Intracellular distribution of the ORF4 gene product of varicella-zoster virus is influenced by the IE62 protein

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INTRODUCTION

The course of productive infection by varicella-zoster virus (VZV), the causative agent of chickenpox and shingles, is most probably orchestrated in a sequential and highly ordered manner, as is the case for the prototype system of the subfamily *Alphaherpesvirinae,* herpes simplex virus type 1 (HSV-1) (Honess & Roizman, 1974, 1975). Since VZV is highly cell-associated *in vitro,* studies of its biology have been hindered. Current insight into the regulation of VZV gene expression has been largely gained by analogy with HSV-1 and the more recent appearance of direct molecular analyses (for a review see Ostrove, 1990; Davison, 1991).

VZV DNA carries four open reading frames (ORF4, ORF61, ORF62 and ORF63) which encode proteins exhibiting similarities in sequence with HSV-1 immediate-early regulatory proteins (Davison & Scott, 1986). Two of these proteins (the gene products of ORF61 and ORF62) can complement their HSV-1 homologues ICPO and ICP4 (Disney & Everett, 1990; Moriuchi *et al,* 1992); VZV ORF4-encoded protein (IE4), on the other hand, appears to be functionally distinct from HSV-1 ICP27 (Moriuchi *el al,* 1994; Perera *et al,* 1994). Little is known regarding the functional relationship between IE63 and HSV-1 ICP22. No HSV-1 ICP47 homologue has been proposed in VZV. These four proteins display regulatory functions in transient assays; moreover, ORF61p excepted, all have been classified as immediate-early proteins (Forghani *et al,* 1990; Debrus *et al,* 1995; S. Debrus, P. Defechereux, B. Rentier and J. Piette, unpublished results). Cooperative interactions amongst VZV putative immediate-early proteins are likely events (Perera *et al,* 1992a; Hay & Ruyechan, 1994). IE62, defined as a major regulatory protein, can transactivate the expression of VZV genes of all three putative kinetic classes (immediate-early, early and late) (Inchauspe *el al.,* 1989; Inchauspe & Ostrove, 1989; Perera *et ah,* 1992 a, b; Baudoux *et al,* 1995) and autoregulate its promoter (Disney *et ah,* 1990; Perera *et ah,* 1992b). IE4, which possesses transactivating properties on various promoters when transfected alone, can enhance activation by IE62 when both proteins are expressed (Inchauspe *et ah,* 1989; Inchauspe & Ostrove, 1989; Perera *et ah,* 1992a; Defechereux *et ah,* 1993; Moriuchi *et ah,* 1994). Depending on the cell line, ORF61p is a transactivator of VZV promoters in the absence of other viral regulators but is a repressor of IE4- and IE62-mediated activation (Nagpal & Ostove, 1991; Moriuchi *et ah,* 1992, 1993; Perera *et ah,* 1992a). IE63 can inhibit activation by IE62 or IE4 (S. Debrus, P. Defechereux, B. Rentier and J. Piette, unpublished data; Hay & Ruyechan, 1994). In the lytic cycle, these different activities could be coordinated to lead to effective virus production. Similar DNA transfection studies performed on HSV-1 have led researchers to investigate cooperativity among immediate-early proteins ICPO, ICP4 and ICP27 (Sekulovich *et ah,* 1988; Su & Knipe, 1989; Zhu *et ah, 1994).* One approach has b een to address intracellular localization of the proteins in the presence of each other. In transient assays, ICPO and ICP4, which are both nuclear when present individually, can act synergistically to transactivate gene expression (Sekulovich *et ah,* 1988); in contrast, ICP27 (also nuclear), which

displays little or no activity on its own, can positively or negatively modulate virus gene expression induced by ICP4 and ICPO (Sekulovich *et ah,* 1988; Su & Knipe, 1989). Immunofluorescence assays (IFA) performed in Vero cells infected with wild-type or various mutant viruses indicated that ICP27 has a significant inhibitory effect on the nuclear localization of ICP4 and ICPO (Zhu *et ah,* 1994; Zhu & Schaffer, 1995). This effect was confirmed in transient assays with pairs of plasmids expressing wild-type forms of the proteins (Zhu & Schaffer, 1995). In contrast, staining pattern analyses performed in cotransfected Vero cells demonstrated (i) that ICP4 promotes the nuclear entry of ICPO and (ii) that ICPO influences the intranuclear localization of ICP4, with both proteins being shown to colocalize in discrete punctate structures (Mullen *et ah,* 1995). Moreover, there is now evidence for a physical interaction between ICPO and ICP4, adding to the observations of mutual influence on their subcellular distribution (Yao & Schaffer, 1994). Hence, another dimension of gene expression regulation has been revealed in HSV-1 infection, possibly linking function to intracellular location. VZV IE62 is located in nuclei of infected (Forghani *et ah,* 1990) and transfected cells (Baudoux *et ah,* 1995), whereas IE4 is mainly distributed in the cytoplasm (Moriuchi *et ah,* 1995). A possible means for IE4 to perform some of its regulatory functions is to be transported into nuclei by other proteins such as IE62. In this report, we have used double-label IFA to investigate the mutual influence of IE4 and IE62 on their intracellular localization in VZV-infected or DNA-transfected Vero cells.

METHODS

Cells, virus and infections.

Vero cells were grown at *37 °C* in Medium 199 supplemented with 10% heat-inactivated fetal calf serum. VZV strain Ellen was propagated by mixing trypsinized VZV-infected cells with non-infected cells at a 1:4 ratio. To perform IFA, Vero cells were plated in 8-5 mm diameter 24-well cluster dishes containing sterile glass coverslips the day before infection. When confluent, cells were overlaid with VZV-infected Vero cells at a 1:4 ratio. Infected monolayers reaching 60% CPE were used for IFA.

Plasmids and antibodies.

Plasmids expressing VZV IE proteins under the control of the cytomegalovirus (CMV) major IE promoter (pCMV4, pCMV61 and pCMV63) were kindly provided by P. Kinchington (University of Pittsburgh, Penn., USA). pGi4 contains a single copy of ORF4 driven by its cognate promoter (Inchauspe *et al,* 1989). IE62 was expressed from pSV62 (Disney *et al,* 1990). pHN570, pHNll37 and p 612-865, which are insertion and deletion mutants of IE62, are described in Baudoux *et al* (1995); mutant pHN570 contains an in-frame insertion after codon 570 of IE62 and mutant pHN1137 contains an in-frame insertion after codon 1137 of IE62. The in-frame deletion mutant p 612-865 was constructed by removing the region extending from codon 612 to 865 in the IE62 protein using *Hpal* and *Nrul* restriction enzymes.

In order to detect IE4, a rabbit polyclonal antiserum (VZVIE4) was raised against a synthetic branched peptide of sequence H-SKRDAR-RERIRKE-OH (aa 169—181) according to described procedures (McLean *et al,,* 1991). To follow IE62, VZVIE62, a mouse monoclonal antibody specific for IE62, was purchased (Viro). A rabbit polyclonal antiserum (iepl278) directed against a synthetic peptide corresponding to the C terminus of IE62 (kindly supplied by P. Jacobs, University of Brussels, Belgium) was also used. Previously generated rabbit antiserum against IE63 was used to follow IE63 intracellular distribution (Debrus *et ah,* 1995). ORF61p was visualized with anti-61 serum generously provided by A. Davison (University of Glasgow, UK).

Transfections.

Vero cells were seeded in 8-5 mm diameter 24-well cluster dishes containing sterile glass coverslips 16 h prior to transfection with LipofectAMINE according to the manufacturer's instructions (Gibco BRL). IE expression plasmids (2 μg) were either transfected alone or in combination, with the total amount of DNA kept constant in each experiment by the addition of sonicated herring sperm DNA. At 24 h post-transfection the medium was replaced and at 48 h post-transfection cells were fixed and processed for single- or double-label IFA.

Immunofluorescence studies.

Transfected or infected Vero cells grown on coverslips as described above were washed twice in cold PBS, fixed for 20 min in 1:1 acetone:methanoI (v/v) and immediately stored at — 20 °C. To perform the single- or double-label indirect IFA, cells were rehydrated in PBS/dry milk (3 % w/v) for 30 min at room temperature before the primary antibodies were added. Dilutions of primary antibodies in PBS/dry milk were: VZVIE4, 1:10; MAb VZVIE62, 1:60; iep1278, 1:250; VZVIE63, 1:100; and VZVIE61, 1:20. Incubation was performed at 3 7 °C for 60 min. Following three washes in PBS, incubation was continued with the secondary antibodies, FITC-conjugated swine anti-rabbit (1:40) (Dako) and/or Texas Red-conjugated sheep anti-mouse (1:30) (Amersham) for 45 min at 37 °C. In single-label IFA, secondary FITC-conjugated swine anti-rabbit antibody was incubated with 1% (w/v) Evans blue as counterstaining. Coverslips were then rinsed three times in water and mounted on glass slides with Fluoprep (bioMérieux) to prevent quenching of fluorescence. Processed coverslips were viewed under a Leitz fluorescence microscope equipped with 40 x and 100 x water objective lenses and fitted with adequate filter sets for detection of the two chromophores used. Pictures were taken with Fuji Sensia 400 film.

RESULTS

IE62 influences the subcellular distribution of the IE4 protein in transfected cells

IE4 has been shown to be a strong transactivator of VZV and heterologous gene expression in transient transfection assays (Inchauspe *et al.,* 1989; Inchauspe & Ostrove, 1989; Perera *et al.,* 1992*a;* Defechereux *et al.,* 1993). Cotransfection experiments demonstrated the capacity of this protein to enhance the activation seen with IE62 on VZV gene promoters refractory to IE4 alone (Perera *et ah,* 1992 *a).* One possible mechanism of such a stimulation is that an interaction occurs between these two proteins, thereby influencing their intracellular localization. This hypothesis was addressed in the absence of other regulatory proteins in cotransfection experiments with plasmids expressing intact IE4 and IE62 in Vero cells. The intracellular distribution of these proteins was examined by single- and double-label IF A.

First, cells were transfected only with plasmid pGi4, which carries ORF4 under the control of its cognate promoter (Inchauspe *et a/.,* 1989). It should be noted that the number and intensity of positively labelled cells was rather low (< 1 %). Such a situation has been repeatedly observed with ICP27 under the control of its cognate promoter (Mears *et al,* 1995; Zhu & Schaffer, 1995). In contrast, transfection with the IE62-expressing plasmid alone led to a higher number of positive cells (> 10%). Since ORF62 was under the control of a simian virus 40 promoter more powerful than the ORF4 cognate promoter, a plasmid expressing IE4 from the strong CMV promoter (pCMV4) was also assayed in an attempt to increase IE4 expression levels and to verify whether IE4 concentrations influence intracellular localization. In single transfections with pCMV4, a marked increase in the number of transfected cells was observed compared to pGi4 (> 10%) without alteration of the intracellular pattern (data not shown). Increasing the amounts of both ORF4 vectors (4 and 10 μg) did not change the subcellular distribution of the protein (data not shown). The distribution of IE4-specific fluorescence was determined by counting immunofluorescence-positive cells and placing them in one of the following categories: cells exhibiting exclusively cytoplasmic staining, cells exhibiting exclusively nuclear staining or cells exhibiting nuclear and cytoplasmic staining with cytoplasmic fluorescence either greater than, equal to, or less than nuclear fluorescence. Table 1 gives the average percentage of cells which fell into each subclass after transfection with pCMV4. IE4 staining (Fig. 1) was predominantly cytoplasmic *(55-8%)* or cytoplasmic and nuclear, with staining in the nucleus weaker than in the cytoplasm *(27.5 %).* Some dots of higher intensity were frequently present in the nuclei of these cells. These differences in staining might be partially explained by the transfection conditions and do not necessarily reflect normal infection patterns because of the absence of interactions and regulation by other virus proteins. Cells individually transfected with pSV62 showed essentially diffuse nuclear fluorescent staining (Baudoux *et al.,* 1995).

In the presence of IE62, IE4 intracellular distribution was modified as shown in Table 1, which

summarizes the categorization of cells exhibiting IE4-specific fluorescence in a set of independent cotransfection experiments conducted with 2 µg pCMV4 and 2 µg pSV62. Monolayers were doubly stained for IE4 and IE62. IE4-expressing cells were counted and placed in categories as described above. IE62-specific staining was extensive and diffusely nuclear in transfected cells with nucleolar exclusion (Fig. *2a, c).* Ten percent of cells exhibited IE62 in the cytoplasm, many of which only displayed intense fluorescence in the endoplasmic reticulum.

Table 1. Intracellular localization of IE4 after cotransfection with plasmids expressing wild-type or mutant forms of IE62

N	N>C	N = C	N <C	c	Plasmids[1]
		Localization of IE4[2]			
0	1.6 ± 1.6	14.1 ± 4.9	27.5 ± 11.5	55.8 ± 9.6	pCMV4 *(n = 10)*
24.4 ± 10.5	33.1 ± 11.7	16 ± 7.4	13.4 ± 7.9	12.7 ± 9.7	pCMV4 + pSV62 *(n = 10)*
12.9 ± 3	34 ±5.6	13.1 ± 3.6	26 ± 4.3	14 ± 3.5	pCMV4 + pHN5 70 *(n = 2)*
0	1.2 ±1.3	8.7 ± 3	31.5 ± 5.2	59 ± 9	pCMV4 + pHN1137 (n = 4)
0	0.9 ± 0.1	9.4 ±0.9	41.4 ±4	48.7 ± 3	pCMV4 + p 612.865 *(n = 3)*

[2] *Percentages of IE4-positive cells in each pattern of staining (as described in legend to Fig. 1) were determined in several independent experiments. Averages and SD (SEM for pCMV4) of n independent experiments are presented.*
[1] *Effector plasmids (2 µg/ml each) used were as follows: IE4, pCMV4; wild-type IE62, pSV62; mutant IE62 polypeptides, pHN570 (insertion in region 2), pHN1137 (insertion at the border of region 4 and 5) and p 612-865 (deletion of region 3 and part of region 4).*
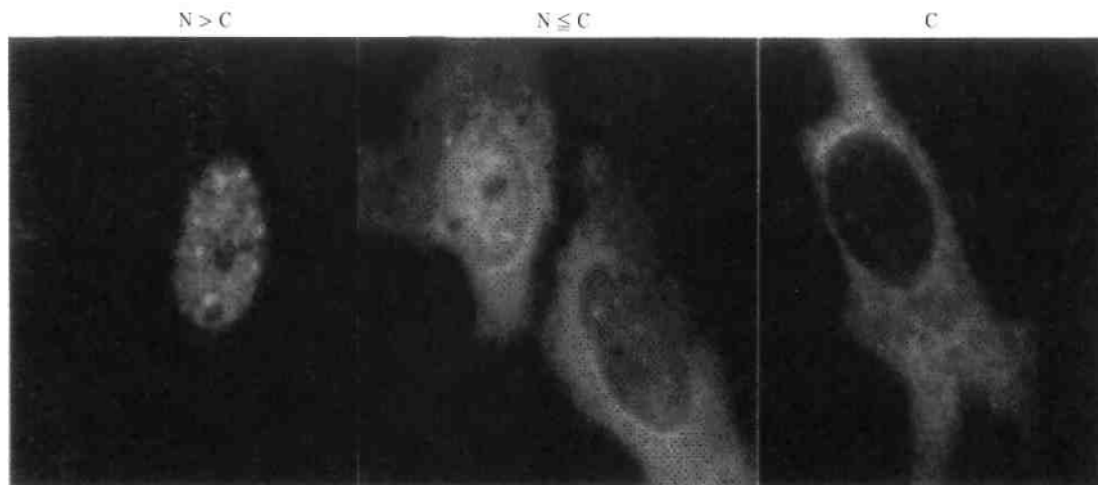
Fig. 1. Intracellular distribution of IE4 protein in Vero cells transfected with IE4 expression vector. Cells were transfected with 2 µg of pCMV4. Indirect IFA was performed with rabbit antiserum VZVIE4 and FITC-conjugated swine anti-rabbit IgG. Immunofluorescence-positive cells were counted and categorized in one of five groups: cells that exhibited only nuclear staining (N); nuclear staining greater (N > C), equal (N = C) or less (N < C) than cytoplasmic staining; or cytoplasmic staining only (C). The average percentage and SEM of cells belonging to each category was calculated from 1 0 independent transfection experiments and is given in Table 1. Examples of each category except N are illustrated; N C, nuclear staining equal to or less than cytoplasm staining.

When IE62 was present in nuclei, the percentage of cells exhibiting IE4 nuclear staining (Fig. *2 b)* and, most predominantly, cells with nuclear staining more intense than cytoplasmic staining (Fig. *2d)* increased significantly, while the percentage of cells with cytoplasmic staining decreased (see Table 1). Statistical analysis of the results of 10 experiments with the χ^2 test for association showed that IE4 and IE62 distributions were not independent. Generally, high levels of IE62 expression in nuclei (very intense fluorescence, Fig. *2 a, c)* correlated with the presence of IE4 in the nucleus, whereas lower levels of IE62 fluorescence were found in cells where IE4 was only cytoplasmic (data not shown). This suggests that a certain critical IE62 concentration could be required for nuclear translocation of IE4.

The CMV promoter in pCMV4 is very poorly stimulated by IE62 (Baudoux *et al.,* 1995) so transactivation of IE4 expression is unlikely to be responsible for the observed changes in its intracellular distribution.

In these experiments, it was very striking that no significant alteration of IE62 intracellular localization was observed in response to the presence of IE4. The proportion of IE62-positive nuclei remained identical whether IE4 was expressed or not.

Intracellular localization of IE4 and IE62 in VZV-infected cells

In order to compare observations made in transient transfection assays with the physiological localization of IE62 and IE4 during infection, Vero cells were infected with VZV strain Ellen and processed for double-label immunofluorescence in order to detect IE4 and IE62 simultaneously. IE62 was most prominent in the nuclei of infected cells, as previously described by Forghani *et al.* (1990). Some nuclei showed intense globular staining, probably correlating with replication compartments. The staining pattern for IE4 in these cells was mainly cytoplasmic but in numerous infected cells, a slight staining with occasionally intense fluorescent dots could be detected in the nuclei (resembling the C and N C patterns seen in Fig. 1). In certain cases, these dots were found also to contain IE62 (data not shown). These observations were reproduced in MRC5 cells, another cell line used to grow VZV in our laboratory (S. Debrus, P. Defechereux, B. Rentier and J. Piette, unpublished results).

ORF61 p and IE63 proteins are not capable of inducing nuclear translocation of IE4

ORF61p and IE63 are two VZV regulatory proteins which could intervene in IE4 distribution, since they both possess transactivating and transrepressing properties and can interfere or synergize with IE4 (S. Debrus, P. Defechereux, B. Rentier and J. Piette, unpublished data; Nagpal & Ostrove, 1991; Perera *et al.,* 1992A; Hay & Ruyechan, 1994). Transfection experiments were undertaken with pCMV61 or pCMV63, which carry the genes under the control of the powerful CMV promoter/enhancer. To detect ORF61p and IE63, IFA were performed in individually transfected cells with rabbit antisera VZVIE61 and VZVIE63, respectively.
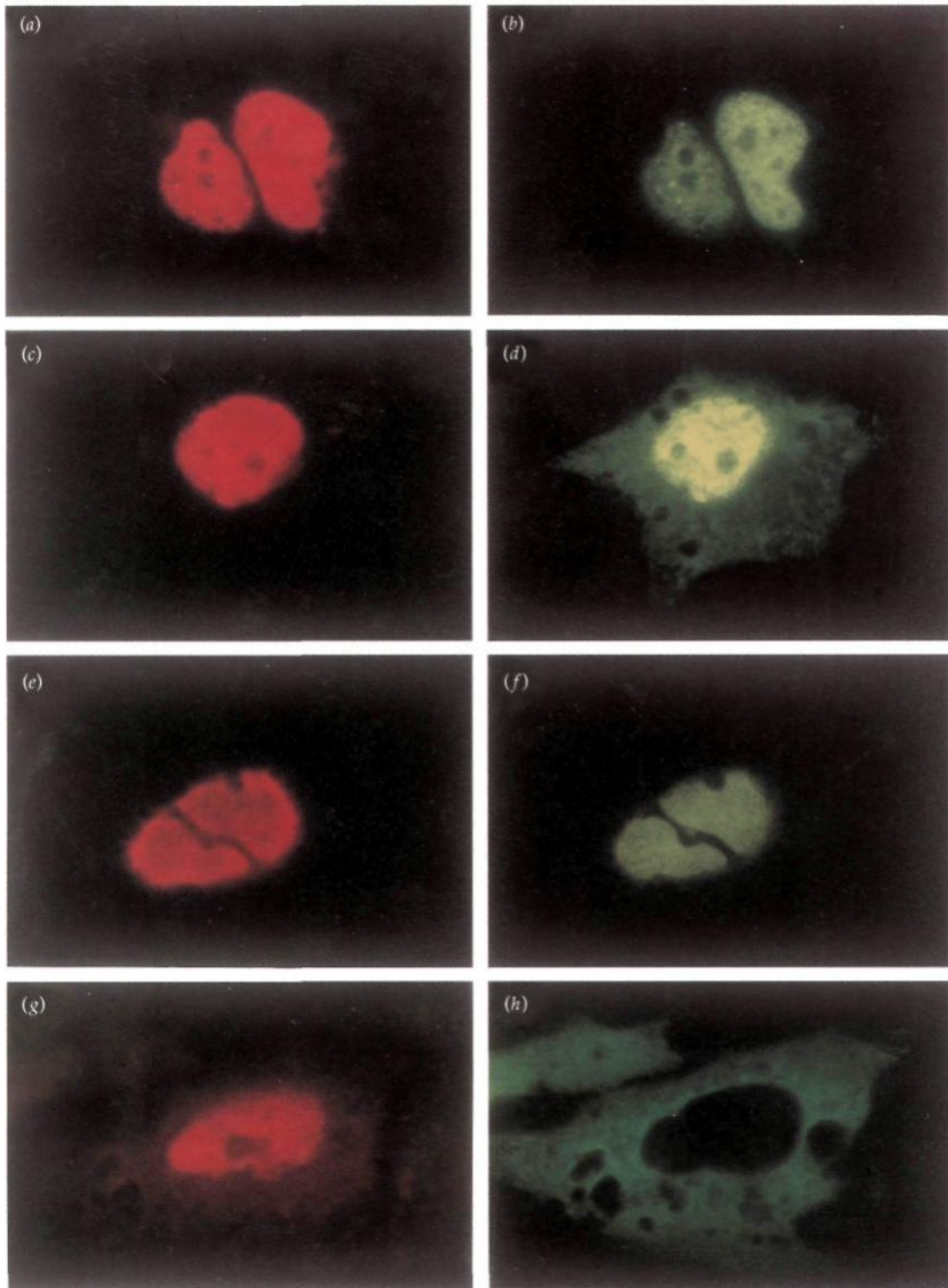
***Fig**. 2. Intracellular distribution of IE4 and IE62 proteins after cotransfection with IE4 and wild-type or mutant IE62 expression vectors. Monolayers were transfected with 2 μg of pCMV4 and 2 μg of pSV62 (a-d), 2 μg pHN570 (e-f) or 2 μg pHN1 1 37 (g-h). Coverslips were incubated with a mixture of MAb VZVIE62 (a, c, e, g) and antiserum VZVIE4 (b, d, f, h) as primary antibodies, then incubation was pursued with FITC-conjugated swine anti-rabbit IgG and Texas Red-conjugated sheep anti-mouse IgG as secondary antibodies.*

Table 2. Intracellular localization of IE4 after cotransfection with plasmids expressing ORF61p or lE63

| Expt | Number[t] | Localization of IE4* | | | | | Plasmids[f] |
		N	N >C	N = C	N <C	c	
1	434	0	0'7	10	19	70.3	pCMV4 + pCMV61
	506	0	1	11.9	48	39.1	pCMV4 + pCMV63
2	263	0	1.4	14.1	36.4	48.1	pCMV4 + pCMV61
	283	0.4	3.8	10.7	28.1	57	pCMV4 + pCMV63

* Percentages of IE4-positive cells in each pattern of staining were determined for two independent experiments.
[t] Total number of immunofluorescence-positive cells examined in each transfection.
[f] Effector plasmids (2 μg/ml each) used were as follows: IE4, pCMV4; IE63, pCMV63; ORF61p, pCMV61.

In transfected cells, ORF61p-associated fluorescence displayed a diffuse pattern with intense dots, reminiscent of that of infected cells (Stevenson *et al.*, 1992) and of its HSV-1 homologue ICP0 (Mullen *et al.,* 1995). IE63 staining was found with greater intensity in the nuclear compartment than in the cytoplasm, as had previously been shown in infected cells (Debrus *et al.,* 1995). The number of positive cells was usually high, reflecting efficient transient expression. Cotransfection experiments involving pCMV63 or pCMV61 and pCMV4 were then conducted. To ensure the conditions of transfection were appropriate for IE4 translocation, a control cotransfection with pSV62 was conducted in parallel. Cotransfected coverslips were analysed in parallel with antibodies VZVIE63 or VZVIE61 and VZVIE4 (data not shown). IE4-positive cells were counted and assigned to the different categories (Table 2). In neither case was IE4 found to be transported in nuclei, in contrast to the situation with wild-type IE62. Therefore, translocation to the nucleus is not an artifact resulting from cotransfection with regulatory proteins and seems specific to cotransfection with the major regulatory protein IE62. The presence of IE4 did not influence IE63 or ORF61p intracellular localizations in our cotransfection system (data not shown).

Effect of IE62 mutants on IE4 intracellular localization

In an attempt to elucidate the mechanism through which IE62 affects IE4 subcellular localization and to distinguish between promoter transactivation and other direct or indirect influences, cotransfection experiments with three plasmids (derived from pSV62) encoding mutant IE62 polypeptides and the pCMV4 vector were carried out. Expression vector pHN5 70 contains an in-frame insertion after the 5 70 codon of ORF62. This mutation is located in region 2 (the DNA-binding domain; Wu & Wilcox, 1991) and results in the loss of IE62 transactivating properties (Baudoux *et al.,* 1995). IFA on pHN5 70-transfected cells showed diffuse nuclear staining with two or more intense fluorescent regions, somewhat different to the diffuse nuclear staining observed with wild-type IE62 (Fig. *2e).* When pHN570 and pCMV4 were cotransfected, the overall distribution of IE4-specific fluorescence resembled that of cells cotransfected with wild-type IE62 and pCMV4 (Table 1). IE4 colocalized with the mutant polypeptide in the nuclear regions (Fig. 2 *e, f*). The influence of IE62 on IE4 localization was clearly not dependent on its transactivating property.

To confirm this observation, pHN1137, another IE62 mutant which carries an insertion at codon 1137 in the C-terminal end of region 4 (Baudoux *et al*., 1995) and has lost most its transactivating activity, was tested. The mutant protein encoded by pHNH37 exhibited similar diffuse nuclear staining to wild-type IE62 (Fig. *2g).* Interestingly, pHNH37 seemed incapable of directing IE4 to the nuclei of transfected cells (Fig. *2h).* The distribution of IE4-positive cells was identical to that of IE4 vector alone (Table 1). This suggested that the region around amino acid 1137 of IE62 might play an important role in the translocation mechanism.

Finally, p 612-865, a deletion mutant lacking the whole region 3, which contains a putative nuclear localization signal (NLS; Baudoux *et al.,* 1995), and part of region 4, was cotransfected with IE4. p 612-865 has lost the epitope recognized by MAb VZVIE62, since no fluorescence was detected in double-label IFA. Therefore, coverslips were treated separately with iepl278 and VZVIE4. p 612-865

is clearly cytoplasmic (Baudoux *et al.*, 1995) and unable to induce nuclear translocation of IE4 (Table 1). Therefore, loss of this region, either intrinsically or through the influence it has on overall IE62 integrity, prevents the translocation of IE4 into the nucleus.

DISCUSSION

The present study focused on characterizing VZV IE4 protein intracellular distribution in the presence of IE62. It showed that IE62 could influence the intracellular localization of IE4 in transfected cells: when IE62 was present, IE4 became more nuclear.

In the course of productive infection, IE4 was mainly cytoplasmic, although a slight IE4-specific staining could also be found in nuclei. In nuclei of infected Vero and MRC5 cells (S. Debrus, P. Defechereux, B. Rentier and J. Piette, unpublished results), IE62 was frequently distributed in dots or speckles which were sometimes also found to contain IE4. One explanation as to why not all cells within foci displayed overlapping fluorescent signals might be that such an event occurs only at precise times during the course of virus replication and is dependent upon regulatory protein concentrations. To understand the apparent differences between the observations made in infected and in cotransfected cells, the particular conditions associated with analysis of protein functions *in vitro* should be taken into consideration. Most importantly, the absence of other virus proteins which could influence IE4 and/or IE62 expression and localization must be acknowledged. Recent data acquired in our laboratory clearly indicate a down-regulatory activity of IE63 on IE4 trans-activation (S. Debrus, P. Defechereux, B. Rentier and J. Piette, unpublished results), which could influence IE4 expression levels in VZV-infected cells. Availability of VZV mutants should help to clarify the physiological relevance of such *in vitro* observations.

ICP4 and ICP27, which both possess NLS, are found predominantly in nuclei of HSV-1-infected cells, with ICP4 present in replication compartments (Mears *et al.,* 1995; Zhu & Schaffer, 1995). In light of our observations, VZV IE4 locates differently from HSV-1 ICP27. Therefore, despite sequence similarities, these proteins are different in their localization and, at least partially, in their functions (Moriuchi *et ah,* 1995).

In transfected cells, IE4 tends to locate in the cytoplasm and in the nucleus, with intensity of nuclear staining weaker than in the cytoplasm. Recent findings suggest that the N-terminal portion (1—403) of the molecule contains a NLS, although no sequence has been proposed (Moriuchi *et al.,* 1995). By computer search analysis, we have found a putative bipartite signal, RRSSRSYNTQSSRKHRDR, between amino acids 118 and 134. This sequence is located at a position similar to that of the recently defined ICP27 NLS (aa 110-137) (Mears *et al,* 1995). The function of this putative NLS needs to be experimentally determined. The C terminus (aa 404—452) appears to play a role in the cytoplasmic distribution of the protein, since a truncation mutant lacking this region was exclusively localized in the nucleus in a speckled pattern with nucleolar exclusion (Moriuchi *et al.,* 1995). In our experiments, a speckled arrangement was observed in the nuclei of a few cells transfected with pCMV4 alone; however, in such cases, diffuse cytoplasmic staining was present. Interestingly, no speckles were ever detected in the cytoplasm. The IE4 protein seems to possess an intrinsic capacity to be directed to the nucleus, under undefined conditions, possibly involving its C terminus.

IE4-specific staining observed in nuclei when IE62 was present was usually diffuse, similar to a chimeric construct between IE4 amino acids 1—403 and ICP27 amino acids 465-512 (Moriuchi *et al.,* 1995). Therefore, in cotransfected cells, IE62 might participate in alleviating the influence of the IE4 C-terminal region on cytoplasmic localization. In view of our results, a certain critical IE62 expression level is apparently required for the nuclear localization of IE4, since the presence of IE62 in the nucleus did not always correlate with colocalization. Such dependence has been shown in the case of the influence of ICP27 on ICP4 nuclear localization (Zhu & Schaffer, 1995).

It should be noted that although IE4 becomes more nuclear when IE62 is present, translocation to the nuclei is not complete. The IE62-mediated effect could be direct or indirect. The influence of IE62 on IE4 localization is not dependent on enhancement of IE4 expression, since a mutant IE62 protein expressed from pHN570, which has lost its transactivating properties, was still capable of driving IE4 to the nucleus. Quite interestingly, the other mutants specified by pHN1137 and p 612-865 were not able to influence IE4 distribution. The modification around amino acid 1137 altered the IE62 protein in such a way as to impair the translocation mechanism. Protein stability or folding might be affected, interfering with a direct interaction with IE4 or indirect interactions with cellular proteins required in post-translational modification of IE4. The mutation is situated in region 4, which is homologous to IE62 and ICP4 (McGeoch *et al,* 1986; Baudoux *et al,* 1995). Recently, Yao & Schaffer (1994) have shown that the C-terminal 235 amino acids (1064-1298) of ICP4 are important for efficient protein—

protein interaction with ICP0. Therefore, region 4 of IE62 could possibly be a functional domain involved in physical interaction. Further experiments to provide evidence of such specific interactions are necessary to support this hypothesis. Analysis of p 612-865 confirmed the importance of IE62 functions, as it also seems incapable of directing IE4 to the nucleus from the cytoplasm. The deleted region might contain signals critical for this purpose.

Previous studies conducted with HSV-1 proteins ICP4, ICP27 (Zhu & Schaffer, 1995) and ICP0 (Zhu *et a\., 1994) have shown that (i) ICP27 negatively influences the nuclear localization of ICP4 and ICP0; (ii) the C-terminal half of ICP27 is required for this function; (iii) the localization and level of expression of ICP27 are critical for the influence on ICP4 distribution; and (iv) residues between amino acids 820 and 1029 of the ICP4 molecule are responsive to the negative effect of ICP27. These observations and our results taken collectively provide new evidence of differences between IE4 and ICP27 regarding intracellular localization and interactions with other regulatory proteins. IE4 neither influenced IE62 intracellular localization nor modified that of ORF61p. Major importance is attributed to the C-terminal region of ICP27 in regulatory functions (Hardwicke *et al.,* 1989; McMahan & Schaffer, 1990) and to directing ICP4 to the cytoplasm (Zhu & Schaffer, 1995). This portion of the IE4 molecule also appears to be crucial for its properties (Perera *et al.,* 1994). There is little functional complementarity between the C-terminal portions of ICP27 and IE4 (Moriuchi *et a/.,* 1995), leading to the suggestion that the different behaviours observed could be associated with the specificity of each C-terminal region in the context of their native proteins.

We have shown, in transient transfection assays, that IE62, but not IE63 or ORF61p, can influence IE4 repartition between intracellular compartments. Although the mechanisms are at present unclear, this effect can possibly be correlated with the synergy observed between these two virus regulators in various CAT assays. Interestingly, IE4 seems incapable of influencing IE62 intracellular distribution. Our observations in infected and transfected cells add evidence of differences between closely related IE4 and ICP27 of HSV-1.

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