Role of the cellular protein hDaxx in human cytomegalovirus immediate-early gene expression

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Human cytomegalovirus (HCMV) immediate-early (IE) transcription is stimulated by virion phosphoprotein pp71, the product of gene UL82. It has previously been shown that pp71 interacts with the cellular protein hDaxx and, in the studies presented here, the significance of this interaction was investigated for HCMV IE gene expression. In co-transfection experiments, the presence of hDaxx increased the transcriptional response of the HCMV major IE promoter (MIEP) to pp71, but it was not possible to determine whether the effect was due to an interaction between the two proteins or to stimulation of hDaxx synthesis by pp71. The use of small interfering RNA (siRNA) in long- and short-term transfection approaches reduced intracellular hDaxx levels to no more than 3% of normal. Infection of hDaxx-depleted cells with herpes simplex virus recombinants containing the HCMV MIEP revealed significantly greater promoter activity when hDaxx levels were minimal. Similarly, reducing intracellular hDaxx amounts resulted in greater IE gene expression during infection with an HCMV mutant lacking pp71, but had no effect on IE transcription during infection with wild-type HCMV. The results suggest that hDaxx is not important as a positive-acting factor for the stimulation of HCMV IE transcription by pp71. Instead, it appears that hDaxx acts as a repressor of IE gene expression, and it is proposed here that the interaction of pp71 with hDaxx is important to relieve repression and permit efficient initiation of productive replication.

INTRODUCTION

Human cytomegalovirus (HCMV), a member of the subfamily Betaherpesvirinae, is an important human pathogen that can cause severe fetal damage and organ-transplant rejection. The HCMV genome is predicted to encode 165 genes that are expressed in a regulated manner during infection (Dolan et al., 2004). Synthesis of the immediate-early (IE) proteins precedes the coordinated expression of the remaining viral genes. The major IE promoter (MIEP) controls the production of proteins IE1 and IE2, which have important roles in regulation of viral and cellular transcription. The MIEP itself is responsive to many cellular-signalling pathways and additionally is activated by the viral tegument phosphoprotein pp71, the product of gene UL82 (Liu & Stinski, 1992). Virus mutants deleted for UL82 enter productive replication inefficiently after infection of cells at low m.o.i., demonstrating that, although the MIEP is intrinsically very active, stimulation of IE transcription by pp71 is important for initiation of the viral gene-expression programme (Bresnahan & Shenk, 2000; Cantrell & Bresnahan, 2005).

The mechanism by which pp71 stimulates HCMV IE gene expression is unclear at present. Co-transfection experiments suggested that specific sequence elements in the HCMV MIEP are targets for pp71 (Chau et al., 1999; Liu & Stinski, 1992), whereas other studies inferred that pp71 exerts a general effect on the transcription of the HCMV genome (Balick et al., 1997; Homer et al., 1999). It is likely that pp71 operates in conjunction with cellular proteins and important studies have shown that pp71 interacts with Daxx, the human form of which is named hDaxx (Hofmann et al., 2002; Ishov et al., 2002). By co-transfection, it was demonstrated that expression of hDaxx enhanced pp71-mediated stimulation of expression from the HCMV MIEP (Hofmann et al., 2002). In addition, HCMV infection of mouse fibroblasts lacking Daxx resulted in twofold fewer IE protein-positive cells after infection with HCMV, a result that was tentatively interpreted as suggesting a positive role for Daxx in HCMV IE transcription (Ishov et al., 2002). Recently, HCMV mutants that contain mutations of the Daxx-binding sites in pp71 have been isolated and these exhibit phenotypes indistinguishable from that of a pp71 deletion mutant, reinforcing the importance of the cellular protein for the replication of HCMV (Cantrell & Bresnahan, 2005).

Interaction between hDaxx and pp71 therefore represents a critical step in the initiation of HCMV infection. It is difficult to predict a mechanism of IE gene activation by the two proteins, because the proposed role of Daxx in uninfected cells is complex and controversial at present (Michaelson, 2000). Many studies suggest that the protein...
mediates apoptosis, as overproduction of Daxx resulted in greater sensitivity to certain apoptotic stimuli (Chang et al., 1998; Yang et al., 1997). In apparent contrast, depletion of Daxx by the use of small interfering RNA (siRNA) or by genomic deletion also gave increased apoptosis, suggesting an anti-apoptotic role for the protein (Chen & Chen, 2003; Michaelson & Leder, 2003; Michaelson et al., 1999). Further complexity arises in considering the intracellular location of Daxx. The pro-apoptotic phenotype resulting from overexpression has been attributed to cytoplasmic interaction with the cellular Fas receptor and subsequent activation of apoptosis signal-regulating kinase (Chang et al., 1998; Ko et al., 2001; Yang et al., 1997). However, alternative studies have demonstrated that Daxx can be detected in substructures known as nuclear domain 10 (ND10), at heterochromatin and at centromeres, suggesting that the protein can act in the nucleus (Everett et al., 1999; Ishov et al., 1999, 2004; Pluta et al., 1998; Torii et al., 1999; Xue et al., 2003). Intriguingly, input herpesvirus genomes accumulate at ND10 (Ishov & Maul, 1996; Maul et al., 1996; Rosenke & Fortunato, 2004). There is general agreement that Daxx can act as a repressor of transcription, although most studies performed to date have relied on plasmid-based transfection assays (Li et al., 2000a, b; Michaelson & Leder, 2003; Torii et al., 1999). Two basic hypotheses explain the significance of Daxx for HCMV replication. Daxx may act positively with pp71 to stimulate viral IE transcription or, alternatively, pp71 may be required to prevent Daxx from repressing the transcription of incoming viral genomes.

Our approach to the study of pp71 has relied on the use of IE-deficient herpes simplex virus type 1 (HSV-1) recombinants as vehicles to introduce pp71 and reporter constructs into cells (Homer et al., 1999; Marshall et al., 2002). The parental HSV-1 mutant, int1312, contains mutations that inactivate the virion transactivator VP16 and the IE transactivator proteins ICP0 and ICP4. Further modification of int1312 by introduction of the HCMV UL82-coding sequences yielded a virus, int1324, that is capable of expressing pp71 in a range of cell types. Additionally, int1312 derivatives containing reporter constructs have been produced. By using a co-infection approach, it was possible to demonstrate that pp71 stimulates expression from a variety of promoters (Homer et al., 1999). The system provides an alternative to a plasmid-based method, as the activity of pp71 can be assessed by using a herpesvirus genome as the target template. We have used the co-infection system, in addition to infection with HCMV itself, to investigate the importance of hDaxx for the activity of pp71. Alteration of hDaxx levels was achieved by transfection of plasmids encoding the protein or by the use of siRNA.

**METHODS**

**Cells and viruses.** Human fetal foreskin fibroblasts (HFF2), pp71-transformed human fibroblasts WF28-71-HA (Bresnahan & Shenk, 2000), human glioblastoma (U373) and osteosarcoma (U2-OS) cells and African green monkey (Cercopithecus aethiops) fibroblasts (CV-1) were cultivated in Dulbecco’s medium containing 5% (v/v) fetal calf serum, 5% (v/v) newborn calf serum, 100 units penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹. Wild-type HCMV was strain AD169. Mutant ADsubUL82, containing a cassette encoding green fluorescent protein (GFP) and puromycin resistance in place of pp71-coding sequences (Bresnahan & Shenk, 2000), was kindly provided by Dr T. Shenk, Princeton University, NJ, USA, and propagated on WF28-71-HA cells. A titre, expressed as infectious units ml⁻¹, was obtained by co-infection of HFF2 cells with dilutions of ADsubUL82 plus 1 p.f.u. AD169 per cell and determination of the percentage of GFP-positive cells at 3 days post-infection (p.i.). The HSV-1 mutants int1324, int1372 and int1382 have been described previously (Homer et al., 1999; Preston & McFarlane, 1998; Preston et al., 1998; Rinaldi et al., 1999). They are derived from mutant int1312, which contains a 12 bp insertion in the VP16-coding region, a deletion of the RING domain of ICP0 and the ICP4 temperature-sensitive mutation of HSV-1, tsK (Davison et al., 1984; Preston et al., 1998). Mutant int1324 has an additional insertion of the pp71-coding region, int1372 the Cre recombinase and int1382 the Escherichia coli lacZ sequences. The additional insertions were in the TK region of int1312, with the HCMV MIEP (~750 to +7)controlling expression. Titres were determined on U2-OS cells at 31 °C in the presence of 3 mM hexamethylene bisacetamide, a protocol that largely overcomes the three mutations of int1312-derived viruses.

**Transfection.** Cells were transfected with plasmids or siRNAs by using a Nucleofector 1 (Amaxa), following the protocols provided by the manufacturers and using optimized settings. Transfection efficiencies were routinely 30–70% for plasmids and 90–100% for siRNA. siRNA duplexes (Qiagen) were transfected into cells at 400 nM efficiencies were routinely 30–70 % for plasmids and 90–100 % for siRNA. siRNA duplexes (Qiagen) were transfected into cells at 400 nM final concentration. The sense strands of the duplexes were: hd1, 5’-GGCGUCAUGUCUCAUCAAU (nt 36–384 of hDaxx); hd2, 5’-GGAGUUGGACUCCUCAGGA (nt 626–643 of hDaxx; Michaelson & Leder, 2003); lamin, 5’-CUGGACUCCAGAAAGA (nt 606–627 of lamin A/C; Elbashir et al., 2001). All siRNAs were 3’-tailed with dTdT. Transfected cells were plated in 24-well dishes and maintained at 37 °C for 3 days prior to processing.

**Plasmids.** Plasmid pM1J123 consists of the pp71-coding region in pcDNA4/HisMax (Invitrogen) and thus expresses pp71 tagged at the N terminus with polyhistidine (his). Plasmid pCP37736 was derived by inserting the hDaxx-coding BamHI fragment of cloned hDaxx cDNA (Ishov et al., 1999) into pcDNA4/HisMax. To produce plasmids that encode short hairpin RNA (shRNA) specific for hDaxx or lamin A/C, two approaches were used. In the first of these, two oligodeoxyribonucleotide duplexes corresponding to separate regions of hDaxx or one corresponding to lamin A/C were cloned into pSilencer 2.1-U6 neo (Ambion). The target regions were as described above for hd1, yielding pM1J168, hd2, yielding pM1J169, and lamin A/C, yielding pM1J167. In the second approach, an oligodeoxyribonucleotide duplex encoding shRNA corresponding to hd2 was cloned into pSKU6, a vector containing the mouse U6 promoter (constructed and kindly provided by A. Patel, MRC Virology Unit, Glasgow, UK). The cassette consisting of the U6 promoter plus hd2 sequence was cloned between the MluI and KpnI sites of pcDNA5/FRT (Invitrogen), thereby removing the HCMV MIEP from pcDNA5/FRT, to yield pCP4920.

**Selection of cell lines.** U373 cells were transfected with pM1J167, pM1J168 or pM1J169, linearized by cleavage in the vector sequences. After 2 days, monolayers were trypsinized and replated at low concentrations in medium containing 500 μg G418 ml⁻¹. Single colonies were amplified and passaged routinely in medium containing 100 μg G418 ml⁻¹. CV-1(F) cells, containing a single integrated copy of sequences specifying a lacZ–zeocin-resistance fusion protein (lacZeo) preceded by an FRT site, were obtained from Invitrogen as a component of the ‘Flip-In’ system. These cells were transfected
with pCP44920 plus pOG44 (Invitrogen), which specifies the Flp recombinase. Flp-mediated site-specific recombination between FRT sites in the cell genome and pCP44920 resulted in integration of the mouse U6 promoter–hd2 cassette, with the concomitant acquisition of hygromycin resistance and loss of lacZeo production. Two days after transfection, monolayers were trypsinized and replated at low density in medium containing 100 μg hygromycin ml⁻¹. Single colonies were amplified and passaged in medium containing 100 μg hygromycin ml⁻¹. For cell line 18, routine screening during passage revealed that a proportion of cells had become positive for expression of Daxx. This culture was replated at low concentration and single colonies yielding lines negative and positive for Daxx (18/11 and 18/23, respectively) were isolated. Both lines were resistant to hygromycin, but sensitive to zeocin, indicating that 18/23 arose by shutdown of shRNA expression. The characteristics of the cell lines used in this study are summarized in Table 1.

**Western blots.** Samples were analysed on protein blots as described previously (Marshall et al., 2002). Primary antibodies were rabbit anti-pp71 (Preston & Nicholl, 2005) diluted 1:100; mouse anti-Daxx (Pharmingen and a gift from G. Maul, Wistar Institute, Philadelphia, PA, USA) diluted 1:300; mouse anti-lamin A/C (Santa Cruz) diluted 1:500, and mouse anti-β-actin (Sigma) diluted 1:1000. Detection was achieved by using Amersham ECL blotting detection agents, or the ECL Plus system for detection of Daxx.

**Immunofluorescence.** Mouse anti-HCMV IE1 (Biogenesis) was used at 1:500, with fluorescein isothiocyanate-conjugated goat anti-mouse (Sigma, 1:100) as secondary. Nuclei were stained with 1 μg propidium iodide ml⁻¹. Images were collected by using a Zeiss LSM510 confocal microscope.

**Expression assay.** Monolayers in 24-well plates were infected in a total volume of 0·1 ml. After adsorption of virus for 1 h, 0·9 ml medium was added and cells were incubated at 38·5°C until harvested for β-galactosidase assays using 4-methylumbelliferone-β-d-galactoside as substrate, as described previously (Preston & Nicholl, 1997).

### Histochemical detection of β-galactosidase

This assay was performed as described previously (Jamieson et al., 1995).

**RNA analysis.** RNA analysis by electrophoresis and blotting was performed as described previously (Nicholl & Preston, 1996). Radiolabelled probes were an HCMV IE1-specific fragment excised from pcDNA3IE72 (Bryant et al., 2000) and a glyceraldehyde phosphate dehydrogenase (GDH)-specific fragment excised from the cloned GDH cDNA (a gift from S. Milligan, Division of Virology, University of Glasgow, UK).

### Table 1. siRNA-expressing cell lines

<table>
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<th>Line</th>
<th>Cell type</th>
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<tbody>
<tr>
<td>1/17, 1/4</td>
<td>U373</td>
<td>siLamin</td>
</tr>
<tr>
<td>3/2</td>
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<td>shiD1</td>
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<tr>
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<td>shiD2</td>
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<tr>
<td>18/11</td>
<td>CV-1(F)</td>
<td>shiD2</td>
</tr>
<tr>
<td>18/23</td>
<td>CV-1(F)</td>
<td>None†</td>
</tr>
</tbody>
</table>

*CV-1(F) cells are derived from CV-1 cells.
†18/23 is a revertant of 18/11 that is assumed to lack siRNA.

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### RESULTS

#### Co-expression of hDaxx and pp71 in human fibroblasts

Previous studies have shown that expression of hDaxx increased pp71-mediated stimulation of HCMV MIEP activity in co-transfection assays (Hofmann et al., 2002). To investigate whether this also occurred when the MIEP was present in the ir1312 genome rather than on a plasmid, HFF2 cells were transfected with plasmids and subsequently infected with ir1382. Cells positive for β-galactosidase were recorded (Fig. 1a). Transfection of a pp71-encoding plasmid increased expression by 12-fold in this assay. Transfection of pCP37736, a plasmid that encodes his-tagged hDaxx, surprisingly increased expression by fivefold and the two plasmids together gave a 42-fold stimulation. Examination of protein levels showed that the expression of hDaxx did not affect the amount of pp71 present (Fig. 1b). However, the amount of his-hDaxx specified by pCP37736 exceeded the endogenous levels (Fig. 1c, lane 3) and expression of pp71 gave a further increase (Fig. 1c, lane 4). As approximately 30% of cells were transfected successfully, those cells that received the plasmids contained significantly raised levels of hDaxx. We concluded that overexpression of hDaxx per se stimulated expression from the MIEP in ir1382, and that the greater effect in the presence of pp71 might signify a response to the further increased levels of

![Fig. 1. Transfection of plasmids expressing hDaxx and pp71 increases expression from the HCMV MIEP. HFF2 cells were transfected with 2·5 μg pMJ123 (expresses pp71), 2·5 μg pCP37736 (expresses his-hDaxx) or 2·5 μg of both plasmids. Total DNA was adjusted to 5 μg in all cases by the addition of pcDNA4/Hismax DNA. (a) At 24 h post-transfection, 2×10⁶ cells were infected with 3×10⁴, 1×10⁵ or 3×10⁶ p.f.u. in1382 and incubated at 38·5°C for 24 h. Mean numbers of β-galactosidase-expressing cells per 10⁵ p.f.u. in1382 were calculated, excluding plates with more than 150 positive cells. (b) Extracts from cells transfected with pCP37736 without (+) or with (+) pMJ123 were made at 24 h post-transfection and analysed for pp71 levels. (c) Extracts from cells transfected with pcDNA4/Hismax (lane 1), pMJ123 (lane 2), pCP37736 (lane 3) or pCP37736 plus pMJ123 (lane 4) were analysed for hDaxx levels.](http://vir.sgmjournals.org)
hDaxx, rather than an interaction between the two proteins. In view of these considerations, the effect of reducing hDaxx levels was investigated.

**Use of siRNA to decrease hDaxx levels**

A number of approaches were taken to deplete hDaxx from cells by use of siRNA. In initial experiments, siRNAs specific for hDaxx were transfected into HFFF2 cells. Two target regions of hDaxx were selected. One of these (sihD2) was equivalent to the region used successfully in previous studies (Michaelson & Leder, 2003), whilst the other (sihD1) was to a separate part of the coding region. The previously characterized lamin A/C-specific siRNA (siLamin) was used as a control. Pilot experiments showed that transfection of either hDaxx-specific siRNA reduced hDaxx levels by 80–90% after 3 days. Unfortunately, the use of HFFF2 cells was compromised by the observation that the 80–90% reduction in hDaxx levels was transient; at days 2 and 4 the effects were not so great, presumably reflecting the intracellular stabilities of hDaxx and the siRNAs.

As an alternative approach, U373 cells were transfected with plasmids that expressed shRNAs specifying sihD1, sihD2 or siLamin and cloned cell lines were selected. Lines were screened for the presence of hDaxx or lamin A/C (Fig. 2a). Depletion of the cognate protein was observed, although the reduction of hDaxx was greater than that of lamin A/C. Lines 3/2 (expressing sihD1), 2/22 (expressing sihD2) and 1/4 or 1/17 (expressing siLamin) were chosen for further study. Lines 1/4 and 1/17 gave equivalent results. As described later, it became necessary to reduce further the amounts of hDaxx and this was achieved by transfecting lines 3/2 and 2/22 with the alternative siRNA (sihD2 or sihD1, respectively), followed by analysis at 3 days post-transfection. Pilot experiments established that, in U373 cells, the reduction in hDaxx levels was stable between 2 and 4 days after transfection (results not shown). The efficacy of this approach is shown in Fig. 2(b). U373 cells transfected with sihD2 (lane 1) show a clear reduction in hDaxx levels compared with U373 cells transfected with siLamin (lane 2). To assess the degree of reduction, dilutions were made of the siLamin-transfected cell lysate (lanes 3–5). The intensity of the residual band in lane 1 is approximately equivalent to that of a 10-fold dilution of siLamin-transfected cell lysate (lane 3), suggesting a 90% reduction by transient transfection of sihD2. The hDaxx level in line 1/17 was reduced to a similar extent (lanes 8 and 9). Line 3/2 transfected with siLamin (lane 7) contained approximately 10% of the amount of Daxx in U373 cells (lane 3), and this amount was further reduced by transfection of sihD2 to a value approximately equivalent to 3% of that in U373 cells (lanes 5 and 6).

Cell line 2/22 also contained approximately 10% of U373 quantities of hDaxx (Fig. 2c, lanes 3 and 6) and transfection of sihD1 reduced this value to approximately 3% (lane 7). It should be noted that values for residual hDaxx are maximum estimates, as a proportion of the signal could arise from non-specific cross-reaction with other cellular proteins. This semi-quantitative analysis was carried out.

![Fig. 2. Reduction of Daxx levels with siRNA. (a) U373 cells or derived stable lines were analysed for expression of hDaxx or lamin A/C. The cells were untransformed U373 (lane 1), sihD1-transformed line 3/2 (lane 2), sihD2-transformed line 2/22 (lane 3), siLamin-transformed lines 1/4 (lane 4) or 1/17 (lane 5) or cells transformed with empty vector (lane 6). (b) U373 cells (lanes 1–5), 3/2 (lanes 6 and 7) or 1/17 (lanes 8 and 9) were transfected with sihD2 (2) or siLamin (L) and extracts were made after incubation at 37°C for 3 days. Tenfold, 20-fold or 30-fold dilutions of the siLamin-transfected U373 lysate are shown in lanes 2, 3 and 4, respectively. (c) CV-1(F) (lanes 1–5), 18/11 (lanes 6 and 7) or 18/23 (lanes 8 and 9) cells were transfected with sihD1 (1) or siLamin (L) and extracts were made after incubation at 37°C for 3 days. Tenfold, 20-fold or 30-fold dilutions of the siLamin-transfected CV-1(F) lysate are shown in lanes 2, 3 and 4, respectively.](image-url)
Routine to assess the levels of hDaxx during experiments with transfected cell lines. Even without further transfection of siRNA, the presence of hDaxx at ND10 was not detectable in 3/2 or 2/22 cells by immunofluorescence (see Supplementary Fig. S1, available in JGV Online).

A third approach, aided by the fact that the shhD1 and shhD2 targets were present in the Cercopithecus aethiops Daxx sequence, was the use of specific insertion shhD2-encoding sequences by Flp recombinase into the genome of CV-1 cells engineered to contain an FRT site [CV-1(F) cells] and subsequent selection of cell lines. This approach yielded lines with very low Daxx levels. During passage of line 18, cells that expressed Daxx emerged due to shutdown of shRNA synthesis and the line was recloned to yield Daxx-negative (18/11) and Daxx-positive (18/23) cells. Line 18/23 thus provided an ideal control for the effects of Daxx, as it was a clonally related ‘revertant’ of line 18/11. Transfection of parental CV-1(F) cells with sihD1 reduced Daxx levels to approximately 3% of those in siLamin-transfected cells (Fig. 2d, lanes 1 and 5) and similar results were obtained for line 18/23 (lanes 9 and 8). Daxx was not detectable in 18/11 cells transfected with siLamin (lane 7) or shhD1 (lane 8), showing that, in CV-1 cells, Daxx levels could be effectively reduced to <3% of normal (lane 4).

**Functional activity of pp71 in hDaxx-depleted cell lines**

We utilized a short-term expression assay based on the use of the IE-impaired HSV-1 mutant in1312 to investigate the activity of pp71 in hDaxx-depleted cells (Homer et al., 1999) (Table 2). Cells were first infected with in1324, an in1312-based mutant that expresses pp71, and subsequently infected with in1382, expressing the β-galactosidase reporter. As a control, cells were co-infected with in1382 and the HSV-1 mutant tsK, which expresses ICP0 and fully derepresses the in1382 genome. Levels of β-galactosidase were expressed as percentages of the fully derepressed value.

In the U373-derived cell lines, irrespective of the siRNA transfected, the introduction of pp71 by infection with in1324 gave expression levels that were approximately 50% of those in cells co-infected with tsK. In all cases, pp71 stimulated expression over the values from cultures that were mock-infected or infected with in1372 (a control virus identical to in1324, except that Cre was expressed instead of pp71). Control experiments showed that pp71 levels were similar in all cell lines and that the expression of pp71 did not alleviate the effects of siRNA (see Supplementary Figs S2 and S3, available in JGV Online). Therefore, pp71 increased expression from the HCMV IE promoter even when hDaxx levels were no more than 3% of normal. These experiments revealed, however, a significant effect of reducing hDaxx levels on the activity of the HCMV MIEP in the absence of pp71. In cell lines 3/2 and 2/22, which contained approximately 10% of normal hDaxx levels, expression was greater than in U373 or 1/17 cells by approximately twofold and, additionally, transient transfection of shhD1 or shhD2

<table>
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<tr>
<th>Cell line</th>
<th>siRNA</th>
<th>Expression of β-galactosidase preinfection (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mock</td>
</tr>
<tr>
<td>U373</td>
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<tr>
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<td>siL</td>
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</tr>
<tr>
<td>2/22</td>
<td>shhD1</td>
<td>16·8</td>
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</table>

*Transfected with shhD1 or shhD2.
†ND, Not determined.

Further increased expression by approximately threefold. As a consequence, the expression of β-galactosidase in hDaxx-reduced cell lines reached levels 11·3% (line 3/2) or 16·8% (line 2/22) of the derepressed values, an increase of 4·7- to 7·0-fold over the value in siLamin-transfected U373 cells.

The data from Table 2 relating to expression in the absence of pp71 are documented more thoroughly in Table 3 because the differences detected were relatively small. For the four U373 cell lines, the increases in expression between transfection with shhD1 or shhD2, compared with siLamin, were all highly significant (P<0·01). To confirm this finding in an alternative cell system, the CV-1 line 18/11 and its revertant 18/23 were transfected with shhD1 or siLamin and subsequently infected with in1382. As found with U373 cells, transfection of shhD1 resulted in a greater relative level of β-galactosidase expression than transfection of siLamin for both lines. Expression in 18/11 cells transfected with shhD1, with the greatest deficit of Daxx, was fourfold greater than in 18/23 cells transfected with siLamin, which had normal Daxx levels. Therefore, in two different cell types, reducing intracellular Daxx levels by use of siRNA resulted in increased activity of the HCMV IE promoter when delivered as a component of the in1312 genome.

**HCMV IE gene expression in hDaxx-reduced cells**

To investigate the effects of reducing hDaxx levels on IE gene expression from the HCMV genome, U373 lines 3/2, 2/22,...
and 1/17 were infected with HCMV AD169 at low m.o.i. (0.5 p.f.u. per cell) and analysed by immunofluorescence (Fig. 3a) or protein expression (Fig. 3b). There were no significant differences in IE protein production between cell lines and no effect of reducing hDaxx levels by transfection of sihD1 or sihD2. Analogous results were obtained during infection with 0.05 p.f.u. HCMV AD169 per cell for 14 h (see Supplementary Fig. S4, lanes 5–8, available in JGV Online), and in HFFF2 cells transfected with sihD1 plus sihD2 (results not shown). In a further test, cell lines were infected with 1 p.f.u. HCMV per cell in the presence of cycloheximide and IE RNA levels were analysed (Fig. 3c). Relative to the loading-control signal (GDH hybridization), no differences in IE RNA levels were detected between cell lines or in response to reduction of hDaxx levels. Thus, by three approaches, no decrease in HCMV IE gene expression was detected when hDaxx levels were reduced to approximately 3% of the normal value.

The experiments with the in1312-based recombinants suggested that removal of hDaxx increased expression from the MIEP in the absence of pp71 (Table 3). To investigate whether this is also the case for HCMV, cultures were infected with the pp71 deletion mutant ADsubUL82 and IE protein synthesis was analysed (Fig. 4). Low amounts of IE1 protein were detected in U373 cells transfected with siLamin (lane 2) and transfection of sihD2 resulted in increased expression (lane 3). Lines 3/2 and 2/22 supported greater IE expression than U373 cells when transfected with siLamin (lanes 4 and 6) and the protein levels increased further upon transfection of sihD2 or sihD1 (lanes 5 and 7). Additional experiments demonstrated that pp71 stimulated ADsubUL82-directed IE gene expression in U373 cells and that the degree of response to pp71 was lower in hDaxx-depleted cells because the basal IE expression was increased (see Supplementary Fig. S4, available in JGV Online). Therefore, the investigation with ADsubUL82 demonstrates a clear inverse correlation between hDaxx levels and IE

**Table 3. Expression of β-galactosidase in the absence of pp71**

U373-derived cell lines, transfected with siRNAs, were infected with in1382, with or without tsK, as described in Table 2 and harvested at 9 h p.i. CV-1 lines 18/23 and 18/11 were infected with $5 \times 10^5$ p.f.u. in1382, with or without $1 \times 10^6$ p.f.u. tsK, and harvested at 11 h p.i. β-Galactosidase levels were expressed as percentages of the value for tsK-infected cultures. Values for U373-derived lines are taken from Table 2.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>siRNA</th>
<th>No.*</th>
<th>Expression (%D)</th>
<th>SD</th>
<th>Range</th>
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<td>10</td>
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*Number of experiments.
†SD, Standard deviation.
‡Transfected with sihD1 or sihD2.
expression in the absence of pp71, supporting the data in Tables 1 and 2 using in1312-based recombinants.

**DISCUSSION**

The studies reported here were designed to investigate the significance of hDaxx for the activity of pp71. Plasmid co-transfection demonstrated that expression from the HCMV MIEP, when delivered to cells as a component of in1312, was stimulated by overproduction of hDaxx and that the effect of hDaxx was additive with the activation by pp71. This observation is in general agreement with that of Hofmann et al. (2002), who used an entirely plasmid-based transfection approach to monitor activation of the HCMV MIEP in U373 cells. In detail, however, there are differences between the data presented here and those of Hofmann et al. (2002). We observed an increase in the amount of expressed hDaxx when a plasmid encoding pp71 was additionally present, whereas Hofmann et al. (2002) did not. In addition, we compared the level of plasmid-directed hDaxx with that of the endogenous protein and found, not surprisingly, that transfection resulted in a large increase in the quantity of intracellular hDaxx. Also, in contrast with the findings of Hofmann et al. (2002), we detected a fivefold increase in expression from the MIEP when hDaxx alone was overexpressed in human fibroblasts. In view of these results, we concluded that overexpression of hDaxx per se stimulated MIEP activity and that the additional increase in MIEP activity when pp71 and hDaxx were expressed together might be an indirect consequence of the further-increased hDaxx levels rather than an interaction between the two proteins. Nonetheless, the two interpretations remain equally valid and further investigation of this question is required. The differences between our findings and those of Hofmann et al. (2002) could be due to the different transfection methods employed, the cell type or differences in the response of the MIEP when used as part of the HSV-1 in1312 genome or on a plasmid.

At present, we have no explanation for the stimulation of MIEP activity by overexpression of hDaxx, an observation that is difficult to reconcile with the finding that reducing hDaxx levels has an analogous effect. There is, however, an interesting parallel with the situation regarding the involvement of hDaxx in apoptosis, in which both over- and underexpression of the protein sensitize cells to apoptotic signals (Chang et al., 1998; Chen & Chen, 2003; Michaelson & Leder, 2003; Michaelson et al., 1999; Yang et al., 1997).

Conceptually, analysing the effects of reducing hDaxx levels is possibly a more straightforward approach than investigating the consequences of overexpression. The use of siRNA is liable to a number of pitfalls, mainly centred on concerns regarding whether the effects observed are specifically due to the depletion of the chosen target protein. We obtained equivalent results by using two hDaxx target sequences, overcoming the objection that off-target effects on other gene products explained the observed results. As a further control, lamin A/C-specific siRNA, which was demonstrated to be functional, was included. The use of a revertant CV-1 cell line also provided a useful control, enabling a comparison to be made between two lines that were clonally related and would be expected to differ primarily only in the production of hDaxx-specific siRNA.

It was not possible to maintain stable U373 lines containing <10 % of normal hDaxx levels, suggesting that the protein is very important for growth of these cells. This supposition was reinforced by experiments in which 3/2 cells were retransformed with a plasmid that expressed shhD2, using a different selection system. Initially, colonies that were devoid of detectable hDaxx were isolated, but they rapidly changed to contain the same level as parental 3/2 cells upon expansion to usable cell lines (unpublished results). However, by transient transfection, we were able to produce cell populations with approximately 97 % reduction of hDaxx levels, a value exceeding that obtained in most siRNA-based studies.

Despite the severe reduction in hDaxx levels achieved in transformed, transfected U373 cells, no effect was noted on wild-type HCMV IE gene expression at the level of protein or RNA synthesis or on the activity of the MIEP when pp71 was present. The results therefore do not support the concept that hDaxx acts positively with pp71 to increase IE transcription, unless 3 % of normal levels of the protein is sufficient for full activation. The severe phenotype of HCMV pp71 deletion mutants indicates that any impairment of pp71 activity would be detected readily as a diminution of IE gene expression (Bresnahan & Shenk, 2000; Cantrell & Bresnahan, 2005).

Reducing hDaxx levels resulted in an increase in the activity of the HCMV MIEP in the absence of pp71, as shown by the increased expression after infection with ADsubUL82 or in1382. In the case of in1382, the increase was only approximately twofold when approximately 10 % of normal hDaxx levels remained, but reduction to approximately 3 % (3/2...
cells transfected with sihD2 or 2/22 with sihD1) gave values four- to sevenfold greater than background (U373 or 1/17 cells transfected with siLamin). It is clear, however, that the basal expression levels from in1382 achieved by depleting cells of hDaxx did not reach those observed when pp71 was present or when HSV-1 ICP0 was provided by co-infection with rK. This finding suggests either that hDaxx is only a component of the repression mechanism, with additional factors also involved, or that the small amounts of hDaxx remaining were sufficient to repress MIEP activity to some extent in the absence of pp71. During infection with ADsubUL82, reducing hDaxx levels resulted in increased IE1 protein synthesis, supporting the conclusions from the studies with in1382. Therefore, by two approaches that analyse the activity of the HCMV MIEP in different settings, severe reduction in the amount of hDaxx resulted in a significant increase in IE promoter activity when pp71 was not present.

The results, therefore, suggest an involvement of hDaxx in repression of HCMV MIEP activity, a conclusion that is consistent with reports that hDaxx acts as a transcriptional repressor in other systems (Greger et al., 2005; Hollenbach et al., 1999; Li et al., 2000a, b; Michaelson & Leder, 2003; Torii et al., 1999). Repression is thought to occur through chromatin modification, based on the recruitment of histone deacetylases (HDACs) that interact with hDaxx (Hollenbach et al., 2002; Tang & Maul, 2003). It is possible that hDaxx and HDACs are targeted to HCMV DNA in interaction with factors that bind to the MIEP, such as ETS family proteins, which are known to bind hDaxx (Li et al., 2000b; Wright et al., 2005). Alternatively, the location of viral genomes at ND10 may render them generally susceptible to hDaxx-mediated repression in a manner that is independent of target sequence. It must also be recognized that, despite the evidence of a direct interaction between hDaxx and pp71, the effects that we observed may be indirect. As in many experiments in which protein levels are altered, factors that act downstream of hDaxx, and hence are affected by its depletion, may actually be the critical components that influence the activity of the HCMV MIEP.

In conclusion, our results suggest that hDaxx represses transcription from the HCMV MIEP rather than acting positively. Inherent in this result is the implication that pp71, either directly or indirectly, is involved in overcoming the repressive action of hDaxx and thereby enabling IE transcription to occur efficiently. It will be important to investigate the effects of pp71 on hDaxx and associated proteins.

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