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Control of cytomegalovirus lytic gene expression by histone acetylation

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Permissiveness for human cytomegalovirus (HCMV) infection is dependent on the state of cellular differentiation and has been linked to repression of the viral major immediate early promoter (MIEP). We have used conditionally permissive cells to analyze differential regulation of the MIEP and possible mechanisms involved in latency. Our data suggest that histone deacetylases (HDACs) are involved in repression of the MIEP in non-permissive cells as inhibition of HDACs induces viral permissiveness and increases MIEP activity. Non-permissive cells contain the class I HDAC, HDAC3; super-expression of HDAC3 in normally permissive cells reduces infection and MIEP activity. We further show that the MIEP associates with acetylated histones in permissive cells, and that in peripheral blood monocytes the MIEP associates with heterochromatin protein 1 (HP1), a chromosomal protein implicated in gene silencing. As monocytes are believed to be a site of viral latency in HCMV carriers and reactivated virus is only observed upon differentiation into macrophages, we propose that chromatin remodeling of the MIEP following cellular differentiation could potentially play a role in reactivation of latent HCMV.

Keywords: cytomegalovirus/heterochromatin protein 1/histone acetylation/immediate early/latency

Introduction

Following primary infection, human cytomegalovirus (HCMV) establishes life-long latent infection of the host. Most infections are asymptomatic; however, primary infection or reactivation in the immunocompromised can result in life-threatening disease (Rubin, 1990). In addition, infection *in utero* can result in mental retardation, hearing loss or even death of the fetus (Fowler *et al*., 1992).

HCMV is able to replicate in a variety of cell types, including fibroblasts, neuronal cells, epithelial cells, endothelial cells and macrophages (for review see Sinzger and Jahn, 1996). Upon infection of these permissive cells, the virus undergoes an ordered cascade of gene expression. The major immediate early (IE) genes are the first to be transcribed, resulting in two abundant proteins, IE1 p72 and IE2 p86 (Stenberg *et al*., 1989), which are able to autoregulate the major immediate early promoter (MIEP; Pizzorno *et al*., 1988; Cherrington and Mocarski, 1989). In addition to activating the promoters of viral early genes, the IE proteins can potently activate some cellular promoters (Hermiston *et al*., 1987; Hunninghake *et al*., 1989; Hagemeier *et al*., 1992; M.Wade *et al*., 1992; Walker *et al*., 1992; Schwartz *et al*., 1994, 1996; Hayhurst *et al*., 1995; Caswell *et al*., 1996). The resulting viral early gene expression is required for DNA replication and ultimately leads to the expression of the late virus-assembly genes (for reviews see Anders and McCue, 1996; Gibson, 1996; Spector, 1996).

The control of viral IE gene expression is believed to play a pivotal role in determining viral replication and appears to occur in a differentiation-dependent manner. Peripheral blood monocytes are believed to be a major site of carriage of HCMV (Taylor-Wiedeman *et al*., 1991) and the virus is likely to be latent in these cells due to restricted IE gene expression (Ibanez *et al*., 1991; Lathey and Spector, 1991). However, when monocytes terminally differentiate into macrophages, reactivation of lytic gene expression and productive infection occurs (Ibanez *et al*., 1991; Lathey and Spector, 1991; Minton *et al*., 1994; Taylor-Wiedeman *et al*., 1994). Indeed, latent HCMV has been shown to be reactivated *in vitro* by allogeneic stimulation of peripheral blood mononuclear cells (Soderberg-Naucler *et al*., 1997). In addition, latently infected granulocyte–macrophage progenitor cells and bone marrow cells of seropositive individuals have been shown to give rise to HCMV IE1 sense and antisense transcripts, initiated at novel promoters (Kondo *et al*., 1996; Hahn *et al*., 1998; Slobedman and Mocarski, 1999); however, no role has been ascribed to these transcripts (White *et al*., 2000).

In order to understand better the possible mechanisms involved in latency, model systems that differentially regulate the MIEP have been studied. These include conditionally permissive human N-teratocarcinoma (T2) cells and peripheral blood-derived monocytes (Gonczol *et al*., 1984; LaFemina and Hayward, 1986; Nelson and Groudine, 1986; Shelbourn *et al*., 1989a; Ibanez *et al*., 1991; Lathey and Spector, 1991; Minton *et al*., 1994; Taylor-Wiedeman *et al*., 1994). These cell types represent good model systems for differentiation-dependent HCMV lytic gene expression (Andrews *et al*., 1984; Ibanez *et al*., 1991; Lathey and Spector, 1991). Following differentiation with either retinoic acid (RA) or hydrocortisone and phorbol ester, the IE gene expression block is lifted, resulting in HCMV-permissive cells (Nelson *et al*., 1987; Lubon *et al*., 1989; Shelbourn *et al*., 1989a; Ibanez *et al*., 1991; Lathey and Spector, 1991; Minton *et al*., 1994; Taylor-Wiedeman *et al*., 1994).

It is known that expression from the MIEP, comprising enhancer, modulator and unique regions (for review see

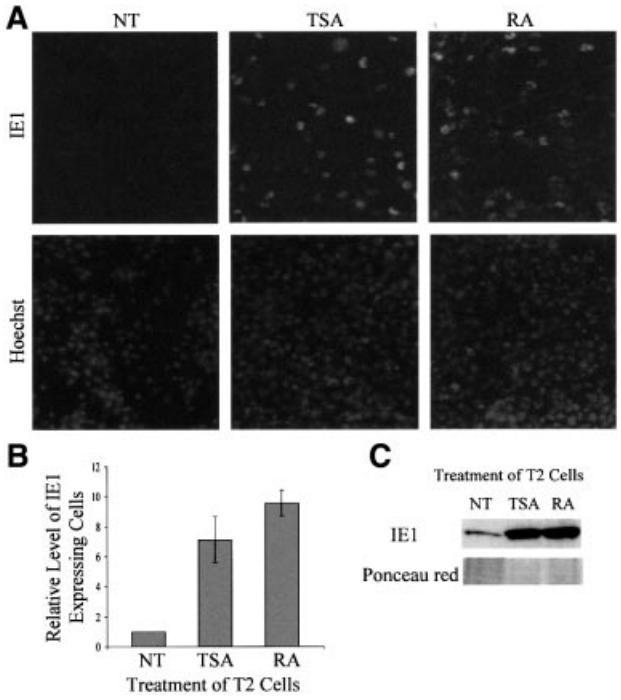

Fig. 1. T2 cells either untreated (NT), treated with deacetylase inhibitor (TSA) or differentiated into T2RA cells with RA were infected with HCMV CR[IE1–GFP] for 24 h. (**A**) The GFP fluorescence of a typical field from each cellular population is shown (IE1), along with the corresponding Hoechst 33342 stained nuclei (Hoechst). (**B**) Cells (50 000) of each population were analyzed by FACS and the relative number of IE1-expressing cells, from three independent experiments, is shown, along with the 95% confidence interval. (**C**) Western blot analysis of 10 μg of cellular extracts probed with an anti-IE antibody and stained with 0.5% Ponceau red in 1% TCA.

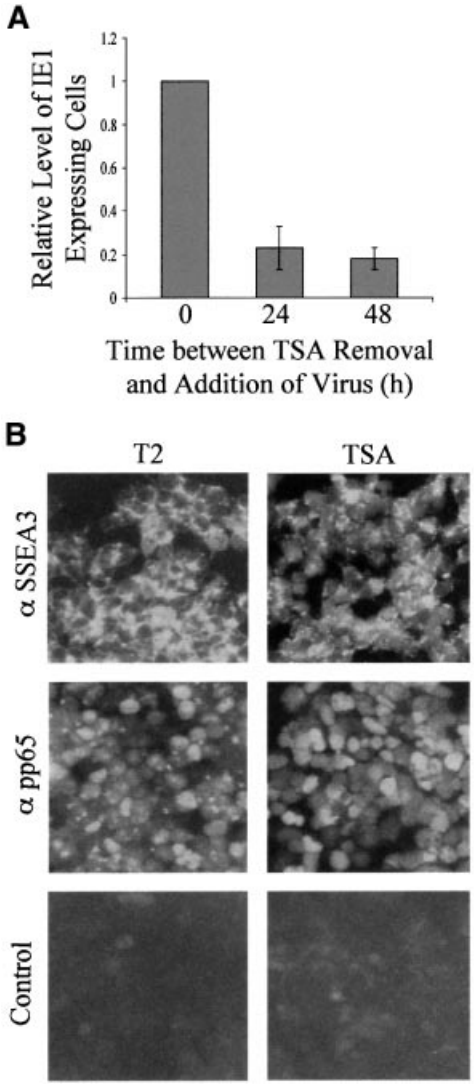

Fig. 2. (**A**) Following TSA treatment, cells were incubated in the absence of inhibitor for 0, 24 and 48 h, prior to infection with HCMV CR[IE1–GFP] for 24 h. The average relative level of IE1-expressing cells, from three independent experiments, is shown, along with the 95% confidence interval. (**B**) Indirect immunofluorescence of HCMV-infected, T2 and TSA-treated cells detected with either αSSEA3 or αpp65 monoclonal antibodies. An immunoglobulin control is also shown.

Meier and Stinski, 1996), is at least partly determined by cellular factors such as NF-κB/rel, CREB/ATF, SP-1, AP1, serum response factor and ELK-1, which all promote activity by binding to one or more sites in the enhancer (Sambucetti *et al*., 1989; Lang *et al*., 1992; Liu and Stinski, 1992; E.J.Wade *et al*., 1992; Kowalik *et al*., 1993; Chan *et al*., 1996). The activity of the MIEP can also be enhanced in normally non-permissive T2 cells when liganded RA receptor (RAR) homodimers or RAR–retinoid X receptor heterodimers occupy the RA response elements within the enhancer region (Angulo *et al*., 1996). In contrast, the MIEP is also regulated by cellular repressors, such as MBFs, YY1, MRF and Gfi-1, which bind to the MIEP and are all preferentially expressed in undifferentiated cells (Shelbourn *et al*., 1989a; Liu *et al*., 1994; Huang *et al*., 1996; Zweidler-Mckay *et al*., 1996). Although these factors clearly regulate the MIEP in transfection assays, their role in the context of virus infection is unclear (Meier, 2001).

Differentiation of T2 cells induces changes in specific DNase I hypersensitivity between nucleotides –650 and –775 of the modulator region, suggesting that the chromatin structure of the MIEP may effect differentiation specific viral replication (Nelson and Groudine, 1986). Chromatin-based repression mechanisms are believed to result from distinct modifications of histone N-termini. Reduced acetylation of the histone H4 N-terminus mediated by histone deacetylases (HDACs), in conjunction with methylation at lysine 9 of histone H3, has been predicted to define the key histone signals for heterochromatinization (Rea *et al*., 2000). Moreover, it has been shown that histones specifically methylated at lysine 9 of histone H3 associate with heterochromatin protein 1 (HP1; Bannister *et al*., 2001; Lachner *et al*., 2001). HP1 has been shown to preferentially associate with heterochromatin regions of chromosomes and has been implicated in gene silencing (for review see Eissenberg and Elgin, 2000).

In this study, we investigate whether inhibition of HDACs relieves the infection block of normally non-permissive undifferentiated cells, whether non-permissive cells contain more HDACs than permissive cells and whether the permissiveness of differentiated cells can be reduced by the overexpression of HDAC3. Using chromatin immunoprecipitation (ChIP) assays, we also analyze differences in MIEP-associated chromatin proteins

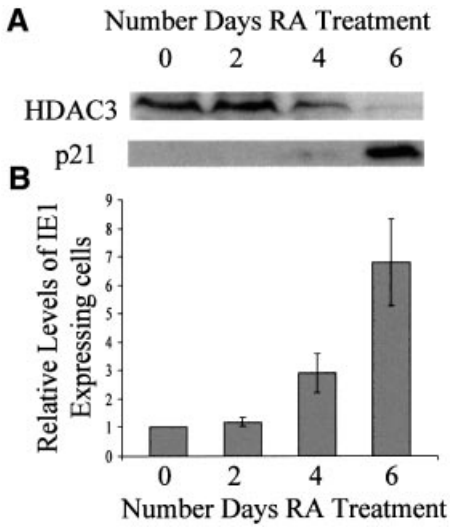

Fig. 3. T2 cells treated with RA for 0, 2, 4 and 6 days were either analyzed on a western blot, probed with anti-HDAC3 antiserum and anti-p21 antibody (**A**) or infected with HCMV CR[IE1–GFP] for 24 h and analyzed by FACS (**B**). Shown in (B) is the average relative level of IE1 expression in 50 000 infected cells, along with the 95% confidence interval. Data are from three independent experiments.

between HCMV-infected non-permissive and permissive cells. The results lead us to believe that the chromatin structure around the MIEP changes with cellular differentiation and thus may play a role in controlling HCMV latency and reactivation.

Results

HDAC inhibition induces HCMV permissiveness of T2 cells

Undifferentiated T2 cells are known to be non-permissive for IE gene expression. However, following treatment with RA, these cells differentiate into a permissive phenotype (Nelson *et al*., 1987; Lubon *et al*., 1989; Shelbourn *et al*., 1989a). A similar observation is noted with CR[IE1–GFP], a recombinant HCMV carrying IE1 fused to enhanced green fluorescent protein (GFP; J.Gawn, M.Denson, E.Sherratt, G.Wilkinson, R.Caswell and R.Greaves, in preparation). This virus infects undifferentiated T2 cells poorly, as determined by GFP fluorescence or by detection of IE using western blot analysis, but shows good levels of infection in differentiated T2RA cells (Figure 1). Interestingly, when T2 cells are treated with the deacetylase inhibitor trichostatin A (TSA), substantial increases in levels of infection, approaching those seen after RA treatment, are observed (Figure 1). Identical results are also seen when TSA-treated T2 cells are infected with the AD169 strain of HCMV and IE expression is detected by indirect immunofluorescence (data not shown).

TSA has been shown to induce differentiation of a variety of cells, including Friend leukemia and Neuro 2a cells (Yoshida *et al*., 1990; Inokoshi *et al*., 1999); therefore, we investigated whether TSA-induced permissiveness of T2 cells was maintained following the removal of the deacetylase inhibitor. In contrast to RA-induced permissiveness, which does not decrease following withdrawal of RA prior to HCMV infection, Figure 2A shows that the level of infection decreases significantly when T2 cells, pre-treated with TSA, are incubated in the absence of an inhibitor for $\geqslant$24 h, prior to infection. This result suggests that TSA-induced transient permissiveness for HCMV is not a result of cellular differentiation. However, to preclude the possibility that TSA induces transient cellular differentiation, the level of the differentiation-specific antigen SSEA3 was investigated. Differentiation with RA has previously been shown to reduce SSEA3 expression (Andrews *et al*., 1984; Shelbourn *et al*., 1989b). However, treatment of T2 cells with TSA for 24 h results in no decrease in SSEA3 expression (Figure 2B), arguing that TSA does not invoke transient differentiation of these cells.

To exclude the possibility that TSA treatment acts at the level of virus entry, we analyzed the level of virus uptake by determining nuclear expression of the viral tegument protein pp65, as reported previously (Revello *et al*., 1992). After inoculation for 16 h with HCMV, pp65 could be detected in the nuclei of both untreated and TSA-treated cells, to similar levels (Figure 2B). Taken together, the results suggest that TSA does not alter the entry of HCMV into T2 cells and that TSA-induced viral permissiveness does not primarily result from cellular differentiation. It seems most likely that TSA transiently inhibits HDACs that would normally limit HCMV infection in non-permissive T2 cells.

HDAC3 protein levels decrease upon differentiation of T2 cells to a permissive phenotype

We next asked whether differences in repression of the MIEP between permissive and non-permissive cells might be due to the relative levels of HDACs in T2 and T2RA cells. To answer this, we analyzed the levels of HDAC3 in T2 cells treated with RA for 0, 2, 4 and 6 days. Figure 3A shows that the level of HDAC3 decreases following incubation with RA for $\geqslant$4 days, with very limited expression being observed after 6 days of treatment. The decrease in HDAC3 expression correlates with the onset of detection of the cyclin-dependent kinase inhibitor $p21^{WAF1/cip1}$ (p21). Expression of p21 has previously been shown to be induced when T2 cells are differentiated with RA into a neuronal phenotype (Hromas *et al*., 1999; Bartkova *et al*., 2000) that is permissive for HCMV (Figure 3B). Importantly, Figure 3 also shows that there appears to be an inverse relationship between the number of cells expressing IE proteins upon HCMV infection and the level of HDAC3 expression. This observation is consistent with HDAC3 limiting infection in non-permissive T2 cells.

Super-expression of HDAC3 in permissive cells reduces HCMV infection

As HDAC3 decreases considerably in permissive T2RA cells, we asked whether permissiveness for HCMV could be reduced by overexpression of HDAC3 in these cells. Prior to infection with HCMV, T2RA cells were transiently transfected with pDsRED-N1, as an indicator plasmid, together with an equal amount of either pcDNA3, pcDNA3-HDAC3 or pcDNA3-HDAC3Δ, which encodes for a non-functional form of the enzyme. Cells expressing HDAC3 show a substantial decrease in infection efficiency compared with cells expressing the mutant HDAC3

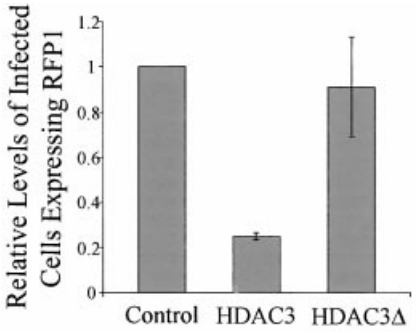

Fig. 4. Prior to infection with HCMV CR[IE1–GFP], T2RA cells were transiently co-transfected with equal concentrations of pDsRED-N1 and either pcDNA3 (control), pcDNA3-HDAC3 (HDAC3) or pcDNA-HDAC3Δ (HDAC3Δ). Cells were analyzed for green and red fluorescence on a Beckton Dickinson FACSort. The data show the average relative levels of RFP1 in 5000 infected cells.

(HDAC3Δ) or cells transfected with vector-only control (Figure 4). Similar to infection with CR[IE1–GFP], infection with AD169 HCMV was also inhibited in T2RA cells expressing HDAC3 (data not shown).

Histone deacetylase inhibition induces activity of the MIEP in T2 cells

The non-permissiveness of T2 cells for HCMV has been linked to repression of the viral MIEP by a differentiation specific factor (Liu *et al*., 1994). If viral infection is limited by HDAC-mediated repression of the MIEP region, deacetylase inhibition should increase the activity of the MIEP. Like permissiveness for HCMV, the activity of pESCAT, an MIEP-driven chloramphenicol acetyl transferase (CAT) construct, was significantly increased in T2 cells following TSA treatment (Figure 5A). This suggests that the limited HCMV infection observed in T2 cells results from HDAC-mediated repression of the MIEP region of the virus. In contrast, the activity of pIEP1CAT, which contains a major deletion of the MIEP and shows no differentiation-dependent expression (Liu *et al*., 1994), is only minimally alleviated by TSA (Figure 5B).

Super-expression of HDAC3 in permissive cells reduces activity of the MIEP in T2 cells

As the viral MIEP region appears to be important for limiting HCMV infection in T2 cells and HDAC3 expression substantially decreases infection efficiency of normally permissive T2RA cells, we asked whether overexpression of HDAC3 could reduce the activity of the MIEP in permissive cells. T2RA cells were transiently transfected with either pcDNA3, pcDNA3-HDAC3 or pcDNA-HDAC3Δ, along with pESCAT or pIEP1CAT. The activity of pESCAT was substantially reduced in cells transfected with pcDNA3-HDAC3 compared with the other controls (Figure 6A). In contrast, pIEP1CAT activity was only marginally affected by pcDNA3-HDAC3 (Figure 6B). This result is again consistent with specific repression of the full-length MIEP by HDAC3.

The HCMV MIEP is associated with acetylated histones in permissive cells

The transcriptional capacity of chromatin has been linked to the acetylation and deacetylation status of histones

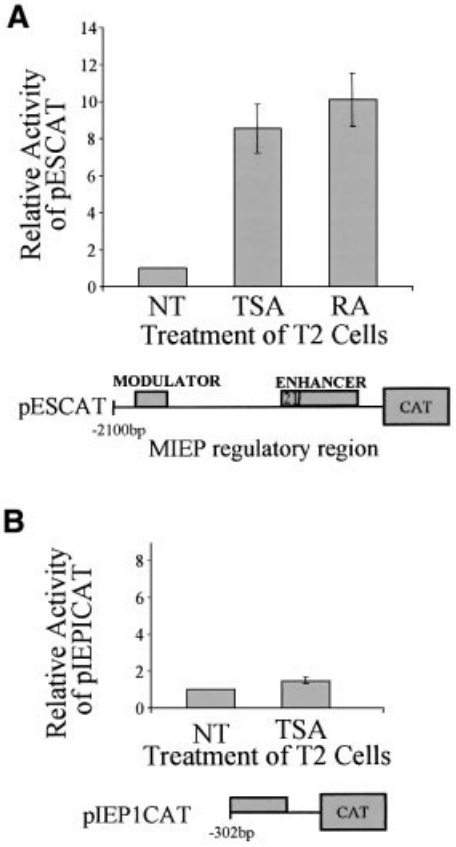

Fig. 5. (**A**) T2 cells either untreated (NT), treated with deacetylase inhibitor (TSA) or differentiated into T2RA cells with RA were transfected with pESCAT, which contains the CAT gene under the control of the HCMV MIEP from nucleotide –2100 to +72. Thirty-six hours post-transfection, CAT assays were performed and standardized to β-galactosidase (β-gal) activity expressed from the co-transfected plasmid pSV-β-gal. Shown are the average relative CAT activities from three independent experiments and the 95% confidence interval. (**B**) The average relative CAT activities from pIEP1CAT-transfected cells.

(Hebbes *et al*., 1988). As super-expression of HDAC3 renders T2RA cells non-permissive for HCMV, and HDAC inhibition of T2 cells results in an increase in both the MIEP activity and HCMV permissiveness, we asked whether the acetylation status of the chromatin structure around MIEP differed in T2 and T2RA cells. Therefore, we performed ChIP assays using normal serum or an anti-acetylated histone H4 antiserum to immunoprecipitate DNA associated with histones from infected T2 and T2RA cells. The relative levels of acetylated histones associated with the viral MIEP were assessed by a PCR assay using primers complementary to the MIEP.

HCMV appears to enter both T2 and T2RA cells equally (Figure 7A, lanes 1 and 2). This is consistent with limited viral infection in T2 cells resulting from differentiation-specific cellular factors repressing the MIEP and affirms previous observations that limited viral infection of T2 cells does not result from a virus entry block (LaFemina and Hayward, 1986; Nelson and Groudine, 1986; Meier, 2001). Importantly, more PCR product results from T2RA cells than T2 cells, following immunoprecipitation with anti-acetylated histone H4 antiserum (Figure 7A, lanes 4 and 3, respectively, and B). This suggests that the HCMV MIEP is associated with significantly more acetylated

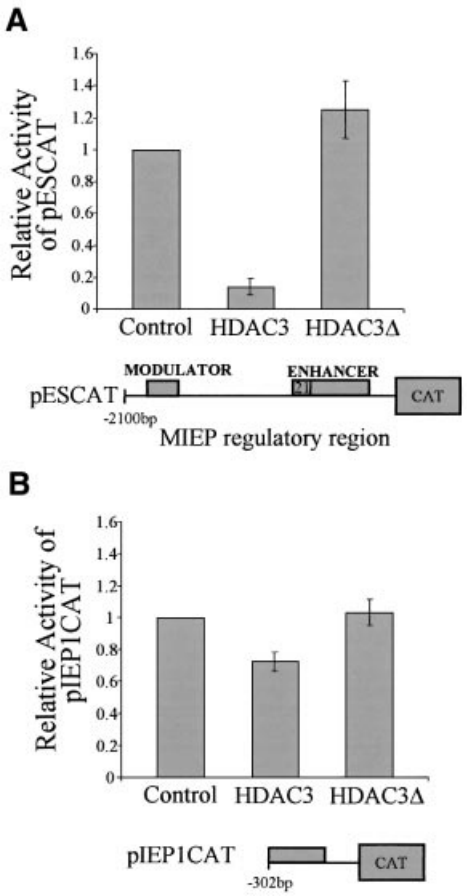

Fig. 6. T2RA cells were transfected with either pcDNA3 (control), pcDNA3-HDAC3 (HDAC3) or pcDNA-HDAC3Δ (HDAC3Δ), along with pESCAT or pIEP1CAT, shown in (**A**) and (**B**), respectively. The average relative CAT activities, measured 36 h post-transfection, from three experiments and the 95% confidence intervals are shown.

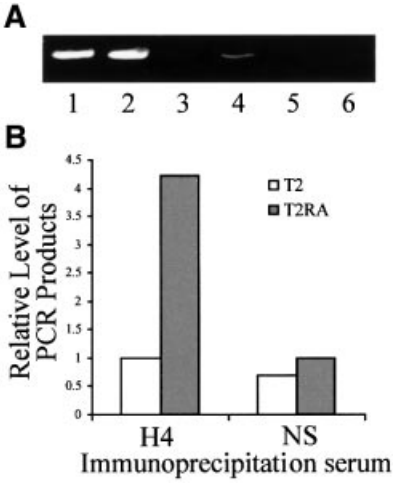

Fig. 7. ChIP assay on HCMV-infected T2 and T2RA cells. Twenty-four hours post-infection, DNA associated with histones was immunoprecipitated and used in PCR assays with primers complementary to the MIEP. (**A**) Lanes 1, 3 and 5 of the 3% agarose gel contain products from T2 cells, whilst lanes 2, 4 and 6 are from T2RA cells. PCR products from viral-input controls, removed prior to immunoprecipitation, are in lanes 1 and 2; anti-acetylated histone H4 (H4) antiserum-precipitated samples are in lanes 3 and 4, and normal serum (NS)-precipitated samples are in lanes 5 and 6. (**B**) NIH Image 1.62 was used to measure the relative level of the PCR products. The data in (B) represent the average from two independent experiments, standardized to the viral-input controls.

histones in T2RA cells than in their undifferentiated, non-permissive phenotype.

Monocytic cells are believed to be a major site of carriage of HCMV in normal healthy seropositives (Taylor-Wiedeman *et al*., 1991) and differentiation of monocytes has been linked to activation of the MIEP (Ibanez *et al*., 1991; Lathey and Spector, 1991; Taylor-Wiedeman *et al*., 1994). Consequently, peripheral blood monocytes, which are generally not permissive for IE expression upon infection (Figure 8A), and monocyte-derived macrophages (MDMs), which are permissive for HCMV (Figure 8B), were analyzed for the presence of HDAC3. Figure 8C shows that the level of this deacetylase decreases following differentiation into MDMs. This observation is consistent with HDAC3 limiting infection in non-permissive monocytes, as well as in T2 cells. Therefore, we asked whether the differentiation-specific changes in acetylation of histones associated with the MIEP in T2RA cells were mirrored in monocytic cells. Further ChIP assays were performed on infected peripheral blood monocytes and MDMs. As with T2RA cells, the MIEP in permissive MDMs is associated with hyper-acetylated histones compared with undifferentiated, non-permissive monocytes (Figure 8E, lanes 6 and 2, respectively, and F). Again, this suggests that the viral MIEP becomes associated with acetylated histones following differentiation of monocytes to a permissive phenotype.

HCMV MIEP is associated with HP1 in monocytes

Recent studies predict that both reduced acetylation of the histone H4 N-terminus and histone H3 methylation are key events in DNA silencing (Rea *et al*., 2000). Methylated lysine 9 of histone H3 binds HP1 (Bannister *et al*., 2001; Lachner *et al*., 2001), which has been implicated in gene silencing (for review see Eissenberg and Elgin, 2000). Therefore, we asked whether the MIEP was associated with HP1 in non-permissive monocytes. As shown, the MIEP-specific PCR products from monocytes immunoprecipitated with anti-HP1 antiserum are more abundant than MIEP-specific products from permissive MDMs (Figure 8E, lanes 3 and 7, respectively, and F). This suggests that the MIEP is significantly more associated with HP1 in monocytes and is consistent with the silencing of this viral promoter in these cells.

Discussion

The state of cellular differentiation appears critical for permissive infection with HCMV. T2 cells are normally non-permissive for HCMV infection; however, differentiation with RA results in a permissive phenotype. We show here that T2 cells can be rendered transiently permissive for HCMV following treatment with TSA, a histone deacetylase inhibitor, suggesting that HDACs play a role in the repression of viral replication. This is consistent with recent data showing that TSA treatment of pre-infected T2 cells resulted in productive HCMV infection (Meier, 2001). Our observations clearly

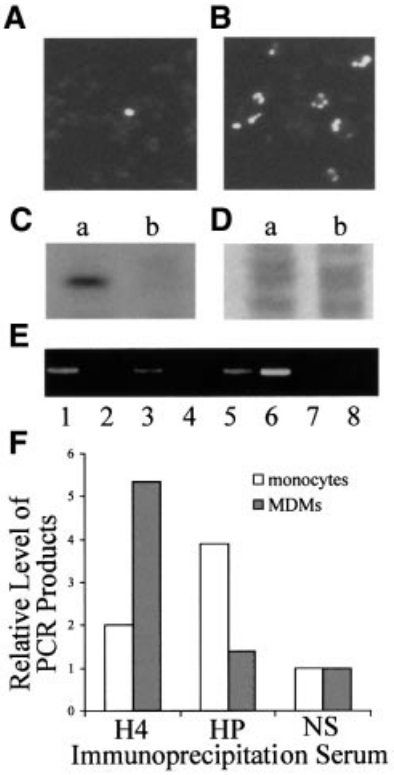

Fig. 8. The relative levels of HCMV TB40/E infection of monocytes and MDMs, detected by specific IE-indirect immunofluorescence, are shown in (**A**) and (**B**), respectively. (**C**) Western blot analysis of uninfected monocyte (lane a) and MDM (lane b) cellular extracts probed with anti-HDAC3 antiserum. (**D**) Following detection, the total protein present on nitrocellulose was visualized with 0.5% Ponceau red in 1% TCA. (**E**) ChIP assay on HCMV-infected monocytes and MDMs. PCR reactions, using primers complementary to the MIEP, were performed on immunoprecipitated histone protein-associated DNA from monocytes (lanes 1–4) and MDMs (lanes 5–8). Lanes 1 and 5 contain PCR products from viral-input controls and additional PCR products are from samples immunoprecipitated with anti-acetylated histone H4 antiserum (H4; lanes 2 and 6), anti-HP1 antiserum (HP; lanes 3 and 7) and normal serum (NS; lanes 4 and 8). (**F**) NIH Image 1.62 was used to measure the relative level of the PCR products. The data in (F) represent the average from two independent experiments, standardized to the viral-input controls.

implicate HDACs in limiting HCMV infection as the relatively high level of HDAC3 in T2 cells and monocytes correlates with non-permissiveness for HCMV. Furthermore, fibroblasts, which are fully permissive for HCMV, contain little HDAC3 (data not shown), implying that the relative level of this HDAC may be important for HCMV permissiveness in a range of cell types. The levels of HDAC1 and HDAC2 also appear to decrease upon differentiation into permissive phenotypes (data not shown), suggesting that other HDACs may also play a role in limiting viral permissiveness. However, we pursued HDAC3 as a repressor of the HCMV MIEP since superexpression of this HDAC appeared most effective at inhibiting viral infection in permissive T2RA cells.

HDAC-mediated repression of HCMV appears to occur within the MIEP, primarily between nucleotides –2100 and –302, which includes the modulator and 21 bp repeat elements of the enhancer. Deacetylases, including HDAC3, may act to modulate the transcriptional activity of MIEP by affecting the acetylation status of transcription factors. For instance, YY1, which binds to the 21 bp repeats present in the modulator and enhancer regions of the MIEP (Liu *et al*., 1994), was recently found to be acetylated in two regions (Yao *et al*., 2001). HDACs are further able to regulate transcriptional activity by modifying the acetylation status of histones (for review see Kouzarides, 2000). Since histone deacetylase inhibition of T2 cells substantially increases the MIEP activity to a level similar to that seen in permissive T2RA cells, we asked whether the acetylation status of histones associated with the MIEP differed in non-permissive cells compared with permissive cells. ChIP assays clearly show that the MIEP is more often associated with acetylated histone H4 in permissive T2RA cells and monocyte-derived macrophages than in their undifferentiated non-permissive counterparts. Furthermore, in non-permissive monocytes, the MIEP is associated with HP1, which is known to localize at sites of heterochromatinization, where it mediates gene silencing (for review see Eissenberg and Elgin, 2000).

The availability of cellular transcription factors involved in recruitment of chromatin remodeling factors to the MIEP and the remodelers themselves probably change during differentiation of a cell to a permissive phenotype. One potential candidate for the recruitment of HDAC3 to the MIEP in normally non-permissive T2 cells is YY1. As well as binding to the 21 bp repeat elements in the MIEP (Liu *et al*., 1994), this protein binds to HDAC3 and other class I HDACs (Yang *et al*., 1997). However, as a recombinant virus lacking both the 21 bp repeats and the modulator is repressed in T2 cells (Meier, 2001), and as a MIEP-CAT reporter plasmid lacking these regions is slightly activated by TSA, other MIEP-binding cellular repressors, besides YY1, may be capable of recruiting HDACs to the HCMV MIEP.

Recruitment of chromatin silencing factors to the MIEP may play an important role in repressing gene expression during HCMV latency. Indeed, changes in chromatin structure have been implicated in the control of latency in other herpesviruses. Latent herpes simplex virus type 1 (HSV-1) DNA is associated with nucleosomes *in vivo* (Deshmane and Fraser, 1989). Following TSA treatment of neurons latently infected with a recombinant HSV-1, the activity of the viral immediate early promoter increased significantly (Arthur *et al*., 2001), suggesting that HDACs may play a role in controlling HSV-1 latency. Like HSV-1, in latent Epstein–Barr virus (EBV), DNA is organized in nucleosomes (Shaw *et al*., 1979; Dyson and Farrell, 1985; Jenkins *et al*., 2000) and the switch to productive EBV infection is believed to involve activation of the IE gene *BZLF1* (Countryman and Miller, 1985). Moreover, the promoter of *BZLF1* is more associated with acetylated histones following activation of EBV-positive AK6 cells (Jenkins *et al*., 2000). The HCMV genome has also been shown to be associated with nucleosomal proteins (Kierszenbaum and Huang, 1978; St Jeor *et al*., 1982) and therefore chromatin arrangement may also play a role in controlling HCMV latency. Consequently, latent HCMV may result from cellular chromatin remodeling factors causing the MIEP in monocytes to become heterochromatinized.

In view of the data presented here, we propose the following model. The high levels of HDACs present in non-permissive cells result in the inhibition of HCMV infection due to the deacetylation of the histones around the MIEP, the recruitment of HP1 (and perhaps other silencing factors) and the subsequent silencing of the promoter. Upon differentiation into a permissive phenotype, the balance of cellular chromatin remodeling factors changes. Histones around the MIEP may become acetylated, allowing viral transcription and productive HCMV infection to occur. Rearrangement of the chromatin to an active structure in permissive cells is unlikely to be simply due to a lack of HDACs and methylases, but probably involves increases of histone acetylases and other activators. Thus, the balance of positive and negative factors may act as a tightly regulated molecular switch, controlling the chromatin arrangement of the MIEP and hence the permissiveness of cells for HCMV infection.

Materials and methods

Cell culture

The human teratocarcinoma cell line NTERA-S cl.D1 (T2 cells; Gonczol *et al*., 1984) was split 1:3 every 3 days into minimum essential medium with Earle's salts, supplemented with 10% fetal calf serum (FCS). For differentiation into HCMV-permissive phenotype, cells were split 1:10 and maintained in medium containing 10^{-6} M all-*trans*-RA (Sigma) for 5 days. Histone deacetylase inhibition was achieved by inclusion of 330 nM TSA (Wako Pure Chemical Industries) in the medium for 16 h. Following Lymphoprep (Nycomed Pharma AS) gradient centrifugation, adherent peripheral blood monocytes from healthy adults were purified as described previously (Taylor-Wiedeman *et al*., 1994). Monocytes were maintained in Iscove's modified Dulbecco's medium supplemented with 20% FCS or differentiated into MDMs with 5×10^{-5} M hydrocortisone for 6 days and 2×10^{-8} M phorbol 12-myristate 13-acetate for the final 16 h.

Viruses

Unless specified, experiments were performed using the AD169 strain of HCMV. CR[IE1–GFP], a kind gift of Richard Greaves (Department of Infectious Diseases, Imperial College School of Medicine, London, UK), is a recombinant Towne strain of HCMV expressing a fusion of IE1 to GFP (J.Gawn, M.Denson, E.Sherratt, G.Wilkinson, R.Caswell and R.Greaves, in preparation). HCMV TB40/E was isolated from a bone marrow recipient and passaged in HUVEC cells (Sinzger *et al*., 1999).

Plasmids

pDsRED-N1 (Clontech) encodes a red fluorescent protein (RFP1). pcDNA3-HDAC3 contains a sequence encoding HDAC3 fused to a C-terminal FLAG, cloned in the *Bam*HI and *Xba*I sites of pcDNA3.1 (Invitrogen). pcDNA3-HDAC3Δ was cloned in a similar way but has an internal deletion of the HDAC enzymatic core domain from residue 399 to 527. pESCAT contains the CAT gene under the control of the HCMV MIEP from nucleotide –2100 to +72 relative to the transcriptional start site, whilst pIEP1CAT contains CAT under the control of the MIEP from nucleotide –302 to +72 (Shelbourn *et al*., 1989a).

Western blot analysis

Approximately 10 μg of cellular extracts were analyzed by SDS–PAGE and transferred to Hybond C nitrocellulose (Amersham). Proteins were detected using E13 (PARIS), which recognizes HCMV IE1 p72 and IE86 p86 proteins, anti-HDAC3 antiserum (Emiliani *et al*., 1998) and anti-p21 antibody (Fredersdorf *et al*., 1996). Horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) and ECL reagents (Amersham) were used as described in the manufacturers' instructions. Following detection, the filters were stained with 0.5% Ponceau red in 1% trichloroacetic acid (TCA) to ensure equal protein transfer.

Indirect immunofluorescence

Sixteen hours post-HCMV infection, cells grown overnight on spot slides were fixed and stained as described previously (Shelbourn *et al*., 1989b). Proteins were detected with anti-SSEA3 antibody (Shelbourn *et al*., 1989b), anti-pp65 antibody (Revello *et al*., 1992) or, as a control, with total immunoglobulins (Sigma), followed by conjugated secondary antibody (Sigma).

Transient transfection and infection of T2RA cells

pDsRED-N1 (20 μg) along with 20 μg of pCDNA3, pCDNA3-HDAC3 or pCDNA3-HDAC3Δ were transiently transfected into 2×10^6 T2RA cells using Lipofectin (Life Technologies). Thirty-six hours post-transfection, the cells were infected with CR[IE1–GFP] for a further 24 h. Cells were analyzed at the standard setting for FL1 and FL2 on a Becton Dickinson FACSort.

CAT assays

Cells (2×10^6) were transfected with either pESCAT or pIEP1CAT by calcium phosphate co-precipitation and the cells were harvested 36 h post-transfection. It was necessary to dilute extracts from pIEP1CAT transfections 1:10 compared with pESCAT before assaying for CAT activity (Gorman *et al*., 1982), which was detected using a Hewlett-Packard Instant Imager.

ChIP assay

Twenty-four hours post-infection with HCMV AD169, 10^6 T2 or T2RA cells were lysed and aliquots stored as viral-input controls. DNA associated with histones was immunoprecipitated with either normal serum (Sigma) or anti-acetyl histone H4 antiserum (ChIP grade; Upstate Biotechnology) using the manufacturer's protocol. DNA from disrupted nucleosomes was precipitated and used in PCR with primers TGGGACTTTCCTACTTGG and CCAGGCGATCTGACGGTT, complementary to positions –272 and +13, relative to the MIEP start site. Similar ChIP assays were also performed on 10^6 monocytes or MDMs infected with HCMV TB40/E; however, histone-associated DNA was also immunoprecipitated with anti-HP1 antiserum (Pak *et al*., 1997), in addition to anti-acetyl histone H4 antiserum.

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