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Activity and intracellular localization of the human cytomegalovirus protein pp71

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The human cytomegalovirus (HCMV) tegument phosphoprotein pp71 activates viral immediate early (IE) transcription and thus has a role in initiating lytic infection. Protein pp71 stimulates expression from a range of promoters in a sequence-independent manner, and in this respect behaves similarly to the herpes simplex virus type 1 (HSV-1) IE protein ICP0. The intracellular localization of pp71 was investigated after its expression from transfected plasmids or from HSV-1 mutants constructed to produce pp71 transiently. The protein colocalized with the cell promyelocytic leukaemia (PML) protein at nuclear domain 10 (ND10) structures but, unlike ICP0, pp71 did not induce disruption of ND10. The activity of pp71 in mouse sensory neurons *in vivo* was investigated after co-inoculation of animals with pairs of HSV-1 mutants, one expressing pp71 and the second containing the *E. coli lacZ* gene controlled by various promoters. In this system, pp71 stimulated β-galactosidase expression from a range of viral IE promoters when mice were analysed at 4 days postinoculation. At later times, expression of pp71 resulted in a reduction in numbers of neurons containing β-galactosidase, indicating cytotoxicity or promoter shutoff. The HSV-1 latency-active promoter was not responsive to pp71, demonstrating specificity in the activity of the protein. Pp71 was as active in mice lacking both copies of the PML gene (PML−/−) as in control animals, and in PML−/− fibroblasts pp71 stimulated gene expression as effectively as in other cell types. Therefore, neither the PML protein nor the normal ND10 structure is necessary for pp71 to stimulate gene expression.

Introduction

The synthesis of immediate early (IE) gene products is an important control point in the replication of herpesviruses. IE proteins are crucial for expression of early and late genes and hence the successful progress of infection. Indeed, the balance between lytic and latent outcomes of infection may be determined at the level of IE gene expression (Preston, 2000). In many alphaherpesviruses studied to date, transcription of the IE genes is activated by proteins in the incoming virus particle. The best studied example is that of herpes simplex virus type 1 (HSV-1), in which the tegument protein VP16 interacts with cellular proteins Oct-1 and HCF to form a multiprotein complex that binds to TAATGARAT elements present in HSV-1 IE promoters (reviewed by O'Hare, 1993). Stimulation of HSV-1 IE transcription by VP16 is therefore achieved by a sequence-specific mechanism. The importance of this event for virus replication is demonstrated by the finding that HSV-1 mutants encoding nonfunctional VP16 initiate productive infection inefficiently at low m.o.i. in cell culture and are attenuated for virus replication *in vivo* (Ace *et al.*, 1989; Steiner *et al.*, 1990; Harris & Preston, 1991; Jamieson *et al.*, 1995; Mossman & Smiley, 1999; Tal-Singer *et al.*, 1999).

The human cytomegalovirus (HCMV) tegument phosphoprotein pp71, encoded by gene UL82, appears to be a functional counterpart of VP16. In transfection assays, expression of pp71 stimulates transcription from the HCMV major IE promoter and from heterologous promoters (Liu *et al.*, 1992; Baldick *et al.*, 1997; Chau *et al.*, 1999). Protein pp71 also exerts a general influence on gene expression since it increases the infectivity of transfected HCMV DNA through an effect that is separable from increased IE protein production (Baldick *et al.*, 1997). An HCMV mutant that lacks the pp71 coding

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sequences initiates infection poorly at low m.o.i., exhibiting a phenotype similar to that of HSV-1 strains deficient for VP16 function (Bresnahan & Shenk, 2000). In a separate approach to studying pp71, an HSV-1 recombinant named *in*1324 was constructed (Homer *et al*., 1999). The parental virus for *in*1324 was *in*1312, an HSV-1 mutant containing three mutations: an insertion that inactivates transactivation by VP16, a deletion that abrogates the function of the IE protein ICP0, and a temperature sensitive mutation that renders a major transcription factor, the IE protein ICP4, non-functional at temperatures greater than 38 °C (Preston *et al*., 1998). The three proteins that are crucial for HSV-1 transcription are functionally impaired, and as a consequence the *in*1312 genome is converted to a repressed, quiescent state within the first few hours after infection (Preston & Nicholl, 1997; Samaniego *et al*., 1998). These features enable *in*1312 to be used as a vector, in which an inserted foreign gene is expressed transiently and is dependent on the intrinsic strength of the promoter controlling it. A cassette containing the HCMV UL82 coding sequences, controlled by the HCMV major IE promoter, was inserted into the *in*1312 genome to yield *in*1324, a virus that produces pp71 in infected cells (Homer *et al*., 1999). Since *in*1312 can infect a wide range of cells in culture, the recombinant provides a means of producing pp71 in a variety of cell types. By use of *in*1324, it was shown that many viral promoters, themselves cloned into *in*1312, are activated by pp71 (Homer *et al*., 1999). In addition expression of the adenovirus virus associated RNA I (VAI) was stimulated by pp71. The observation that the responsive promoters contain no obvious sequence homologies, together with the fact that VAI is transcribed by RNA polymerase III rather than polymerase II, indicates that pp71 is able to activate expression from a wide range of promoters when they are present in the HSV-1 genome.

In its broad promoter specificity pp71 resembles the HSV-1 IE protein ICP0, which increases expression from viral and cellular promoters indiscriminately in cotransfection assays. ICP0 is crucial for the onset of HSV-1 gene expression after infection of tissue culture cells at low m.o.i. (reviewed by Everett, 2000a). Although ICP0 stimulates expression from all classes of promoter within the HSV-1 genome, it does not function as a classical transcription factor. ICP0 locates to and disrupts cellular nuclear domain 10 (ND10) structures and centromeres within the cell nucleus, due at least in part to stimulating the degradation of the ND10 proteins promyelocytic leukaemia (PML) and Sp100, and the centromere proteins CENP-A and CENP-C (Everett & Maul, 1994; Maul & Everett, 1994; Everett *et al*., 1999; Lomonte *et al*., 2001). Input HSV-1 DNA is located at ND10 (Ishov & Maul, 1996; Maul & Everett, 1996), and disruption of ND10 by ICP0 correlates strongly with activation of the viral gene expression programme (Everett *et al*., 1998a, b). HCMV DNA localizes to ND10 (Ishov *et al*., 1997), and disruption of the structures during HCMV infection is achieved primarily by expression of the IE72 protein (Kelly *et al*., 1995; Korioth *et al*., 1996; Ahn & Hayward, 1997; Ishov *et al*., 1997; Wilkinson *et al*., 1998).

In the studies described here, we investigated further the properties of pp71 and its functional similarity to ICP0 using *in*1312-based recombinants. We took a novel approach to investigation of pp71 function, namely the inoculation into mice, via the footpad, of *in*1324 together with an *in*1312-based recombinant containing the indicator gene lacZ. Gene expression was examined by histochemical staining for β-galactosidase activity in dorsal root ganglion (DRG) cells. Our previous studies showed that a foreign gene product (Cre recombinase) synthesized in neurons can act in *trans* during the first few days after inoculation, and that replication of *in*1312-based viruses is not detectable at the mouse body temperature (Rinaldi *et al*., 1999; Marshall *et al*., 2000). When HSV-1 is inoculated into mice, the virus eventually becomes latent in sensory neurons and thus a long term interaction is established. Expression from the HSV-1 latency-active region, which specifies the latency-associated transcript (LAT), can be monitored by the use of a construct in which the β-geo gene (a fusion of lacZ and neomycin phosphotransferase coding sequences) with an encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) immediately upstream is inserted into the LAT transcription unit. This construct has been shown to direct expression of β-galactosidase with latent kinetics when cloned into the genomes of wild-type HSV-1 or *in*1312 (Lachmann & Efstathiou, 1997; Marshall *et al*., 2000).

We describe here the use of tissue culture and *in vivo* assays to investigate the activity of pp71 and its interaction with cellular ND10 structures.

Methods

■ **Cells.** Human foetal lung (Flow 2002) and human foetal foreskin (HFFF2) fibroblasts were propagated in Dulbecco's medium supplemented with 5% (v/v) foetal calf serum, 5% (v/v) newborn calf serum, nonessential amino acids (Life Technologies), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Vero cells, and immortalized fibroblasts from PML null or wild-type mice (Ishov *et al*., 1999), were propagated in the above medium supplemented with 10% (v/v) foetal calf serum and no newborn calf serum.

■ **Plasmids.** Plasmid pCP7991 was produced by cloning a 700 bp *Acc*I–*Hin*dIII fragment from pJ 7Ω containing the simian CMV (SCMV) IE promoter (Morgenstern & Land, 1990) upstream of the *E. coli lac*Z coding sequences in place of the HCMV IE promoter in plasmid pMJ101 (Nicholl & Preston, 1996). Plasmid pAR37 was constructed from pCP43937 (Homer *et al*., 1999), which contains the pp71 coding sequences, controlled by the HCMV major IE promoter, embedded in the HSV-1 TK coding region. A double-stranded oligonucleotide (top strand: 5′ AGCTTCCATGGGCCATCATCATCATCATCATGGCCTGGTTCCGCGTGGATCCGG), with 4 bp extensions compatible with *Hin*dIII sites (5′end) or *Nco*I sites (3′end) was cloned into a plasmid containing pp71 coding sequences with the initiator ATG sequences changed to CCATGG, an *Nco*I site. A fragment with six N-terminal histidines [(his)6] fused to the pp71 coding region was cloned into pCP1802 (Homer *et al*., 1999) to yield plasmid pAR37, in which the (his)6–pp71 protein was controlled by the HCMV major IE promoter and

Table 1. *in*1312-based mutants used in the study

All insertions were at the TK locus, with the exception that *in*1388 contained the IRES-*β*-geo insertion in the LAT locus.

Name	Insertion
*in*1316	HCMV IE-(YFP-pp71)
*in*1320	HCMV IE-(his)6-pp71
*in*1324	HCMV IE-pp71
*in*1325	HCMV IE-GFP
*in*1357	SCMV IE-lacZ
*in*1372	HCMV IE-Cre
*in*1382	HCMV IE-lacZ
*in*1383	HSV-1 ICP0-lacZ
*in*1388	IRES-*β*-geo

Table 2. Preinfection with *in*1324 complements the replication of *dl*1403

Virus added	Insertion*	Titre of *dl*1403/48lacZ	
		Preinfection†	Postinfection‡
None	—	$1{\cdot}7 \times 10^6$	$2{\cdot}0 \times 10^6$
*ts*K	—	$8{\cdot}3 \times 10^7$	$9{\cdot}0 \times 10^7$
*in*1324	pp71	$2{\cdot}1 \times 10^7$	$1{\cdot}9 \times 10^6$
*in*1325	GFP	$1{\cdot}4 \times 10^6$	ND
*in*1372	Cre	$1{\cdot}7 \times 10^6$	ND

* Insertions contained the indicated coding sequences controlled by the HCMV IE promoter.
† Monolayers of 8×10^5 HFL cells were preinfected with 5×10^5 p.f.u. of *ts*K, or $1{\cdot}5 \times 10^4$ p.f.u. of *in*1312-based mutants (representing equivalent inocula, as described in Methods), maintained at 38·5 °C for 6 h, and infected with various dilutions of *dl*1403/48lacZ. Monolayers were maintained at 38·5 °C in growth medium containing 2% human serum for 20 h and plaques identified by X-Gal staining.
‡ Monolayers of HFL cells were infected with various dilutions of *dl*1403/48lacZ, incubated overnight at 38·5 °C in growth medium containing 2% human serum, washed extensively and infected with *ts*K (5×10^5 p.f.u. per plate) or *in*1324 ($1{\cdot}5 \times 10^4$ p.f.u. per plate). After incubation for a further 20 h at 38·5 °C in the presence of 2% human serum, plaques were identified by X-Gal staining.
ND, Not determined.

embedded in the HSV-1 TK coding region. DNA sequencing confirmed that the oligonucleotide was inserted correctly. Plasmid pCP74126 was constructed by inserting a 380 bp *Taq*I–*Eco*RV fragment containing the promoter for the HSV-1 UL48 (VP16) gene (Dalrymple *et al.*, 1985) upstream of *lacZ* in pMJ27 (Jamieson *et al.*, 1995). The resultant plasmid, pCP74126, contains *lacZ* controlled by the early/late UL48 promoter, embedded in the TK coding region. Plasmid pYFPpp71 was constructed by inserting pp71 coding sequences in the *Sma*I site of pEYFP-C1 (Clontech), such that a yellow fluorescent protein (YFP)–pp71 fusion protein was produced. The sequences encoding the fusion protein were cloned as an *Age*I–*Not*I fragment into pCP1802 to yield pMJ129, in which the YFP–pp71 protein was controlled by the HCMV major IE promoter and embedded in the HSV-1 TK coding region

■ **Viruses.** HSV-1 (strain 17) mutants *in*1312, *in*1324, *in*1372, *in*1382, *in*1383 and *in*1388 have been described previously (Preston *et al.*, 1998; Homer *et al.*, 1999; Rinaldi *et al.*, 1999; Preston & McFarlane, 1998; Marshall *et al.*, 2000). Mutant *in*1357 was constructed by cotransfecting *Sca*I-cleaved pCP7991 with *in*1312 DNA. Thymidine kinase (TK)-deficient plaques that expressed *β*-galactosidase were purified, and an isolate containing no detectable parental virus when analysed by Southern hybridization was named *in*1357. Mutants *in*1316 and *in*1320 were constructed from pMJ129 and pAR37, respectively. *Sca*I-cleaved plasmids were cotransfected with *in*1312 DNA, TK-deficient plaques were purified, and isolates containing no detectable parental genomes by Southern hybridization were named *in*1316 and *in*1320. Virus *dl*1403/48lacZ was constructed by cotransfection of *Sca*I-cleaved pCP74126 with *dl*1403 DNA (Stow & Stow, 1986). Plaque isolates that were TK negative and expressed *β*-galactosidase were analysed by Southern hybridization, and an isolate containing no parental DNA was purified and named *dl*1403/48lacZ. The inserted sequences of the *in*1312-based mutants used in the studies described here are summarized in Table 1. Titres of *in*1312-based recombinants are expressed here as the value on baby hamster kidney clone 13 cells at 31 °C, in the presence of 3 mM hexamethylene bisacetamide (HMBA) (McFarlane *et al.*, 1992). This value underestimates the potential number of infectious units by a factor of approximately 50 due to the ICP0 mutation. The titre of *dl*1403/48lacZ was determined after fixing cells and staining with 5-bromo-4-chloro-3-indolyl *β*-D-galactoside (X-Gal), as described previously (Jamieson *et al.*, 1995).

■ **Immunofluorescence.** Monolayers of cells on coverslips were fixed, permeabilized and treated with antibodies as described previously (Everett *et al.*, 1999; Parkinson & Everett, 2000). Primary antibodies were mouse monoclonal anti-polyHistidine Peroxidase Conjugate (Sigma) diluted 1:200; mouse monoclonal anti-PML antibody 5E10 (Stuurman *et al.*, 1992, kindly provided by R. Everett) diluted 1:10; and rat monoclonal anti-Sp100 antibody Sp26 (Grotzinger *et al.*, 1996; kindly provided by R. Everett) diluted 1:5000. Secondary antibodies were FITC-conjugated goat anti-mouse IgG (Sigma) diluted 1:100; cy3-conjugated goat anti-rat IgG (Amersham) diluted 1:2000; and cy5-conjugated goat anti-mouse IgG (Amersham) diluted 1:1000. Confocal microscopy was carried out using a Zeiss LSM510 confocal microscope, as described by Everett (2000b). The scanning conditions used ensured that signal overlap was essentially eliminated.

■ **Immunoblot analysis.** Proteins in cell extracts were analysed as described by Everett *et al.* (1999). Detection was achieved by use of mouse monoclonal anti-polyHistidine peroxidase-conjugated antibody diluted 1:1000 or rabbit anti-GFP serum (Abcam) diluted 1:1000 and processing for enhanced chemiluminescence.

■ **Transfection.** Cell monolayers on coverslips were transfected with 0·2 μg of plasmid DNA, using Lipofectamine (Life Technologies) as carrier according to the manufacturer's instructions. Monolayers were fixed and processed for immunofluorescence at 6 h after transfection.

■ **Inoculation of mice.** Female BALB/c mice, 4–5 weeks old, were inoculated in the footpad as described previously (Marshall *et al.*, 2000). Control or homozygous PML null mutant (PML −/−) mice (Wang *et al.*, 1998a), both strain 129Sv, were used at 4–6 weeks old and in these cases, due to variability in the breeding of animals, both males and females were used. Preliminary experiments showed that there were no significant differences in HSV-1 gene expression between males and females, and within the age range taken. Lumbar 3, 4 and 5 dorsal root ganglia (DRG) were dissected and stained with to X-Gal reveal *β*-galactosidase expressing cells, as described previously (Marshall *et al.*, 2000).

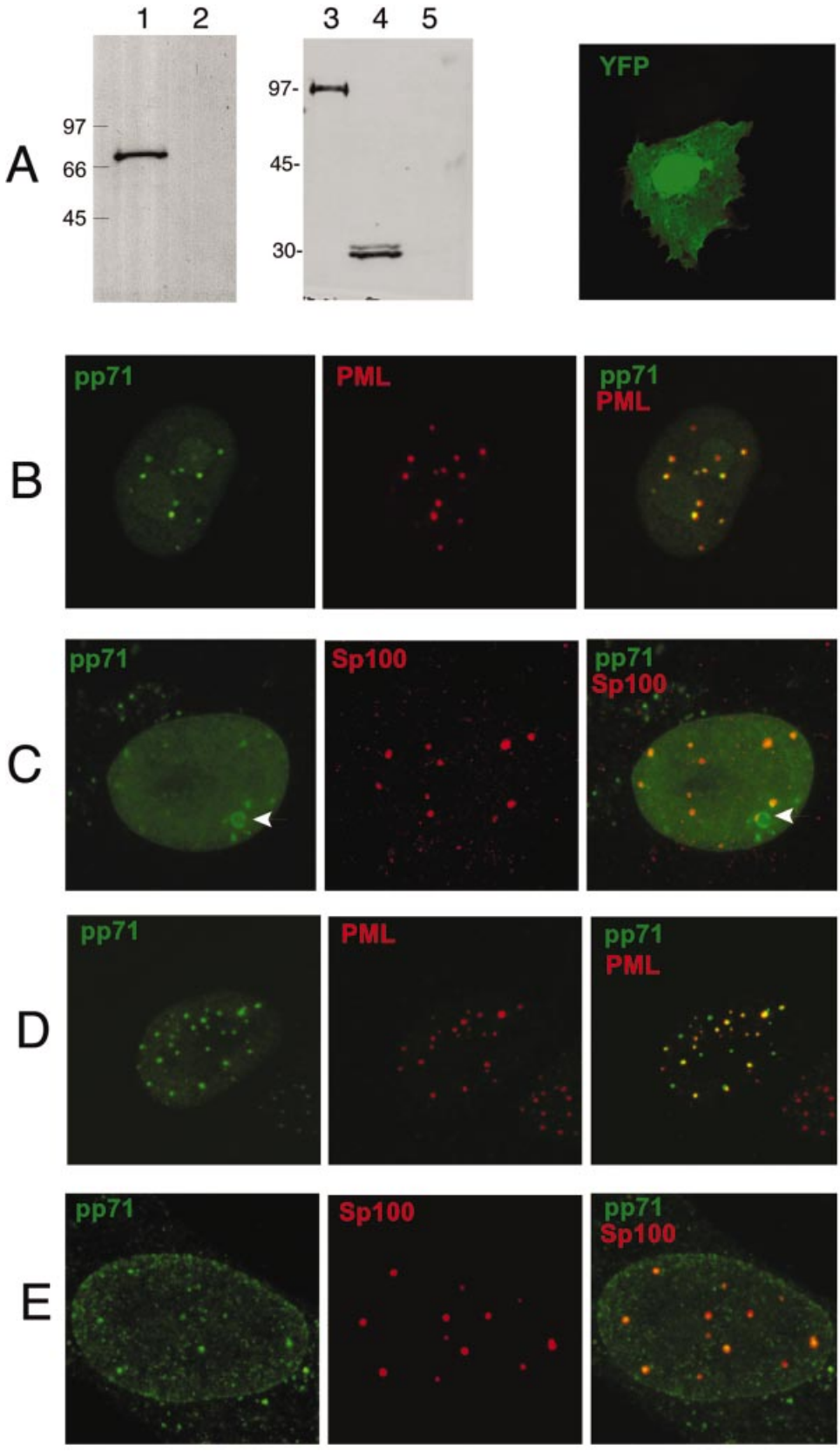

Fig. 1. For legend see facing page.

■ **Assay for pp71 activity.** Activation of gene expression was determined as described by Homer *et al.* (1999). Monolayers were infected with *in*1312 derivatives that express pp71, incubated at 38·5 °C for 3 h, and infected with *in*1382. After a further 5 h at 38·5 °C, cell extracts were made and assayed for β-galactosidase activity, using 4-methylumbelliferyl β-D-galactoside as substrate (Preston & Nicholl, 1997).

Results

Protein pp71 partially complements the absence of ICP0

HFL cells were preinfected with *in*1324, *in*1325 (a rescuant of *in*1324 that expresses GFP instead of pp71; Homer *et al.*, 1999) or *in*1372 (which expresses Cre recombinase; Rinaldi *et al.*, 1999). After incubation at 38·5 °C for 6 h, to permit the production of pp71, monolayers were superinfected with *dl*1403/48lacZ, an HSV-1 mutant deleted for the ICP0 coding sequences. After further incubation overnight at 38·5 °C, plaques were counted. Preinfection with *in*1324 resulted in a 12-fold increase in the titre of *dl*1403 whereas *in*1325 or *in*1372 had no effect. Preinfection with the HSV-1 ts mutant *ts*K, which provides ICP0, resulted in a 49-fold increase in titre. In the experiment shown in Table 2, titres were calculated from plates containing approximately equal numbers of plaques, because titrations of ICP0 mutants do not show linear responses to dilution (Stow & Stow, 1986; Everett, 1989). Thus, despite uncertainties regarding the degree of complementation, pp71 expressed from *in*1324 partially overcomes the absence of ICP0. In a further investigation, monolayers were infected with dilutions of *dl*1403/48lacZ and superinfected with *in*1324 or *ts*K the following day (Table 2). Superinfection with *ts*K resulted in complementation but superinfection with *in*1324 did not, showing that pp71 cannot reverse the silencing of the HSV-1 genome in tissue culture cells once it has occurred.

Protein pp71 localizes to but does not disrupt ND10

Hensel *et al.* (1996) showed that pp71, produced by transfection of cells or present early after infection with HCMV, could be detected in discrete nuclear foci by immunofluorescence. The possibility that these were ND10 was investigated, and to facilitate the studies two tagged versions of pp71 were produced. A plasmid that expresses a YFP–pp71 hybrid protein, pYFPpp71, was constructed for use in transfection experiments. *In*1312-based recombinants analogous to *in*1324 but expressing YFP–pp71 (*in*1316) or N-terminally his-tagged pp71 (*in*1320) were produced in order to detect protein synthesized after infection. These new mutants stimulated expression from the HCMV IE promoter to the same extent as *in*1324, confirming that YFP–pp71 and (his)6-tagged pp71 are functional (results not shown, and see Table 5). In addition, proteins of the correct size were detected in cells infected with *in*1316 or *in*1320 (Fig. 1). The intracellular distribution of YFP–pp71 was analysed by confocal microscopy, after transfection of Vero cells with pYFPpp71 or infection of HFFF2 cells with *in*1316. ND10 was identified by co-staining with an anti-PML antibody. The YFP–pp71 fusion protein and PML colocalized at ND10 (Fig. 1), whereas YFP alone was dispersed through the cell. During infection of Vero or HFFF2 cells with *in*1320, pp71 was detected by immunoreactivity with anti-polyHistidine antibody, and anti-Sp100 was used to detect ND10 (Fig. 1). Sp100 and pp71 colocalized in discrete foci, although pp71 was also dispersed through the nucleus. The patterns shown in Fig. 1 are typical of the appearance of most positive cells, but under conditions in which higher levels of pp71 expression occurred large masses accumulated in the nucleus (arrowed in Fig. 1), reminiscent of the structures reported upon overproduction of ICP0 (Wu *et al.*, 1996). No disruption of ND10 was observed after transfection of pYFPpp71 or after infection within *in*1316 or *in*1320 during the time period examined. These results suggest that the structures observed by Hensel *et al.* (1996) were ND10, an observation that has also been made by T. Stamminger and co-workers in transfected cells (personal communication).

HCMV pp71 is active in mouse DRG neurons *in vivo*

Infection with *in*1324 increased expression of *lacZ* when the reporter gene was present in a coinfected *in*1312 genome, not only in human fibroblasts (Homer *et al.*, 1999) but also in monkey (Vero) and Syrian hamster (BHK-21) cells (results not shown), demonstrating that the action of pp71 is not species specific. In addition, the HCMV IE promoter, when present in the *in*1312 genome, was capable of directing functionally relevant amounts of a gene product (Cre recombinase) in DRG neurons of mice (Rinaldi *et al.*, 1999). Based on these findings, the possibility that pp71 was functional in the *in vivo* environment provided by DRG neurons was investigated.

Fig. 1. Intracellular localization of pp71. Row A includes immunoblots of extracts from Vero cells infected with 0·1 p.f.u. of *in*1320 per cell for 24 h at 38·5 °C (lane 1) or mock infected (lane 2) and probed with anti-polyHistidine antibody, and extracts of Vero cells transfected with pYFPpp71 (lane 3), pEYFP-C1 (lane 4) or mock transfected (lane 5) and probed with anti-GFP serum. Molecular mass markers are shown to the left of the panels. Row A also shows a representative image of Vero cells transfected with pEYFP-C1. Row B shows a representative confocal image of a Vero cell transfected with pYFPpp71 and costained for PML. Row C shows a representative confocal image of a Vero cell infected with 0·01 p.f.u. of *in*1320 at 38·5 °C for 24 h and stained with anti-polyHistidine and anti-Sp100 antibodies. A large mass is labelled with an arrowhead. The fact that the anti-polyHistidine antibody was peroxidase conjugated is irrelevant to this experiment. Row D shows a representative image of an HFFF2 cell infected with 0·01 p.f.u. of *in*1316 at 38·5 °C for 24 h and processed as for row B. Row E shows HFFF2 cells, infected and processed as for row C. Images are z-series optical slices projected onto a single plane to include all ND10 structures in the cell.

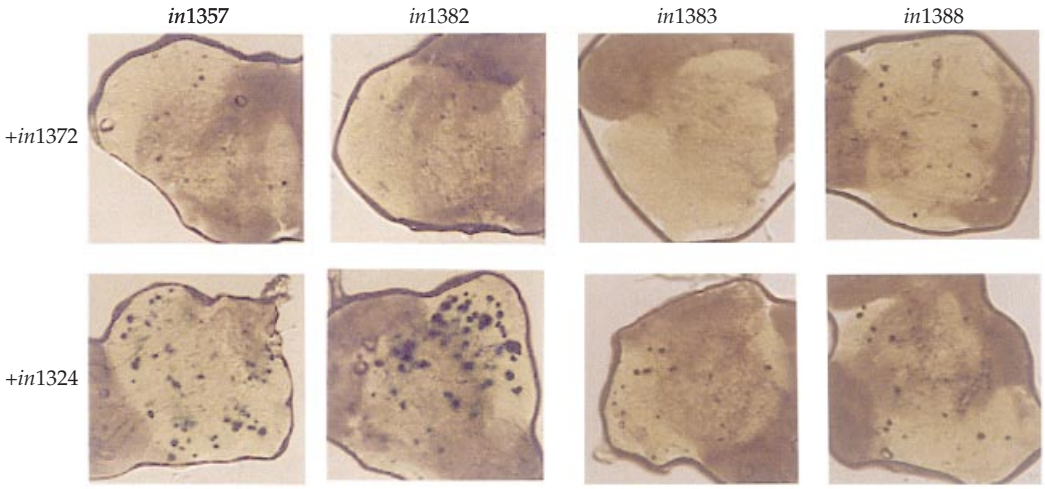

Fig. 2. Expression of β-galactosidase in mouse DRG neurons. Whole mounts of representative L4 DRG dissected from mice at 4 days postinoculation and stained for β-galactosidase are shown. Mice were inoculated with 2×10^5 p.f.u. of *in*1372 or *in*1324, plus 2×10^5 p.f.u. of *in*1357, *in*1382 or *in*1383, or plus 4×10^4 p.f.u. of *in*1388.

Table 3. Effects of *in*1324 on β-galactosidase expression in BALB/c mouse DRG

Virus 1*	Promoter†	Virus 2‡	Insertion§	Days p.i.	β-Gal-positive neurons‖	No. of mice
*in*1382	HCMV IE	*in*1372	Cre	4	19·9 (10·6)	8
*in*1382	HCMV IE	*in*1324	pp71	4	89·4 (32·1)	8
*in*1382	HCMV IE	*in*1372	Cre	42	20·0 (9·6)	4
*in*1382	HCMV IE	*in*1324	pp71	42	2·8 (2·2)	4
*in*1357	SCMV IE	*in*1372	Cre	4	25·1 (13·2)	8
*in*1357	SCMV IE	*in*1324	pp71	4	113·2 (16·9)	8
*in*1357	SCMV IE	*in*1372	Cre	42	20·2 (10·3)	4
*in*1357	SCMV IE	*in*1324	pp71	42	6·2 (4·1)	4
*in*1383	HSV-1 ICP0	*in*1372	Cre	4	0	8
*in*1383	HSV-1 ICP0	*in*1324	pp71	4	18·1 (9·7)	8
*in*1388 (4×10^3)	LAT-IRES	*in*1372	Cre	4	10·8 (4·3)	4
*in*1388 (4×10^3)	LAT-IRES	*in*1324	pp71	4	13·0 (5·0)	4
*in*1388 (4×10^4)	LAT-IRES	*in*1372	Cre	4	21·9 (9·2)	16
*in*1388 (4×10^4)	LAT-IRES	*in*1324	pp71	4	34·0 (15·0)	16
*in*1388 ($2{\cdot}5 \times 10^5$)	LAT-IRES	*in*1372	Cre	4	47·7 (25·4)	6
*in*1388 ($2{\cdot}5 \times 10^5$)	LAT-IRES	*in*1324	pp71	4	38·2 (10·9)	6

* Viruses *in*1382, *in*1357 and *in*1383 were inoculated at a dose of $2{\cdot}5 \times 10^5$ p.f.u. per mouse and *in*1388 was inoculated at the dose stated.
† Promoter controlling *lacZ* in virus 1.
‡ Viruses *in*1372 and *in*1324 were inoculated at a dose of $2{\cdot}5 \times 10^5$ p.f.u. per mouse.
§ Insertion in virus 2.
‖ The mean number of β-galactosidase-positive neurons per mouse in DRG L3, L4 and L5 combined, with standard deviation in parentheses.

Mice (BALB/c) were inoculated via the footpad with mixtures containing equal amounts of *in*1382 and either *in*1324 or *in*1372, and after 4 days ganglia were removed and stained for β-galactosidase activity. As shown in Fig. 2 and Table 3, co-inoculation of *in*1324 resulted in a 4- to 5-fold increase in the numbers of positive neurons in fixed whole mounts of DRG, compared with the numbers observed upon co-inoculation of *in*1372. The same degree of increase was observed when *in*1357, in which the SCMV IE promoter controls *lacZ*, was co-inoculated with *in*1324 (Fig. 2 and Table 3). In the case of *in*1383, in which the HSV-1 ICP0 promoter controls *lacZ* expression, no β-galactosidase-expressing neurons were detected when the virus was inoculated alone (results not shown) or with *in*1372, but when *in*1324 was co-inoculated approximately 18 positive neurons per mouse were observed (Fig. 2 and Table 3). The presence of *in*1324 enabled detection of

Table 4. Effect of *in*1324 on β-galactosidase expression in PML−/− and 129Sv mice

Mouse strain	Virus 1*	Promoter†	Virus 2‡	Insertion§	Days p.i.	β-Gal-positive neurons‖	No. of mice
129Sv	*in*1357	SCMV IE	*in*1372	Cre	4	27·1 (15·8)	9
129Sv	*in*1357	SCMV IE	*in*1324	pp71	4	84·0 (24·7)	9
129Sv	*in*1357	SCMV IE	*in*1372	Cre	42	50·0 (21·3)	9
129Sv	*in*1357	SCMV IE	*in*1324	pp71	42	25·2 (12·7)	9
PML−/−	*in*1357	SCMV IE	*in*1372	Cre	4	25·4 (13·4)	9
PML−/−	*in*1357	SCMV IE	*in*1324	pp71	4	111·3 (31·6)	9
PML−/−	*in*1357	SCMV IE	*in*1372	Cre	42	47·4 (12·0)	9
PML−/−	*in*1357	SCMV IE	*in*1324	pp71	42	25·0 (8·5)	9
129Sv	*in*1388	LAT-IRES	*in*1372	Cre	4	10·0 (4·1)	5
129Sv	*in*1388	LAT-IRES	*in*1324	pp71	4	10·8 (4·7)	5
PML−/−	*in*1388	LAT-IRES	*in*1372	Cre	4	19·4 (15·8)	5
PML−/−	*in*1388	LAT-IRES	*in*1324	pp71	4	16·8 (13·0)	5

* Virus *in*1357 was inoculated at a dose of $2{\cdot}5 \times 10^5$ per mouse and *in*1388 was inoculated at 4×10^4 p.f.u. per mouse.
† Promoter controlling *lacZ* in virus 1.
‡ Viruses *in*1372 and *in*1324 were inoculated at a dose of $2{\cdot}5 \times 10^5$ p.f.u. per mouse.
§ Insertion in virus 2.
‖ The mean number of β-galactosidase-positive neurons per mouse in DRG L3, L4 and L5 combined, with standard deviation in parentheses.

expression from the ICP0 promoter, an element that is not strong enough to give detectable β-galactosidase production in DRG neurons. Therefore, as in tissue culture cells, the expression of pp71 increased expression from herpesviral IE promoters when they were present in the *in*1312 genome.

Expression from all promoters examined to date is stimulated in tissue culture cells upon coinfection with *in*1324. The ability to observe an analogous effect in DRG neurons *in vivo* provided a means to extend the analysis of promoter specificity, using the HSV-1 LAT promoter that is active in this cell type. Mutant *in*1388 is an *in*1312-based recombinant that contains β-geo controlled by the HSV-1 latent promoter, with the EMCV IRES present to enable translation of β-galactosidase (Lachmann & Efstathiou, 1997; Marshall *et al.*, 2000). The LAT promoter is stronger than viral IE promoters in neurons, and previous studies showed that β-galactosidase expression, in terms of numbers of positive neurons, was essentially maximal upon injection of 2×10^5 p.f.u. of *in*1388 (the amount of *in*1382, *in*1357 and *in*1383 used in the studies described here; Table 3). Different concentrations of *in*1388 were therefore tested, to avoid saturation of the response in neurons. For three doses of *in*1388 tested (4×10^3, 4×10^4 and $2{\cdot}5 \times 10^5$ p.f.u. per mouse), no significant differences in the numbers of positive neurons were observed between co-inoculation of *in*1324 or *in*1372. This result indicates that the HSV-1 LAT promoter is not responsive to pp71 in neurons, and rules out trivial explanations for the effect of *in*1324 on the other viruses tested, such as alteration of the delivery of viral genomes to the ganglia.

The finding that co-inoculation of *in*1324 increased expression from the IE promoters tested raised the interesting possibility that pp71 may be able to improve long-term expression during HSV-1 latency in neurons. Ganglia were therefore examined at 42 days postinoculation, a time when HSV-1 is latent. In mice that received *in*1372 and either *in*1382 or *in*1357, the numbers of positive neurons observed were equivalent at 4 and 42 days, as previously found with the HCMV IE promoter in *in*1382 (K. R. Marshall & C. M. Preston, unpublished observations). By contrast, co-inoculation of *in*1357 and *in*1324, or co-inoculation of *in*1382 and *in*1324, resulted in 18- or 32-fold *decreases*, respectively, in the numbers of positive neurons compared with the 4 day values. Therefore, although expression of pp71 stimulated promoter activity in the short term, during latency it had a negative effect that was presumably due either to loss of neurons or promoter shutoff.

HCMV pp71 is active in the absence of PML

A transgenic homozygous mouse strain null for PML has been produced (Wang *et al.*, 1998a). The availability of this strain provided an opportunity to investigate whether PML is required for the activity of pp71, a possibility in view of the colocalization of the two proteins in tissue culture cells. PML−/− or control strain 129Sv mice were co-inoculated with *in*1357 plus either *in*1324 or *in*1372 (Table 4). As found in BALB/c mice, the presence of *in*1324 gave a 3- to 5-fold increase in the number of positive neurons in both PML−/− and 129Sv mice. Greater variation was observed with the PML−/− and129Sv mice, compared with BALB/c, presumably due to the less stringent matching of age and sex at the time of inoculation. Nonetheless, the stimulatory effect of *in*1324 was highly significant in both PML−/− and 129Sv animals. As with BALB/c mice, expression from the LAT

Table 5. Activity of pp71 in PML−/− fibroblasts

Preinfection*	m.o.i.†	Insertion‡	β-Gal activity§	
			PML−/−	129Sv
None			216	139
*in*1324	1	pp71	554 (2·6)	310 (2·2)
*in*1324	3	pp71	1032 (4·8)	506 (3·6)
*in*1320	1	(his)6-pp71	597 (2·8)	318 (2·3)
*in*1320	3	(his)6-pp71	922 (4·3)	416 (3·0)
*in*1325	1	GFP	192 (0·9)	136 (1·0)
*in*1325	3	GFP	140 (0·6)	95 (0·7)
*in*1372	1	Cre	200 (0·9)	120 (0·9)
*in*1372	3	Cre	190 (0·9)	116 (0·8)
*in*1316	1	YFP-pp71	837 (3·9)	ND
*in*1316	3	YFP-pp71	1070 (5·0)	ND

* Monolayers of 5×10^5 cells were infected, maintained at 38·5 °C for 3 h and infected with 4×10^4 p.f.u. of *in*1382. After further incubation at 38·5 °C for 5 h, cells were harvested.
† M.o.i. as p.f.u. per monolayer $\times 10^4$, taking titres on BHK cells at 31 °C in the presence of 3 mM HMBA.
‡ Inserted sequences, controlled by HCMV IE promoter.
§ β-Galactosidase activity of extracts, with the degree of stimulation over mock-infected cultures in parentheses.
ND, Not determined.

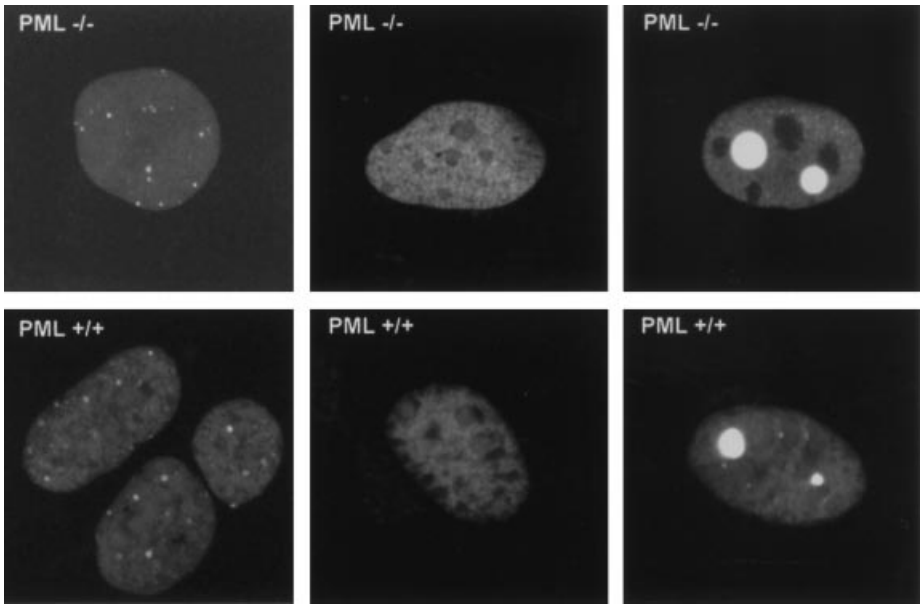

Fig. 3. Distribution of YFP-pp71 in PML−/− or 129Sv (PML+/+) mouse fibroblasts transfected with pYFPpp71. The three patterns of fluorescence described in the text are shown: localized on the left, dispersed in the centre, and large masses on the right. Images are z-series optical slices projected onto a single plane to include all structures in the cell.

promoter was equivalent when *in*1388 was co-inoculated with either *in*1324 or *in*1372 (Table 4). When β-galactosidase-positive neurons were counted at 42 days postinoculation, the numbers after co-inoculation of *in*1357 with *in*1372 surprisingly increased by 2-fold in both PML−/− and 129Sv mice, and the reasons for this difference from the findings in BALB/c are not clear. In mice co-inoculated with *in*1357 and *in*1324, however, the numbers decreased between 4 and 42 days, although not to the extent seen in BALB/c mice, and again there was no difference in the responses of PML−/− and 129Sv animals.

The *in vivo* results demonstrate that PML is not absolutely

required for activation of gene expression by pp71. Using this experimental approach, however, it is not possible to control the virus load of individual neurons since HSV-1 genome copy number can vary considerably (Sawtell, 1997). The activity of pp71 in primary mouse embryo fibroblasts, transformed by transfection with a plasmid expressing SV40 T antigen, was therefore examined. Monolayers of PML−/− or control fibroblasts were infected with *in*1320, *in*1324, *in*1325 or *in*1372 and, after incubation at 38·5 °C for 3 h, infected with *in*1382. Production of *β*-galactosidase after a further 5 h was determined (Table 5). As found in mice, expression of pp71 stimulated expression from the HCMV IE promoter by 3- to 5-fold in both cell lines. The PML−/− cell line expressed *β*-galactosidase from *in*1382 approximately 1·5 times more efficiently than the 129Sv line, probably accounting for the slightly higher degree of response to pp71. This variation in absolute levels of expression is assumed to be due to the clonal nature of the cell lines rather than their PML contents. The intracellular localization of YFP–pp71 was investigated after transfection of pYFPpp71 into PML−/− and control fibroblasts (Fig. 3). Three patterns of fluorescence were observed: some cells contained large masses, some exhibited diffuse nuclear staining, but in others specific loci were superimposed on the diffuse pattern, suggesting that pp71 can assume a localized distribution within nuclei even in the absence of PML. When transfections were carried out in parallel under identical conditions, a greater proportion of pp71-containing cells contained at least one large mass in PML−/− cultures compared with 129Sv cultures (60 % vs 29 %), possibly due to the greater activity of the HCMV IE promoter in the null cells. In single plane scans of cells that contained specific loci of pp71 (as in Fig. 3), PML−/− cells contained 8·5 (SD 3·1) 'dots' compared with 5·8 (SD 2·3) for 129Sv cells.

Discussion

The utilization of virion proteins to stimulate IE transcription by herpesviruses with diverse cell tropisms *in vivo* is in some ways surprising, since the IE promoters of HSV-1 and, particularly, HCMV are highly active in transfection assays when compared with most cellular promoters. Nonetheless, the importance of VP16 and pp71 is underscored by the impairment for initiation of replication at low m.o.i. in virus mutants in which the proteins are not functional (Ace *et al*., 1989; Bresnahan & Shenk, 2000). For HSV-1, the significance of transactivation by VP16 can be viewed as ensuring that ICP0 is produced rapidly to counteract repression of the viral genome by the host cell. The HCMV IE72 protein appears to play a role in preventing repression, since viral mutants fail to initiate replication efficiently at low m.o.i. (Greaves & Mocarski, 1998). The studies presented here, together with those of Baldick *et al*. (1997), suggest that pp71 directly affects the expression of the entire genome in addition to its role in stimulating the synthesis of IE72.

The fact that expression of pp71 partially complements the replication of an ICP0-null HSV-1 mutant implies a degree of functional interchangeability although, as shown previously using HCMV particles as the source of pp71 (Preston & Nicholl, 1997), this does not extend to reversal of the quiescent state. Similarity between pp71 and ICP0 is also apparent in the localization of the two proteins to ND10. Many viral proteins translocate to these sites, and the translocation usually correlates with positive effects on gene expression (Maul, 1998; Everett, 2000a). Most striking is HSV-1 ICP0, which exhibits reduced activation of gene expression if sequences important for accumulation at ND10 are altered by mutation (Everett *et al*., 1998a). The fact that stimulation of gene expression occurs normally in the absence of PML indicates that pp71 does not need intact ND10 for its activity. The localized areas of pp71 staining in PML−/− fibroblasts are intriguing, as their existence may suggest that other components of ND10, which remain in discrete loci even when PML is absent, are binding sites for pp71. The significance of pp71 migration to ND10 is unclear at present. Our transfection studies with pYFPpp71 show that localization to ND10 is not dependent on the presence of a viral genome, although it is likely that colocalization of pp71, IE proteins and viral genomes to a specific site would be advantageous for activation of gene expression.

Since there are no animal models for HCMV infection, attempts to elucidate the activities of HCMV gene products *in vivo* rely on studies with their homologues in animal cytomegaloviruses. No functional information is available on the properties of pp71 homologues; thus the demonstration of activity in mouse DRG neurons is the only evidence to date that pp71 activates HCMV IE transcription *in vivo*. Although this conclusion could be anticipated, the known changes to the genome structure and growth characteristics of HCMV once in tissue culture always raise a suspicion that the virus may behave differently *in vivo*. In addition, our results demonstrate that cellular factors which mediate the activity of pp71 are present even in highly differentiated, postmitotic neurons. The effect is possibly most dramatic when the response of *in*1383 is considered: the ICP0 promoter in this virus is not sufficiently active to direct levels of *β*-galactosidase that are detectable by X-Gal staining, but in the presence of pp71 many neurons were positive for the enzyme.

The LAT promoter, in virus *in*1388, is currently the only promoter known to be unresponsive to pp71 in the coinfection assays we have used. The regulation of this element is complex, however, with downstream regions contributing to its activity and in addition maintaining latent expression while the remainder of the genome is silenced (Lachmann & Efstathiou, 1997; Lokensgard *et al*., 1997). There are a number of possible interpretations for the failure of *in*1324 to stimulate expression of *β*-galactosidase upon coinfection with *in*1388. These include the following: (1) sequence motifs in the LAT promoter, especially in the downstream region, may block or

override pp71 activity; (2) pp71 may prevent or delay repression of the HSV-1 genome (and perhaps that of HCMV) – thus LAT, which escapes repression, is not affected by the protein; (3) the LAT promoter in neurons may be transcribed at maximal levels, such that further stimulation is not possible due to limiting amounts of one or more transcription factors; (4) stimulation of transcription may be matched by an increase in cleavage of the primary transcript, a possibility since the splice donor site for the major LAT is present in the mRNA that encodes *β*-geo (Farrell *et al.*, 1991; Arthur *et al.*, 1998; Alvira *et al.*, 1999). Arguing against this final possibility is the observation that levels of the spliced mRNAs for HSV-1 ICP0 and ICP22 were increased by pp71 in tissue culture cells (Homer *et al.*, 1999).

The reduction in the numbers of positive neurons at 42 days after co-inoculation with *in*1324 suggests that expression of pp71 is, in the long term, toxic or causes a shutdown of transgene expression. Taking the former possibility, it may be that increased levels of HSV-1 IE proteins in neurons that receive *in*1324 cause damage or, alternatively, that pp71 stimulates the synthesis of toxic cellular gene products. It is unlikely that the decrease in positive neuron numbers is due to induction of apoptosis, since PML −/− cells are defective in the execution of certain apoptotic signals (Wang *et al.*, 1998b; Zhong *et al.*, 2000). If promoter shutdown occurs it is unlikely to be due directly to the activity of pp71, since the protein stimulates rather than represses gene expression, but may result from the synthesis of inhibitory cell factors.

Although not directly relevant to the action of pp71, our finding with *in*1388 shows that HSV-1 latency occurs apparently normally in the absence of PML. It is not known whether ND10 exists in mouse neurons *in vivo*, but on the assumption that any such structures would require PML, it appears that HSV-1 does not require ND10 as a site to harbour latent genomes *in vivo*. In the human neuron-like NT2 line, the permissiveness to HSV-1 infection was not significantly changed when PML levels were increased by manipulation of cell culture conditions (Hsu & Everett, 2001).

Our results show that pp71 shares some properties with the HSV-1 IE protein ICP0, essentially in the ability to stimulate expression from a variety of promoters and migration to ND10. The functional significance of the intranuclear localization of pp71 is unclear, however, and the availability of cell lines that lack PML will be useful in unravelling the properties that are important for its effects on viral gene expression.

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