repressing phenotype of insertion and deletion mutants was examined on the IE62 promoter. Transfec-tions were performed with p62CAT (4 µg) and pSV62 or its derivatives (4 µg). The results of these transfections are summarized in Table 1 and in Figure 4. To measure repression of the IE62 promoter activity, at least five transfection experiments were performed for each mutant and in all transfection assays two positive controls were included (see above). Transfections were performed in parallel with pMC1, which expresses the HSV-1 Vmw65 protein, and gave similar results to those obtained without pMC1 (data not shown).
These data show that the two phenotypes exhibited by IE62 (repression and transactivation) could be separated in some cases and were not determined by exactly the same protein regions. Most mutations introduced in IE62 did not greatly affect its repressing activity and only region 2 turned out to be highly sensitive to disruption (pHN570 and pΔ90-570; Table 1 and Fig. 4C). Proteins deleted in region 1 (pΔ90-124 and pΔ90-416) were still able to repress the ORF62 promoter (Fig. 4C). The mutant expressing only the first 859 residues (pSN859) did not completely lose its repressing activity (40% of the wild-type protein; Table 1), whereas it no longer transactivated. On the other hand, the polypeptide containing the first 758 amino acids (pHN758), spanning regions 1-3, failed to repress the IE62 promoter (Table 1). However, deletion of amino acids 612-865 retained >70% of the repressive activity (Fig. 4C), so the difference between the repressing properties of the truncated mutants pSN758 and pSN859 could be due to an improper conformation of the protein expressed by pSN758. Unexpectedly, a large deletion encompassing region 4 (pΔ865-1097; Fig. 4C) led to an IE62 mutant with higher repressive capability than the smaller deletion made in this region (pΔ865-971; Fig. 4C).



Published in : Nucleic acids research (1995), vol. 23, iss. 8, pp. 1341-1349
Status : Postprint (Author's version)

Figure 4. Relative repressing activities of IE62 mutants on p62CAT compared with the wild-type protein as described in the legend to Figure 3. Vero cells were transfected with 4 µg p62CAT and 4 µg pSV62. (A) Transrepression exhibited by each insertion mutants. (B) Map of the IE62 protein. (C) Relative repressing activity of each deletion mutant.

It should be noted that deletion of region 3 (pΔ636-733 and pΔ612-865) only partially affected the repressing phenotype (Fig. 4C), although these deleted proteins were located mainly in the cytoplasm of the transfected cells (Fig. 2c and d).

Discussion

Regulatory functions of IE62

The ORF62 gene product of VZV is a major regulatory protein capable of activating viral genes of all three putative kinetic classes. Some authors (19,21) have shown transactivation of the ORF4, ORF61 and TK gene promoters by IE62. However, most of the transient expression experiments reported in the literature were performed using only one concentration of IE62 expression plasmid (19,21). The IE62 protein can also repress or activate its own expression, depending on the cell lines used (22,23), and the activation is clearly dependant on the amount of IE62 expressed in the system (23). To determine whether or not IE62 concentration directly influences its regulatory activities, dose-response experiments were performed in Vero cells. Under our experimental conditions, IE62 was shown to be able to transactivate the expression of all putative IE genes (ORF4, ORF61 and ORF63) and E genes (TK, Pol and MDBP) in a dose-dependent fashion. Since the maximum CAT stimulations were recorded at low amounts of transfected pSV62, we postulated that transactivating effects due to IE62 could only occur when this protein was weakly expressed in the infected cells; probably mainly during the initial steps of the replicative cycle. This hypothesis is supported by the fact that during similar experiments, IE62 could activate its own promoter only at weak concentrations of transfected pSV62, an important repressing effect being observed at higher concentrations. This could be explained by the fact that the high affinity.
DNA binding site(s) involved in transactivation could be rapidly saturated and at higher IE62 intracellular concentrations it bound to other sites, leading to repression of its own expression. It was recently shown (16) that both the ORF10 and ORF62 gene products were present in the virion tegument. However, the ORF10 product seemed only able to stimulate the ORF62 promoter (56) and could not affect expression of ORF4 and ORF61 (57), unlike its HSV counterpart, Vmw65, which is a potent transactivator of all HSV IE genes (5,6,58). It was then proposed that this incorporation of ORF10 and ORF62 products into the virion might be a compensatory mechanism to ensure rapid expression of VZV IE genes upon entry into a susceptible cell (16,56,57). After release in the host cell, the virion-associated transactivator ORF10 would enhance the initiation of VZV replication by increasing the expression of IE62, which could then rapidly transactivate the other IE genes and enhance the efficiency of VZV infection (57). This possibility has been illustrated nicely in recent works (21,23). In the present study we have demonstrated the ability of the IE62 protein to up-regulate expression of the ORF4, ORF61 and ORF63 genes of VZV at low intracellular concentrations.
The role of IE62 on L gene expression still appears controversial. In transient expression assays, a 6-fold stimulation of the gE promoter was observed using the pGi26 effector plasmid expressing the IE62 protein under the control of the ORF62 promoter (19). Other authors (21,59) observed a stimulation of the jgl promoter with pCMV62 (containing the IE62 gene under the control of the HCMV IE promoter), but no effects were recorded using the pGi26 expression vector. No regulatory effects were detected on the gC promoter (21). Under our experimental conditions, the gE and gB promoters were consistently refractory to any activation by IE62. This difference could be due partly to use of the SV40 early promoter in the effector plasmid used in this study, instead of the strong HCMV IE promoter, so that IE62 would not be expressed at sufficient intracellular concentrations to transactivate gE and gB gene promoters. Alternatively, it should be noted that the pgpIICAT plasmid, which carries the gB gene promoter, might lack critical cis-elements essential for activation by the IE62 protein, even though this construct bears putative regulatory sequences extending from nucleotide -258 to -8 (relative to the gB ATG codon) (51).

Functional domains of the IE62 molecule

Published in : Nucleic acids research (1995), vol. 23, iss. 8, pp. 1341-1349
Status : Postprint (Author's version)

IE62 is a large phosphoprotein which is structurally and functionally complex. Five intramolecular regions have been proposed, mainly based on the amino acid sequence conservation between VZV and HSV-1 homologs (24). In order to define which regions of IE62 contribute to the regulatory functions of the molecule, a panel of 14 truncated, 18 in-frame insertion and seven deletion mutants were assayed for their respective abilities to transactivate the ORF61 and the putative early MDBP gene promoters, as well as to repress the ORF62 promoter.

Our results show that the two large regions of homology between VZV IE62 and HSV-1ICP-4, regions 2 and 4, do indeed correspond to functional entities, as defined by the sensitivities of their activities to disruption. In particular, region 2 represented a very critical domain for all phenotypic effects studied. Integrity of the highly conserved region 2 is essential for DNA binding and transcriptional regulatory functions of HSV-1 ICP4 (34,38). It was shown that this IE62 region expressed in bacteria as a fusion or a non-fusion polypeptide, particularly amino acids 472-633, was able to bind to numerous DNA sequences throughout the VZV gene 62 promoter region (42,48), the HSV-1 IE3 promoter (42,60) and the HSV-1 gD promoter (60).

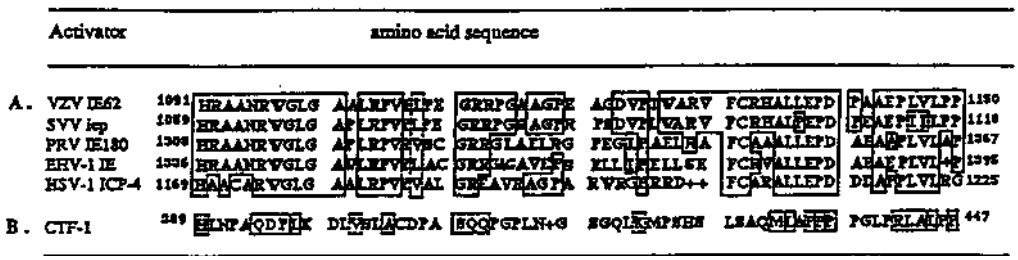


Figure 5. Amino acid sequence of a putative activating domain of IE62 situated at the border between regions 4 and 5. (A) Amino acids of this IE62 sequence protein are aligned with the corresponding regions found among other alphaherpesvirus IE62 homologs (SVV, PRV, HSV-1, EHV-1). (B) Sequence comparison of the C-termini of CAAT binding proteins. Numbers indicate the boundaries of the domains in the context of the native proteins. Boxed amino acids are identical residues and hatched boxes show similar residues. Gaps (+) have been introduced into the sequence for the best alignment.

A region was identified within the DNA binding domain of IE62 and ICP4 that showed striking similarity to the DNA recognition helix of the homeodo-main, with a highly conserved quartet of residues, WLQN (543-546) (60). Moreover, one IE62 mutant containing an insertion mutation in the codon Gly471 was unable to repress its own promoter (22). Recently it was demonstrated that substitution of IE62 Lys548, just adjacent to the WLQN region, drastically reduced the DNA binding activity of the IE62 DNA binding domain and this protein failed to activate gene expression (60). Substitutions of Gln545 or Asp546 resulted in alteration of the DNA binding interaction and reduced transactivation (60). Our results are in good agreement with these previous studies. The complete loss of regulatory activities of a mutant protein containing a deletion in region 2 confirmed that region 2 of IE62 was the most critical region for both repression and transactiva-tion. Moreover, we showed that an insertion in codon Gly570 completely abolished these activities, confirming that the sequences around this amino acid or the residue itself were critical for the regulatory properties. However, the molecule containing an insertion after Ala472 remained partially transactivating and totally repressing, whereas insertion at position 471 completely modified the repressive functions (22). In view of the mutational dissection of the HSV-1ICP4 molecules (34,35,37-39) and other studies (22,48,60), we suggest that these mutations in region 2, which affect the regulatory activities, are responsible for extensive modifications of the DNA binding properties of the protein. Our results show that regions 4 and 5 are clearly of high significance for transactivation. Phenotypes of truncated mutants in the C-terminus of the protein suggest that no residue in the 1263-1310 stretch plays a role in IE62 functions. However, peptides truncated within region 4 and insertion mutants at Leu 1137 are severely impaired in their transactivating activities. Interestingly, they were not impaired to the same extent in their ability to repress gene expression, integrity of this region being much less important for autoregulation. Curiously, a larger deletion, pΔ865-1097, had greater repressing activity than the smaller deletion pΔ865-971. One explanation for this phenomenon could be that an altered part of region 4 which was still present in pΔ865-971 might have disordered the overall protein shape, affecting distant functional sites involved in repression but not in transactivation. Another observation from the results presented above was the importance of amino acids 971-1263 for transactivating the early MDBP gene promoter,

rather than for activating the ORF61 gene promoter. Some authors have made a similar observation in the case of HSV-1 (37), demonstrating that region 4 of ICP4 was more important for transactivation than for repression. Studies of nonsense and deletion mutations in both copies of the ICP4 gene in HSV-1 demonstrated that alterations in region 4 and/or 5 affected late gene expression, DNA synthesis and/or intranuclear localization of the protein (34). Since deleted proteins in region 4 exhibited a nuclear distribution identical to that of the full-length protein, we excluded the possibility that this IE62 region is involved in intranuclear localization. Moreover, as the DNA binding domain of IE62 is located in region 2 (42,48), it seems very unlikely that the IE62 proteins exhibiting a loss of transactivation by mutation in region 4/5 are defective in DNA binding.

Interestingly, the sequence around Leu 1137 of DE62, located between the C-terminus of region 4 and the N-terminus of region 5, is very well conserved among the closely related regulatory proteins of alphaherpesviruses, such as the HSV-1 ICP4, SVV IE62 and PRV IE180 proteins and the EHV-1 IE gene product (Fig. 5 A). This sequence appears to be rich in bulky hydrophobic residues. Moreover, alignment of these amino acid sequences with the CAAT box binding protein (Fig. 5B), which exerts its regulatory effects through a proline-rich region (61), reveals a motif characterized by a preponderance of carbonyl-containing amino acids flanking bulky hydrophobic residues (62). We are currently investigating possible involvement of this border between regions 4 and 5 in a tripartite complex with basal cellular transcriptional factors like TBP and/or TF1ID, as has been proposed for the CAAT binding factor (63) and for HSV-1 ICP4 (30).

Residues 90-416 in region 1 clearly had no essential effect on the regulatory functions of IE62, except perhaps amino acids 124-416, which might contain a sequence required for full transactivation of the ORF29 promoter. Previously, using yeast GAL4 protein chimeras, other authors have shown that the N-terminal 161 amino acids of the molecule contain a potent activator domain and that much of the transactivation activity was contained within the first 90 acidic-rich residues. Mutant proteins lacking this activation domain were unable to transactivate the ORF4 promoter in transient expression assays (49,50). Our results show that the remaining residues of region 1 (90-416) do not play a significant role in the regulatory functions of IE62.

Deletion of region 3 completely abolished the stimulating activities of the IE62 protein and left the protein with a cytoplasmic localization in transfected cells, suggesting that nuclear localization is essential for transactivation. Similar results were observed when the homologous region in HSV-1ICP4 was deleted (34,37). That part of region 3 which is present in both proteins is a single stretch of five perfectly conserved amino acids (Fig. 2h). This sequence shows remarkable similarities to the nuclear localization signal of SV40 large T antigen (55). Recently this sequence of the ICP4 protein was precisely identified as the nuclear localization signal (47). The functional analysis performed in this study, together with the similarity to the SV40 and ICP4 nuclear localization signals, suggest strongly that this sequence could be essential for nuclear localization.

In conclusion, this paper describes a mutational analysis of the IE62 protein in order to define its functional regulatory domains. Besides the DNA binding domain located in region 2 and the acidic transcriptional activation domain in region 1, our results defined a new functional domain situated in region 4/5 playing a crucial role in the IE62 transactivation functions and showing similarity with the proline-rich activator domain of the CAAT box binding protein. By immunofluorescence experiments, a nuclear localization signal has been mapped to region 3.

Acknowledgements

We are grateful to Dr Everett (MRC Glasgow, UK) and Dr J. Ostrove (Bethesda, MD, USA) for providing plasmids and Dr R Jacobs (University of Brussels, Belgium) for the IE62 antibody. This work was supported by grants from the Belgian National Fund for Scientific Research (NFSR), SmithKline Beecham Biologicals (SKBB) and the Belgian National Lottery. LB is supported by an IRSIA grant, PD by a NFSR-SKBB grant and SS by a SKBB grant. JP and M-PM are respectively Research Director and Research Associate of the Belgian NFSR.

References

[1] Davison,A.J. (1991) *J. Gen. Virol,* 72,475-186.
[2] Ostrove,J.M. (1990) *Adv. Virus Res.,* 38,45-98.
[3] Davison,AJ. and ScottJ.E. (1986) *J. Gen. Virol,* 67, 1759-1816.
[4] Honess,R.W. and Roizman,B. (1974) /. *Virol,* 14, 8-19.

[5] Batterson,W. and Roizman,B. (1983) *J. Virol,* 46, 371-377.
[6] Campbell,M.E.M, PalfreymanJ.W. and Preston.C.M. (1984) *J. Mol. Biol,* 180, 1-19.
[7] Gerster,T. and. Roeder,R.G. (1988) *Proc. Natl. Acad. Sci. USA,* 85, 6347-6351.
[8] Machem,S. and Roizman,B. (1982) *J. Virol,* 44, 939-949.
[9] 0'Hare,P. and Goding,C.R. (1988) *Cell,* 52,453-445.
[10] Preston,C.M., Frame,M.C. and Campbell,M.E.M. (1988) *Cell,* 52, 425^34.
[11] Honess,R.W. and Roizman,B. (1975) *Proc. Natl. Acad. Sci. USA,* 72, 1276-1280.
[12] Everett,R.D. (1987) *Anticancer Res., 1,* 589-604.
[13] Shiraki,K. and Hyman,R.W. (1987) *Virology,* 156,423-426.
[14] FelserJ.M., Kinchington,P.R., Inshauspe,G., Straus,S.E. and OstroveJ.M. (1988) *J. Virol,* 62, 2076-2082.
[15] Forghani,B., Mahalingam,R., Vafai,A., Hurst J.W. and Dupuis,K.W. (1990) *Virus Res.,* 16, 195-210.
[16] Kinchington,P.R., HouglandJ.K., Arvin,A.M, Ruyechan,W.T. and HayJ. (1992) *J. Virol,* 66, 359-366.
[17] Cabirac, G.F., Mahalingam, R., Wellish, M. and Gilden, D.H. (1990) *Virus Res.,* 15, 57-68.
[18] Everett,R.D. (1984) *EMBO J.,* 3, 3135-3141.
[19] Inchauspe,G., Nagpal,S. and OstroveJ.M. (1989) *Virology,* 173,700-709.
[20] Inshauspe,G. and OstroveJ.M. (1989) *Virology,* 173,710-714.
[21] Perera,L.R, MoscaJ.D., Ruyechan,W.T. and Hay,J. (1992) *J. Virol,* 66, 5298-5304.
[22] Disney,G.H., McKee,T.A., Preston,C.M. and Everett,R.D. (1990) *J. Gen. Virol,* 71, 2999-3003.
[23] Perera,L.R, Mosca,R.D., Sadeghi-Zadeh,M., Ruyechan,W.T. and Hay,J. (1992) *Virology,* 191, 346-354.
[24] Mc Geoch,D.J., Dolan,A., Donald,S. and Braver,D.H.K. (1986) *Nucleic Acids Res.,* 14, 1727-1745.
[25] Felser,J.M, Straus,S.E. and Ostrove,J.M. (1987) *J. Virol,* 61, 225-228.
[26] Disney,G.H. and. Everett,R.D. (1990) *J. Gen. Virol,* 71, 897-906.
[27] Longnecker,R. and Roizman,B. (1986) *J. Virol,* 58, 583-591.
[28] Preston,C.M. (1979) *J. Virol,* 29, 275-284.
[29] Dixon,R.A.F. and Schaffer,P.A. (1980) *J. Virol,* 36, 189-203.
[30] Smith.CA., Bates,R, Rivera-Gonzales,R., Gu,B. and Deluca,N.A. (1993) *J. Virol,* 67,4676-4687.
[31] Cheung,A.K. (1989) *Nucleic Acids. Res.,* 17,4637-4646.
[32] Vleek,C, Paees,V.A. and Schwyzer-M. (1989) *Virus Genes,* 2, 335-346.
[33] Grundy,F.J., Bauman,R.P. and O'CallaghanJλJ. (1989) *Virology, 111,* 223-236.
[34] DelucaJST.A. and Schaffer,P.A. (1988) /. *Virol,* 62, 732-743.
[35] Deluca,N.A. and Schaffer,P.A. (1988) *Nucleic Acids. Res.,* 15,4491-4511.
[36] Paterson,T. and Everett,R.D. (1990) *J. Gen. Virol,* 71,1775-1783.
[37] Paterson,T. and Everett,R.D. (1988) *Virology,* 166, 186-196.
[38] Paterson,T. and Everett,R.D. (1988) *Nucleic Acids Res.,* 16, 11005-11025.
[39] Shepard,A.A., Imbalzano,A.M. and Deluca,N.A. (1989) *J. Virol,* 63, 3714-3728.
[40] Faber,S.W. and Wilcox,K.W. (1986) *Nucleic Acids Res.,* 14, 6067-6083.
[41] Wu,C.L. and Wilcox,K.W. (1990) *Nucleic Acids. Res.,* 18, 531-538.
[42] Wu,C.L. and Wilcox,K.W. (1991) /. *Virol,* 65,1149-1159.
[43] ResnickJ., Boyd,B.A. and Haffey,M.L. (1989) *J. Virol,* 63, 2497-2503.
[44] Roberts-M.S., Boundy,A., 0'Hare,R, Pizzomo,M.C, Ciufo,D.M. and Hayward,G.S. (1988) *J. Virol,* 62,4307-4320.
[45] BeardJP., Faber,S., Wilcox,K.W. and Pizer,L.I. (1986) *Proc. Natl. Acad. Sci. USA,* 83,4016-4020.
[46] Tedder,D.G., Everett,R.D., Wilcox,K.W., Beard,R and Pizer,L.I. (1989) *J. Virol,* 63, 2510-2520.
[47] Mullen,M.-A, Ciufo,D.M. and Hayward,G.S. (1994) *J. Virol,* 68, 3250-3266.
[48] TylerJ.K. and Everett,R.D. (1993) *Nucleic Acids Res.,* 21, 513-522.
[49] CohenJ.L, Heffel,D. and Seidel,K. (1993) *J. Virol,* 67,424^4251.
[50] Perera,L.R, MoscaJ.D., Ruyechan,W.T., Hayward,G.S., Straus,S.E. and HayJ. (1993) *J. Virol,* 67,4474-4483.
[51] Defechereux,R, Melen,L., Baudoux,L., Merville-Louis,M.-R, Rentier,B. and PietteJ. (1993) *J. Virol,* 67,4379-4385.
[52] McKee,T.A., Disney,G.H., Everett,R.D. and Preston,C.M. (1990) *J. Gen. Virol,* 71, 897-906.
[53] SambrookJ., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[54] NeumanJ.R., Morency,C.A. and Russian,K.O. (1987) *BioTechniques,* 5, 444.

Published in : Nucleic acids research (1995), vol. 23, iss. 8, pp. 1341-1349
Status : Postprint (Author's version)

[55] Kalderon,D., Roberts,B., Richardson,W.D. and SmithA.E. (1984) *Nature,* 311, 33-38.
[56] Moriuchi,H., Moriuchi,M., Straus,S.E. and CohenJ.I. (1993) *J. Virol,* 67, 2739-2746.
[57] Moriuchi,H., Moriuchi,M., Straus,S.E. and Cohen J.I. (1994) *Virology,* 200,297-300.
[58] Pellett,RE., McKnightJ.L.C., Jenkins,F.J. and Roizman,B. (1985) *Proc. Natl. Acad. Sci. USA,* 82, 5870-5874.
[59] Ling,R, Kinchington,RR., Sadeghi-Zadeh,M., Ruyechan,W.T. and HayJ. (1992) *J. Virol,* 66, 3690-3698.
[60] TylerJ.K., Allen,K.E. and Everett,R.D. (1994) *Nucleic Acids Res.,* 22, 270-278.
[61] Mermod-N., 0'Neill,E.A., Kelly,T.J. and Tjian,R. (1989) *Cell,* 58,741-753.
[62] Cress,W.D. and Triezenberg,S.J. (1991) *Science,* 251, 87-90.
[63 Kim,T. *K.* and Roeder,RJ. (1994) *Proc. Natl. Acad. Sci. USA,* 91, 4170-4174.