Varicella-zoster-virus IE63 protein represses the basal transcription machinery by disorganizing the pre-initiation complex.

Emmanuel Di Valentin[1], Sébastien Bontems[1], Lionel Habran[1], Olivier Jolois[3], Nicolas Markine-Goriaynoff[2], Alain Vanderplasschen[2], Catherine Sadzot-Delvaux[1] and Jacques Piette[1]*

[1] Laboratory of Virology and Immunology, University of Liège, B-4000 Liège, Belgium

[2] Laboratory of Immunology and Vaccinology, University of Liège, B-4000 Liège, Belgium

[3] Laboratory of Histology, University of Liège, B-4000 Liège, Belgium

*To whom correspondence should be addressed: Jacques Piette, Laboratory of Virology and Immunology, Institute of Pathology B23, University of Liège, B-4000 Liège, Belgium.

Tel: 32-4-366 2442, Fax: 32-4-366 9933, E-mail: jpiette@ulg.ac.be

Short title: VZV IE63 regulatory properties

Abstract

Using transient transfection assays, regulation properties of Varicella-Zoster Virus (VZV)-encoded IE63 protein were analyzed on several VZV immediate early (ORF4), early (ORF28) and late (ORF67) promoters. IE63 was shown to repress the basal activity of most of the promoters tested in epithelial (Vero) and neuronal (ND7) cells to various extents. Trans-repressing activities were also observed on heterologous viral and cellular promoters. Since a construct carrying only a TATA box sequence and a series of wild-type or mutated IL-8 promoters were also repressed by IE63, the role of upstream regulatory elements were ruled out. Importantly, the basal activity of a TATA-less promoter was not affected by IE63. By using a series of IE63 deletion constructs, amino-acids 151 to 213 were shown to be essential to the trans-repressing activity in Vero cells, while in ND7 cells, the essential region extended to a much larger carboxy-terminal part of the protein. We also showed that IE63 was capable of disrupting the transcriptional pre-initiation complex and of interacting with several general transcription factors. The central and carboxy-terminal domains of IE63 are important for these effects. Altogether, these results demonstrated that IE63 protein is a transcriptional repressor whose activity is directed towards general transcription factors.

Key words: down-regulation ; general transcription factors; repression; transcription; varicella-zoster.

Abstract words: 197

Introduction

Varicella-Zoster-Virus (VZV) is a human alphaherpesvirus that causes chickenpox (varicella) and becomes latent in dorsal root ganglia (DRG). Upon reactivation from latency, VZV is responsible for zoster (shingles). The VZV genome is a double-stranded DNA molecule composed of 71 open reading frames (ORFs) encoding 68 proteins that seem to be regulated in a manner similar to other alphaherpesviruses. After entry into the cells, VZV genes are expressed in a temporal cascade. The immediate early (IE) genes are expressed first. They stimulate early (E) gene expression, providing most of the proteins necessary for viral DNA replication. After DNA synthesis has occurred, genes of the late (L) class encoding structural proteins are expressed. In contrast to Herpes Simplex Virus type 1 (HSV-1), the VZV latency is characterized by the expression of several IE (ORFs 4, 62 and 63) and E (ORFs 21, 29, 66) genes (Cohrs et al., 1996, Cohrs et al., 2003, Grinfeld and Kennedy, 2004, Mahalingam et al., 1996, Meier et al., 1993). However, the biochemical and molecular processes controlling latency and reactivation of VZV are still largely unknown.

VZV open reading frames 63 and 70 (ORF63/70) encode a 278-amino-acid long protein with a predicted molecular mass of 30.5 kDa but produce an extensively modified 45 kDa protein in infected cells (Debrus et al., 1995). ORF63 is expressed as an IE protein (IE63) and exhibits a limited homology with the HSV-1 IE protein ICP22 (Davison and McGeoch, 1986, Davison and Scott, 1986) and the EHV-1 EICP22 protein (Derbigny et al., 2000). IE63 most closely resembles HSV-1 Us1.5, which is a 274-amino-acid protein encoded by a gene that is co-linear with the HSV-1 ICP22 (Baiker et al., 2004).

IE63 is of particular interest in VZV pathogenesis since it is abundantly expressed during acute infection as well as during latency (Debrus et al., 1995, Grinfeld and Kennedy, 2004, Kennedy et al., 2000, Mahalingam et al., 1996). During lytic infection, its localization is mostly nuclear but it can also be faintly detected in the cytoplasm. However, during latency, IE63 is almost exclusively localized in the cytoplasm of infected neuronal cells (Grinfeld and Kennedy, 2004, Lungu et al., 1998, Mahalingam et al., 1996). IE63 protein interacts with VZV IE62 protein (Baiker et al., 2004, Lynch et al., 2002) and is essential for VZV replication (Baiker et al., 2004, Sommer et al., 2001) and latency (Cohen et al., 2004).

Transcription of viral genes during a productive infection is mediated by interaction between viral activator or repressor proteins and various components of the cellular transcription machinery. This interaction is important for increasing or decreasing the assembly of the preinitiation complex (PIC) necessary for transcription of genes by RNA polymerase II (RNA POL II). Activators or repressors may act directly or indirectly on the PIC formation. The PIC is composed of the RNA POL II and general transcription factors (GTFs) (for recent review, see (Hahn, 2004) that can be attached on promoter templates *in vitro* (Buratowski, 1994, Grondin and DeLuca, 2000, Hampsey, 1998). In most cases, the recognition of promoters is mediated by TFIID through the binding of the TATA binding protein (TBP) subunit to TATA box elements. The action of several viral proteins has been shown to be directed toward the PIC assembly (Choy and Green, 1993, Grondin and DeLuca, 2000, Gu et al., 1995, Huang and McCance, 2002, Jang et al., 2001, Kim et al., 2003, Long et al., 1999, Manet et al., 1993).

The gene regulatory properties of IE63 protein are not very well understood and remain controversial. Some authors have reported that IE63 protein could

repress the VZV IE62 promoter, stimulate the VZV thymidine kinase promoter but could not affect expression of late genes in Vero cells (Jackers et al., 1992). Another team suggested that expressed under the control of its own promoter, IE63 did not exert any regulatory properties on IE, E and L genes in the same cell line (Kost et al., 1995). Conversely, IE63 was shown to up-regulate IE62 effect on VZV gI promoter in A3.01 cells, a CD4+ T-cell line and to interact with VZV IE62 protein (Lynch et al., 2002). Recently, IE63 has been shown to exert important trans-repressive properties on VZV DNA polymerase gene (ORF28) promoter both in Vero and ND7 cells (Bontems et al., 2002). Therefore, an extensive series of transient transfection studies were performed in order to better understand IE63 gene regulatory properties and its mechanism of action. From the data presented in this paper, it appears that IE63 is capable of repressing the basal activity of several promoters and is able to interfere with the components of the pre-initiation complex.

Results

IE63 is a transcriptional repressor targeting the TATA-box. For many years, the transcriptional regulatory properties of IE63 remained unclear, being described either as a transcriptional repressor or activator depending on the promoter tested or without any transcriptional effect (Jackers et al., 1992, Kost et al., 1995, Lynch et al., 2002). Recently, it was unambiguously shown that IE63 represses transcription initiated from the promoter controlling the VZV DNA polymerase gene (Bontems et al., 2002). In order to better characterize IE63 regulatory properties, we decided to extend this important observation to other VZV promoters and to compare IE63 activity in two cell lines where VZV leads to either a productive (monkey kidney epithelial cells, Vero) or non-productive (immortalized rat sensory neurons, ND7) cycle (Bontems et al., 2002). Vero and ND7 cells were transfected with a plasmid expressing IE63 together with various constructs (Table 1) where the reporter gene was under the control of autologous (Figure 1) as schematically represented in Figure 1A or heterologous viral and cellular promoters (Figures 2 and 3) as schematically represented in Figures 2A and 3A. Figure 1B and 1C show that in both Vero and ND7 cells, IE63 exerts transcriptional repression properties towards autologous VZV promoters belonging to two classes: ORF4 (p4-Luc) which encodes a gene regulator expressed as an IE protein, ORF 28 (pPOL-Luc) which encodes the viral DNA polymerase and is an E protein. The basal activity of these promoters was dose-dependently decreased with IE63 expression (pcDNA-IE63). IE63 did not show any repressive activity on the promoter belonging to the L class (p67-Luc) that controls expression of the glycoprotein I (Figure 1B and C). The presence of IE63

expression in the two cell lines was detected by western blot analysis (Figure 1B and C, lower part).

In order to identify elements of these promoters that could help us to clarify the mechanism of repression mediated by IE63, sequence alignment analysis was carried out. The only motives shared by these promoters were a TATA-box and the Initiator element (InR) suggesting that IE63 could exert its repression on basal transcription initiation. Then, computer search for TATA-boxes was carried out on the promoter sequences using three different programs (Table 1). The scores were arbitrarily set on the same scale from 0 to 1; 1 being the best score. TATA-boxes of ORF4 (p4-Luc), ORF28 (pPOL-Luc), and ORF67 (p67-Luc) were previously mapped (Kinchington et al., 1994, Kost et al., 1995, Ling et al., 1992, Meier et al., 1994). As expected, sequence analysis of these 3 TATA-boxes revealed that ORF4 and 28 obtained good scores, while the score of the ORF67 TATA box was much lower and could be classified as an atypical TATA-box (Table 1). We then extended the sequence analysis to other VZV promoters (Table 1). Strikingly, one of the lowest scores was obtained for a TATA-box from the ORF63 promoter (p63-Luc) which apparently had also an atypical TATA-box (Table 1). Transient transfection of Vero cells was therefore carried out with this promoter (Figure 1D). As shown in Figure 1D, IE63 exhibited a lesser repressing activity against its own promoter either under an extended or a short (163 bp) version (p63(163bp)-Luc) (Figure 1A). A similar behaviour was observed in ND7 cells transfected with the extended version of the ORF63 promoter (data not shown).

We also analyzed different heterologous viral and cellular promoters known to have good TATA-boxes (Table 1). These were: the major IE promoter from human cytomegalovirus (CMV, pCMV-Luc) (Foecking and Hofstetter, 1986) and the HSV-1

UL44 (gC, pEL-Pgc-Luc) promoters (Lium and Silverstein, 1997). As expected, the scores obtained with the various programs were very close to 1 (Table 1). IE63 repression property of these promoters was also examined (Figure 2). In that respect, these two promoters also had their basal activities down-regulated by IE63, both in Vero and ND7 cells (Figure 2B, C). The observation that both VZV and heterologous promoters could have their basal activity repressed by IE63 in a similar extend and independently of the cell type used is an interesting information that clarifies the regulatory properties of this viral protein.

To further clarify the importance of TATA-box and/or InR elements in the repression mediated by IE63, we used a reporter plasmid whose transcription was under the control of a TATA box (pFR-Luc). The scores obtained by this sequence were quite good (Table 1). Figure 3B shows that IE63 reduced the activity of this promoter by about 60% both in Vero and ND7 cells, reinforcing the idea that IE63 targeted TATA-boxes and/or InR. In order to confirm that IE63 targets only TATA-box motif, we have analyzed the effect of IE63 on the human polymerase alpha promoter (ph.POLα-Luc) which lacks this motive but bears an InR (Figure 3A and C). As expected, no TATA-box motive within this promoter was detectable by computer analysis (Table 1). Even without any detectable TATA-box, this promoter exhibited a high basal activity. Although IE63 protein was dose-dependently expressed (Fig 3c, lower part), the basal activity of the human polymerase alpha promoter was not decreased both in Vero and ND7 cells (Fig 3c). These results confirmed, on the one hand, that several upstream regulatory elements such as AP-2, AP-1, EF2, SP1, ATF and CAAT box were not involved in IE63 repression and, on the other hand, IE63 protein targets only the TATA-box sequence. This experiment also ruled out the role of the InR sequence in the IE63-mediated repression. The lack of importance of

several upstream regulatory elements was further confirmed by using various constructs of the cellular IL-8 promoter which has been very well-characterized (Roebuck, 1999). The wild-type version of the promoter was dose-dependently repressed in both cell lines (Figure 3D, Table 1) and this repression was independent on the mutations of various upstream regulatory elements. This demonstrated that AP-1, NF-IL-6 and NF-κB elements were not required for IE63 activity.

IE63 central and carboxy-terminal domains are important for repression. Recently, we showed that IE63 is a highly phosphorylated protein and the phosphorylation sites situated in the carboxy-terminal regions are necessary for repression (Bontems et al., 2002). In order to better characterize IE63 domains that are important for repression, IE63 deletion mutants were constructed: (i) IE63 Δ1-75 with the amino-terminal region removed, (ii) IE63 Δ76-150 with the central region partly deleted, (iii) IE63 Δ151-213 with the region rich in CK1-CK2 phosphorylation sites was deleted and (iv) IE63 Δ214-278 where the carboxy-terminal domain containing two putative phosphorylation sites for CDK1 (Baiker et al., 2004, Bontems et al., 2002) and a nuclear localization signal were removed (Figure 4A). Western blot analysis demonstrated that these mutant proteins were stably expressed after transfection of Vero (Figure 4B) and ND7 (Figure 4C) with 1 to 3 µg of expression vectors. It should be noted that the removal of domain 151-213 of IE63 modified the electrophoretic migration of the protein which appeared somewhat smaller than expected (25 kDa instead of 34 kDa) (Figure 4B). This is likely due to the removal of a domain which is known to be highly phosphorylated in the wild-type protein (Bontems et al. 2002). Co-transfection of cells with IE63 wild-type or the deleted constructs and VZV DNA polymerase reporter vector (pPOL-Luc) led to unexpected

results (Figure 4D and E). Indeed, in Vero cells, the removal of either the amino-terminal (IE63 Δ1-75), the second domain (IE63 Δ76-150) or the carboxy-terminal domain (IE63 Δ214-278) of IE63 did not alter its transrepression activity on the ORF28 (pPOL-Luc) promoter (Figure 4D) in comparison with IE63 wild-type. However, IE63 domain 3 was found essential for repression since its removal led to the loss of the repressive activity of the protein. Surprisingly, even the basal activity of the promoter was increased with this construct. In ND7 cells, the pattern of repression obtained with these mutant proteins was not identical (Figure 4E). The proteins lacking domain 3 (IE63 Δ150-213) or domain 4 (IE63 Δ214-278) turned out to be less efficient in repressing the ORF28 promoter, demonstrating the importance of the carboxy-terminal part of IE63 in this cell type (Figure 4E). Similar results were obtained with the IL-8 promoter (data not shown).

IE63 co-localizes in the cell nucleus with TFIIE. As it is likely that IE63 targeted TATA-boxes, we decided to characterize the cellular localization of IE63 with a component of the basal transcription machinery. In the classical model of transcriptional pre-initiation complex (PIC) assembly, the transcription factor TFIIE (composed of two subunits, TFIIEα and TFIIEβ) binds the RNA POL II at a late stage of initiation. Therefore, TFIIE was found to be a good marker for PIC localization in the cell nucleus. Confocal microscopy analysis was carried out on Vero and ND7 cells transfected with the various constructs leading to wild-type or mutant IE63 expression (Figure 5). Co-localization between IE63 and endogenous TFIIEα subunit was analyzed by the use of secondary antibodies linked to Fluorescein (TFIIE, in green) and Texas red (IE63, in red) (Figure 5A to 5F). IE63 wild- type protein was mainly observed in the nucleus of Vero and ND7 cells. As shown in Figure 5A and

5B, both IE63 and TFIIE are perfectly localized in the nucleus of the two cell types except in the nucleolus where IE63 was absent. Even if these results did not demonstrate on their own the interaction between IE63 and TFIIE, they showed that these proteins exhibited a very similar distribution in the nucleus. A similar co-localization pattern was observed in cells expressing IE63 Δ1-75 and IE63 Δ76-150 (data not shown).

Analysis of the cells transfected with pcDNA-IE63 Δ151-213 revealed that the mutant protein expressed was mainly localised in the nucleus (Figure 5C and 5D). However, significantly less IE63 co-localization with TFIIE was observed compared with the wild-type protein. In the case of the IE63 Δ214-278, due to the removal of the nuclear localisation signal, this protein was exclusively localised in the cytoplasm of the two cell lines (Figure 5E and 5F). Surprisingly, although in all conditions TFIIE was exclusively located in the nucleus, a minor fraction of TFIIE could be detected in the cytoplasm of Vero cells expressing IE63 Δ214-278 (Figure 5E). In that case, the two proteins were shown to co-localize. This observation suggested that IE63 could partly sequester TFIIE or other associated GTFs in this cellular compartment. In ND7 cells, IE63 was also mainly localized in the cytoplasm but not TFIIE (Figure 5F). Importantly, IE63Δ214-278 and TFIIE were never co-localizing in the nucleus of ND7 cells (Figure 5F). A positive control of nuclear co-localization in identical experimental conditions was carried out by co-transfecting IE63 and IE62 expression vectors in the two cell lines. IE62 was chosen since this protein was previously shown capable of interacting with IE63 (Lynch et al., 2002) (Baiker et al., 2004). As shown in Figures 5G and 5H, IE62 (in green) and IE63 (in red) were detected in the cell nucleus and perfectly co-localized.

IE63 disorganizes the transcriptional pre-initiation complex. We then decided to examine whether IE63 could influence the PIC assembly when formed on a DNA probe. In that respect, a PIC assembly assay (Grondin and DeLuca, 2000) was performed involving a probe encompassing well-described transcription initiation sites (from HSV-1 gC or IL-8 promoters) bound to magnetic beads and extracts from Vero and ND7 cells either transfected or not with an IE63 expression vector. After PIC formation, the DNA-protein complexes were extensively washed and analyzed by SDS PAGE and silver-stained. Due to a weak binding efficiency of the Vero cell extracts to the gC or IL-8 probes, these experiments were only carried out with ND7 cells. As shown in Figure 6A, SDS-PAGE analysis reveals several bands with a diminished intensity after IE63 expression (shown by the arrows), whereas others were totally unchanged (shown by stars). Examples of putative GTFs that could correspond to proteins having a reduced band intensity are listed in Figure 6A: TFIIE subunits: α (56 kDa), β (34 kDa), RNA POL II subunits: POLR2A (220 kDa), POLR2C (33 kDa), TAFs: TAF1 (250 kDa), TAF7 (55 kDa), TAF10 (30 kDa), TFIIH: polypeptide 2 (44 kDa), polypeptide 3 (34 kDa) and TFIIB (33 kDa). The mean reduction in the amount of putative transcription factors by IE63 expression was calculated after gel photodensitometry: TAF1: 39 %, POLR2A: 41 %, TFIIEα: 18 %, TFIIH2: 25 %, TFIIEβ: 50%. It should be noted that no increase in the band intensity was observed after IE63 expression in ND7 cells. In order to identify the factors incorporated within the pre-initiation complex whose concentration was lowered by IE63 on the gC probe, western blot analysis was carried out on several GTFs [RNA POL II, TFIIE, TFIIH, TFIID (TBP subunit) and TFIIB] and IE63. PIC assembly assays were performed in parallel on nuclear proteins bound to the probe and taken from cells transfected either with an empty vector (pcDNA3.1$^-$) or with an IE63 expression

vector (pcDNA-IE63) (Figure 6B). Positive control was the protein input unbound to the probe ("not bound"). The first important information obtained by Western blot analysis was that only a very marginal fraction of IE63 could be found associated to the basal transcription machinery. The vast majority of IE63 was found in the flow through. Among the factors found to be associated with the pre-initiation complex, three of them (TFIIEα, TFIIH and TFIIB) had their concentration lowered when IE63 was expressed (Figure 6B, central lane). The content in the RNA POL II large subunit (POLR2A) was also lowered but to a lesser extent. Finally, TBP was found to be unaffected by the presence of IE63. This last result could be explained by the weak interaction between these two proteins observed with a two-hybrid system (data not shown) and by the fact that TBP is buried inside the pre-initiation complex with no external exposure. We have also carried out a PIC assembly assay on the IL-8 probe and similar results were obtained (data not shown). Overall, these data demonstrated that IE63 is capable of interfering with several important factors of the transcriptional pre-initiation complex and therefore explained why IE63 represses transcription.

In order to correlate the data obtained in transient transfection assays with transcriptional pre-initiation complex assembly, PIC assays were also carried out on extracts from ND7 cells transfected with plasmids expressing IE63 where the domains 3 or 4 were removed and compared to wild-type IE63 or un-transfected cells (Figure 6C). Western blot analysis of the PIC content showed that TFIIE and TFIIB were not affected when domain 4 of IE63 was deleted (Figure 6C, lane 4). A slight decrease in the content of TFIIE was also observed with IE63 lacking the domain 3 (Figure 6C). This demonstrates that the region of IE63 that encompassed most of the phosphorylation sites was crucial for removing these factors. Again, the IE63 deletion mutants were found stable and well-expressed in these conditions and the level of

TFIIE expressed was similar in cells that either expressed IE63 (wild-type or deletion mutants) or not (Figure 6D).

To further confirm IE63 interaction with several GTFs, immunoprecipitation assays of IE63 were carried out (Figure 6E). ND7 cells were transfected with pcDNA-IE63-IRES2-EGFP (allowing the expression of IE63 and GFP) and lysed 24 hours later. Western blot analyses of the proteins immunoprecipated with IE63 revealed the presence of TFIIH (Figure 6E). The presence of RNA POL II (POLR2A) and TFIIE in the immunoprecipitates could also be faintly detected (Figure 6E). As a control, IE63 was also revealed by western blotting among the immunoprecipated proteins. Conversely, TFIIH immunoprecipitation also allowed the detection of IE63 in the transfected cells (Figure 6F).

From these data, we can conclude that IE63 disorganizes the basal transcription initiation complex by interfering with the binding of several important GTFs on promoters through interaction with its phosphorylated domain leading to a repression of gene transcription.

Discussion

In this paper, IE63 protein was shown to repress the basal activity of IE and E VZV promoters in a dose dependent manner. However, IE63 seems to repress the L promoter tested (gI promoter) and its own promoter to a far lesser extent. Heterologous promoters such as those controlling the CMV major IE gene and the human IL-8 were also repressed. When all the promoter sequences used were analyzed, the only element common to the tested promoters was the TATA-box. Its importance for IE63 repression was demonstrated by using promoters either bearing solely a TATA-box or having no TATA-box: IE63 efficiently repressed the first construct while it had no effect on the second. In conclusion, IE63 was shown to be a transcriptional repressor acting on different promoters but its efficiency depends on the presence and on the nature of the TATA-box. Several authors, including our group, have not always observed this property of IE63 to act as a transcriptional repressor (Jackers et al., 1992; Kost et al., 1995; Lynch et al., 2002). This can be explained by the use of less sensitive transfection technologies by several authors or by suspecting that IE63 repressive activities could not be observed in every cell type. By deletion analysis, we have also demonstrated that amino acids 151 to 213 of IE63 were essential for the repression activity in Vero cells. This domain of IE63 encompasses the S/T-rich region shown to be phosphorylated by both CK1 and –2. Therefore, it can be suspected that the active form of IE63 in these assays is the phosphorylated form. Unexpectedly, and for unknown reasons, the removal of this domain converts IE63 into a slight gene activator in Vero cells. In ND7 cells, the essential domain extended from the central part to the carboxy-terminal end of the protein. This carboxy-terminal domain encompasses a nuclear localization signal and

two amino-acids (T222 and S224) that could be phosphorylated by CDK1. Therefore, we can suspect that in undifferentiated neuronal cells the phosphorylation of these two amino-acids could be a pre-requisite for transcriptional repression.

Several authors reported that viral proteins can interact with GTFs for gene transactivation or repression (Carrozza and DeLuca, 1996, Grondin and DeLuca, 2000, Gu et al., 1995, Hampsey, 1998, Jang et al., 2001, Kawaguchi et al., 1997, Kim et al., 2003, Long et al., 1999). Three different mechanisms of repression via the basal transcription machinery have been proposed (for review, see Gaston and Jayaraman, 2003). The first involves a modification of the RNA POL II large subunit C-terminal domain (CTD). HSV-1 ICP22 and UL13 were shown capable of altering RNA POL II CTD phosphorylation to promote late viral transcription (Long et al., 1999). The second mechanism is an interference with the binding of TBP to the TATA-box leading to a transcriptional repression. The adenovirus E1 protein represses transcription by making direct contact with TBP that interfere with the formation of the TBP-TATA complex (Song et al., 1997). Finally, a number of repressors either sequester GTFs or bind to GTF creating a steric hindrance preventing a correct PIC assembly. Our results revealed that this last mechanism may be applicable to IE63. The first step of PIC assembly is the TBP fixation on the TATA-box. We have first looked at this step and IE63 did not seem capable of targeting TBP. This was confirmed by (i) the demonstration that IE63 interacted poorly with TBP when using a two-hybrid system (data not shown) and (ii) TBP was not removed from the immobilized promoters in the presence of IE63. However, the content of RNA POL II, TFIIB, TFIIE and TFIIH bound to the probe was altered by IE63. Immunoprecipitation experiments confirmed these effects. Indeed, TFIIH, and to a lesser extent TFIIE and RNA POL II, were found to be immunoprecipitated with

IE63. Therefore, we believe that IE63 directly interacts with one of these proteins leading to the destabilisation of the PIC and the removal of several GTFs from the promoter. The PIC destabilisation explains how IE63 is a transcriptional repressor. These results confirmed those obtained using infected cells where IE63 was found to immunoprecipitate with RNA POL II (Lynch et al., 2002). Although the nature of the GTF(s) targeted by IE63 has not yet been unambiguously identified, it is obvious from the data presented above that IE63 sequestered one or several GTFs and by this way destabilized the PIC assembly.

This hypothesis is reinforced by the observation made by confocal microscopy. Indeed, IE63 was always observed in the nucleus and co-localized with TFIIE, an important GTF. IE63 deleted of its region encompassing amino acids 151 to 213, important for repression, did not co-localize so clearly with TFIIE. Moreover, the expression of the cytoplasmic mutant of IE63 (IE63 Δ214-278) in Vero cells co-localized with a small fraction of TFIIE present in the cytoplasm. This unexpected finding suggests that even present in the cytoplasm, IE63 could sequester a small fraction of neo-synthesized GTF(s). That could explain the preserved repressing capacity of this deleted IE63 protein. The cytoplasmic sequestration of TFIIE seems to be a special feature of IE63 expressed in Vero cells, since TFIIE was never found in the cytoplasm of ND7 cells. Interestingly, promoter repression by the carboxy-terminal mutant of IE63 was partially lost in ND7 cells.

In summary, this study demonstrated that IE63 is a transcriptional repressor effective on every promoter bearing a good TATA-box consensus. The repression occurs after IE63 had disorganized the formation of the pre-initiation complex by sequestering one or several GTFs. The capacity of IE63 to repress transcription has been observed in two different cell lines where VZV has a productive (Vero) or a non-

productive cycle (ND7), demonstrating that this property is independent of the cell-type and does not explain the non-productive infection observed in non differentiated neuronal cells by itself. To further clarify the role of IE63 in the latent infectious cycle, it would be interesting to analyze the repressive properties of this protein in differentiated neurons and to investigate the influence of phosphorylation by cellular kinases.

Materials and methods

Plasmids- IE63 activity on different promoters was performed by transient transfection assays. Reporter plasmids with the luciferase (Luc) gene are under the control of VZV or heterologous promoters using the pGL3-Basic vector (Promega): p4-Luc (VZV ORF4 promoter), pPOL-Luc (VZV ORF28), p67-Luc (VZV ORF67), p63-Luc (VZV ORF63). To construct the p4-Luc, the VZV ORF4 promoter was amplified by PCR from the p4-CAT (Defechereux et al., 1993) and then cloned in the Sma I and Bgl II sites. The pPOL-Luc was constructed as described previously (Bontems et al., 2002). To obtain the p67-Luc, pgI-CAT plasmid (Ling et al., 1992) was digested by Bgl II to excise the VZV ORF67 promoter and cloned in the pGL3-Basic vector into the Bgl II sites. The p63-Luc plasmid was generated by inserting the intergenic region between ORF62 and ORF63 in the pGL3-basic vector. A shorter version containing 163bp of IE63 promoter driving luciferase gene was also performed; p63(163bp)-Luc. The pFR-Luc contains the luciferase gene and a TATA box sequence (Stratagene, USA). Plasmid pIL-8-Luc, a human IL-8 promoter fragment of 133bp and plasmid pIL-8-NF-κB-mut-Luc, a human IL-8 promoter fragment of 133bp with the NF-κB site GGAATTTCCT (-80 to -71bp), mutated to TAACTTTCCT. Plasmid pIL-8-AP-1-mut-Luc contained a human IL-8 promoter fragment of 133 bp with the AP-1 site TGACTCA (-126 to -120bp) mutated to TATCTCA. Plasmid pIL-8-NF-IL-6-mut-Luc [containing a human IL-8 promoter fragment of 133bp with the NF-IL6 site CAGTTGCAAATCGT (-94 to -81bp) mutated into AGCTTGCAAATCGT]. All these IL-8-Luc plasmids were kindly provided by Dr. W. Vandenberghe and Dr. G. Haegeman (Ghent University, Ghent, Belgium). The pEL-Pgc-Luc contains the promoter of HSV-1 UL44 (gC) gene and the luciferase reporter gene (Lium and Silverstein, 1997).

Plasmid ph.POLα-Luc contains the human DNA polymerase alpha promoter and was described elsewhere (Moon et al., 2001, Truscott et al., 2003). For the expression of IE63 or IE62, we cloned the ORF63 or ORF62 (under the control of the CMV promoter) in the pcDNA3.1$^-$ (InVitrogen) in order to obtain pcDNA-IE63 (Bontems et al., 2002) or the pcDNA-IE62. ORF63 was also cloned in the pcDNA-IRES2-EGFP (BD Biosciences, Clonetech) for dual expression of IE63 and EGFP (pcDNA-IE63-IRES2-EGFP). Four plasmids expressing IE63 deletion mutants were constructed by PCR from pcDNA-IE63. The first, pcDNA-IE63Δ1-75, led to the expression of IE63 lacking, the first 75 amino acids. pcDNA-Δ75-150, pcDNAΔ151-213, pcDNAΔ214-278 are plasmids that express IE63 deleted of amino acids 75 to 150, 151 to 213 and 214-278, respectively. The ORF63Δ214-278 was also cloned in frame with the amino c-myc tag in the pCMV Tag3 (Stratagene) to obtain the pmyc-IE63Δ214-278. All these expression constructions were sequenced.

TATA-box analysis – The promoter sequences tested were submitted to three different software that search for the core promoter and/or the TATA-box (the score was reported on a scale between 0.5 and 1.0): http://www.fruitfly.org/seq_tools/promoter.html (Reese, 1996), http://tfbind.ims.u-tokyo.ac.jp (Heinemeyer et al., 1998) , http://motif.genome.ad.jp/ (Heinemeyer et al., 1999).

Antibodies – The antibodies used in this work are against -IE63 (monoclonal and polyclonal, (Bontems et al., 2002, Debrus et al., 1995, Kennedy et al., 2001)), -TBP (polyclonal, Santa Cruz Biotechnology, SC-273), -TFIIB (monoclonal, Transduction Laboratories, T41520), -TFIIEα (polyclonal, Santa Cruz Biotechnology, SC-237), TFIIH –CDK7 (monoclonal, Santa Cruz Biotechnology, SC-7344) and RNA POL II largest subunit (monoclonal, Santa Cruz Biotechnology, sc-17798), -c-Myc

epitope (monoclonal, Santa Cruz Biotechnology, sc-40), -IE62 (polyclonal, (Baudoux et al., 1995)).

Cells - Vero cells (a monkey kidney cell line, ATCC CCL-81) were grown in EMEM medium (Biowhittaker) supplemented with L-glutamine and 10% foetal bovine serum (Biowhittaker). ND7 cells (ECACC n° 92090903) were obtained from a fusion of murine neuroblastoma cells with primary nerve cells from rat dorsal root ganglia. They were grown in RPMI-1640 (Biowhittaker) supplemented with L-glutamine and 5% foetal bovine serum (Invitrogen) (Wood et al., 1990).

Cytoplasmic and nuclear protein extracts - Briefly, cells from confluent 175 cm^2 dishes were harvested, washed in cold PBS (137 mM NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH7.4), pelleted and resuspended in 180 µL of cold hypotonic buffer (10mM HEPES-KOH pH7.9, 0.1 mM EDTA 0.1 mM, 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF, 0.5% IGEPAL and protease inhibitors [Complete Protease Inhibitors, Roche Molecular Biochemicals]). The cells were then incubated for 15 min on ice, vortexed 10 s and centrifuged 30 s at 14.000 rpm. The supernatants corresponding to cytoplasmice extract were stored at –80°C. The pellets were resuspended in 120 µL of hypertonic buffer containing 50 mM HEPES-KOH pH 7.9, 50 mM KCl, 300 mM NaCl, 1 mM DTT, 0.5 mM PMSF and protease inhibitors [Complete Protease Inhibitors, Roche Molecular Biochemicals]). Cells were allowed to swell on ice for 30 min. After centrifugation (15 min at 20,000 g at 4 °C), the supernatants containing the nuclear proteins were stored at –80 °C. Protein concentrations were measured by the Bradford method (reagent from Biorad).

Total cellular protein extracts (RIPA lysis) and Western blot analysis - Total cellular extracts from Vero cells were obtained by RIPA lyses. Briefly, cells from 35-mm diameter six-wells cluster dishes were harvested, washed in PBS (137 mM

NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH7.4), pelleted and resuspended in 200 µL of RIPA lyses buffer (PBS supplemented with 1% Nonited P-40, 0.5% Tween 20, 0.1% (w/v) SDS, 5 mM EDTA, 1 mM dithiothreitol, and protease inhibitors [Complete protease inhibitors, Roche Molecular Biochemicals]). Cells were allowed to swell on ice for 30 min, vortexed, then transferred on ice for an additional 30 min, and centrifuged 20 min at 20,000 x g at 4 °C. Protein concentrations of the supernatants were measured by the Bradford method (reagent from Biorad) and analyzed by western blotting (15 µg of protein was loaded).

Transient transfection assays - Transfections were carried out with cells (Vero or ND7) seeded into 35-mm diameter six-wells cluster dishes using the FUGENE 6 transfectant reagent according to the manufacturer's prescriptions (Roche Molecular Biochemicals). For each experiment, cells were co-transfected with 2 µg of luciferase reporter plasmids and 0 to 3 µg of IE63 plasmid expression (pcDNA-IE63) The amounts of DNA were adjusted with herring sperm DNA. Special attention was made to obtain an equimolar ratio of CMV promoters in each independent experiment. Twenty-four hours post-transfection, cells were harvested and Luciferase assays were performed using the "Luciferase Reporter Gene Assay, high sensitivity" kit (Roche Molecular Biochemicals), according to the instructions of the manufacturer. For each experiment, the concentration of proteins in each sample was measured in order to normalize the results. Data from luciferase assays were collected from at least three independent transfection experiments. P values were calculated using graphpad quickcalcs software (*www.graphpad.com*). To compare observed and expected means, one sample *t*-test was used. For comparison of two means, unpaired *t*-test was chosen. For western-blot analysis, 15 µg of protein extracts (RIPA lysis) were loaded on a 10% SDS-PAGE gel. After migration and

transfer, detection of IE63 was made using a monoclonal antibody as previously described (Bontems et al., 2002, Kennedy et al., 2001).

Confocal microscopy **–** Cells, transfected by pcDNA-IE63 grown on coverslips were rinsed with warmed PBS and fixed with 4% (w/v) paraformaldehyde/PBS for 10 min at room temperature and 20 min at 37 °C. After washing with PBS, the cells were permeabilized with PBS containing 0.1 % Triton X-100 for 10 min at room temperature and 20 min at 37 °C. Cells were then incubated with a rabbit serum anti-TFIIE endogenous and a monoclonal antibody anti-IE63 (9A12) (Debrus et al., 1995) (to detect IE63wt, IE63 Δ1-75, IE63 Δ76-150 and IE63 Δ151-213) or monoclonal antibody anti c-myc (for detection of IE63Δ214-278) in PBS + 1% fetal bovine serum (FBS) for 1 hour at 37 °C. After washing with PBS + FBS 1%, coverslips were incubated with FITC-conjugated anti-rabbit secondary antibodies (DAKO A/S, Denmark) and a Texas Red-conjugated anti-mouse secondary antibody (Molecular Probes, Leiden, The Netherlands) for 1 hour at 37 °C. For IE62 and IE63 staining, cells were first co-transfected with the same amount of expression plasmids pcDNA-IE62 and pcDNA-IE63. To detect IE62, polyclonal antibody was used and then FITC-conjugated anti-rabbit secondary antibodies (DAKO A/S, Denmark). The staining for IE63 was the same as described above. Following a PBS rinse, coverslips were mounted with SlowFade Light Anti-fade reagent (Molecular Probes, Leiden, The Netherlands). Confocal microscopy analyses were performed with a TCS SP confocal microscope (Leica) as described previously (Vanderplasschen et al., 2000). Pictures were collected using a 63x0.9HCX APO L objective and electronic amplifications giving rise to square pictures corresponding to 39.7 x 39.7 µm of the specimen. Cross-talking was avoided by sequential acquisition of the green and the red signals and by setting appropriate spectral windows. The absence of cross-

talking between channels was demonstrated by the analysis of single positive specimens (data not shown).

PIC assembly assays - A 70 bp of HSV-1 gC (UL44) promoter and a 100 bp IL-8 promoter were synthesized and biotinylated (Eurogentec, Belgium) on the 5' end. The purified biotinylated promoters were then immobilized on a magnetic resin conjugated with streptavidin (Dynal) at 10 µg of DNA biotinylated per 200 µg of beads by assay. A 175 cm^2 T-flask containing cells was transfected with 24 µg of plasmids (pcDNA3.1-, pcDNA-IE63wt). Cells were treated for a period of 36 hours with G418 (1mg ml-1) (In Vitrogen) in order to select only the transfected cells. About 500 µg of the total cell extracts (estimated by the Bradford assay) either containing IE63 protein or not was added to 50 µg of immobilized promoters in 400 µL of binding buffer as described by (Grondin and DeLuca, 2000). The samples were then incubated for 1 hour at room temperature on a rotating mixer. After incubation, the immobilized templates with the proteins were concentrated with a magnetic concentrator (Dynal), resuspended and washed 5 times in 400 µL of binding buffer. The whole samples were finally analyzed by western blotting and silver staining (Invitrogen, SilverXpress) after SDS-PAGE 10 or 12% gels. Silver-stained gels densitometry was done using ImageQuant software.

Immunoprecipitations – Mouse monoclonal antibody against IE63 (9A12, 30 µl) and 30 µl of protein A-Sepharose (Amersham Pharmacia Biotech) were incubated in buffer C (Yamamoto et al., 2001) (20 mM Tris-HCl [pH 7.9 at 4°C], 0.5 mM EDTA, 20% [vol/vol] glycerol, 0.5 mM PMSF, 10 mM 2-mercaptoethanol, 0.002% [vol/vol] Nonidet P-40) containing 100 mM KCl (BC100) and 200 µg of bovine serum albumin (BSA)/ml for 2 h at 4°C with rotation. The protein A-Sepharose beads were precipitated and washed twice with 1 mL of buffer C containing 0.5 M KCl

(BC500) and twice again with 1 ml of BC100. ND7 cells transfected with pcDNA-IE63-IRES-EGFP or with pcDNA-IRES-EFGP were lysed with protocole lysis using Buffer I and buffer II (described above, extract) containing 20% of glycerol. About 400 µg of cellular extracts were then incubated with the prepared anti-IE63 antibody-protein A beads in a 1.5 mL of BC500 overnight at 4°C with rotation. The beads were washed three times with 1 mL of BC500 and boiled in SDS sample buffer. Then the immunoprecipitated proteins released from the beads were analyzed by Western blotting. The membranes were either cut into several pieces before being incubated with the various antibodies or reprobed.

Acknowledgments. We thank Dr. S. Silverstein (Columbia University, New York, USA), Dr. Truscott and Dr. M. Nepveu (McGill University, Montreal, Quebec) and Dr. W. Vandenberghe and Dr. G. Haegeman (Ghent University, Ghent, Belgium) for kindly providing us plasmids pEL-Pgc-Luc, ph.POLα-Luc and pIL-8-Luc, respectively. This work was supported by grants from the Belgian National Fund for Scientific Research (F.N.R.S.). Sébastien Bontems and Emmanuel Di Valentin are supported by Télévie (F.N.R.S., Brussels). Lionel Habran is supported by “Fonds pour la Recherche en Industrie et Agriculture" (F.R.I.A., Brussels). Jacques Piette is Research Director from the F.N.R.S. (Brussels, Belgium). We also thank N. Renotte for technical assistance.

References

Baiker, A., Bagowski, C., Ito, H., Sommer, M., Zerboni, L., Fabel, K., Hay, J., Ruyechan, W. and Arvin, A. M. (2004). The Immediate-Early 63 Protein of Varicella-Zoster Virus: Analysis of Functional Domains Required for Replication In Vitro and for T-Cell and Skin Tropism in the SCIDhu Model In Vivo. J. Virol. *78*, 1181-1194.

Baudoux, L., Defechereux, P., Schoonbroodt, S., Merville, M. P., Rentier, B. and Piette, J. (1995). Mutational analysis of varicella-zoster virus major immediate-early protein IE62. Nucleic Acids Res. *23*, 1341-1349.

Bontems, S., Di Valentin, E., Baudoux, L., Rentier, B., Sadzot-Delvaux, C. and Piette, J. (2002). Phosphorylation of varicella-zoster virus IE63 protein by casein kinases influences its cellular localization and gene regulation activity. J. Biol. Chem. *277*, 21050-21060.

Buratowski, S. (1994). The basics of basal transcription by RNA polymerase II. Cell 77, 1-3.

Carrozza, M. J. and DeLuca, N. A. (1996). Interaction of the viral activator protein ICP4 with TFIID through TAF250. Mol. Cell. Biol. *16*, 3085-3093.

Choy, B. and Green, M. R. (1993). Eukaryotic activators function during multiple steps of preinitiation complex assembly. Nature *366*, 531-536.

Cohen, J. I., Cox, E., Pesnicak, L., Srinivas, S. and Krogmann, T. (2004). The varicella-zoster virus open reading frame 63 latency-associated protein is critical for establishment of latency. J. Virol. *78*, 11833-11840.

Cohrs, R. J., Barbour, M. and Gilden, D. H. (1996). Varicella-zoster virus (VZV) transcription during latency in human ganglia: detection of transcripts mapping to genes 21, 29, 62, and 63 in a cDNA library enriched for VZV RNA. J. Virol. *70*, 2789-2796.

Cohrs, R. J., Gilden, D. H., Kinchington, P. R., Grinfeld, E. and Kennedy, P. G. (2003). Varicella-zoster virus gene 66 transcription and translation in latently infected human Ganglia. J. Virol. *77*, 6660-6665.

Davison, A. J. and McGeoch, D. J. (1986). Evolutionary comparisons of the S segments in the genomes of herpes simplex virus type 1 and varicella-zoster virus. J. Gen. Virol. *67 (Pt 4)*, 597-611.

Davison, A. J. and Scott, J. E. (1986). The complete DNA sequence of varicella-zoster virus. J. Gen. Virol. *67 (Pt 9),* 1759-1816.

Debrus, S., Sadzot-Delvaux, C., Nikkels, A. F., Piette, J. and Rentier, B. (1995). Varicella-zoster virus gene 63 encodes an immediate-early protein that is abundantly expressed during latency. J.Virol*. 69*, 3240-3245.

Defechereux, P., Melen, L., Baudoux, L., Merville-Louis, M. P., Rentier, B. and Piette, J. (1993). Characterization of the regulatory functions of varicella-zoster virus open reading frame 4 gene product. J.Virol*. 67*, 4379-4385.

Derbigny, W. A., Kim, S. K., Caughman, G. B. and O'Callaghan, D. J. (2000). The EICP22 protein of equine herpesvirus 1 physically interacts with the immediate-early protein and with itself to form dimers and higher-order complexes. J. Virol. *74*, 1425-1435.

Foecking, M. K. and Hofstetter, H. (1986). Powerful and versatile enhancer-promoter unit for mammalian expression vectors. Gene *45*, 101-105.

Grinfeld, E. and Kennedy, P. (2004). Translation of varicella-zoster virus genes during human ganglionic latency. Virus Genes *29*, 317-319.

Grondin, B. and DeLuca, N. (2000). Herpes simplex virus type 1 ICP4 promotes transcription preinitiation complex formation by enhancing the binding of TFIID to DNA. J. Virol. *74,* 11504-11510.

Gu, B., Kuddus, R. and DeLuca, N. A. (1995). Repression of activator-mediated transcription by herpes simplex virus ICP4 via a mechanism involving interactions with the basal transcription factors TATA-binding protein and TFIIB. Mol. Cell. Biol. *15,* 3618-3626.

Hahn, S. (2004). Structure and mechanism of the RNA polymerase II transcription machinery. Nat. Struct. Mol. Biol. *11*, 394-403.

Hampsey, M. (1998). Molecular genetics of the RNA polymerase II general transcriptional machinery. Microbiol. Mol. Biol. Rev. *62*, 465-503.

Heinemeyer, T., Chen, X., Karas, H., Kel, A. E., Kel, O. V., Liebich, I., Meinhardt, T., Reuter, I., Schacherer, F. and Wingender, E. (1999). Expanding the TRANSFAC database towards an expert system of regulatory molecular mechanisms. Nucleic Acids Res. *27*, 318-322.

Heinemeyer, T., Wingender, E., Reuter, I., Hermjakob, H., Kel, A. E., Kel, O. V., Ignatieva, E. V., Ananko, E. A., Podkolodnaya, O. A., Kolpakov, F. A., Podkolodny,

N. L. and Kolchanov, N. A. (1998). Databases on transcriptional regulation: TRANSFAC, TRRD and COMPEL. Nucleic Acids Res. *26*, 362-367.

Huang, S. M. and McCance, D. J. (2002). Down Regulation of the Interleukin-8 Promoter by Human Papillomavirus Type 16 E6 and E7 through Effects on CREB Binding Protein/p300 and P/CAF. J. Virol. *76*, 8710-8721.

Jackers, P., Defechereux, P., Baudoux, L., Lambert, C., Massaer, M., Merville-Louis, M. P., Rentier, B. and Piette, J. (1992). Characterization of regulatory functions of the varicella-zoster virus gene 63-encoded protein. J. Virol. *66*, 3899-3903.

Jang, H. K., Albrecht, R. A., Buczynski, K. A., Kim, S. K., Derbigny, W. A. and O'Callaghan, D. J. (2001). Mapping the sequences that mediate interaction of the equine herpesvirus 1 immediate-early protein and human TFIIB. J. Virol. *75*, 10219-10230.

Kawaguchi, Y., Bruni, R. and Roizman, B. (1997). Interaction of herpes simplex virus 1 alpha regulatory protein ICP0 with elongation factor 1delta: ICP0 affects translational machinery. J. Virol. *71*, 1019-1024.

Kennedy, P. G., Grinfeld, E. and Bell, J. E. (2000). Varicella-zoster virus gene expression in latently infected and explanted human ganglia. J. Virol. *74*, 11893-11898.

Kennedy, P. G., Grinfeld, E., Bontems, S. and Sadzot-Delvaux, C. (2001). Varicella-Zoster virus gene expression in latently infected rat dorsal root ganglia. Virology *289*, 218-223.

Kim, S. K., Jang, H. K., Albrecht, R. A., Derbigny, W. A., Zhang, Y. and O'Callaghan, D. J. (2003). Interaction of the equine herpesvirus 1 EICP0 protein with the immediate-early (IE) protein, TFIIB, and TBP may mediate the antagonism between the IE and EICP0 proteins. J. Virol. *77*, 2675-2685.

Kinchington, P. R., Vergnes, J. P., Defechereux, P., Piette, J. and Turse, S. E. (1994). Transcriptional mapping of the varicella-zoster virus regulatory genes encoding open reading frames 4 and 63. J. Virol. *68*, 3570-3581.

Kinchington, P. R., Vergnes, J. P. and Turse, S. E. (1995). Transcriptional mapping of varicella-zoster virus regulatory proteins. Neurology *45*, S33-35.

Kost, R. G., Kupinsky, H. and Straus, S. E. (1995). Varicella-zoster virus gene 63: transcript mapping and regulatory activity. Virology *209*, 218-224.

Ling, P., Kinchington, P. R., Sadeghi-Zadeh, M., Ruyechan, W. T. and Hay, J. (1992). Transcription from varicella-zoster virus gene 67 (glycoprotein IV). J. Virol. *66*, 3690-3698.

Lium, E. K. and Silverstein, S. (1997). Mutational analysis of the herpes simplex virus type 1 ICP0 C3HC4 zinc ring finger reveals a requirement for ICP0 in the expression of the essential alpha27 gene. J. Virol. *71*, 8602-8614.

Long, M. C., Leong, V., Schaffer, P. A., Spencer, C. A. and Rice, S. A. (1999). ICP22 and the UL13 protein kinase are both required for herpes simplex virus-induced modification of the large subunit of RNA polymerase II. J. Virol. *73*, 5593-5604.

Lungu, O., Panagiotidis, C. A., Annunziato, P. W., Gershon, A. A. and Silverstein, S. J. (1998). Aberrant intracellular localization of Varicella-Zoster virus regulatory proteins during latency. Proc. Natl. Acad. Sci. U. S. A. *95*, 7080-7085.

Lynch, J. M., Kenyon, T. K., Grose, C., Hay, J. and Ruyechan, W. T. (2002). Physical and functional interaction between the varicella zoster virus IE63 and IE62 proteins. Virology *302*, 71-82.

Mahalingam, R., Wellish, M., Cohrs, R., Debrus, S., Piette, J., Rentier, B. and Gilden, D. H. (1996). Expression of protein encoded by varicella-zoster virus open reading frame 63 in latently infected human ganglionic neurons. Proc. Natl. Acad. Sci. U. S. A. *93*, 2122-2124.

Manet, E., Allera, C., Gruffat, H., Mikaelian, I., Rigolet, A. and Sergeant, A. (1993). The acidic activation domain of the Epstein-Barr virus transcription factor R interacts in vitro with both TBP and TFIIB and is cell-specifically potentiated by a proline-rich region. Gene. Expr. *3*, 49-59.

Meier, J. L., Holman, R. P., Croen, K. D., Smialek, J. E. and Straus, S. E. (1993). Varicella-zoster virus transcription in human trigeminal ganglia. Virology *193*, 193-200.

Meier, J. L., Luo, X., Sawadogo, M. and Straus, S. E. (1994). The cellular transcription factor USF cooperates with varicella-zoster virus immediate-early protein 62 to symmetrically activate a bidirectional viral promoter. Mol. Cell. Biol. *14*, 6896-6906.

Moon, N. S., Premdas, P., Truscott, M., Leduy, L., Berube, G. and Nepveu, A. (2001). S phase-specific proteolytic cleavage is required to activate stable DNA binding by the CDP/Cut homeodomain protein. Mol. Cell. Biol. *21*, 6332-6345.

Reese, M. G., N. L. Harris, and F. H. Eeckman. (1996). Large scale sequencing specific neural networks for promoter and splice site recognition. L. Hunter and T. E. Klein (ed.), Biocomputing. Proceedings of the 1996 Pacific Symposium. World Scientific Publishing Co., Singapore.

Roebuck, K. A. (1999). Regulation of interleukin-8 gene expression. J Interferon Cytokine Res. *19*, 429-438.

Sommer, M. H., Zagha, E., Serrano, O. K., Ku, C. C., Zerboni, L., Baiker, A., Santos, R., Spengler, M., Lynch, J., Grose, C., Ruyechan, W., Hay, J. and Arvin, A. M. (2001). Mutational analysis of the repeated open reading frames, ORFs 63 and 70 and ORFs 64 and 69, of varicella-zoster virus. J. Virol*. 75*, 8224-8239.

Song, C. Z., Loewenstein, P. M., Toth, K., Tang, Q., Nishikawa, A. and Green, M. (1997). The adenovirus E1A repression domain disrupts the interaction between the TATA binding protein and the TATA box in a manner reversible by TFIIB. Mol. Cell. Biol. *17*, 2186-2193.

Truscott, M., Raynal, L., Premdas, P., Goulet, B., Leduy, L., Berube, G. and Nepveu, A. (2003). CDP/Cux stimulates transcription from the DNA polymerase alpha gene promoter. Mol. Cell. Biol. *23*, 3013-3028.

Vanderplasschen, A., Markine-Goriaynoff, N., Lomonte, P., Suzuki, M., Hiraoka, N., Yeh, J. C., Bureau, F., Willems, L., Thiry, E., Fukuda, M. and Pastoret, P. P. (2000).

A multipotential beta -1,6-N-acetylglucosaminyl-transferase is encoded by bovine herpesvirus type 4. Proc. Natl. Acad. Sci. U. S. A. *97*, 5756-5761.

Wood, J. N., Bevan, S. J., Coote, P. R., Dunn, P. M., Harmar, A., Hogan, P., Latchman, D. S., Morrison, C., Rougon, G., Theveniau, M. and . (1990). Novel cell lines display properties of nociceptive sensory neurons. Proc. R. Soc. Lond. B Biol. Sci. *241*, 187-194.

Yamamoto, S., Watanabe, Y., van der Spek, P. J., Watanabe, T., Fujimoto, H., Hanaoka, F. and Ohkuma, Y. (2001). Studies of nematode TFIIE function reveal a link between Ser-5 phosphorylation of RNA polymerase II and the transition from transcription initiation to elongation. Mol. Cell. Biol. *21*, 1-15.

Table legend

Table 1. Synthesis of VZV IE63 effect on all promoters tested.

A. Schematic representation of VZV (IE4, p4-Luc; DNA polymerase, pPOL-Luc; gC, p67-Luc; IE63, p63-Luc and p63(163bp)-Luc; ORF66, p66-Luc) and heterologous (HSV-1 gC, pEL-Pgc-Luc; CMV IE, pCMV-Luc, pFR-Luc, human DNA polymerase alpha, ph.POLα-Luc; human IL-8, pIL-8-Luc) promoters tested.

B. Computer analysis of TATA-boxes mapped for all promoters using three different programs: Fruitfly.org (http://www.fruitfly.org/seq_tools/promoter.html), TFIIbind (http://tfbind.ims.u-tokyo.ac.jp) and Transfac (http://motif.genome.jp). For p63-Luc, the computer analysis score corresponds to the first TATA-box (TTATTCATAGA). The percentage of IE63 repression obtained on each promoter in Vero or ND7 cells transfected with 3 µg of pcDNA-IE63 and 2 µg of reporter plasmid is shown (right part). The results shown are the average obtained with a minimum of three independent experiments. nd = not done.

Figure legends

Figure 1. Effect of VZV IE63 protein on the basal activity of VZV promoters.

(A) Schematic sequence organization of VZV ORF4 (p4-Luc), ORF28 (pPOL-Luc), ORF67 (p67-Luc) and ORF63 (p63-Luc and p63(163bp)-Luc) promoters used as reporter plasmids. The arrows indicate the transcription start sites and the previously mapped TATA-box are framed. Luciferase gene ATG position is circled. The reporter plasmid p63-Luc contains IE63 full length promoter. ORF63 promoter contains two potential transcription start sites (cloned into p63-Luc) located at positions –88 and –157 relative to the ORF63 ATG. These two potential TATA-boxes, proposed by these authors are TATAAA and TTATTCATAGA. p63(163bp)-Luc vector encompasses only the first TATA-box (TTATTCATAGA) and contains 163 bp relative to the ORF63 ATG. Known upstream regulatory elements (Kinchington et al., 1995) are shown, on IE4 promoter: SP1 binding site (GC box) and CAAT (CAAT box) binding site.

(B), (C) and (D). Effect of VZV IE63 protein on VZV IE4 promoter (p4-Luc), VZV DNA polymerase promoter (ORF28, pPOL-Luc), VZV gI promoter (p67-Luc) and VZV ORF63 promoter (p63-Luc and p63(163bp)-Luc). Vero (B, D) or ND7 (C) cells were co-transfected with luciferase reporter plasmids and increasing concentrations of plasmids expressing IE63 (pcDNA-IE63) as described in the material and methods section. Results are presented in percentage of stimulation with respect to the basal expression of the promoter used (100%). At least three independent experiments were conducted for each condition and standard errors of the mean are shown as error bars. The expression of IE63 protein was confirmed by western blotting (Figure 1B, 1C and 1D, lower part). ✯ =p value<0.002; ✯✯ = p value≥0.002.

Figure 2. Effect of IE63 protein on heterologous promoters.

(A) The sequence organization of CMV (IE) and HSV-1 UL44 promoters used as reporter plasmids are shown. The arrows indicate the transcription start sites and the previously mapped TATA-box.

(B) Effect of VZV IE63 protein on CMV (major IE) and HSV-1 UL44 (gC) promoters. Vero (B) or ND7 (C) cells were co-transfected with reporter plasmids (pCMV-Luc or pEL-Pgc-Luc) and an increasing concentration of plasmid expressing IE63 (pcDNA-IE63). Results are presented in percentage of stimulation with respect to the basal expression of the promoter used (100%). At least three independent experiments were conducted for each condition. Standard errors of the mean are shown as error bars. ✯ =p value<0.002; ✯✯= p value≥0.002.

Figure 3. Importance of the TATA-box and upstream elements for IE63 repressive activity.

(A). Schematic sequence organization of a promoter containing only a TATA-Box element (pFR-Luc), a TATA-box-less promoter (the human DNA polymerase alpha, ph. POLα-Luc) and the human interleukin-8 promoter (pIL-8-Luc) reporter plasmids. The arrows indicate the transcription start sites and the previously mapped TATA-box. Several known upstream regulatory elements of human DNA polymerase alpha and IL-8 promoters are shown: AP-1, AP-2, E2F, SP1 binding site (GC box), ATF, CAAT (CAAT box) binding sites and AP-1, NF-IL-6, Oct-1, NF-κB binding sites, respectively.

(B) and (C). VZV IE63 protein regulation on the basal activity of the pFR-Luc and ph.POLα-Luc. Vero or ND7 cells were co-transfected with reporter plasmids: pFR-

Luc (B) or ph.POLα-Luc (C) and with 1 to 3 µg of plasmids pcDNA-IE63. Results are presented as percentage of stimulation with respect to the basal expression of the promoter used (100%). At least three different experiments were conducted and standard errors of the mean are shown as error bars.

(D). IE63 effects on the pIL-8-Luc. Vero cells were co-transfected with 2 µg of reporter plasmids (pIL-8-Luc, pIL-8-NF-κB-mut-Luc, pIL-8-NF-IL-6-mut-Luc or pIL-8-AP-1-mut-Luc) and 1, 2, and 3 µg of pcDNA-IE63. 24 hours post-transfection, cells were harvested and luciferase assays were performed. Results are presented as percentage of stimulation with respect to the basal expression of the promoter used (100%). At least three different experiments were conducted. ✯ =p value<0.002; ✯✯ = p value≥0.002.

Figure 4. Mapping of VZV IE63 protein repressive properties.

(A). Schematic representations of IE63 wild-type and four IE63 deletion constructs (IE63 Δ1-75, IE63 Δ76-150, IE63 Δ151-213 and IE63 Δ214-218). Two prediction phosphorylation sites for CDK1 and the NLS (black box) are shown. Amino-acids 151 to 278 contain potential sites for cellular kinases (CK1, CK2 and CDK1).

(B) and (C). Expression of wild type and mutated IE63 in Vero (B) or ND7 (C) cells was analyzed by western blotting. Vero or ND7 cells were co-transfected with 2 µg of reporter plasmids (pPOL-Luc) with 1, 2, and 3 µg of plasmids pcDNA-IE63 wild-type or deletion constructs. Cells were harvested and then a RIPA lysis was done. Cellular protein extracts were loaded on a 10% SDS-PAGE gel. After migration and transfer on a PVDF membrane, IE63 proteins were detected using a polyclonal antibody. Secondary antibodies used were coupled to peroxidase. The membranes were then revealed with ECL kit (Amersham Pharmacia).

(D) and (E). Vero and ND7 cells were co-transfected with VZV DNA polymerase promoter reporter plasmid (pPol-Luc) with 1-3 µg of plasmids pcDNA-IE63 wild type or deletion constructs (pcDNA-IE63wt, pcDNA-IE63Δ1-75, pcDNA-IE63Δ76-150, pcDNA-IE63Δ151-213, pcDNA-IE63Δ214-278) constructs. Results are presented as percentage of stimulation with respect to the basal expression of the promoter (reporter alone=100%). Three different experiments or more were conducted, and standard errors of the mean are shown as error bars.

Figure 5. Confocal microscopy analysis of wild-type and deleted IE63 proteins in Vero and ND7 cells.

Vero and ND7 cells were transfected with 2 µg of plasmids expressing either wild-type (A,B) or mutant IE63 (C, D, E, and F) and then fixed before being incubated with antibodies against IE63 and TFIIE. Confocal microscopy analysis was performed after incubation with anti-IE63 or anti-TFIIE antibodies and staining with Texas Red- or FITC-conjugated secondary antibodies, respectively. Vero (G) and ND7 (H) cells were co-transfected with the same amount of pcDNA-IE62 and pcDNA-IE63. Polyclonal antibody directed against IE62 and monoclonal antibody directed against IE63 were used. Then a staining with anti-mouse Texas Red- (showing IE63 in red) or anti-rabbit FITC- (showing IE62 in green) conjugated secondary antibody was performed. These images were merged to study co-localization.

Figure 6. Effect of VZV IE63 on the pre-initiation complex formation in ND7 cells.

Cells were transfected with pcDNA3.1$^-$ or with pcDNA-IE63 wild type or mutated. Nuclear extracts were incubated for 1h with immobilized HSV-1 gC promoters (as described in the experimental procedures). The assembled PIC was then washed

and the proteins bound onto the promoters were analyzed by SDS-PAGE and subsequent silver-staining (A) or western blotting (B).

(A). Silver stained SDS-PAGE analysis. Molecular weight corresponding to SDS6H-1VL (Sigma) is indicated, Lane 1: PIC assembly with extracts from ND7 transfected with pcDNA3.1, Lane 2: PIC assembly with extracts from ND7 transfected with pcDNA-IE63. Arrow points indicate a decrease in staining when lane 2 was compared to lane 1. Putative corresponding GTFs are shown next to the arrows. Stars correspond to proteins whose amounts are unchanged by IE63.

(B). The presence of the RNA POL II largest subunit (POLR2A), TFIIEα, TFIIH, TBP, TFIIB and IE63 after PIC assembly on the gC probe with extracts from ND7 transfected with pcDNA3.1$^-$ (lane 1) and from ND7 transfected with pcDNA-IE63 (lane 2) were assessed by Western blotting analysis. The unbound extracts were used as a positive control (lane 3).

(C) The presence of TFIIEα, TBP and TFIIB after PIC assembly with extracts from ND7 transfected with pcDNA3.1 (lane 1), pcDNA-IE63 (lane 2), pcDNA-IE63Δ151-213 (lane 3) or pcDNA-IE63Δ214-278 (lane 4) were assessed by Western blotting analysis. The excess of extracts that were not bound to the gC promoter was used as positive control (lane 5).

(D). The expression levels of IE63 (wild type or mutated forms) and of TFIIEα were verified by western blot (12% SDS-PAGE) of extracts from ND7 transfected with pcDNA-IE63wt (lane 1), pcDNA-IE63Δ151-213 (lane 2), pcDNA-IE63Δ214-278 (lane 3) and pcDNA3.1$^-$ (lane 4).

(E). Immunoprecipitation of IE63 protein. Immunoprecipitation was performed in a 175 cm^2 T-flask of ND7 transfected with 12 µg of pcDNA-IE63-IRES2-EGFP (lane 1) or pcDNA-IRES2-EGFP (lane 2). IE63 protein was immunoprecipitated using an IE63

monoclonal antibody (9A12). After washing, immunoprecipitated proteins were loaded on 10% SDS PAGE and western blotting analysis was carried out with antibodies directed against POLR2A, TFIIEα, TFIIH and IE63.

(F). Immunoprecipitation of TFIIH from ND7 cells transfected with 12 µg of pcDNA-IE63-IRES2-EGFP. After washing, immunoprecipitated proteins were analyzed by western blotting with the IE63 monoclonal antibody (9A12).

Figure 1

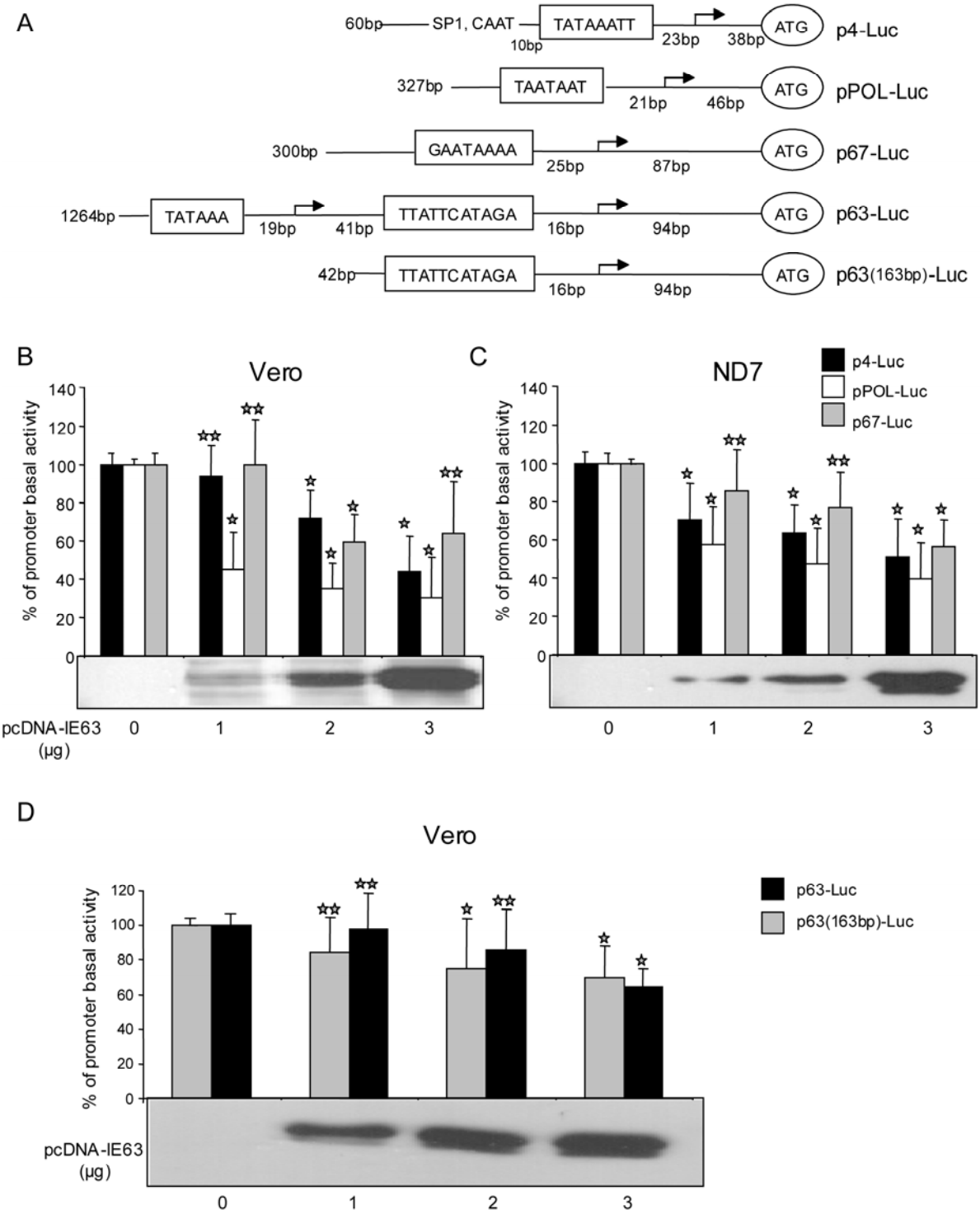

A
60bp
SP1, CAAT
TATAAATT
10bp
23bp
38bp
ATG
p4-Luc
327bp
TAATAAT
21bp
46bp
ATG
pPOL-Luc
300bp
GAATAAAA
25bp
87bp
ATG
p67-Luc
1264bp
TATAAA
19bp
41bp
TTATTCATAGA
16bp
94bp
ATG
p63-Luc
42bp
TTATTCATAGA
16bp
94bp
ATG
p63(163bp)-Luc
B
Vero
% of promoter basal activity
pcDNA-IE63 (μg)
0
1
2
3
C
ND7
p4-Luc
pPOL-Luc
p67-Luc
D
Vero
p63-Luc
p63(163bp)-Luc

Figure 2

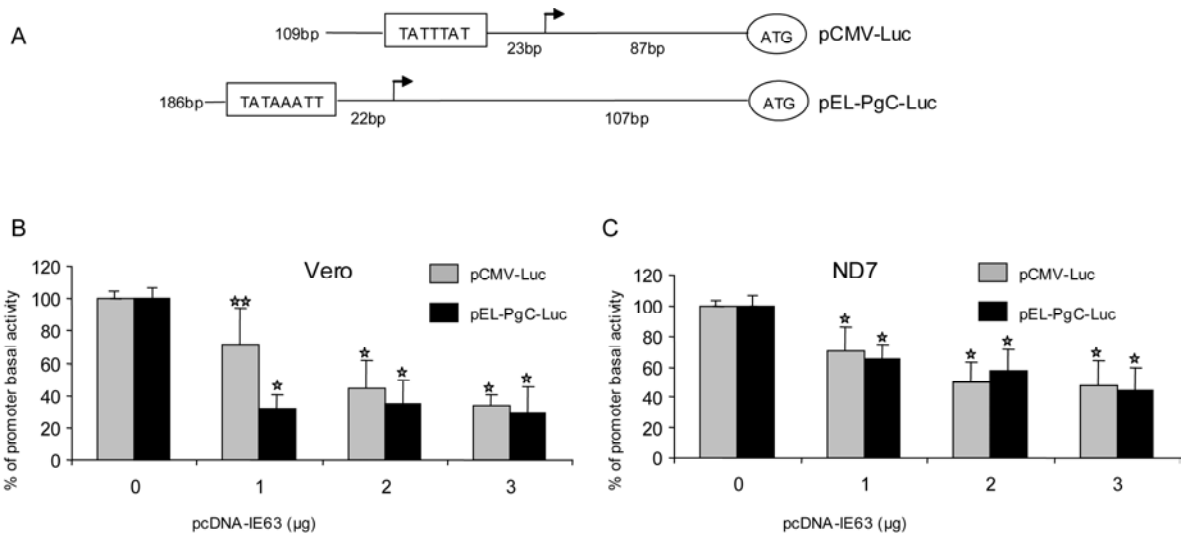

A
109bp
TATTTAT
23bp
87bp
ATG
pCMV-Luc
186bp
TATAAATT
22bp
107bp
ATG
pEL-PgC-Luc
B
Vero
pCMV-Luc
pEL-PgC-Luc
% of promoter basal activity
120
100
80
60
40
20
0
0
1
2
3
pcDNA-IE63 (μg)
C
ND7
pCMV-Luc
pEL-PgC-Luc
% of promoter basal activity
120
100
80
60
40
20
0
0
1
2
3
pcDNA-IE63 (μg)

Figure 3

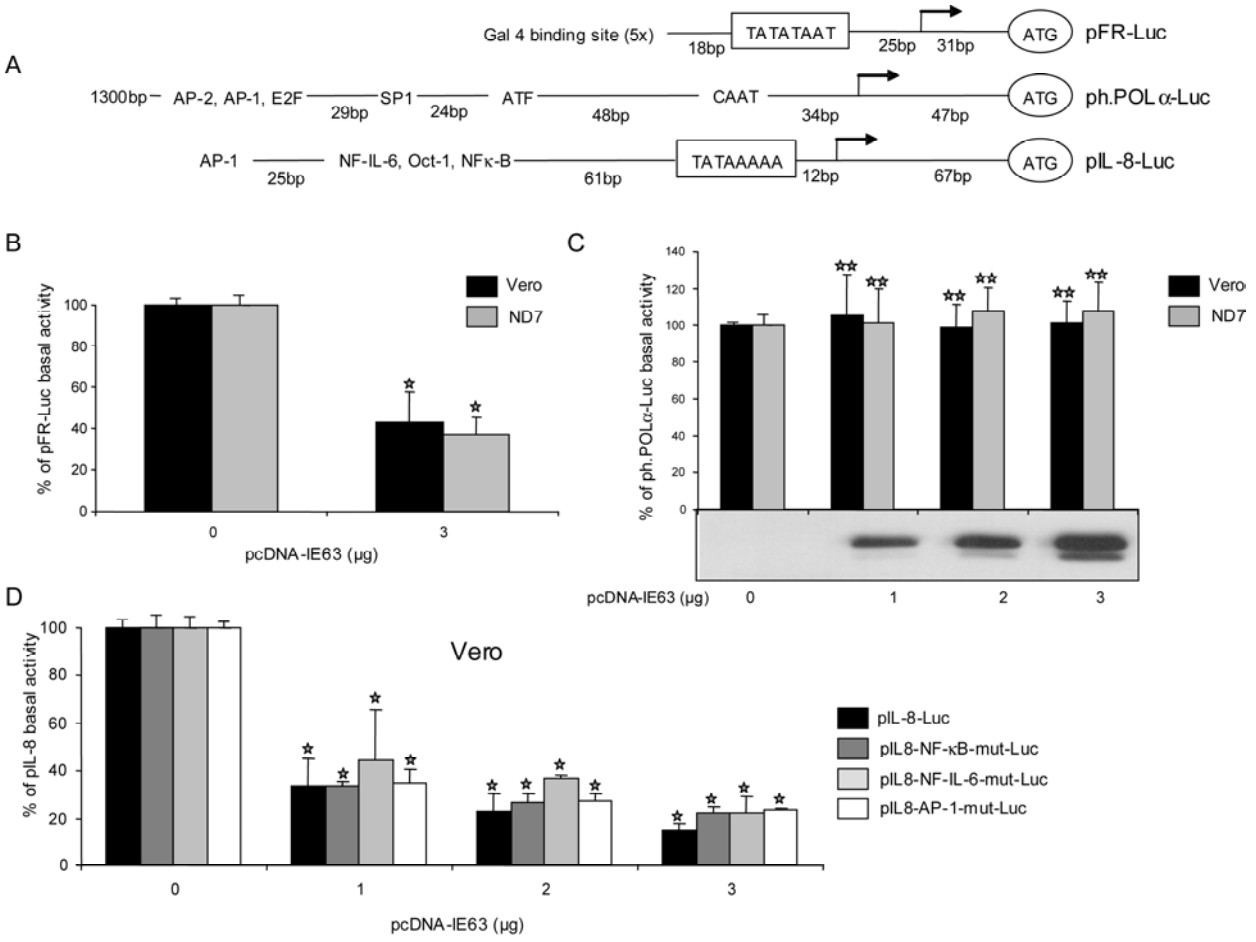

A
Gal 4 binding site (5x)
18bp
TATATAAT
25bp
31bp
ATG
pFR-Luc
1300bp
AP-2, AP-1, E2F
29bp
SP1
24bp
ATF
48bp
CAAT
34bp
47bp
ATG
ph.POL α-Luc
AP-1
25bp
NF-IL-6, Oct-1, NFκ-B
61bp
TATAAAAA
12bp
67bp
ATG
pIL-8-Luc
B
% of pFR-Luc basal activity
Vero
ND7
pcDNA-IE63 (μg)
C
% of ph.POLα-Luc basal activity
Vero
ND7
pcDNA-IE63 (μg)
D
Vero
% of pIL-8 basal activity
pIL-8-Luc
pIL8-NF-κB-mut-Luc
pIL8-NF-IL-6-mut-Luc
pIL8-AP-1-mut-Luc
pcDNA-IE63 (μg)

Figure 4

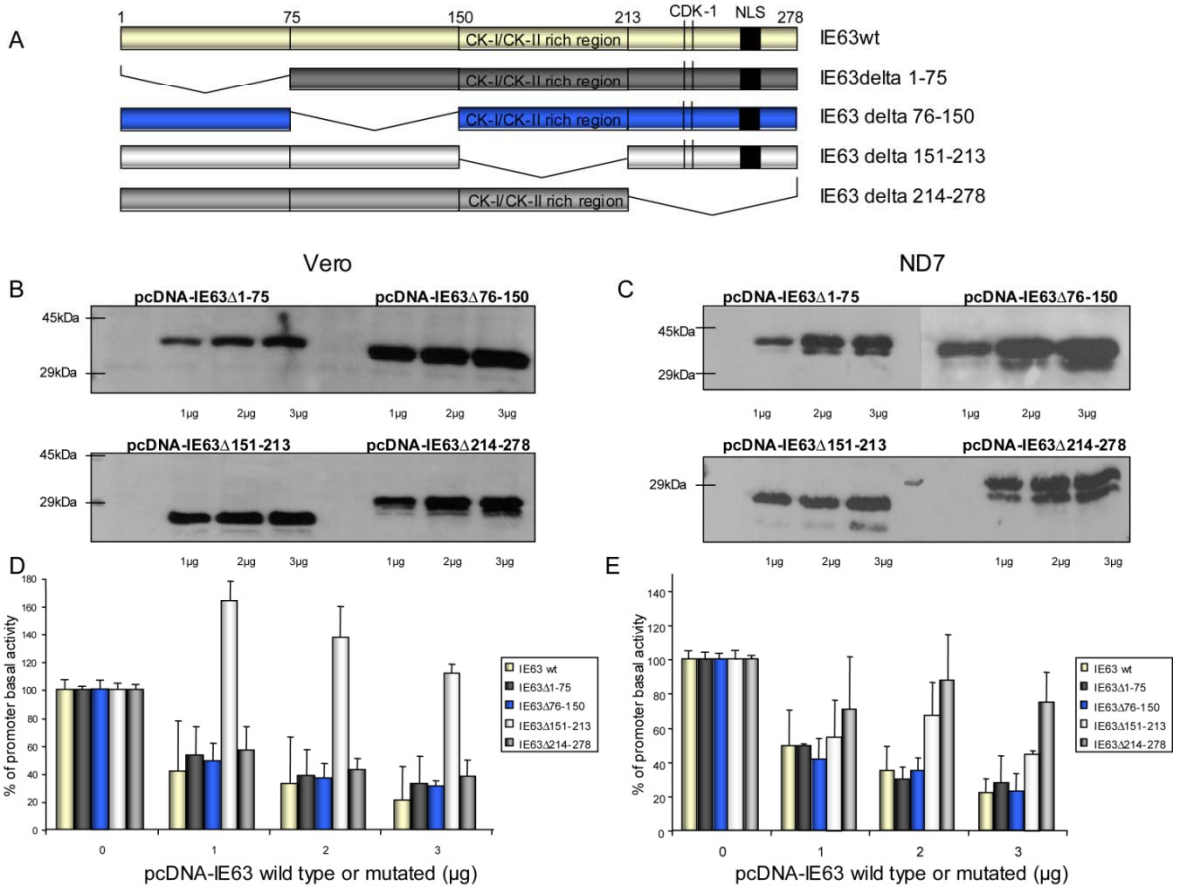

A
1
75
150
213
CDK-1
NLS
278
CK-I/CK-II rich region
IE63wt
IE63delta 1-75
IE63 delta 76-150
IE63 delta 151-213
IE63 delta 214-278
B
Vero
pcDNA-IE63Δ1-75
pcDNA-IE63Δ76-150
pcDNA-IE63Δ151-213
pcDNA-IE63Δ214-278
45kDa
29kDa
1µg
2µg
3µg
C
ND7
D
E
% of promoter basal activity
pcDNA-IE63 wild type or mutated (µg)
IE63 wt
IE63Δ1-75
IE63Δ76-150
IE63Δ151-213
IE63Δ214-278

Figure 5

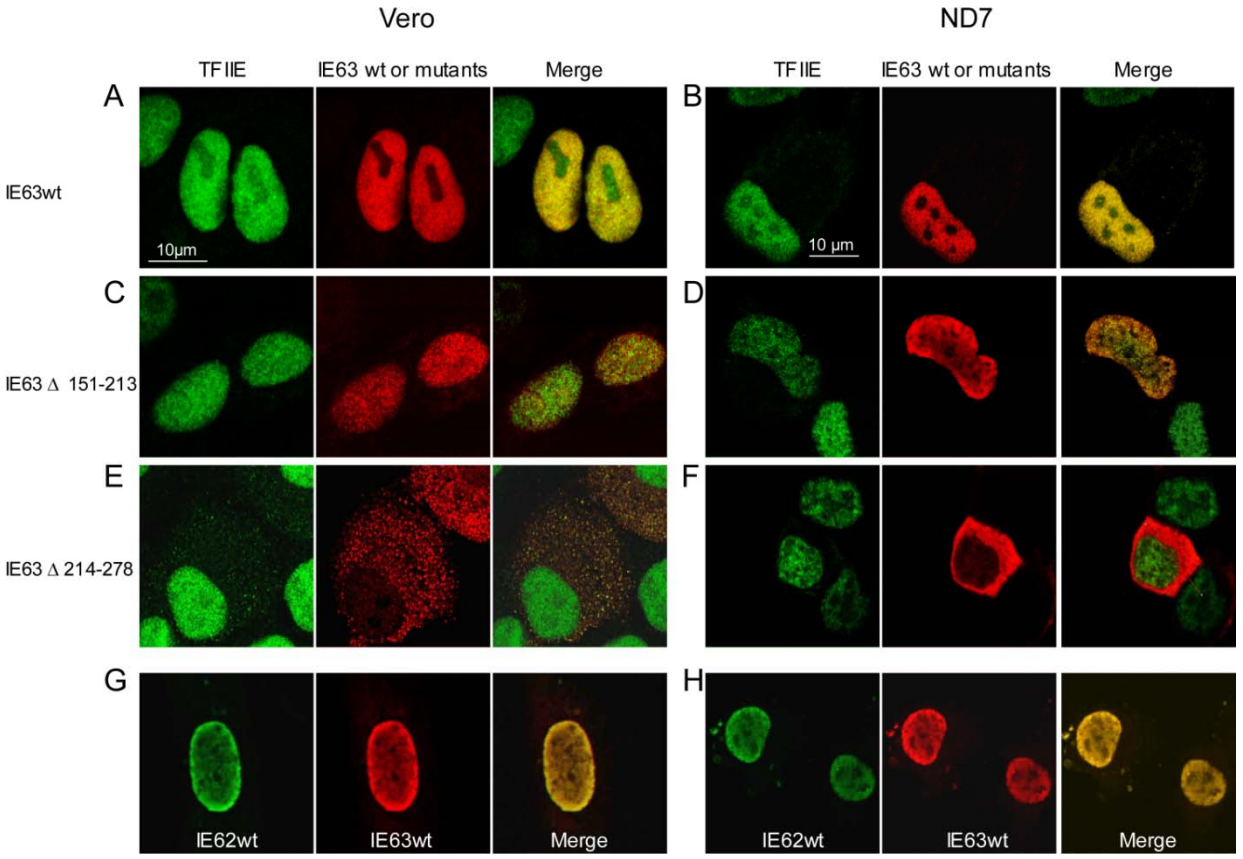

Vero
ND7
TF IIE
IE63 wt or mutants
Merge
TF IIE
IE63 wt or mutants
Merge
A
B
IE63wt
10μm
10 μm
C
D
IE63 Δ 151-213
E
F
IE63 Δ 214-278
G
H
IE62wt
IE63wt
Merge
IE62wt
IE63wt
Merge

Figure 6

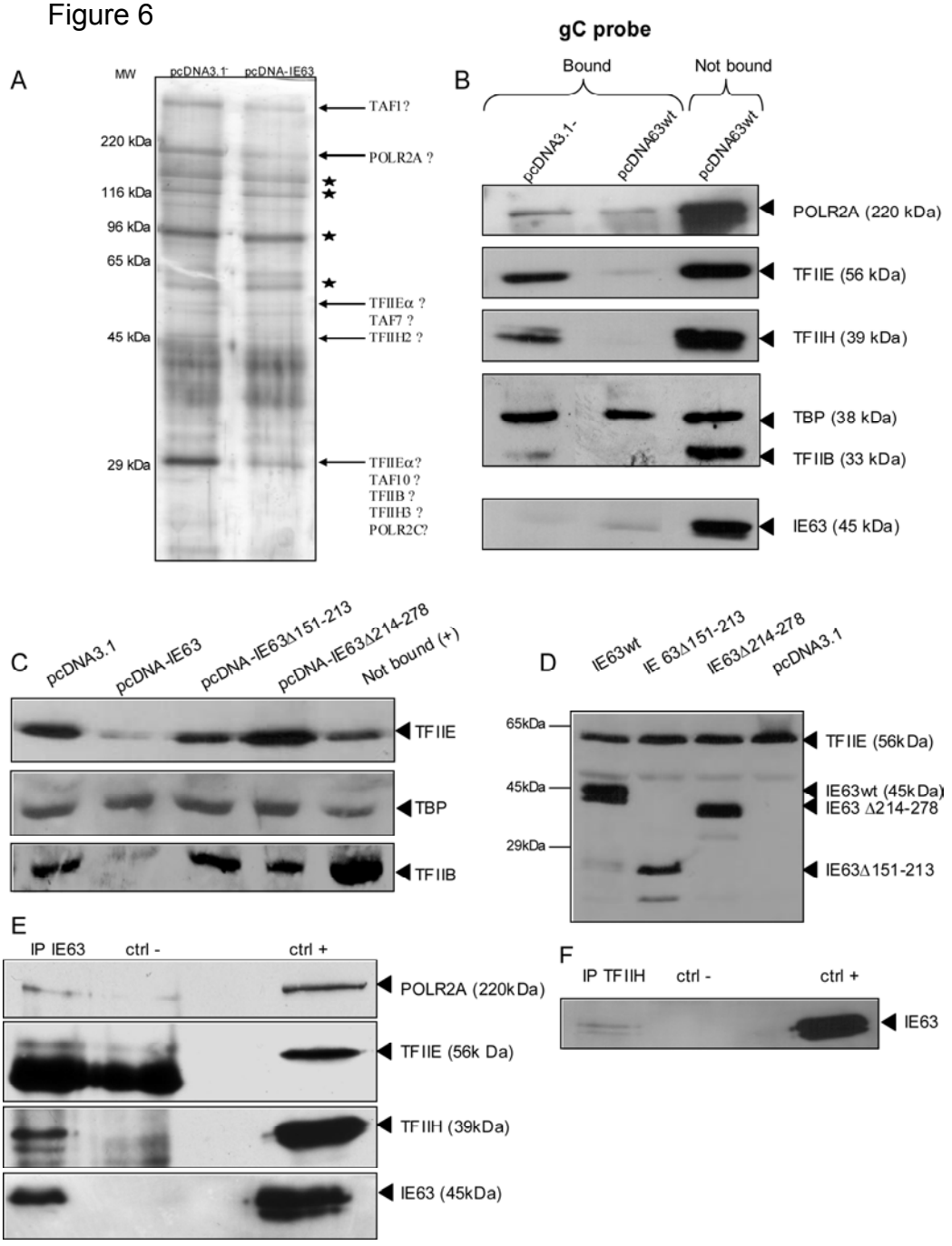
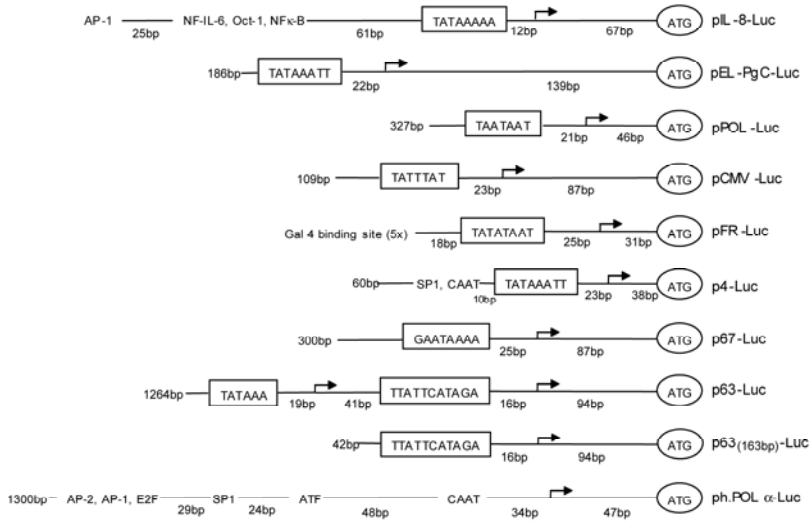

A
MW
pcDNA3.1-
pcDNA-IE63
220 kDa
116 kDa
96 kDa
65 kDa
45 kDa
29 kDa
TAF1?
POLR2A ?
TFIIEα ?
TAF7 ?
TFIIH2 ?
TFIIEα?
TAF10 ?
TFIIB ?
TFIIHB ?
POLR2C?
B
gC probe
Bound
Not bound
pcDNA3.1-
pcDNA63wt
pcDNA63wt
POLR2A (220 kDa)
TFIIE (56 kDa)
TFIIH (39 kDa)
TBP (38 kDa)
TFIIB (33 kDa)
IE63 (45 kDa)
C
pcDNA3.1
pcDNA-IE63
pcDNA-IE63Δ151-213
pcDNA-IE63Δ214-278
Not bound (+)
TFIIE
TBP
TFIIB
D
IE63wt
IE 63Δ151-213
IE63Δ214-278
pcDNA3.1
65kDa
45kDa
29kDa
TFIIE (56kDa)
IE63wt (45kDa)
IE63 Δ214-278
IE63Δ151-213
E
IP IE63
ctrl -
ctrl +
POLR2A (220kDa)
TFIIE (56k Da)
TFIIH (39kDa)
IE63 (45kDa)
F
IP TFIIH
ctrl -
ctrl +
IE63

Table 1

A



B

Fruitfly score	tfbind score	Transfac score	Mean percentage of repression at 3 µg of pcDNA-IE63	
			Vero	ND7
1	0.95	0.92	83% ±7	67% ±14
1	0.9	0.87	71% ±15	56% ±14
0.92	0.84	0.74	70% ±11	61% ±17
0.8	0.86	0.89	67% ±6	52% ±15
0.96	0.89	0.83	57% ±14	63% ±8
0.97	0.85	0.81	56 % ±17	49% ±18
0.65	0.85	0.81	36 % ±25	43% ±13
0.65	0.85	0.81	31% ±11	31% ±5
0.27	<0.5	<0.70	30% ±17	nd
<0.15	<0.5	<0.7	0% ±12	0% ±15