Human Daxx-mediated Repression of Human Cytomegalovirus Gene Expression Correlates with a Repressive Chromatin Structure around the Major Immediate Early Promoter*

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Upon herpesvirus infection, viral DNA becomes associated with nuclear structures known as nuclear domain 10 (ND10). The role of ND10 during herpesvirus infection has long been contentious; data arguing for a role for ND10 in repression of infection have been countered by other data showing little effect of ND10 on virus infection. Here we show that knockdown of human Daxx (hDaxx) expression, an important component of ND10, prior to infection with human cytomegalovirus resulted in increased levels of viral immediate early RNA and protein expression and that this correlated with an increased association of the major immediate early promoter with markers of transcriptionally active chromatin. Conversely, we also show that stable overexpression of hDaxx renders cells refractory to cytomegalovirus immediate early gene expression. Intriguingly, this hDaxx-mediated repression appears to be restricted to cells stably overexpressing hDaxx and is not recapitulated in transient transfection assays. Finally, hDaxx-mediated repression of cytomegalovirus major immediate early gene expression was overcome by infecting at higher virus titers, suggesting that an incoming viral structural protein or viral DNA is responsible for overcoming the repression of viral gene expression in hDaxx superexpressing cells. These data suggest that hDaxx in ND10 functions at the site of cytomegalovirus genome deposition to repress transcription of incoming viral genomes and that this repression is mediated by a direct and immediate effect of hDaxx on chromatin modification around the viral major immediate early promoter.

Human cytomegalovirus (HCMV) is an extremely widespread, opportunistic pathogen of considerable clinical significance. Primary infection of healthy, immunocompetent individuals with HCMV is normally asymptomatic. However, as is the case with all herpesviruses, after the initial control of primary infection by the immune system, HCMV establishes lifelong latency in the host. During latency and throughout the lifetime of the infected individual, the herpesviruses can reactivate. Although generally asymptomatic, such reactivation can result in severe disease. As such, the disease burden of herpesviruses can be life-long and particularly severe when associated with immunosuppression, such as in transplant patients and in human immunodeficiency virus-infected patients who have developed immunodeficiency.

After infection with herpes simplex virus-1, the viral genomes appear to co-localize with cellular nuclear structures known as nuclear domain 10 (ND10) and give rise to replication centers in close proximity to these sites (3, 4). ND10 are discrete interchromosomal accumulations of a number of proteins, many of which are transcriptional repressors. Intriguingly, many of the human herpesviruses encode an immediate early protein, which functions to destroy or redistribute components of ND10 (5−17), and a number of studies have attempted to address the role of ND10 during infection. One of the first observations was that ICP0 (intracellular protein 0) of herpes simplex virus-1 was sufficient for the disruption of ND10 (14) and that disruption occurred via proteosomal degradation of the promyelocytic leukemia (PML) protein, an essential structural component of ND10 (15, 18, 19).

Parallel studies with HCMV have identified that an immediate early protein, IE72, is similarly necessary and sufficient for the disruption of ND10 (5, 6, 11, 12, 16), although this disruption is mediated through an unknown mechanism and does not occur through ubiquitination and degradation via the proteosome (5, 20). In HCMV-infected cells, despite its relocation, PML has not been shown to be altered biochemically. Experiments that analyzed HCMV infection of a fully permissive astrocytoma cell line engineered to stably express PML (21), showed that disruption of ND10 by virus was delayed by up to 6 h compared with controls. Concomitant with this, viral DNA replication compartment formation was delayed, and the expression of early and late viral proteins were suppressed.
Overall, these data supported the suggestion that ND10 represents a nuclear environment which is unsupportive of viral gene transcription and that may function in an antiviral capacity. Consistent with this, a recent report has shown that the targeted knockdown of PML expression (and thus ND10 integrity) prior to viral infection results in the release of increased titers of progeny virus following productive infection (22).

The interaction of HCMV proteins with ND10 does not appear to be exclusive to IE72. The viral tegument protein and transactivator, pp71, accumulates at ND10 in a process mediated by another major ND10 component, the hDaxx protein (23–25). This results in a transient pp71-mediated degradation of hDaxx between 3 and 12 h of infection, arguing for a role of hDaxx in repression of infection (26). Consistent with this, hDaxx can function as a promiscuous transcriptional repressor, possibly via the recruitment of histone deacetylases to transcriptionally active chromatin (27), resulting in a closed chromatin structure and the repression of transcription (28–30).

It is becoming increasingly clear that herpesvirus gene expression may also be regulated by chromatin structure (31, 32). For example, the regulation of the major immediate early promoter (MIEP) is likely to involve higher order chromatin structure (32, 33). In experimentally infected monocytes and CD34+ cells, the MIEP is associated with HP-1 (heterochromatin protein 1), a classical marker of transcriptionally repressed chromatin (34), which is consistent with a lack of IE gene expression in these cells. In contrast, following infection of macrophages or mature dendritic cells, the MIEP is associated with acetylated histones, a marker of transcriptional activation (29, 30), and this correlates with detectable IE gene expression in these cells (32). Furthermore, this regulation of the MIEP by chromatin structure is also likely to be important during HCMV latency and reactivation in cells isolated from naturally infected seropositive individuals (35).

Although chromatin structure of the viral MIEP is clearly associated with latency and reactivation of viral IE gene expression, there is increasing evidence that chromatinization of the MIEP may also play a role during productive infection of fully permissive cell types. Although the virus and its major IE promoter exhibit only limited sequence homology with HCMV, in mouse cytomegalovirus there is evidence that chromatinization of the viral genome accompanies productive infection (36). The addition of the general histone deacetylase inhibitor TSA, with the histone deacetylase inhibitor TSA, suggesting that an intermediate early gene expression in cells stably overexpressing hDaxx was overexpressed by infecting at higher MOIs or by preincubating the cells with the histone deacetylase inhibitor TSA, suggesting that an incoming viral structural protein or viral DNA can overcome this hDaxx-mediated repression and that the observed Daxx-mediated repression of IE gene expression requires the recruitment of chromatin-modifying enzymes to the MIEP.

**Experimental Procedures**

**Cells and Viruses**—Human foreskin fibroblasts and human malignant glioma cells (U373 MG), were grown in Eagle’s minimal essential medium (EMEM) (Invitrogen), supplemented with 10% (v/v) fetal calf serum with added penicillin/streptomycin (EMEM-10) at 37 °C in a humidified 5% CO2 atmosphere. The low passage strain Toledo or AD169 was used for all HCMV infections. For infection with HCMV, cells were infected at room temperature for 1 h, with rocking, before the virus-containing medium was aspirated and replaced with fresh EMEM-10.

**Plasmids and Transfections**—pCDNA3-hDaxx contains a full-length cDNA of hDaxx in the pCDNA3 expression vector. pDsRed (Clontech) encodes a novel RFP under the control of the minimal HCMV MIEP. pDsRed-hDaxx (a kind gift of Richard Caswell, Cardiff University) consists of the full-length hDaxx cDNA, minus the stop codon, cloned into EcoRI/BamHI-digested sites in pDsRed1-N1. To create cell populations transiently or stably expressing plasmid constructs, 25-cm2 flasks were seeded with ~1 × 10^6 cells 24 h prior to transfection. 2 h prior to transfection, cells were washed once with phosphate-buffered saline and overlaid with 4 ml of fresh EMEM-10. Each flask was transfected with 20 μg of plasmid DNA using the CaCl2 method. To select for stable transfectants, 24 h later, the EMEM-10 was replaced with fresh EMEM-10 containing 1 mg/ml G418 (Invitrogen), to select for cells expressing the Geneticin resistance cassette.

**Immunofluorescent Microscopy**—For immunofluorescence, 5 × 10^4 U373 cells/well were seeded on 8-well slides (Nunc) and infected at an MOI of 0.5–1, as described above. To detect pp65 protein at 8 h post-infection, cells were fixed in 4% paraformaldehyde for 15 min at room temperature and then permeabilized in 70% EtOH (−20 °C) for 15 min. Following washing, a mouse monoclonal anti-pp65 antibody (described in Ref. 41 and a kind gift of Bodo Plachter, Universität Mainz, Germany) diluted 1:10 in phosphate-buffered saline containing 1% bovine serum albumin was added to cells for 1 h at room temperature. Cells were then washed twice in phosphate-buffered saline and stained with rabbit.
anti-mouse fluorescein isothiocyanate (1:100 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA).

For PML, hDaxx, and IE72/IE86 staining, cells were stained as described above with either a rabbit anti-hDaxx antisemur (1:100 dilution; Upstate Biotechnology, Charlottesville, VA) for 1 h, a mouse anti-IE72/IE86 antibody (1:100 dilution; clone E13, Argene, Varilhes, France) or a mouse anti-PML antibody (1:200 dilution; Santa Cruz Biotechnology). hDaxx expression was then detected by staining with a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibody (1:30 dilution; Sigma) or donkey anti-rabbit Alexafluor 594 (1:100 dilution; Sigma) for 1 h. PML or IE72/IE86 expression was detected using a fluorescein isothiocyanate-conjugated goat anti-mouse IgG antiserum for 1 h (1:30 dilution; Sigma). All nuclei were counterstained with Hoechst 33242 (Sigma).

Cells were examined by UV microscopy, using the appropriate narrow band filters, in a Nikon Eclipse TE300 inverted microscope (Nikon UK Ltd.). Images were captured using a Hamamatsu color chilled 3CCD camera (Hamamatsu City, Japan), linked to the microscope, and Image Pro 4.1 software (Media Cybernetics UK).

Where specified, a minimum of 10 representative fields of view were counted for total cell number and the corresponding number of cells that were positive for hDaxx, RFP-hDaxx, or IE antigen expression as well as co-expression of IE antigen and hDaxx or RFP-hDaxx.

Chromatin Immunoprecipitation (ChIP) Assay—Chromatin immunoprecipitations were carried out essentially as previously described (32, 35). Briefly, fibroblasts were fixed with 1% formaldehyde to cross-link the DNA–protein interactions and then lysed. DNA associated with histones was immunoprecipitated with rabbit control serum (Sigma), anti-acetyl histone H4 antiserum (ChIP grade; Upstate Biotechnology), or anti-di-methylated (K9) histone H3 antiserum (Upstate Biotechnology) at 1:200 dilution overnight at 4 °C.

DNA from disrupted nucleosomes was precipitated and amplified by PCR using the following conditions. The MIEP was amplified with sense primer (5'-GAT GCA TTT CCT ACT TGG-3') and antisense primer (5'-CCA GGC GAT CTG ACG GTT-3') complementary to positions −272 and +13 relative to the MIEP start site. The cycle parameters for amplification of immediate early cDNA were 20 cycles at 94 °C (40 s), 55 °C (40 s), and 72 °C (90 s). Cellular chromatin remodeling are localized to ND10 bodies, including histone deacetylases and histone methyltransferases (27, 38, 39). The activity of the MIEP is regulated by higher order chromatin structure in both cell lines (32, 43) and primary myeloid cells during productive infection (32, 33) and during natural latency (35). More recently, we have observed that the MIEP is also associated with specific forms of chromatin during productive infection of fibroblasts, and thus we wished to determine whether repression of IE gene expression could be due to hDaxx mediating a repressive chromatin structure around the viral MIEP.

To do this, we used siRNAs to specifically target the down-regulation of endogenous hDaxx protein expression in primary human fibroblasts. Western blot analysis of hDaxx protein in siRNA-treated cells (Fig. 1A) clearly shows good down-regulation of hDaxx protein expression in cells transfected with sihDaxx (Fig. 1, A (lane 3) and D (lane 4)) and not in untreated

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Transient down-regulation of Daxx correlates with changes in the activity of the viral MIEP in permissive cells. A, Western blot analysis of hDaxx expression in mock-transfected (lane 1), scramble-transfected (lane 2), and sihDaxx-transfected (lane 3) primary human foreskin fibroblast cells. A Coomassie Blue-stained gel is shown as a protein loading control. B, Western blot analysis of mock-transfected (lane 1), scramble-transfected (lane 2), and sihDaxx-transfected (lane 3) fibroblasts for IE72/IE86 protein expression 24 h posttransfection at an MOI of 0.5. A Coomassie Blue-stained gel is shown as a protein loading control. C, reverse transcription-PCR analysis of total RNA isolated from mock-transfected (lane 1), scramble-transfected (lane 2), and sihDaxx-transfected (lane 3) fibroblasts for IE72 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. D, Western blot analysis of hDaxx and IE72/IE86 expression in mock-transfected (lane 1), scramble-transfected (lane 2), simDaxx-transfected (lane 3), and sihDaxx-transfected (lane 4) primary human foreskin fibroblast cells. A Coomassie Blue-stained gel is shown as a protein loading control. E, Western blot analysis of mDaxx expression in mock-transfected (lane 1), scramble-transfected (lane 2), and simDaxx-transfected (lane 3) primary mouse embryo fibroblast cells. A Coomassie Blue-stained gel is shown as a protein loading control.

Having established that the transfection of sihDaxx resulted in the substantial knockdown of hDaxx expression, cells transfected with siDaxx or scramble siRNA duplexes were subsequently infected with HCMV to determine the effect of hDaxx on HCMV infection. Western blot analysis for IE gene expression was performed on mock-, scramble siRNA-, hDaxx-, and simDaxx-transfected cells at 24 h postinfection at an MOI of 0.5. Infected fibroblasts transfected with hDaxx-specific siRNAs show increased levels of IE72 and IE86 gene expression (Fig. 1, B (lane 3) and D (lane 4)), compared with mock-transfected (lane 1), scramble siRNA-transfected (lane 2), or simDaxx-transfected (Fig. 1D, lane 3) controls. This increase in IE protein expression correlated with increased abundance of the IE72 RNA as detected by a comitochond reverse transcription-PCR analysis for IE expression (Fig. 1C). The data also show that there are increased quantities of IE RNA present in cells at 24 h postinfection in sihDaxx-transfected fibroblasts (Fig. 1C, lane 3) when compared with either mock (Fig. 1C, lane 1) or scramble siRNA-transfected (Fig. 1C, lane 2) cells.

The levels of the IE72 and IE86 mRNAs detected in sihDaxx-treated cells during HCMV infection is consistent with a model in which hDaxx acts to suppress transcription from the MIEP. Consequently, we asked whether knock-down of hDaxx in these cells correlated with an increase in the proportion of MIEPs associated with transcriptionally active chromatin. Chromatin was isolated from infected cells (MOI = 1) and analyzed by a ChIP assay specific for the viral MIEP (Fig. 2). At 3 h postinfection, the MIEP in untransfected control cells was associated with acetylated histones (Fig. 2, bar 3), as might be predicted for an immediate early promoter. We observed a similar pattern following the infection of scrambled siRNA-transfected fibroblasts (Fig. 2, bar 7). Interestingly, a substantial proportion of the viral MIEPs were also immunoprecipitated with histone H3 dimethylated at lysine residue 9 (H3-K9) (Fig. 2, bars 4 and 8). The methylation of H3-K9 is a highly characterized marker of transcriptionally silenced chromatin, and thus this observation suggests that a proportion of incoming viral genomes at this MOI are associated with repressive chromatin at 3 h postinfection. However, when we performed the same analysis in infected fibroblasts, which had been transfected with hDaxx-specific siRNAs, a different pattern of chromatinization was observed. Fig. 2 shows that a greater proportion of the MIEPs in sihDaxx-treated cells were associated with an increased level of acetylated histones (Fig. 2, bar 11) and with much lower levels of dimethylated histones (Fig. 2, bar 12). Down-regulation of hDaxx prior to infection, therefore, appears to result in a discernible increase in the level of acetylation and concomitant decrease in the level of methylation of histones bound to the MIEP at 3 h postinfection.

hDaxx knock-down experiments strongly suggested that hDaxx was involved in repression of HCMV major IE gene expression as early as 3 h postinfection. We decided to determine, conversely, if overexpression of hDaxx could inhibit HCMV infection. To do this, we overexpressed hDaxx in U373 cells by transient transfection. Surprisingly, cells transiently overexpressing hDaxx were as permissive for HCMV (Fig. 3A), as control DsRed-transfected cells (data not shown) or the untransfected cells in the pcDNA3hDaxx-transfected popula-
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FIGURE 3. Transient overexpression of hDaxx has little effect on levels of superinfection with HCMV. A, U373 cell transiently transfected with pcDNA3hDaxx were infected with HCMV at an MOI of 1 and then stained for hDaxx expression (Daxx) and viral IE gene expression (IE) 24 h postinfection. B and C, uninfected U373 cells transiently transfected with pcDNA3hDaxx were also stained for hDaxx (Daxx) and PML (PML) expression. In A–C, Hoechst 33342 staining was used to identify cell nuclei.

FIGURE 4. U373 cells constitutively overexpressing Daxx are refractory to infection with HCMV. A, U373 cells stably transfected with pcDNA3hDaxx were superinfected with HCMV at an MOI of 1. Cells were fixed and stained for hDaxx expression (Daxx) and viral IE gene expression (IE). B, these cells fixed and stained for hDaxx expression (Daxx) were also stained for PML expression (PML). A and B, Hoechst 33342 staining was used to identify cell nuclei. C, cells stably transfected with DsRed or pcDNA3hDaxx, superinfected with HCMV, were also co-stained for hDaxx and viral IE gene expression and analyzed by counting of representative fields; a minimum of 100 cells were counted from each of two independent experiments. The percentage of red cells (RFP- or TRITC-stained hDaxx-expressing cells) co-fluorescing green (IE-expressing cells) was quantified. Error bars, S.D.

tion (monored cells in Fig. 3A), as determined by IE gene expression. Consistent with observations from other workers, commercial anti-hDaxx antibodies are unable to detect endogenous hDaxx by indirect immunofluorescence but can detect hDaxx overexpressed by transfection. However, overexpressed hDaxx in transiently transfected cells was seldom detected associated with ND10 (see Fig. 3B), as might be expected. Co-localization of transiently expressed hDaxx with PML appeared only to occur in cells expressing relatively low levels of hDaxx (Fig. 3C). Such cells made up ~10% of the transiently transfected population (data not shown).

Since it is becoming increasingly clear that hDaxx function can be profoundly affected by its location, and in particular whether it is free or ND10-associated, we decided to also overexpress hDaxx by stable co-transfection (Fig. 4). Cautions of the differences we observed in hDaxx localization during transient transfection, we generated G418/ cells stably expressing hDaxx. To avoid nonrepresentative clones, a polyclonal population of G418/ hDaxx-expressing cells was used. In contrast to transiently expressed hDaxx, U373 cells stably transfected with hDaxx all exhibited punctuate fluorescence, which co-localized with PML-stained ND10 (Fig. 4B). Interestingly, these stably transfected hDaxx cells were refractory to infection with HCMV (MOI = 1) as determined by viral IE gene expression (Fig. 4A). Direct quantification of the immunofluorescence shown in Fig. 4A revealed that stably superexpressing hDaxx cells exhibited an approximate 10-fold decrease in HCMV IE gene expression after superinfection (Fig. 4, A and C). We also quantified the level of overexpression of hDaxx in these cells by Western blot analysis (see Fig. 7A). On the basis that ~20% of cells discernably overexpressed hDaxx by indirect immunofluorescence (Fig. 4A), Image J analysis of hDaxx band intensities of stably transfected cDNA3hDaxx versus stably transfected DsRed cells (Fig. 7A, tracks 1 and 4, respectively) suggest that hDaxx was overexpressed 3–4-fold in stably transfected cDNA3hDaxx cells.

In order to expedite analysis of co-expression of superexpressed hDaxx and viral IE gene expression, we also constructed a plasmid vector in which hDaxx was expressed in frame with red fluorescent protein, generating RFP-tagged hDaxx (RFP-hDaxx). To do this, we stably transfected U373 cells with DsRed and DsRed-hDaxx (Fig. 5) and investigated the effects of RFP-hDaxx stable expression on infection with a HCMV. Once again, we generated G418/ nonclonal populations of cells stably expressing RFP and RFP-hDaxx to minimize the risk of selection of nonrepresentative cell clones. Stably, overexpressed RFP-hDaxx exhibited punctate nuclear expression (Fig. 5, A and B) characteristic of ND10, consistent with our observations in U373 cells stably transfected with untagged hDaxx. Interestingly, transiently transfected DsRed-hDaxx again showed that, in most cells, the RFP-hDaxx did not co-localize with PML except in cells expressing very low amounts of transfected RFP-hDaxx (data not shown), again suggesting that analyses in which hDaxx is transiently transfected into cells may be difficult to interpret. In contrast to our transient transfection data, we clearly observed that stably, overexpressed RFP-hDaxx localized to ND10 by immunofluorescent staining for endogenous PML protein (Fig. 5B). The punctate staining of both RFP-hDaxx and PML fully co-localized, confirming that in DsRed-hDaxx stably transfected cells RFP-hDaxx localized predominantly to ND10 bodies (Fig. 5B). In contrast, RFP
expressed in DsRed-transfected cells showed diffuse cytoplasmic and nuclear expression (Fig. 5A), entirely different from RFP-hDaxx localization.

To test whether expression of other ND10 components was affected by RFP or RFP-hDaxx in stably transfected cells, Western blot analyses were performed on untransfected U373 cells or U373 cells transfected with DsRed or DsRed-hDaxx. Analysis of nuclear extracts showed that endogenous levels of hDaxx were equal in all of the three cell lines, as were levels of Sp100 and PML (data not shown). Based on a comparison of the number of cells in the stably transfected DsRed-hDaxx population overtly expressing RFP-hDaxx (~16%; see Fig. 5A) and Western blot analysis of endogenous hDaxx versus RFP-hDaxx (as was carried out for stably transfected cDNA3hDaxx cells, above), RFP-hDaxx was overexpressed ~2-fold with respect to endogenous Daxx in stably transfected DsRed-hDaxx cells (Fig. 7B).

We then investigated the effects of this RFP-hDaxx overexpression on HCMV infection. U373 DsRed- and U373 DsRed-hDaxx-transfected cells were infected with HCMV at an MOI of 1. At 18 h postinfection, cells were fixed and stained for IE gene expression and visualized by fluorescence microscopy for the co-expression of IE72, RFP, and RFP-hDaxx proteins. The U373 DsRed cells were fully permissive for HCMV IE gene expression (Fig. 6A, b). However, in U373 cells overexpressing RFP-hDaxx, no IE72 expression could be detected (Fig. 6A, e). This effect was specific to DsRed-hDaxx-transfected cells, since in the same field of view, cells that were not overexpressing RFP-hDaxx expressed detectable levels of IE72. Consistent with our observations with untagged hDaxx, these data suggested that HCMV IE gene expression is precluded by high levels of RFP-hDaxx expression (Fig. 6A, e) but not RFP (Fig. 6A, b).

In order to rule out the possibility that U373 DsRed-hDaxx-transfected cells were refractory to virus binding and internalization, we also infected and analyzed them for expression of HCMV tegument protein, pp65. By indirect immunofluorescence, pp65 tegument protein could clearly be visualized within U373 RFP-Daxx-expressing cells (Fig. 6A, h), precluding the possibility that the repressive effects of RFP-Daxx overexpression on virus IE gene expression results from effects on virus binding or entry.

We also quantified the number of RFP- or RFP-hDaxx-expressing cells co-expressing viral IE72 in the polyclonal populations of U373 cells transfected with either DsRed or DsRed-hDaxx, respectively. Fig. 6B shows that cells that stably overexpressed RFP-hDaxx also showed a 7-fold decrease in their ability to support HCMV IE gene expression after superinfection. This repression was less pronounced in cells infected at higher MOIs, such that the 7-fold decrease in HCMV IE gene expression observed in hDaxx-RFP-expressing cells infected at an MOI of 1 was substantially reduced by infection at an MOI of 10 (Fig. 6C), suggesting that hDaxx-mediated repression of the MIEP in cells stably expressing RFP-hDaxx can be titrated away by increasing MOIs. Indeed, consistent with this, a Western blot analysis of hDaxx protein levels at 3 h postinfection of cDNA3hDaxx and DsRed stably transfected cells showed that HCMV-induced degradation of hDaxx protein required higher viral titers in cells stably transfected with cDNA3hDaxx (Fig. 7A). Consistent with published data (26), hDaxx degradation was induced by HCMV infection of control U373 cells stably transfected with DsRed at both 0.5 and 5 MOI (Fig. 7A, lanes 2 and 3) when compared with mock-infected cells (Fig. 7A, lane 1). In contrast, HCMV-induced hDaxx degradation in cells stably overexpressing hDaxx was only observed at higher MOIs (Fig. 7A, lane 3) and not lower MOIs (Fig. 7A, lane 2) when compared with mock-infected controls (Fig. 7A, lane 1). Similarly, in RFP-hDaxx-expressing cells, only high titers of virus were able to result in degradation of RFP-hDaxx or endogenous hDaxx in these cells (Fig. 7B).

Our ChIP analyses of the MIEP (Fig. 2) suggested that hDaxx-mediated repression of the IE gene expression could be associated with the known interaction of hDaxx with chromatin-modifying enzymes, since the knockdown of hDaxx resulted in a concomitant increase in the proportion of acetylated promoters following infection. Therefore, we reasoned that if hDaxx requires chromatin remodeling enzymes to repress the MIEP then inhibition of histone deacetylase activity could abrogate the ability of hDaxx to repress the MIEP.

First, a polyclonal population of RFP-hDaxx cells was infected at an MOI of 0.2, and the level of infection was calculated in the red and nonred populations. As expected, ~15% of nonred cells (i.e. not overexpressing hDaxx) expressed IE72 (Fig. 6D, bar 1). In contrast, we observed that 0.3% of RFP-hDaxx cells were expressing IE72 (Fig. 6D, bar 2), entirely consistent with data in Figs. 4 and 6. The same analysis was then performed on cells incubated with TSA prior to infection. Our analysis of the nonred cells showed that 60% of cells were infected and thus TSA itself induced a ~4-fold increase in the

![Image 50x464 to 299x734]
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**FIGURE 6.** U373 cells constitutively overexpressing RFP-hDaxx are refractory to infection with HCMV. A, U373 stably transfected with DsRed (RFP) (a–c) or DsRed-hDaxx (RFP-hDaxx) (d–f) and g–h) were infected with HCMV at an approximate MOI of 1. Cells were then stained with an anti-IE antibody (IE) (b–e). The arrows identify cells overexpressing RFP-hDaxx in the appropriate panels. Alternatively 6–8 h postinfection RFP-hDaxx expressing (g) cells were fixed and stained for pp65 expression (pp65) (h). Hoechst 33342 staining was used to identify cell nuclei. B, RFP- and RFP-hDaxx-expressing cells, infected with HCMV at an MOI of 1, were also stained for viral IE gene expression and analyzed by counting of representative fields; a minimum of 100 cells were counted from each of two independent experiments. The percentage of red cells (RFP- or RFP-hDaxx-expressing cells) co-fluorescing green (IE-expressing cells) was quantified. The error bars represent S.D. C, U373 stably transfected with DsRed-hDaxx cells were also superinfected with HCMV at an MOI of 10 and analyzed as described in B, above. D, a polyclonal population of RFP-hDaxx cells (20% expressing RFP-hDaxx) were incubated with control (lanes 1 and 2) or TSA (lanes 3 and 4) 18 h prior to infection. 24 h postinfection, the number of RFP-hDaxx (lanes 2 and 4) and non-RFP-hDaxx cells (lanes 1 and 3) expressing IE72 was quantified by fluorescent microscopy. Error bars, 1 S.D. DMSO, Me2SO.

level of infection (Fig. 6D, bar 3), consistent with previous observations made during murine (36) and human CMV infection. Interestingly, however, 33% of RFP-hDaxx were also expressing IE72, representative of a 10-fold increase in the level of permissiveness of the RFP-hDaxx cells (Fig. 6D, bar 4). Although the addition of TSA did result in a 4-fold increase in infection in nonred cells, this was modest compared with the effect on RFP-hDaxx cells (100-fold increase). Thus, these data support the hypothesis that the observed hDaxx mediated repression of the MIEP is regulated, at least in part, by chromatin remodeling enzymes, since their inhibition with TSA counteracts the effects of hDaxx overexpression. Furthermore, these data confirm that the RFP-hDaxx cells retain the capacity to be productively infected and are not irreversibly compromised by RFP-hDaxx overexpression.

**DISCUSSION**

The hDaxx protein physically interacts with various proteins of the chromatin matrix, including histone deacetylase II, H2A, H2B, H3, and H4, and with the chromatin-associated protein Dek (27, 38) based upon the phosphorylation status of hDaxx (27), and hDaxx is thought to recruit histone deacetylases to sites of active transcription by direct interaction with the transcription machinery. Histone tails are subsequently deacetylated. Histone deacetylation then results in a closed chromatin structure and the repression of transcription. Consistent with this, we observed that the HCMV MIEP was associated with dimethylated histones following the infection of synchronous cells. In hDaxx knockdown cells, histones were still associated with the MIEP upon infection; however, there was an overt difference in the pattern of chromatinization of the MIEP such that the MIEP was predominantly associated with acetylated histones. Consistent with this, analysis of the levels of viral IE gene expression also showed that hDaxx knockdown cells expressed considerably higher levels of viral major IE RNA and protein.

Conversely, overexpressed hDaxx could inhibit viral IE gene expression after virus infection. Either wild-type Daxx or RFP-Daxx when stably overexpressed in U373 cells was shown to localize predominantly to ND10 bodies. Both indirect immunofluorescence and Western blot analysis showed that these cells overexpressing hDaxx were substantially less able to support viral IE gene expression when infected at low multiplicities. This would be consistent with the proposed role of hDaxx as a promiscuous transcriptional repressor (38, 44–47), and when overexpressed, hDaxx would be present at high levels at ND10, a location in close proximity to herpesvirus genome deposition (3, 4). This hDaxx-mediated repression of viral IE gene expression was not due to an inability of cells to bind and internalize virus, since hDaxx superexpression did not affect uptake of HCMV tegument protein pp65. Furthermore, we could render these cells permissive for HCMV by direct interaction with the transcription machinery. Histone deacetylases to sites of active transcription by direct interaction with the transcription machinery. Histone tails are subsequently deacetylated. Histone deacetylation then results in a closed chromatin structure and the repression of transcription. Consistent with this, we observed that the HCMV MIEP was associated with dimethylated histones following the infection of synchronous cells. In hDaxx knockdown cells, histones were still associated with the MIEP upon infection; however, there was an overt difference in the pattern of chromatinization of the MIEP such that the MIEP was predominantly associated with acetylated histones. Consistent with this, analysis of the levels of viral IE gene expression also showed that hDaxx knockdown cells expressed considerably higher levels of viral major IE RNA and protein.

Intriguingly, we saw no such inhibition of viral IE gene expression after superinfection when hDaxx was transiently overexpressed in cells. We are not sure how the functionality of hDaxx differs between cells that are transiently expressing or stably overexpressing this protein. However, it has been suggested that hDaxx function can be profoundly affected by its
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Our experiments also indicated that repression of HCMV IE gene expression by stable overexpression of hDaxx could be overcome by increasing HCMV titers. Whether this titration of Daxx-mediated inhibition by increasing virus titers is mediated by viral structural proteins or simply increasing copy number of viral genomes will require further investigation. However, the pp71 tegument protein of HCMV, encoded by UL82, interacts with hDaxx (23), and this interaction is important for efficient IE gene expression and viral replication (25, 49, 50). Recently, it has also been reported that pp71 mediates the transient proteasomal degradation of hDaxx, leading to increased IE expression (26). The authors suggest that degradation of hDaxx by pp71 plays a profound role in regulation of viral IE gene expression. In this study, we show that cells stably overexpressing hDaxx are refractory to infection at an MOI of 1, a titer of virus that would normally result in 60% of cells infected. Since one consecutive of infection is the transient degradation of endogenous hDaxx between 3 and 12 h of infection (26), this suggests that, in cells overexpressing hDaxx or RFP-hDaxx, the incoming pp71 tegument protein is unable to overcome this stable overexpression of hDaxx. However, our observations that overexpressed hDaxx or RFP-hDaxx degradation does occur at higher MOIs suggest that this effect of overexpression hDaxx is not due to an intrinsic inability of overexpressed hDaxx to be degraded by pp71.

Our siRNA knock-down data also argue that the presence of hDaxx prior to the onset of its degradation by pp71, which starts at 2–3 h postinfection (26), as well as when it returns to wild-type levels at 12–24 h postinfection (49) still has profound effects on the level of IE gene expression despite the pp71-mediated transient degradation of endogenous hDaxx; specifically, we observe that even in the presence of pp71, inhibition of hDaxx expression prior to infection has profound effects on IE gene expression. Clearly, the presence of hDaxx in the cells within the first 2–3 h of infection, prior to its degradation by pp71, has an immediate effect at the chromatin level on the viral MIEP consistent with established interactions between hDaxx and repressive components of higher order chromatin structure formation (27). Indeed, we observed that the observed repression of the MIEP by hDaxx in these cells was alleviated by the addition of TSA, suggesting that chromatin remodeling enzymes are in an important factor in hDaxx repression of IE gene expression. Consequently, the knock-down of hDaxx prior to infection could result in the establishment of a chromatin structure around the MIEP that is more conducive to transcriptional activation prior to any delivery of pp71. Similarly, maintaining a knock-down of hDaxx expression by siRNA until later than 24 h postinfection (the time by which pp71-mediated degradation of hDaxx no longer occurs and hDaxx returns to wild-type levels) also profoundly increased IE gene expression in our study. Thus, it is likely that HCMV needs to employ multiple mechanisms over the immediate early time course of infection to overcome the intrinsic repression mediated by hDaxx; this probably also includes ND10 disruption by IE72 (6, 21, 22).

hDaxx then appears to act as an intrinsic inhibitor of efficient viral IE gene expression, which is mediated by packaging of the viral genome into a transcriptionally repressive chromatin state almost immediately upon infection, but this repressive function of hDaxx can be partially “overcome” by incoming viral factors, such as pp71, and is probably augmented by de novo expression of IE72 as the infection proceeds. During the course of this work, Preston and Nicholl (51) reported that siRNA knock-down of hDaxx results in increased IE expression after HCMV infection but only in the absence of incoming pp71 (using a pp71 deletion mutant). We are not sure why this discrepancy exists between our observations and theirs, but we note that, in contrast to our use of transiently siRNA transfected primary fibroblast cells, which virtually eliminated endogenous hDaxx (see Fig. 1A), they used G418-selected clones of immortalized U373 cells stably transfected with siRNA constructs in which endogenous hDaxx needed to be further reduced by a subsequent transfection protocol.

It would appear that the imposition of a transcriptionally inactive chromatin structure on the HCMV MIEP immediately

**Figure 7. Western blot analysis of hDaxx protein expression in transfected cells.** A, Daxx3Daxx stably transfected U373 cells (tracks 1–3) or control DsRed stably transfected U373 cells (tracks 4–6) were left uninfected (mock) or infected at MOIs of 0.5 or 5 for 6 h and analyzed by Western blot for hDaxx expression. B, DsRed-hDaxx stably transfected U373 cells expressing RFP-hDaxx were left uninfected (mock) or infected at MOIs of 0.5 or 5 for 6 h and analyzed by Western blot for hDaxx expression. The arrows designate endogenous Daxx- and RFP-tagged Daxx.
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upon infection would be intrinsically unfavorable for the virus. However, herpesvirus gene expression is classically regulated in a temporal cascade, and this may be achieved, in part, using dynamic chromatin structure, a chromatin structure that is imparted on the incoming viral genome immediately upon infection. Similarly, if chromatinization of the viral MIEP into a more repressive structure occurs preferentially under cellular conditions that are suboptimal for replication, this may provide the virus an opportunity to synchronize its phase of IE gene expression to when the cellular conditions are more favorable. For example, cells that have progressed into late S phase do not appear to be conducive to viral IE expression, and viral replication does not occur until the cell cycle returns back into G1/S phase, when the cell is believed to be an optimal environment for viral DNA replication (52). One possibility is that the virus utilizes chromatin structure to temporarily repress viral gene expression until the environment is more favorable for replication.

Given the dynamic nature of ND10 and ND10 components, it is likely that they may have a number of roles in infection, some supportive and others repressive. Most human herpesviruses have evolved mechanisms to disperse or destroy ND10 during the IE phase of the lytic cycle. A model in which ND10 functions in a repressive role prior to IE gene expression but in which their components then become beneficial to viral replication once released from the ND10 structure might explain such apparent contradictions in the literature. Indeed, it has been noted that infection of ND10-deficient cells with HCMV promotes the formation of rudimentary ND10-like structures of hDaxx and Sp100 (22). Further experimental approaches that critically examine specific ND10-virus interactions are required to clarify their role in virus infection.

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