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Herpes simplex virus type 1 tegument protein VP22 interacts with TAF-I proteins and inhibits nucleosome assembly but not regulation of histone acetylation by INHAT

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Affinity chromatography was used to identify cellular proteins that interact with the herpes simplex virus (HSV) tegument protein VP22. Among a small set of proteins that bind specifically to VP22, we identified TAF-I (template-activating factor I), a chromatin remodelling protein and close homologue of the histone chaperone protein NAP-1. TAF-I has been shown previously to promote more ordered transfer of histones to naked DNA through a direct interaction with histones. TAF-I, as a subunit of the INHAT (inhibitor of acetyltransferases) protein complex, also binds to histones and masks them from being substrates for the acetyltransferases p300 and PCAF. Using *in vitro* assays for TAF-I activity in chromatin assembly, we show that VP22 inhibits nucleosome deposition on DNA by binding to TAF-I. We also observed that VP22 binds non-specifically to DNA, an activity that is abolished by TAF-I. However, the presence of VP22 does not affect the property of INHAT in inhibiting the histone acetyltransferase activity of p300 or PCAF *in vitro*. We speculate that this interaction could be relevant to HSV DNA organization early in infection, for example, by interfering with nucleosomal deposition on the genome. Consistent with this possibility was the observation that overexpression of TAF-I in transfected cells interferes with the progression of HSV-1 infection.

INTRODUCTION

Following adsorption of virions to the host cell membrane, fusion and penetration, herpes simplex virus (HSV) capsids are transported across the cytoplasm to the nuclear pore where, after a poorly understood reorganization of structural components, the viral DNA is transported across the pore to be transcribed in the nucleus by the host cell RNA polymerase II. Among the first viral proteins encountering the host cell after infection are those of the tegument, a proteinaceous layer assembled between the viral capsid and envelope and comprising at least 12 virally encoded proteins (Haarr & Skulstad, 1994). Tegument proteins are known to be involved in a variety of activities that promote virus infectivity, including, for example, shut-off of host protein synthesis (Kwong & Frenkel, 1989; Smibert *et al.*, 1992) or the induction of transcription of the incoming genome in the nucleus (O'Hare, 1993). One of the most abundant components of the HSV-1 tegument is VP22, with approximately 2000 copies per virus particle (Leslie *et al.*, 1996). The exact role of VP22 during the virus replication cycle remains unclear. Deletion of the gene for VP22 has a significant effect on replication of bovine herpesvirus type 1 (BHV-1) (Liang *et al.*, 1995) and Marek's disease virus (Dorange *et al.*, 2002), but little detectable effect on replication of pseudorabies virus (PRV) (del Rio *et al.*, 2002). VP22 of HSV is a phosphorylated protein whose localization has been the subject of some controversy. In HSV recombinants expressing a GFP-tagged version of VP22, the protein was seen virtually exclusively in the cytoplasm (Elliott & O'Hare, 1997), while other workers have observed by immunofluorescence studies the protein to be present both in the cytoplasm and in the nucleus (Morrison *et al.*, 1998; Pomeranz & Blaho, 1999), a result consistent with observations of PRV VP22 (del Rio *et al.*, 2002). HSV VP22 has been shown to interact with microtubules, an interaction that leads to microtubule stabilization and consequent acetylation (Elliott & O'Hare, 1998).

With the aim of understanding the functional role(s) of VP22, we wished to identify cellular proteins that interact with VP22. Previously, we used affinity chromatography on columns containing bound, purified VP22 to show that four proteins were retained specifically (van Leeuwen *et al.*, 2002). One protein was identified as non-muscle myosin IIA (NMIIA) and we explored further the possibility that NMIIA may be involved in virus maturation. Here we report on the identity of two of the other VP22-binding proteins as template-activating factor I α and β (TAF-I α and - β), also named SETa and SETb (Adachi *et al.*, 1994; Nagata *et al.*, 1995; von Lindern *et al.*, 1992). TAF-I α and - β , identified originally as host factors required for adenovirus core replication, have been implicated in chromatin remodelling and were shown to promote the deposition of histones on naked DNA (Miyaji-Yamaguchi *et al.*, 1999; Okuwaki & Nagata, 1998). Furthermore, a multiprotein complex containing TAF-I proteins as major subunits was shown recently to bind to histones, thereby preventing their acetylation by the cellular histone acetyltransferases p300 and PCAF (Seo *et al.*, 2001). Because of this histone-masking effect, the TAF-I-containing complex was named INHAT (inhibitor of acetyltransferases) (Seo *et al.*, 2001). TAF-I β was also identified as part

of the putative oncogene associated with acute undifferentiated leukaemia when translocated to the CAN (NUP214) gene (Kraemer *et al.*, 1994; von Lindern *et al.*, 1992).

Using *in vitro* assays for TAF-I activity in chromatin assembly, we show that VP22 prevents nucleosome deposition on DNA by binding to TAF-I. We also observed that VP22 binds non-specifically to DNA, an activity that is blocked by recombinant TAF-I α . However, VP22 had no effect on the HAT-inhibitory activity of the INHAT complex *in vitro*. Finally, we observed that TAF-I α overexpression appears to block the progression of HSV-1 infection. Together with the results on VP22 interaction and repression of chromatin assembly, our data indicate that modulation of TAF-I α -mediated nucleosome deposition and repression may play a role in virus infection.

METHODS

Cells and transfection. COS-1 cells were maintained in Dulbecco's modified minimal essential medium containing 10 % newborn calf serum. Cells were transfected with plasmid DNA (typically 4 μ g per 10⁶ cells) using the calcium phosphate precipitation method with BES-buffered saline, as described previously (Greaves & O'Hare, 1989). Cells were processed for Western blotting or immunofluorescence approximately 40 h after transfection.

Plasmids. To construct a mammalian myc epitope-tagged TAF-I α expression construct, the sequence was amplified by PCR from cDNA (Nagata *et al.*, 1995) using a TAF-I α -specific forward primer (5'-CGGAAGCTTAATATGGCCCCTAAACGCCAGTCTCC-3'), which contained a *Hind*III site, and a reverse primer (5'-GGTCTAGATCATCTTCTCCTTCATCCTCCTCTC-3'), which contained an *Xba*I site. The PCR product was digested with *Hind*III/*Xba*I and ligated into pCDNA3.1mycisB (Invitrogen), digested similarly with *Hind*III/*Xba*I. This resulted in the construction of an expression vector for full-length TAF-I α containing a 10 residue myc epitope tag at its C terminus. Cloning of the mammalian VP22 expression construct, pc49epB, has been described previously (Dilber *et al.*, 1999).

The VP22.C1.his6 bacterial expression construct was made by PCR amplification from pc49epB using VP22-specific primers containing *Hind*III/*Bam*HI sites (forward primer, 5'-TCGGATCCGACCTCTCGCCGCTCCGTG-3'; reverse primer, 3'-TTAAGCTTCTCGACGGGCCGTCTGGG-3'). The PCR product was digested with *Hind*III/*Bam*HI and cloned in pET24b (Novagen).

Immunofluorescence and antibodies. COS cells seeded on glass coverslips were transfected with the appropriate expression vector and approximately 40 h later were washed twice with PBS and fixed for

20 min at -20°C with 100 % methanol. The cells were then blocked in PBS containing 10 % calf serum for 10 min at room temperature. Primary antibodies were added in the same solution and incubated for 45 min at room temperature. Following two 5 min washes with PBS, secondary antibodies were added in blocking buffer and incubated for 15 min. After an additional two washes in PBS, the coverslips were mounted in Mowiol (Sigma) containing 2.5 % 1,4-diazabicyclo-2.2.2-octane to reduce bleaching. Antibodies used in this study and their dilutions were as follows: monoclonal antibody to the myc epitope (R950-25, diluted 1:200; Invitrogen) and polyclonal antibodies to VP22 (AGV30, diluted 1:500), as described before (Elliott & O'Hare, 1997). Polyclonal anti-TAF-I β antibody (Sp1) was a gift from T. Copeland (Adachi *et al.*, 1994). Secondary antibodies were FITC-conjugated anti-rabbit immunoglobulin (F1-2000, diluted 1:100; Vector Laboratories) and TRITC-conjugated anti-mouse (T7782, diluted 1:200; Sigma).

Protein purification. VP22.C1 was purified on an Ni-NTA column as described previously (Normand *et al.*, 2001). In order to purify VP22.C1 to homogeneity, an additional purification step on a Mono S HR 5/5 column (Pharmacia) was performed. Using a linear NaCl gradient, VP22.C1 eluted at approximately 400 mM NaCl of the Mono S column. Recombinant TAF-I α was purified similarly on an Ni-NTA column and a Mono Q HR 5/5 column. To examine direct protein-protein interactions (see Fig. 2), TAF-I α and - β and variants were purified as described previously (Miyaji-Yamaguchi *et al.*, 1999).

To identify VP22.C1-associated proteins, approximately 2×10^{10} HeLa cells were collected and suspended in 100 ml lysis buffer containing 50 mM Tris pH 8.0, 150 mM NaCl, 1 % Nonidet-P40, 0.5 mM PMSF and Complete Protease Inhibitor cocktail (Boehringer Mannheim). After incubation for 45 min on ice with occasional mixing, the lysate was diluted 1:1 in buffer containing 50 mM Tris pH 7, 50 mM NaCl and 0.5 mM PMSF. Cell fragments and insoluble materials were removed by centrifugation at 12 000 *g* for 30 min. Cleared lysates were loaded onto a 50 ml NTA column equilibrated in wash buffer containing 50 mM Tris pH 7.5, 100 mM NaCl, 0.1 % Nonidet-P40 and 0.5 mM PMSF. The flow-through of this column was collected and imidazole (pH 7.0) and NaCl were added to a final concentration of 20 and 150 mM, respectively. This was subsequently loaded onto a 2ml NTA column containing 4 mg bound, purified VP22.C1 protein, bound and equilibrated already in buffer A (50 mM Tris pH 7, 10 % glycerol, 0.1 % Nonidet-P40, 100 mM NaCl, 2.5 mM β -mercaptoethanol and Complete Protease Inhibitor cocktail). After extensive washing in buffer A, associated proteins were eluted with a 30 ml linear gradient of 100–1000 mM NaCl.

Gel retardation assays. Binding reactions were performed in 20 μl buffer containing 20 mM HEPES/KOH (pH 7.5), 1 mM EDTA, 1 mM DTT, 0.025 % Nonidet-P40, 4 % Ficoll and 0.5 nM DNA probe. The probe used was a 147 bp fragment from *MspI*-digested pUC19 that had been end-labelled with

[α -³²P]dCTP using the Klenow fragment. Amounts of purified proteins used in the binding reactions are as indicated in the figures. Incubations were carried out for 60 min at 4 °C and the resulting complexes were resolved on a 5 % non-denaturing polyacrylamide gel run in 0.5× Tris/borate/EDTA.

From the known amounts of input protein and DNA, the DNA-binding constant could be calculated at approximately 1 nM [K_D =(Total protein)–(DNA bound) at 50 % binding].

Nucleosome assembly assay. Assembly and micrococcal nuclease digestion analysis were performed as described previously (Bulger & Kadonaga, 1994) using three components: (1) a core histone; (2) HuCHRAC (human chromatin accessibility complex) (Poot *et al.*, 2000), the ATP-dependent chromatin remodelling complex; and (3) recombinant TAF-I α (rTAF-I α), as histone chaperone. HuCHRAC (30 ng) was incubated in an ATP-containing buffer with relaxed plasmid DNA as substrate in the presence of various combinations of rTAF-I α and VP22, as indicated. After nucleosome assembly had completed on the target DNA, the DNA was digested with micrococcal nuclease. Proteins were then removed by phenol extraction and the DNA fragments were separated on agarose gels and detected by ethidium bromide staining.

HAT assay. HAT (histone and nucleosome acetyltransferase) assays were performed as described previously (Seo *et al.*, 2001).

RESULTS

TAF-I α and - β are retained on a VP22.C1 column

To identify cellular proteins that bind to HSV-1 VP22, we performed the purification strategy as described previously (van Leeuwen *et al.*, 2002). Purified VP22.C1 (residues 159–301), encompassing the core conserved region linked to a His₆ tag (Normand *et al.*, 2001), was bound to an Ni-NTA column and used as an affinity column to pass over HeLa cell lysates. To minimize any non-specific binding of cellular proteins to the Ni-agarose matrix, the HeLa cell extract was first pre-cleared by passing over a large 50 ml Ni-NTA column. This pre-cleared extract (Fig. 1A, lane 2) was then loaded onto the VP22.C1 affinity column. After washing the column extensively, VP22.C1-bound proteins were eluted with a linear gradient of 100–1000 mM NaCl. The elution fraction (Fig. 1A, lane 3) corresponding to 600 mM NaCl was analysed by SDS-PAGE followed by silver staining and showed a similar profile as before, with enrichment of three prominent protein bands with an apparent molecular mass of 41, 39 and 28 kDa, respectively, and one less prominent band of approximately 220 kDa. These bands were not retained by a His₆-tagged protein of aa 1–60 of VP22 (data not shown). We identified previously the large 220 kDa protein as NMIIA and reported the characterization of a possible role of NMIIA in virus replication (van

Leeuwen *et al.*, 2002). Bands corresponding to the other VP22-binding proteins were excised, subjected to trypsin digestion, reverse-phase HPLC separation and microsequencing.

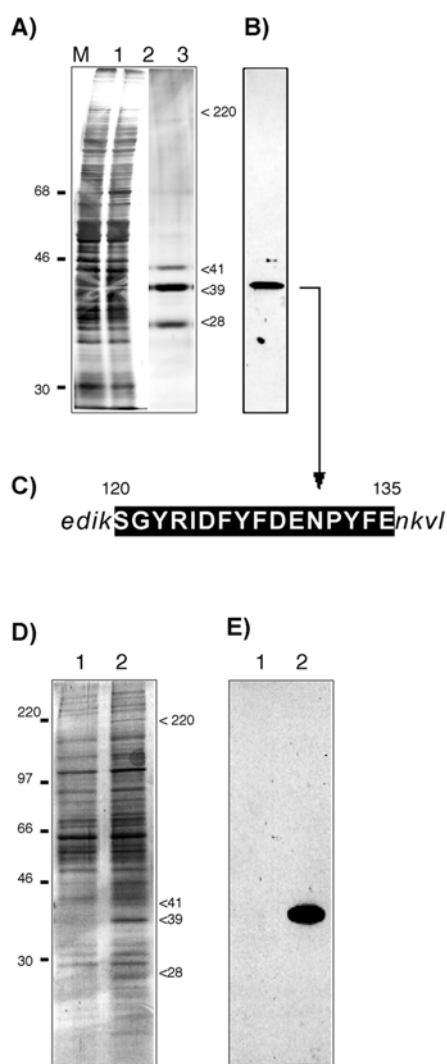


Fig. 1. Affinity purification of VP22-associated proteins from HeLa cell extracts. (A) Cleared cytoplasmic HeLa cell extracts of 10^{10} cells were passed over a VP22.C1 affinity column. Lanes: 1, load sample (2×10^4 cell equivalents); 2, flowthrough sample (2×10^4 cell equivalents); 3, 600 mM NaCl elution fraction (1×10^8 cell equivalents). Proteins were separated by SDS-PAGE and detected by silver staining. (B) The elution fraction was analysed by Western blot using an anti-TAF-I β polyclonal antibody. (C) Isolated protein bands of 41 and 39 kDa were subjected to trypsin digestion and reverse-phase HPLC. Microsequencing yielded the peptide sequence shown in the shaded box. (D) Binding of VP22-associated proteins without pre-clearing. Lanes: 1, 600 mM elution fraction of cytoplasmic proteins binding to the Ni-agarose column; 2, cytoplasmic proteins binding to the Ni-agarose column containing purified VP22.C1. Proteins were separated by SDS-PAGE and detected by silver staining. Protein bands retained specifically on the VP22.C1-containing column are indicated as '<', together with approximate molecular masses. (E) The fractions eluted from the column corresponding to lanes 1 and 2 of (D) were loaded on an SDS-PAGE gel and analysed by Western blot using an anti-TAF-I β -specific polyclonal antibody.

Amino acid sequences from the smallest VP22-associated protein (Fig. 1, lane 3, 28 kDa band) identified it as APRIL/PHAPI2, belonging to a family of acidic, leucine-rich proteins (Mencinger *et al.*, 1998). The possible role of VP22 binding to this protein is currently unknown and has not been pursued yet.

Partial amino acid sequences obtained for both the 41 and 39 kDa bands (Fig. 1, lane 3) were found to be identical (SGYRIDFYFDENPYFE). A database search of the amino acid sequence obtained revealed that the sequence corresponded precisely to human TAF-I α or - β , which have been reported to act as histone chaperone proteins (Nagata *et al.*, 1998). TAF-I α and - β have arisen from a gene duplication event, with TAF-I α located on chromosome 5 and TAF-I β on chromosome 17 (Nagata *et al.*, 1995). These genes differ from each other only in their extreme N-terminal regions (Fig. 2B). To confirm that the peptide sequenced represents TAF-I, the eluted fraction was analysed by Western blotting, in this case using an antibody specific for the TAF-I β form. The results show that the 39 kDa protein was detected specifically

by anti-TAF-I β (Fig. 1B), and indicate that, consistent with the amino acid sequence data, the 41 kDa protein is likely to be TAF-I α .

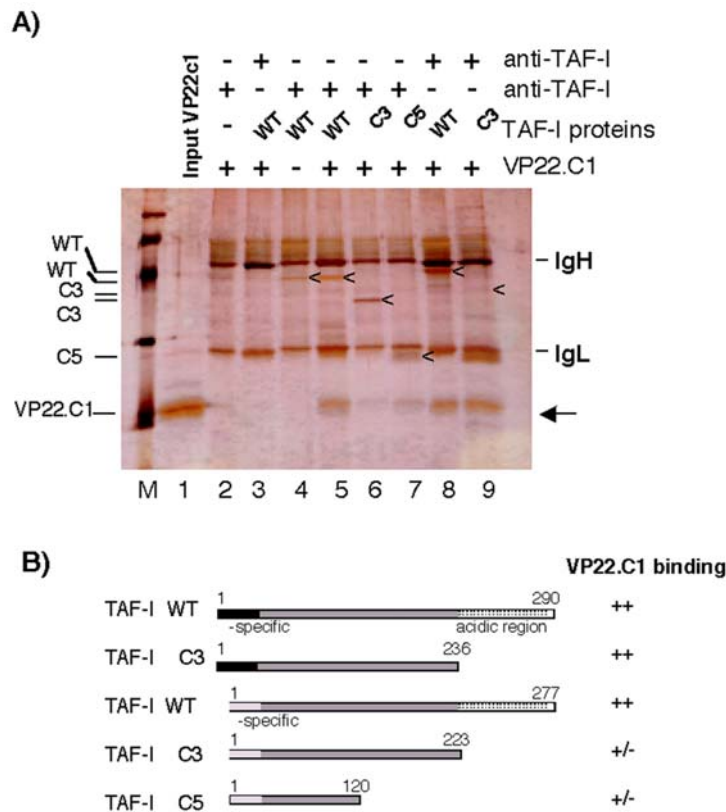


Fig. 2. Interaction between TAF-I proteins and VP22.C1. Protein–protein interactions were carried out using purified recombinant VP22.C1, TAF-I α and TAF-I β , incubated with anti-TAF-I α - or TAF-I β -specific antibodies and the addition of protein A–Sepharose beads. Immunocomplexes were precipitated, separated by SDS-PAGE and detected by silver staining. Separated immunoglobulin heavy (IgH) and light (IgL) chains are indicated. The arrow indicates precipitated VP22.C1. Proteins included in the individual binding reactions were as follows: full-length 1-277 rTAF-I β , indicated as β WT (lanes 3, 4 and 5); full-length 1-291 rTAF-I α , indicated as α WT (lane 8); 1-220 rTAF-I β , indicated as $\beta\Delta$ C3 (lane 6); 1-220 rTAF-I α , indicated as $\alpha\Delta$ C3 (lane 9); 1-120 rTAF-I β , indicated as $\beta\Delta$ C5 (lane 7). Lane M contains molecular mass standards. Total amounts of VP22.C1 used in the binding reactions are as shown in lane 1. Control pull-down in the absence of any rTAF-I protein is shown in lane 2.

Although the HeLa cell extract used to identify VP22-associated proteins was first pre-cleared by passing over a large Ni-NTA column, this pre-clearing step might have been inefficient, with the TAF-I proteins still binding to the VP22.C1 column by some non-specific interaction on the column. Therefore, we repeated the purification on a smaller scale, without any pre-clearing, to assay whether TAF-I β bound to the Ni–agarose matrix at all (Fig. 1D). HeLa cell cytoplasmic extracts were passed directly over a 100 μ l NTA column or an NTA column with bound VP22.C1. Bound proteins were eluted and separated by SDS-PAGE and detected by silver staining. As expected, numerous cellular proteins remain associated with the empty column (Fig. 1D, lane 1) but additional proteins (corresponding to those enriched in Fig.

1A) can be seen on the VP22.C1-containing column (Fig. 1D, lane 2, arrowheads). Western blotting showed that TAF-I β bound only to the VP22.C1-containing NTA column (Fig. 1E, lane 2) with no non-specific binding to the control NTA column (Fig. 1E, lane 1), confirming that the association of TAF-I β with VP22.C1 was specific (although not indicating whether the interaction was a direct or an indirect one).

To confirm the VP22.C1–TAF-I α/β interaction and to examine whether binding was a direct or an indirect association mediated by other bound cellular factors, we tested whether purified rTAF-I α and - β could bind and co-precipitate purified VP22.C1. Therefore, purified VP22.C1 (400 ng) was incubated alone or together with equimolar amounts of purified TAF-I α or - β proteins. Antibodies specific for TAF-I α or - β were then added and immunocomplexes isolated by the addition of protein A–Sepharose beads. Bound proteins were separated by SDS-PAGE and visualized by silver staining. [Note that the TAF proteins and variants are indicated by chevrons within the lanes, and VP22.C1 is indicated by the arrow to the right-hand side (Fig. 2). Bound immunoglobulin heavy and light chains are labelled also.] Control incubations showed that no VP22.C1 was precipitated either by anti-TAF antibody in the absence of TAF-I proteins (Fig. 2, lane 2) or when the TAF-I α -specific antibody was used in combination with the TAF-I β protein (Fig. 2, lane 3). However, in the presence of TAF-I α together with the corresponding anti-TAF-I α antibody (Fig. 2, lane 8), or TAF-I β together with the corresponding anti-TAF-I β antibody (Fig. 2, lane 5), VP22 was co-precipitated efficiently (Fig. 2, arrow). Comparison of the total input VP22.C1 for each of the test samples (Fig. 2, lane 1) with the amount of co-precipitated VP22.C1 indicates that binding was efficient. TAF-I α and - β contain a C-terminal region of approximately 50 residues rich in acidic amino acids, which is important for chromatin remodelling activity (Miyaji-Yamaguchi *et al.*, 1999). We next tested whether this region was involved in the interaction with the largely basic VP22.C1 protein by incubation with variant TAF-I α (Fig. 2, $\alpha\Delta C3$) or TAF-I β (Fig. 2, $\beta\Delta C3$ and $\beta\Delta C5$) lacking the C-terminal region (Fig. 2B). Surprisingly, deletion of the C terminus had different effects on the VP22.C1 interaction for TAF-I α versus TAF-I β . Thus, removal of the C-terminal 54 residues of TAF-I α ($\alpha\Delta C3$) had no effect on the interaction (Fig. 2, lane 9), while the identical truncation of TAF-I β ($\beta\Delta C3$) reduced binding significantly, albeit not abolishing it completely (Fig. 2, lane 6). A similar level of binding was observed for TAF-I β ($\Delta C5$). While we do not fully understand this differential effect, which could, for example, be due to differences in the N terminus of TAF-I α and - β , the result for TAF-I α indicates that VP22.C1 binding is not due to straightforward charge interaction with the C-terminal acidic region.

Transfected TAF-I α and VP22 do not co-localize on condensed chromosomes

VP22 is found both in the cytoplasm and in the nucleus, where nuclear localization represents cells that retain VP22 after nuclear envelope breakdown and division (Elliott & O'Hare, 2000). VP22 has been

shown to localize to condensed chromatin during mitosis. Although TAF-I is a chaperone protein and escorts histones onto DNA through a direct interaction, it does not remain associated with condensed chromosomes (K. Nagata, personal communication). We wished to examine the localization of VP22 and TAF-I α / β when co-expressed by transfection in COS-1 cells. An epitope-tagged (myc) version of TAF-I α was used to detect its localization by indirect immunofluorescence. VP22 was detected with the specific rabbit polyclonal antibody AGV30 (Elliott & O'Hare, 1997). TAF-I α was found largely in the nucleus and, as described previously (Elliott & O'Hare, 2000), transfected VP22 was found in the nucleus where nuclear localization represents cells that have divided. The nuclear staining patterns of VP22 and TAF-I α showed no obvious subnuclear localization pattern, both being excluded from the nucleoli (data not shown). However, in mitotic cells where VP22 was bound to the condensing cellular chromatin (Fig. 3), TAF-I α was excluded from metaphase chromatin (Fig. 3). The presence of VP22 on condensed chromosomes, therefore, does not appear to actively retain TAF-I α chromosomes, at least at detectable levels. These results were, therefore, inconclusive in attempting to substantiate an *in vivo* association between the two proteins, although it remains possible that a subpopulation of TAF-I α could be associated on chromatin (or viral genomes).

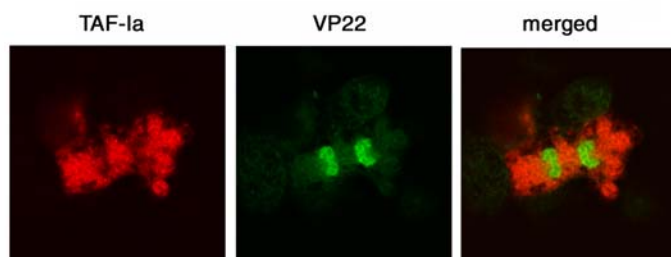


Fig. 3. Localization of VP22 and TAF-I α after co-expression in COS-1 cells. Cells grown on coverslips were co-transfected with plasmid DNA containing the TAF-I α cDNA (red, left panel) and VP22 (green, middle panel). After 40 h, cells were fixed, incubated with the appropriate antibodies and analysed by confocal microscopy. The individual images were merged and are shown in the right panel; co-localized VP22 and TAF-I α appear yellow.

Recombinant TAF-I α prevents *in vitro* VP22 DNA binding

Our observations that VP22, like histones, interacts with a nucleosome assembly protein and is present on condensed chromosomes (Elliott & O'Hare, 2000) prompted us to investigate whether VP22, as with histones, could bind DNA. We assayed the DNA-binding ability of VP22.C1 in a gel mobility shift assay using 32 P-labelled DNA fragments. As shown in Fig. 4(A), we found that purified VP22.C1 is capable of forming protein–DNA complexes, in this case using a 147 bp fragment. Moreover, DNA binding revealed multiple, distinct, shifted complexes, indicating clearly that more than one protein molecule could bind the 147 bp fragment. With increasing concentrations of VP22.C1, at least seven or eight multiple discrete bands were observed (Fig. 4A, lanes 1–6). At higher protein concentrations, a single discrete complex could be observed; a complex that did not enter the gel was also seen (Fig. 4A, lanes 7 and 8). While detailed aspects of the nature of VP22 DNA binding (for example, precise nature of the binding site, sequence specificity and major or minor groove binding) is beyond the scope of this investigation, the

results are consistent with VP22 binding non-specifically once per 15–20 bp. Preliminary competition experiments indicate that VP22 has no strong sequence preference in binding DNA (data not shown). From the known concentration of protein and DNA input, we calculate a K_D for VP22 DNA binding of approximately 1 nM. Although no information is available about the intracellular VP22 concentration, this affinity is certainly within the physiological range to suggest a relevant function for VP22 in DNA binding.

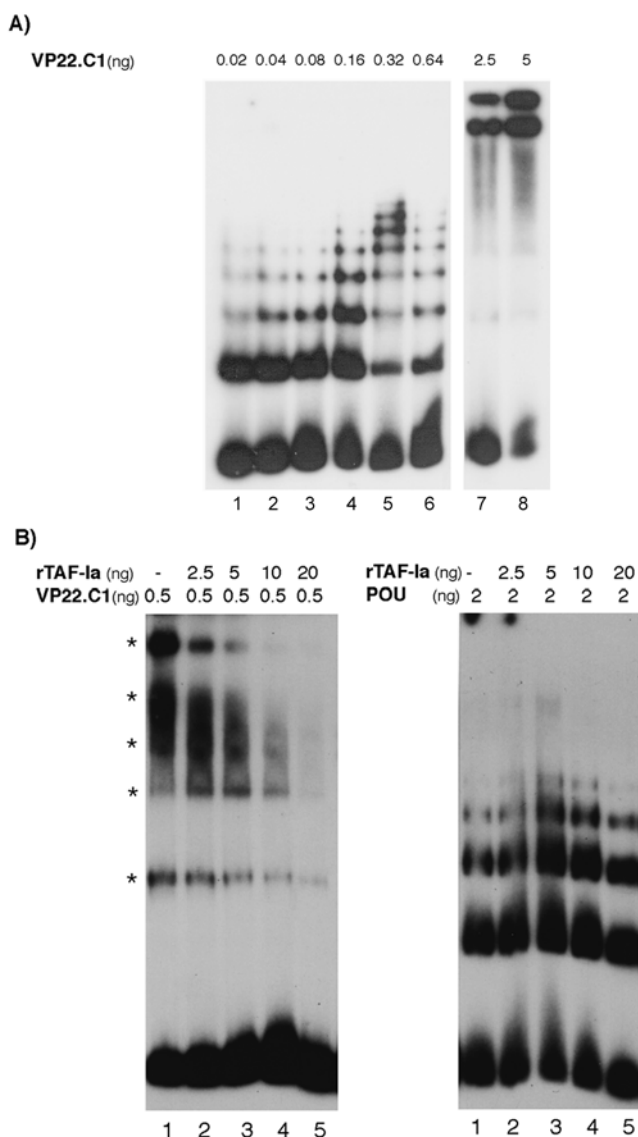


Fig. 4. VP22 DNA binding is blocked by TAF-I α . (A) VP22.C1 was incubated in increasing amounts, as indicated with a 147 bp 32 P-labelled dsDNA probe from pUC19. After 20 min of incubation, complexes were separated in a 5 % non-denaturing polyacrylamide gel, as detailed in Methods. All lanes are from the same gel, although one lane containing an artefact has been removed. (B, C) Different amounts of rTAF-I α in 20 μ l were assayed as indicated with a constant amount of VP22.C1 or the Oct-1 POU domain. Protein–DNA complexes were separated by electrophoresis on a 6 % polyacrylamide gel. Incubation with TAF-I α inhibits VP22.C1 DNA binding selectively.

We next analysed whether the protein–protein interaction between VP22.C1 and TAF-I would have an effect on the formation of VP22.C1–DNA complexes. Addition of purified recombinant TAF-I α to the DNA-binding reaction inhibited VP22.C1 DNA binding effectively (Fig. 4B, left panel, lanes 2–5). Interestingly, inhibition also occurred if TAF-I α was added after VP22.C1–DNA complexes were allowed to form (data not shown). In contrast, and to act as a control, TAF-I α had no effect on DNA binding of the Oct-1 POU domain (Fig. 4B, right panel, lanes 1–5). Note that since the 147 bp fragment used does not

contain a specific octamer binding site for the Oct-1 POU domain, binding here is non-specific and non-cooperative, thereby forming a ladder of multiple protein–DNA complexes very similar to VP22.C1, which has a comparable molecular mass (approx. 20 kDa). These results demonstrate that TAF-I α inhibits VP22 DNA binding selectively, most likely through a specific protein–protein interaction.

VP22.C1 blocks *in vitro* TAF-I α -dependent nucleosome reconstitution

TAF-I α/β has been shown to be a member of a class of proteins involved in chromatin remodelling, reportedly by facilitating nucleosome formation via direct interactions with histones (Matsumoto *et al.*, 1999; Okuwaki & Nagata, 1998). Therefore, we performed *in vitro* nucleosome assembly assays to examine whether VP22.C1 would have any effect on TAF-I α -dependent nucleosome assembly. Core histones (Simon & Felsenfeld, 1979), the ATP-dependent chromatin remodelling complex HuCHRAC (Poot *et al.*, 2000) and rTAF-I α (as a histone chaperone) were incubated in the presence of ATP with relaxed plasmid DNA. After incubation, the DNA was digested with micrococcal nuclease, proteins were removed and the DNA was separated on an agarose gel. Preliminary experiments were performed to optimize conditions for nucleosome formation that is maximally dependent on TAF-I α as the histone chaperone. Results shown in Fig. 5 show the ability of TAF-I α to promote the appearance of 150 bp DNA fragments, corresponding to mono-nucleosomal DNA after nuclease digestion (Fig. 5, compare lanes 1 and 7). When increasing amounts of VP22.C1 were added during the assembly reaction, nucleosome formation was lost progressively until we observed virtually complete inhibition at approximately 1:1 to 2:1 ratio of the proteins (Fig. 5, lanes 2–5). At concentrations of maximum VP22.C1 inhibition (Fig. 5, lane 5), the addition of extra TAF-I α partially restored mono-nucleosome formation (Fig. 5, lane 6), showing a direct interdependence with TAF-I α .

VP22c1 (μ g)	0	0.5	1	2	4	4	-
rTAF-I (μ g)	2	2	2	2	2	4	-
Histones (μ l)	0.5	0.5	0.5	0.5	0.5	0.5	0.5

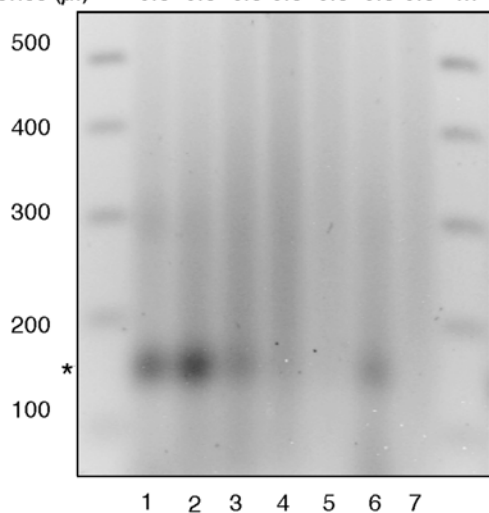


Fig. 5. TAF-I α -dependent nucleosome deposition is blocked by VP22.C1. Micrococcal nuclease digestion of nucleosomes assembled on relaxed plasmid DNA in the presence of indicated factors and 30 ng HuCHRAC. The resulting nucleosome fragments were de-proteinized, subjected to agarose gel electrophoreses and visualized with ethidium bromide staining (a negative image of the stained gel is shown). The molecular mass marker is the 1 kb Plus DNA ladder (Gibco-BRL).

VP22 does not affect INHAT activity

Besides functioning as a histone chaperone, TAF-I proteins have been reported to inhibit p300- and PCAF-mediated acetylation of histones as a subunit of the INHAT complex. To investigate whether VP22 would interfere with this function of TAF-I, we tested the effect of VP22 on INHAT activity *in vitro* (Fig. 6). In agreement with its reported function, the acetylation of core histones by PCAF and p300 was inhibited by addition of increasing amounts of purified INHAT (Fig. 6, compare lane 1 to lanes 2 and 3). Preincubation of the INHAT complex with saturating amounts of VP22 had no significant effect on the inhibiting activity of INHAT (Fig. 6, compare lanes 6–9 with lanes 2 and 3). VP22 alone had no effect of PCAF- or p300-dependent histone acetylation (Fig. 6, compare lanes 4 and 5 with lane 1). These observations demonstrate that the presence of VP22 has little effect on the property of INHAT in inhibiting the HAT activity of P300 and PCAF *in vitro*.

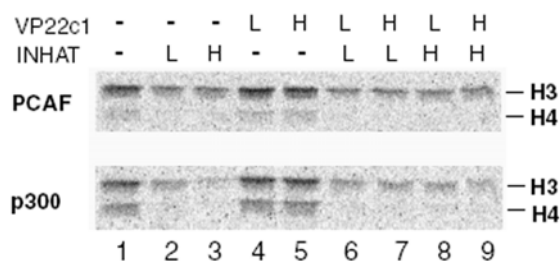


Fig. 6. The presence of VP22 does not change the property of INHAT in inhibiting the HAT activity of p300 or PCAF *in vitro*. 330 ng (L) or 660 ng (H) of INHAT was incubated with 80 ng (L) or 240 ng (H) of VP22 for 5 min before HAT assays were performed with the mixtures in the presence of [¹⁴C]acetyl coenzyme A, p300 or PCAF, and histones. The reaction mixture was subsequently subjected to SDS-PAGE followed by phosphorimager analysis. Bands detected correspond to acetylated histones H3 and H4.

Overexpression of TAF-I α affects progression of HSV-1 infection

In an attempt to study localization of TAF-I α and VP22 during HSV infection, COS-1 cells were transfected with epitope-tagged TAF-I α and infected with HSV-1 at an m.o.i. of 10 p.f.u. per cell and infected cells fixed in paraformaldehyde 10 h after infection to examine localization of VP22 and TAF-I α . Surprisingly, however, we found that transfected cells expressing TAF-I α expressed comparatively little or no VP22 (Fig. 7, arrows). By immunofluorescence, VP22 is found mainly in a cytoplasmic location, combining diffuse and punctate/vesicular pattern together with some nuclear staining (Elliott & O'Hare, 2000). Transfected cells expressing nuclear TAF-I α (Fig. 7, left panel), however, showed minimal staining for this otherwise abundantly expressed protein (Fig. 7, middle panel, arrows). Although only one field is shown, this effect was pronounced and reproducible. Moreover, assaying progress of infectivity with additional markers (for example, anti-VP16 or anti-HSV-1 immediate-early 110 antibodies) showed the same inhibition of virus progression by TAF-I α overexpression (data not shown), indicating that the effect of TAF-I α expression was not affecting VP22 selectively but appeared to result from a generalized inhibition of virus gene expression. We note that in a small percentage of TAF-I α -expressing cells, infection appeared to progress relatively normally (Fig. 7, arrowheads). These cells seem to be dividing, causing TAF-I α to be diluted and thereby possibly reducing its inhibitory effect. One possible cause for

this incomplete virus infection could be a non-specific effect of the presence of transfected plasmid DNA [or the cytomegalovirus (CMV) promoter] in the cells, for example, sequestering essential *trans*-acting factors. To control for this, we examined the effect of transfection and expression of a plasmid containing the CMV promoter and driving β -galactosidase expression. No reduction in virus expression of any of the test proteins was observed in those cells expressing β -galactosidase (data not shown). Finally, the effect on progression of HSV-1 infection might be caused by cellular toxicity caused by TAF-I overexpression. However, TAF-I-expressing cells were, if anything, healthier and did not show cytotoxic effects, and thus far no findings have been reported that any of the TAF/NAP family members are damaging to cells.

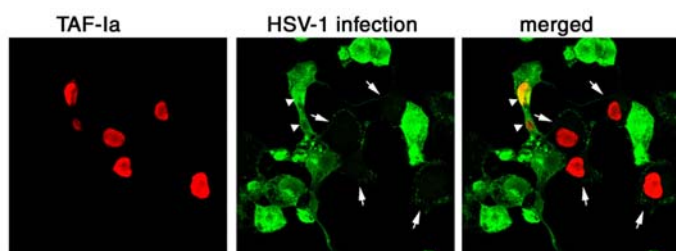


Fig. 7. Overexpression of TAF-I α slows HSV-1 infection. COS-1 cells were transfected with myc epitope-tagged TAF-I α and subsequently infected with HSV-1 at 10 p.f.u per cell. At 10 h post-infection, cells were fixed in paraformaldehyde and stained for VP22 and myc-tagged TAF-I α . Arrows indicate cells in which VP22 was absent or substantially reduced. These cells were expressing TAF-I α selectively.

DISCUSSION

After entering the cell, HSV capsids are transported through the cytosol to nuclear pore complexes where viral genomes are released into the nucleoplasm. Given the dynamics of host cell metabolism and the fact that viral DNA is not thought to be complexed with proteins within the capsid, it might have been expected that the deposition of the incoming naked virus genome in the nucleus would result in recruitment of any of a number of abundant host cell proteins, in particular the basic histones. However, previous studies attempting to address this issue using micrococcal nuclease digestion have shown that the HSV-1 genome in acutely infected cells is not assembled into nucleosomal arrays (Deshmane & Fraser, 1989; Leinbach & Summers, 1980; Muggeridge & Fraser, 1986). Both parental and progeny genomes are thought to be kept free of nucleosomes throughout infection. It is reasonable to propose that any association of incoming viral DNA with cellular nucleosomes would be a repressive one, inhibiting immediate-early viral gene transcription, and that mechanisms may exist to positively block such assembly.

In this work, we show that the HSV-1 structural protein VP22 associates with a cellular histone chaperone, TAF-I α . TAF-I α was shown to bind VP22 both by affinity chromatography of cell extracts on matrices containing bound VP22 and by co-precipitation of VP22 when incubated with purified TAF-I α (or TAF-I β) and immunoprecipitated with anti-TAF-I antibody. We show that interaction with VP22

inhibits the activity of TAF-I α in chromatin assembly, while, conversely, overexpression of TAF-I α suppresses HSV infection *in vivo*. In contrast to these observed effects, we find that the properties of the TAF-I α / β -containing INHAT complex, which blocks histone acetylation effectively by the acetylases p300/CBP and PCAF, seems unaffected by VP22 *in vitro* (Fig. 6). Interestingly, the other main component of the INHAT complex, named pp32/PHAPI (Seo *et al.*, 2002), is extremely similar to the smallest 28 kDa VP22-binding protein we identified as APRIL/PHAPI2 (Mencinger *et al.*, 1998), suggesting that what we have identified as VP22-binding proteins may in fact be identical to the INHAT complex. We note that despite the lack of an effect of VP22 on INHAT activity *in vitro*, others have reported that acetylation of histone H4 is decreased in VP22-expressing cells (Ren *et al.*, 2001).

In considering the possible relevance of these findings in terms of virus replication, several considerations are noteworthy. VP22 is present in approximately 2000 molecules per virion (Leslie *et al.*, 1996) and although the subcellular location of incoming tegument VP22 has not been reported, Morrison *et al.* (1998) have reported that VP22 can be detected in the nucleus early after infection and, indeed, our preliminary studies with large-scale cell biochemical fractionation of infected cells have shown that the majority of the incoming VP22 is located in the nuclear fraction early after infection (unpublished data). In proposing, therefore, that VP22 may be involved in inhibiting nucleosome deposition, two possible, but not mutually exclusive, mechanisms could be envisaged. Assembly could be blocked by VP22 binding to TAF-I α and thus inhibiting TAF-I α binding to and chaperoning histones onto the incoming DNA, or by VP22 binding to viral DNA and subsequently preventing organized nucleosome deposition. In this latter scenario, VP22 DNA binding could itself be blocked by the presence of TAF-I α (Fig. 4), allowing nucleosomes to access DNA. It has not proved possible to date, either by conventional immunofluorescence or in the context of GFP-VP22-expressing recombinant viruses, to visualize incoming VP22 protein adequately enough to examine its association with the incoming genome. The proposal that VP22 may prevent ordered nucleosome deposition on the incoming herpesvirus DNA remains speculative until we develop more sensitive methods for visualization of the incoming particle, but additional approaches such as DNA cross-linking and immunoprecipitation may help answer this question.

We noted upon close examination of the primary amino acid sequence of VP22 that a small conserved part of VP22 has sequence similarity with the C terminus of histone H2A (Fig. 8), which forms a short α -helix and contacts the C-terminal tail of histone H4 in the nucleosome. Interestingly, NAP-1, a close homologue of TAF-I, which also appears to function as a chaperone and facilitates deposition of histones onto DNA (Ishimi & Kikuchi, 1991; Ishimi *et al.*, 1987; McQuibban *et al.*, 1998), has been found to be associated with histone H2A in co-immunoprecipitation analysis (Chang *et al.*, 1997). Analysis of interactions with deletion mutants of VP22 should help define whether this region is important in the

TAF-I interaction. In this context, we also note the recent observation that BHV VP22 associates with histones, as seen in Far-Western assays (Ren *et al.*, 2001), a result that might explain the direct association of VP22 with chromatin in mitotic cells (Fig. 3) (Elliott & O'Hare, 2000).

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                227
VP22 HSV-1   : R T D E D L N E L L G I T T I
Histone H2A  : R N D E D L N K L L G K V T I
                89
                hHHHHHHh         alpha helix

```

Fig. 8. Alignment of the HSV-1 VP22 amino acid sequence with that of human histone H2A. The box denotes conserved amino acids. The H-stretch indicates the α -helix in H2A.

If VP22 binding and inhibition of TAF-I α activity plays a role in virus replication, we believe it is more likely to be relevant early in infection rather than in replication and transcription of progeny genomes. Further work is now under way to develop methods to analyse protein–DNA interactions on the virus genome at the earliest stages of infection.

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