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SHORT COMMUNICATION

Herpes simplex virus type 1 immediate early protein IE63 shuttles between nuclear compartments and the cytoplasm

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Herpes simplex virus type 1 (HSV-1) immediate early protein IE63, an essential nuclear protein, is pleiotropic in function and, at the post-transcriptional level, inhibits RNA splicing, interacts with cellular splicing small nuclear ribonucleoprotein particles (snRNPs), binds RNA and prevents the nucleocytoplasmic transport of intron-containing mRNAs. Here it is reported that IE63 is a nucleocytoplasmic shuttle protein able to travel from snRNP- and RNA-rich nuclear foci to the cytoplasm, where it accumulates during actinomycin D treatment. This newly identified property suggests that IE63 facilitates nuclear export of HSV-1 transcripts, in addition to retaining intron-containing transcripts in the nucleus. The mechanism by which IE63 controls RNA export has yet to be defined.

Infection of mammalian cells with herpes simplex virus type 1 (HSV-1) results in a dramatic alteration of both cellular gene expression and nuclear organization. A key protein which regulates virus–host cell interactions is the 63 kDa nuclear immediate early (IE) phosphoprotein IE63 (ICP27). IE63 is one of two IE proteins essential for lytic virus infection, for the switch to early and late virus gene expression (McGregor *et al.*, 1996) and for efficient virus DNA replication (McCarthy *et al.*, 1989). Homologues of IE63 exist within all herpesviruses so far sequenced, several of which have been shown to modulate gene expression (Perera *et al.*, 1994). IE63 inhibits the splicing of both virus and cellular RNAs (Hardy & Sandri-Goldin, 1994), with concomitant reorganization of the cellular splicing small nuclear ribonucleoprotein particles (snRNPs) from a diffuse speckled pattern to a highly punctate organization (Phelan *et al.*, 1993). As infection proceeds, intron-containing HSV-1 transcripts are increasingly retained in the nucleus in distinct spots which colocalize with the redistributed snRNPs, and IE63 is required for this effect (Phelan *et al.*, 1996). Non intron-containing virus transcripts are exported to the cytoplasm throughout infection (Phelan *et al.*, 1996). A notable feature of the HSV-1 genome is that of some 70 genes expressed during lytic infection, only four contain introns (McGeoch *et al.*, 1988), three of which are IE genes initially expressed with IE63.

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Domains within IE63 include an extreme N-terminal acidic region essential for lytic growth (Rice *et al.*, 1993), an N-terminal nuclear–nucleolar localization signal (Mears *et al.*, 1995), a methylated internal RGG box required for RNA binding (Mears & Rice, 1996) and C-terminal transactivator and transrepressor regions required for IE63 co-immunoprecipitation with anti-Sm antisera (Sandri-Goldin & Hibbard, 1996). IE63 has a putative nuclear export signal (NES; LIDLGLDLSDSDL) located between amino acids 8 and 22 which exhibits homology to the leucine-rich NES of Rev (Kalland *et al.*, 1994) and other proteins known to shuttle from the nucleus (Wen *et al.*, 1995).

Actinomycin D (Act D) inhibits RNA polymerase II activity and prevents nuclear protein import such that shuttling proteins may accumulate in the cytoplasm over time. Here, under inhibitory conditions used to characterize heterogeneous nuclear ribonucleoprotein particle (hnRNP) shuttling proteins (Pinol-Roma & Dreyfuss, 1992), it is demonstrated that IE63 can shuttle from the nucleus to the cytoplasm throughout infection, in contrast to two other HSV-1 proteins present in the nucleus. This remarkable observation defines a new property of IE63 likely to be important for function of this essential protein. It is suggested that, as well as causing nuclear retention of intron-containing RNAs, IE63 promotes the nucleocytoplasmic transport of HSV-1 transcripts.

HeLa cells were grown as monolayers in Dulbecco's modified minimal essential medium supplemented with 5% newborn calf serum and 5% foetal calf serum. Stocks of wild-type HSV-1 (strain 17^+) were grown as described previously (Phelan *et al.*, 1993). All cells were grown as monolayers on sterile glass coverslips previously treated with 10% poly-L-lysine for 10 min after sterilization. Subconfluent monolayers were infected with wild-type HSV-1 at a multiplicity of 10 p.f.u. per cell. After 1 h at 37 °C, the infected medium was

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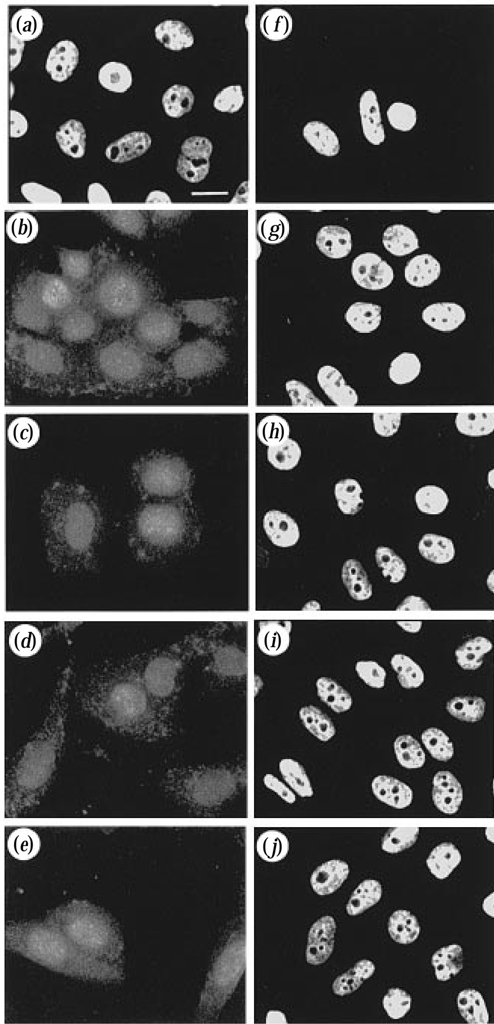

Fig. 1. hnRNP A1 but not hnRNP C can shuttle from the nucleus to the cytoplasm. Mock-infected HeLa cells (*a*, *b*, *d*, *e*, *f*, *g*, *i*, *j*) or HeLa cells infected with HSV-1 for 9 h (*c*, *h*) were labelled by indirect immunofluorescence with antibodies against hnRNP A1 (*a*–*e*) or hnRNP C (*f*–*j*). Mock-infected cells were either untreated (*a*, *f*) or treated with Act D (*b*, *g*), CHX (*d*, *i*) or both Act D and CHX (*e*, *j*) for 3 h prior to fixation and labelling. The HSV-1 infected cells (*c*, *h*) were treated with Act D prior to labelling. Bar, 10 μm.

removed and replaced with fresh prewarmed medium until the time of harvesting. Uninfected cells were treated identically, with the omission of virus. For cycloheximide (CHX) or Act D treatment, the medium from monolayers at 6 or 10 h post-infection was removed and replaced with prewarmed medium containing 10 μg/ml Act D, 40 μg/ml CHX, or both (Pinol-Roma & Dreyfuss, 1992; Kalland *et al*., 1994). The infection was allowed to proceed for a further 3 h before fixation of the cells.

Indirect immunofluorescence experiments were performed as described previously (Phelan *et al*., 1993). HSV-1 IE63 antigen was detected either with an antipeptide antibody '43', raised in rabbits, against the first 16 amino acids of IE63 at a dilution of 1:50 (a gift from H. Marsden, Institute of Virology, Glasgow, UK), or using a commercially available monoclonal antibody, H1113 (Ackermann *et al*., 1984). The hnRNPs A1 and C were detected using monoclonal antibodies 4B10 and 4F4, respectively (a gift from G. Dreyfuss, Howard Hughes Medical Institute, University of Pennsylvania, USA) at a dilution of 1:1000. The U2-splicing snRNPs were labelled with the B″ monoclonal antibody 4G3 (Habets *et al*., 1989) at a dilution of 1:5. The HSV-1 major DNA-binding protein UL29 (Gao & Knipe, 1991) was detected with an anti-UL29 monoclonal antibody (7381) at a dilution of 1:100 and the HSV-1 antigen IE110 was detected with monoclonal antibody 11060 (Everett *et al*., 1993) at a dilution of 1:200 (both provided by A. Cross, Institute of Virology, Glasgow, UK). Cells were examined using a Zeiss fluorescence microscope at ×60 magnification. Photography was performed with Fujicolor ASA 400 film.

The experiments of Pinol-Roma & Dreyfuss (1992) were repeated to establish conditions suitable for the nucleocytoplasmic shuttling ability of a nuclear protein. HeLa cells were treated with Act D and/or CHX prior to fixation and detection of hnRNP A1 or hnRNP C by indirect immunofluorescence. Initially, both proteins were predominantly nuclear (Fig. 1*a*, *f*). Following treatment with Act D, hnRNP A1 accumulated in the cytoplasm (Fig. 1*b*) whereas hnRNP C remained in the nucleus (Fig. 1*g*). Thus, as previously established, hnRNP A1 is capable of shuttling from the nucleus to the cytoplasm, in contrast to hnRNP C. Treatment of cells with CHX prior to Act D treatment (Fig. 1*d*, *i*) had no effect on the cytoplasmic appearance of hnRNP A1, indicating that pre-existing hnRNP A1 in the nucleus is transported to the cytoplasm. Treatment of cells with both Act D and CHX simultaneously generated data indistinguishable from that obtained with Act D treatment alone (compare Fig. 1*e*, *j* with *b*, *g*). Cells infected with HSV-1 for 6 h prior to Act D treatment also showed a cytoplasmic accumulation of hnRNP A1 (Fig. 1*c*), but to a lesser extent than uninfected cells, perhaps due to nuclear retention of intron-containing transcripts; hnRNP C distribution remained nuclear throughout infection (Fig. 1*h*).

Under identical conditions to those described for hnRNP detection, uninfected and HSV-1-infected cells, with or without prior Act D and/or CHX treatment, were labelled to detect IE63 protein by indirect immunofluorescence. IE63 protein was not detected in uninfected cells (Fig. 2*a*), and at 9 h post-infection in the absence of drug treatment IE63 distribution was predominantly nuclear (Fig. 2*b*), forming a largely punctate distribution. A striking feature of cells infected for 6 h then treated with Act D was the high proportion of IE63 protein detected in the cytoplasm using both rabbit polyclonal (Fig. 2*c*) and mouse monoclonal (not shown) antibodies. The punctate nuclear distribution of IE63 was lost and the protein occupied many much smaller sites. Simultaneous treatment with Act D and CHX (Fig. 2*e*) gave an identical pattern to that with Act D treatment alone (compare Fig. 2*e* with *c*); thus, translational

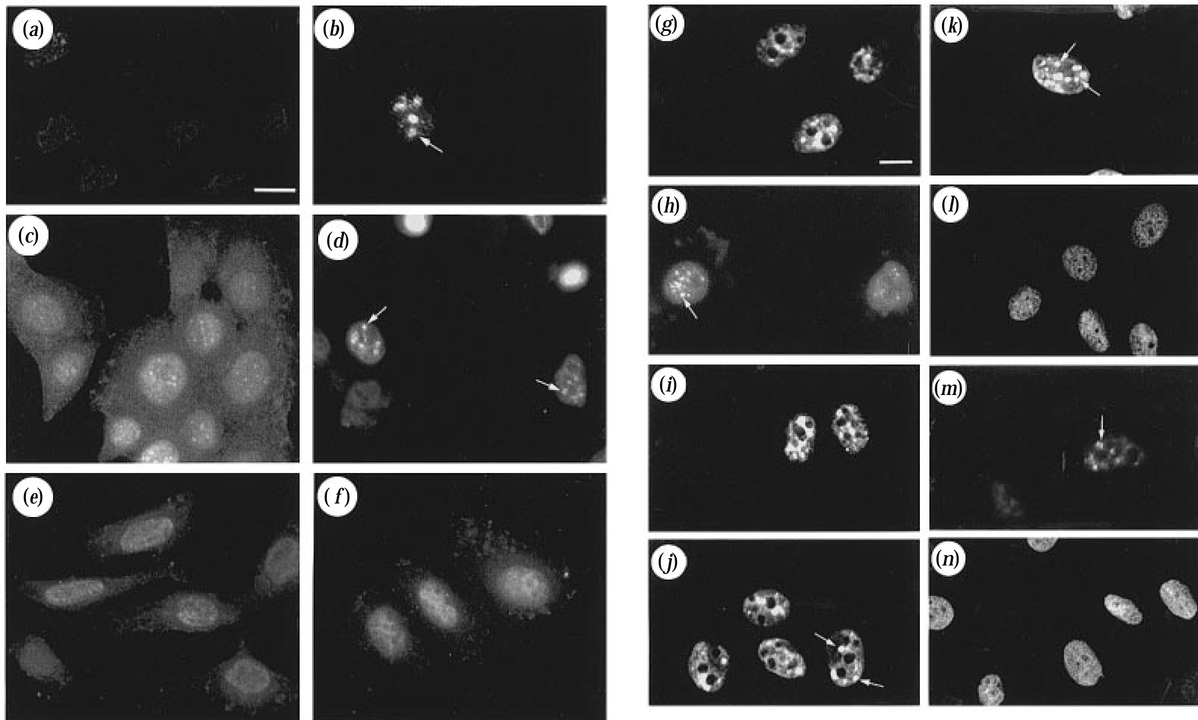

Fig. 2. (*a*–*f*) IE63 can shuttle from the nucleus to the cytoplasm. Mock-infected HeLa cells (*a*) and HeLa cells 9 h (*b*–*e*) or 13 h (*f*) post-infection with HSV-1 (including drug treatment) were labelled by indirect immunofluorescence with a polyclonal antibody to detect IE63. The cells had either undergone no treatment (*a*, *b*) or had been treated for 3 h with Act D (*c*, *f*), CHX (*d*) or both Act D and CHX (*e*) prior to labelling. Bar, 10 μm. (*g*–*n*) The B'' snRNP protein does not shuttle. Mock-infected HeLa cells (*g*–*j*) or cells 9 h post-infection with HSV-1 (*k*–*n*) were labelled with an antibody against the B'' component of the U2 snRNP and detected by indirect immunofluorescence. The cells had either undergone no chemical treatment (*g*, *k*) or had been treated with Act D (*h*, *l*), CHX (*i*, *m*) or both Act D and CHX (*j*, *n*) for 3 h prior to fixation and labelling. Bar, 10 μm.

inhibition did not affect the ability of IE63 to shuttle, and treatment with CHX alone (Fig. 2*d*) did not affect IE63 distribution. To examine whether IE63 could shuttle at later times, cells were infected for 10 h prior to Act D treatment (Fig. 2*f*); IE63 protein was readily detected in the cytoplasm.

HSV-1 infection causes a redistribution of the cellular splicing snRNPs from a widespread diffuse speckled pattern to a highly punctate organization. IE63 is both necessary and sufficient to cause this effect and colocalizes with the redistributed snRNPs and splicing factors (Phelan *et al*., 1993). To examine effects of inhibitor treatments on the B'' component of the U2 snRNP, shown to colocalize with IE63, mock-infected cells (Fig. 2*g*–*j*) and cells 6 h post-infection (Fig. 2*k*–*n*) were treated with Act D and/or CHX for 3 h before detection of B'' by indirect immunofluorescence.

Uninfected cells without treatment demonstrated the normal speckled snRNP pattern (Fig. 2*g*), comprising interchromatin granules and perichromatin fibrils (Spector, 1993). Following infection, the snRNP pattern alters to form a punctate pattern (Fig. 2*k*). By contrast, uninfected cells treated with Act D (Fig. 2*h*) demonstrated a clumping of snRNPs comparable to that observed during infection, a common result of treatments which inhibit transcription (Carmo-Fonseca *et al*., 1992). Infected cells treated with Act D (Fig. 2*l*) lost the punctate distribution of B'' to produce a general nuclear grainy pattern, while CHX treatment (Fig. 2*i*, *m*) had no effect on B'' distribution, and treatment with both Act D and CHX (Fig. 2*j*, *n*) gave the same pattern as that with Act D treatment alone.

To determine whether the ability to shuttle was relatively unique to IE63 or a more common feature of HSV-1 proteins, two other nuclear virus proteins, the 110 kDa transcriptional transactivator IE110 (Everett *et al*., 1991) and the major DNA-binding protein UL29 (Gao & Knipe, 1991) were tested for their ability to shuttle. IE110 (Fig. 3*a*) and UL29 (Fig. 3*f*) were not detected in uninfected cells. Cells infected for 9 and 13 h, respectively, demonstrated IE110 (Fig. 3*b*) and UL29 (Fig. 3*g*) to be nuclear proteins expressed in high abundance. When cells were infected for 6 h (Fig. 3*c*, *d*, *e*) or 10 h (Fig. 3*h*, *i*, *j*) prior to Act D treatment, both IE110 (Fig. 3*c*) and UL29 (Fig. 3*h*) clearly remained nuclear. Treatment with CHX (Fig. 3*d*, *i*) or with both CHX and Act D (Fig. 3*e*, *j*) had no effect on the distribution of these two virus antigens. Treatment of cells with Act D caused an inhibition of transcription with a concomitant intranuclear reorganization of the IE110 antigen. IE110, which is normally associated with virus transcription foci, altered its distribution in response to Act D treatment

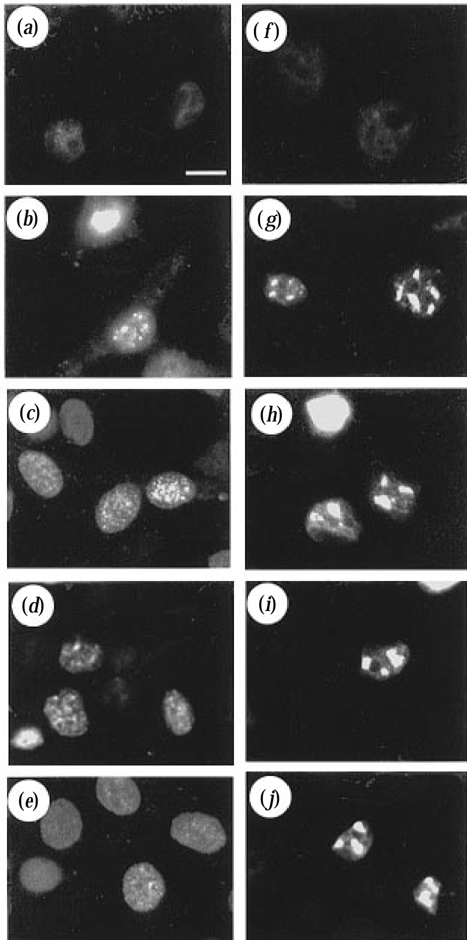

Fig. 3. Other nuclear HSV-1 proteins do not accumulate in the cytoplasm. Mock-infected HeLa cells (*a*, *f*) or HeLa cells 9 h (*b*–*e*) or 13 h (*g*–*j*) post-infection were labelled with antibodies against IE110 (*a*–*e*) or UL29 (*f*–*j*) and detected by indirect immunofluorescence. The cells had either undergone no chemical treatment (*a*, *b*, *f*, *g*) or had been treated with Act D (*c*, *h*), CHX (*d*, *i*) or both Act D and CHX (*e*, *j*) for 3 h prior to fixation and labelling. Bar, 10 μm.

from a relatively small number of nuclear foci to many tiny punctate foci.

The hnRNPs comprise a group of proteins that associate with pre-mRNA molecules and function in promoting post-transcriptional processing and export of mRNA (Dreyfuss *et al*., 1993). Certain hnRNPs, such as hnRNPs A1, K and E (Pinol-Roma & Dreyfuss, 1992), can shuttle from the nucleus to the cytoplasm in a NES-dependent, temperature-sensitive process which transports mRNA through the nucleoplasm and allows translocation across the nuclear pore complex. Other hnRNPs unable to shuttle, such as hnRNPs C1, C2 and U, are restricted to the nucleus by the presence of a nuclear retention sequence which overrides the NES (Nakielny & Dreyfuss, 1996).

Under conditions previously used to show that hnRNPs A1, K and E exhibit nucleocytoplasmic shuttling in a NES-dependent manner, it has been shown that HSV-1 IE63 can shuttle from the nucleus to the cytoplasm, where it accumulates during Act D treatment. IE63 exhibits a predominantly nuclear distribution throughout infection and this feature is also shown by shuttling hnRNP proteins such as A1 and HIV-1 Rev. By contrast, two other HSV-1 proteins present in the nucleus were unable to shuttle under identical conditions.

As expected, the B″ snRNP protein was unable to shuttle; however, during infection with transcription inhibition the snRNPs were dispersed, suggesting that snRNP clumps present during infection are involved in cotranscriptional splicing. Under similar conditions, IE63 was also dispersed from the snRNP clumps to a diffuse pattern, suggesting a cotranscriptional function for IE63 at the clumps which likely correspond to sites of virus transcription (Phelan *et al*., 1997). Co-immunoprecipitation studies have shown a direct physical interaction between IE63 and IE175 (Panagiotidis *et al*., 1997), the other HSV-1 protein essential for lytic function which activates transcription of early and late virus genes, suggesting a direct or indirect effect of IE63 on transcription. Functional interactions between RNA polymerase II and splicing factors (Du & Warren, 1997) have been described; the IE175–IE63 interaction may be analogous to this, and could, via affinity for RNA (Brown *et al*., 1995; Ingram *et al*., 1996), result in cotranscriptional binding of IE63 to pre-mRNAs prior to shuttling.

The very apparent shuttling of IE63 observed following Act D treatment is consistent with the essential role of IE63 in facilitating virus RNA trafficking from the nucleus, perhaps selectively of unspliced RNAs which form the vast majority of virus transcripts. IE63 also causes retention of intron-containing transcripts in the nucleus and these two effects on RNA trafficking could be related. For example, if IE63 trafficking of unspliced RNAs competed for components required for trafficking of spliced RNAs this could lead to inhibition of splicing. A further, not mutually exclusive, possibility is that IE63 may have a cytoplasmic role, such as facilitating translation of virus RNAs, as reported for Rev (Ruhl *et al*., 1993).

References

Ackermann, M., Brown, D. K., Periera, L. & Roizman, B. (1984). Characterisation of herpes simplex virus 1 α proteins 0, 4 and 27 with monoclonal antibodies. *Journal of Virology* **52**, 108–118.

Brown, C. R., Nakamura, M. S., Mosca, J. D., Hayward, G. S., Strauss, S. E. & Perera, L. P. (1995). Herpes simplex virus trans-regulatory protein ICP27 stabilises and binds to 3′ ends of labile mRNA. *Journal of Virology* **69**, 7187–7195.

Carmo-Fonseca, M., Pepperkok, R., Carvalho, M. T. & Lamond, A. I. (1992). Transcription-dependent colocalisation of the U1, U2, U4/U6 and U5 snRNPs in coiled bodies. *Journal of Cell Biology* **117**, 1–14.

Dreyfuss, G., Matunis, M. J., Pinol-Roma, S. & Burd, C. G. (1993). hnRNP proteins and the biogenesis of mRNA. *Annual Reviews in Biochemistry* **62**, 289–321.

Du, L. & Warren, S. L. (1997). A functional interaction between the carboxy-terminal domain of RNA polymerase II and pre-mRNA splicing. *Journal of Cell Biology* **136**, 5–18.

Everett, R. D., Preston, C. M. & Stow, N. D. (1991). Functional and genetic analysis of the role of Vmw110 in herpes simplex virus replication. In *The Control of Herpes Simplex Virus Gene Expression*, pp. 50–76. Edited by E. K. Wagner. Boca Raton, Fla: CRC Press.

Everett, R. D., Cross, A. & Orr, A. (1993). A truncated form of HSV-1 immediate early protein Vmw110 is expressed in a cell-type dependent manner. *Virology* **197**, 751–756.

Gao, M. & Knipe, D. M. (1991). Potential role for herpes simplex virus ICP8 DNA replication protein in stimulation of late gene expression. *Journal of Virology* **65**, 2666–2675.

Habets, W. J., Hoet, M. H., De Jong, B. A. W., Van Der Kemp, A. & Van Venrooij, W. J. (1989). Mapping of B-cell epitopes on small nuclear ribonucleoproteins that react with human autoantibodies as well as with experimentally-induced mouse monoclonal-antibodies. *Journal of Immunology* **143**, 2560–2566.

Hardy, W. R. & Sandri-Goldin, R. M. (1994). Herpes simplex virus inhibits host cell splicing and regulatory protein ICP27 is required for this effect. *Journal of Virology* **68**, 7790–7799.

Ingram, A., Phelan, A., Dunlop, J. & Clements, J. B. (1996). Immediate early protein IE63 of herpes simplex virus type 1 binds RNA directly. *Journal of General Virology* **77**, 1847–1851.

Kalland, K.-H., Szilvay, A. M., Brokstad, K. A., Saetrevik, W. & Haukenes, G. (1994). The human immunodeficiency virus type 1 Rev protein shuttles between the cytoplasm and nuclear compartments. *Molecular and Cellular Biology* **14**, 7436–7444.

McCarthy, A. M., McMahan, L. & Schaffer, P. A. (1989). Herpes simplex virus type 1 ICP27 deletion mutants exhibit altered patterns of transcription and are DNA deficient. *Journal of Virology* **63**, 18–27.

McGeoch, D. J., Dalrymple, M. A., Davison, A. J., Dolan, A., Frame, M. C., McNab, D., Perry, L. J., Scott, J. E. & Taylor, P. (1988). The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. *Journal of General Virology* **69**, 1531–1574.

McGregor, F., Phelan, A., Dunlop, J. & Clements, J. B. (1996). Regulation of herpes simplex virus poly(A) site usage; the early–late switch and the action of immediate early protein IE63. *Journal of Virology* **70**, 1931–1940.

Mears, W. E. & Rice, S. A. (1996). The RGG box motif of the HSV ICP27 protein mediates an RNA binding activity and determines *in vivo* methylation. *Journal of Virology* **70**, 7445–7453.

Mears, W. E., Lam, V. & Rice, S. A. (1995). Identification of nuclear and nucleolar localisation signals in the HSV regulatory protein ICP27. *Journal of Virology* **69**, 935–947.

Nakielny, S. & Dreyfuss, G. (1996). The hnRNP C proteins contain a nuclear retention sequence that can override nuclear export signals. *Journal of Cell Biology* **134**, 1365–1373.

Panagiotidis, C. A., Lium, E. K. & Silverstein, S. J. (1997). Physical and functional interactions between herpes simplex virus immediate early proteins ICP4 and ICP27. *Journal of Virology* **71**, 1547–1557.

Perera, L. P., Kaushal, S., Kinchington, P. R., Mosca, J. D., Hayward, G. S. & Strauss, S. E. (1994). VZV ORF 4 encodes a transcriptional activator that is functionally distinct from that of HSV homolog IC27. *Journal of Virology* **68**, 2468–2477.

Phelan, A., Carmo-Fonseca, M., McLauchlan, J., Lamond, A. I. & Clements, J. B. (1993). A herpes simplex virus type-1 immediate early gene product, IE63, regulates small nuclear ribonucleoprotein distribution. *Proceedings of the National Academy of Sciences, USA* **90**, 9056–9060.

Phelan, A., Dunlop, J. & Clements, J. B. (1996). Herpes simplex virus type 1 protein IE63 affects the nuclear export of virus intron-containing transcripts. *Journal of Virology* **70**, 5255–5265.

Phelan, A., Dunlop, J., Patel, A. H., Stow, N. D. & Clements, J. B. (1997). Nuclear sites of herpes simplex virus type 1 DNA replication and transcription colocalise at early times post infection and are largely distinct from RNA processing factors. *Journal of Virology* **71**, 1124–1132.

Pinol-Roma, S. & Dreyfuss, G. (1992). Shuttling of pre-mRNA binding proteins between the nucleus and cytoplasm. *Nature* **355**, 730–732.

Rice, S. A., Lam, V. & Knipe, D. M. (1993). The acidic amino-terminal region of herpes simplex virus type 1 alpha protein ICP27 is required for an essential lytic function. *Journal of Virology* **67**, 1778–1787.

Ruhl, M., Himmelspath, M., Bahr, G. M., Hammerschmidt, F., Jaksche, H., Wolff, B., Aschauer, H., Farrington, G. K., Pivost, H., Burck, D. A. D. & Hauber, J. (1993). Eukaryotic initiation factor 5A is a cellular target of the human immunodeficiency virus type 1 Rev activation domain mediating trans-activation. *Journal of Cell Biology* **123**, 1309–1320.

Sandri-Goldin, R. M. & Hibbard, M. K. (1996). The herpes simplex virus type 1 regulatory protein ICP27 coimmunoprecipitates with anti-Sm antiserum, and the C-terminus appears to be required for this interaction. *Journal of Virology* **70**, 108–118.

Spector, D. L. (1993). Nuclear organisation of pre-mRNA processing. *Current Opinion in Cell Biology* **5**, 442–448.

Wen, W., Meinkoth, J. L., Tsien, R. Y. & Taylor, S. S. (1995). Identification of a signal for rapid export of proteins from the nucleus. *Cell* **82**, 463–473.

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