Human herpesvirus 6B U19 protein is a PML-regulated transcriptional activator that localizes to nuclear foci in a PML-independent manner

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Human herpesvirus 6B (HHV-6B) contains an IE-B domain spanning open reading frames U16/17–U19, based on homology with human cytomegalovirus. Here, the protein product, U19, of the HHV-6B U19 gene is identified as a 47 kDa transcriptional activator. HHV-6B infection or overexpression of U19 transactivated the RANTES promoter. Mutational analysis of the promoter indicated that transactivation was not critically dependent on the promoter sites CRE, NF-κB, ISRE or NF-IL6. ND10 are nuclear substructures that are involved in several cellular regulatory pathways, including those controlling gene expression. HHV-6B infection resulted in a reduced number of ND10 structures, but with a concomitantly increased level of promyelocytic leukaemia (PML) protein expression and mRNA induction. The U19 protein co-located to ND10 with PML and heterochromatin protein 1 (HP1), but whilst PML formed a ring structure, U19 also localized to the centre of ND10. Knockdown of PML by small interfering RNA did not prevent U19 localization to ND10-like foci, but instead led to a fourfold increase in U19-induced transcription from the RANTES promoter. Generation of four truncated U19 proteins indicated that the N-terminal portion of the protein contains a sequence responsible for nuclear localization; a domain in the N-terminal half of U19 is responsible for its ND10 localization, whereas the C-terminal portion contains the transactivation domain. None of the truncated proteins retained full transactivating ability on the RANTES promoter. Thus, U19 is a transcriptional activator that co-localizes with PML and localizes to ND10-like foci independently of PML, yet is regulated negatively by PML or its associated proteins.

INTRODUCTION

Human herpesvirus 6 (HHV-6) is a betaherpesvirus that was first discovered in 1986 in patients suffering from a lymphoproliferative disorder (Josephs et al., 1986; Salahuddin et al., 1986). Based upon molecular, genetic and epidemiological data, HHV-6 has been divided into two distinct variants, HHV-6A and -6B, which share approximately 90 % nucleotide similarity (Schirmer et al., 1991; Dominguez et al., 1999; Isegawa et al., 1999). Primary HHV-6B infection usually leads to a symptomatic disease in children within 2 years of birth in >90 % of the population (Yamanishi et al., 1988; Okuno et al., 1989; Ward et al., 1993). This disease, exanthema subitum, is characterized by acute febrile illness with high fever lasting for a few days, followed by the appearance of a rash (Yamanishi et al., 1988), and is a frequent cause of emergency visits. Both HHV-6A and HHV-6B are capable of infecting cells in the central nervous system, suggesting that these viruses are neurotropic and could be involved in neuronal disease, although HHV-6A may be more neurotropic than HHV-6B (Chan et al., 2001; Donati et al., 2005). HHV-6B primarily infects T cells and monocytes and enters the cells through a receptor complex involving the ubiquitously distributed molecule CD46. Indeed, HHV-6B displays tropism for a wide range of cells expressing this cell-surface receptor (Lusso et al., 1991; Santoro et al., 1999).

Despite the high level of nucleotide similarity between HHV-6A and HHV-6B, these viruses behave biologically differently, and they may regulate the expression from their homologous open reading frames (ORFs) differently. One gene exhibiting a differential expression pattern is U19, a gene residing in the IE-B locus. Despite a high degree of...
similarity at the nucleotide level, U19 appears to be expressed differentially in HHV-6A and -6B (Mirandola et al., 1998). Depending upon their mRNA expression pattern, HHV-6B genes can be divided into three classes, immediate-early (IE), early (E) and late (L) genes (Øster & Hollberg, 2002). IE genes are transcribed shortly after infection and include a number of known and suspected transcription factors for E and L genes. Based upon homology with the HCMV UL38 gene product, the product of the HHV-6B U19 gene is suspected to be an IE protein with a possible transactivating function, although this remains to be investigated (Colberg-Poley et al., 1992; Nicholas & Martin, 1994; Gompels et al., 1995).

Many IE gene products in a wide range of DNA viruses locate to small nuclear substructures known as nuclear domain 10 (ND10), promyelocytic leukemia (PML) nuclear bodies or promyelocytic oncogenic domains (PODs) (Everett, 2001). Immunofluorescence staining identifies ND10 structures as between five and 50 spherical nuclear dots, ranging from 0.3 to 1.0 μm in diameter (Dellaire & Bazett-Jones, 2004). ND10 domains consist of a large array of more or less loosely associated proteins, which move in and out of the ND10 bodies dependent on e.g. the phase of the cell cycle or signals generated by DNA damage. A number of proteins often present at the ND10 include PML, Sp100, Daxx, BML, ATRX, CBP, TOPOIII and HP1 (Ishov et al., 1999; Luciani et al., 2006). The PML protein is essential for recruitment of many of the ND10-associated proteins to ND10 and plays a key role in complex cellular processes, such as apoptosis, cell-cycle regulation and viral infection (Ishov et al., 1999; Everett, 2006; Tavalai et al., 2006). The presence of both transcriptional repressors and activators in addition to chromatin-remodelling proteins at ND10 has led to the idea that ND10 is important in transcriptional regulation (Boisvert et al., 2001; Wu et al., 2001).

The role of ND10 during viral infection is controversial. After nuclear entry, the genomes of herpes simplex virus type 1 (HSV-1) and human cytomegalovirus (HCMV) are recruited to ND10, followed by the recruitment of viral IE gene products (Ishov & Maul, 1996; Ahn et al., 1999). The targeting of viral genomes and IE proteins to ND10 may suggest that these structures are important for generating the optimal transcriptional environment for viral replication. Alternatively, ND10 may be important as an intracellular antiviral-defence mechanism. The PML protein functions as an inhibitor of viral replication during infection by HSV-1, poliovirus or HCMV (Everett, 2006; Everett et al., 2006; Pampin et al., 2006; Tavalai et al., 2006) and viral proteins may cause disruption of ND10 structures early during infection (Chelbi-Alix & De, 1999; Everett, 2001). The recruitment of IE proteins to ND10 is also seen during infection by HHV-6A and HHV-6B. Importantly, the ND10 structures are not destroyed, but appear to fuse during infection by these viruses (Gravel et al., 2002; Stanton et al., 2002). We show here that the U19 ORF of HHV-6B is transcribed into an unspliced mRNA product. We demonstrate that U19 is indeed a transcriptional activator of RANTES and, moreover, localizes to ND10. Surprisingly, during PML knockdown by small interfering RNA (siRNA), the speckled, ND10-like staining pattern of U19 localization was intact, suggesting that U19 is not recruited to nuclear foci by PML. Furthermore, PML knockdown led to increased U19-dependent transcription of the RANTES promoter. Thus, PML acts as part of the cellular antiviral-defence mechanism by repressing U19-enhanced transcription, but is not responsible for recruiting U19 to nuclear foci.

**METHODS**

**Cell cultures, virus and antibodies.** The human epithelial colon carcinoma cell line HCT116 (a gift from B. Vogelstein and K. W. Kinzler, Johns Hopkins University School of Medicine, Baltimore, MD, USA) was grown in McCoy’s 5A medium (Gibco) supplemented with 10% fetal calf serum (Sigma), glutamine (0.2 g l⁻¹), streptomycin (0.2 g l⁻¹), penicillin (0.2 g l⁻¹) and HEPES (10 mM) (all from the Substrate Department, Bartholin Building, University of Aarhus). HHV-6B (strain PL1) was propagated in MOLT3 cells, and a virus stock at a titre of 3200 TCID₅₀ was produced as described previously (Øster et al., 2006). For infections, the stock was diluted 1:10 in McCoy’s supplemented medium. The following antibodies were used: mouse monoclonal IgG anti-HHV-6B protein p41 (Advanced Biotechnologies Inc.), mouse monoclonal IgG anti-PML (DAKO), rabbit polyclonal anti-PML (Abcam), rabbit polyclonal anti-HP1 (Abcam), Alexa Fluor 488-conjugated rabbit F(ab')₂ anti-mouse (Invitrogen), Alexa Fluor 546-conjugated rabbit F(ab')₂ anti-mouse (Invitrogen), Alexa Fluor 488-conjugated goat F(ab')₂ anti-rabbit (Invitrogen), fluorescein isothiocyanate (FITC)-conjugated rabbit polyclonal anti-DDDDK Flag tag (Abcam), rabbit polyclonal anti-DDDDK Flag tag (Abcam) and the DNA dye TO-PRO-3 iodide 642/661 (Invitrogen).

**Cloning of U19.** All PCRs were performed upon cDNA made from MOLT3 cells infected with HHV-6B (strain PL-1). Normal PCR for cloning into the pcDNA3.1/TOPO-V5-HIS plasmid (Invitrogen) was performed with the end-specific primers 1 and 2 (see Supplementary Table S1), with HindIII or EcoR1 overhangs. Semiquantitative PCR was performed on a LightCycler instrument (Roche Applied Science) with U19 internal primers 5 and 6 and β-actin internal primers 16 and 17 (Supplementary Table S1), using SYBR green detection reagent (Øster & Hollberg, 2002). The relative amount of U19 mRNA, estimated by semiquantitative PCR analysis, was normalized against the amount of β-actin mRNA. The full-length U19 ORF was amplified by PCR and cloned into the pcDNA3.1/TOPO-V5-HIS plasmid by use of a TOPO cloning kit (Invitrogen). Flag-tagged U19 was generated by cloning the full-length U19 ORF into an MCS-modified pcDNA3/FLAG plasmid via restriction enzymes EcoRI and HindIII. The truncated constructs were made by PCR amplification of the pcDNA3.1/TOPO-V5-HIS-U19 plasmid with primers 3, 4, 8, 9, 10, 11, 12 and 13 (Supplementary Table S1) and cloned into the MCS-modified pcDNA3/FLAG plasmid via restriction enzymes EcoRI and HindIII. All constructs were verified by sequencing reactions using primers specific for the T7 and BGH
sequencing sites in the pcDNA3 and pcDNA3.1 vectors, and an internal U19-specific primer (Supplementary Table S1).

**Transfections.** Cells used for immunoﬂuorescence analysis were transfected by using FuGENE-6 transfection solution (Roche). Transfections were performed according to the manufacturer’s instructions. Cells used for Western blotting analysis were transfected by using a Nucleofector device with Nucleofector solution V and program D-37 (all from Amaxa). Transfections were performed according to the manufacturer’s instructions (Amaxa). The stable U19-expressing HCT116 cell line was generated by transfecting HCT116 cells with the pcDNA3.1/TOPO-U19 construct using the FuGENE-6 system, followed by 3 weeks selection in medium containing geneticin G-418. U19-positive clones were identiﬁed by RT-PCR analysis. Cells used for siRNA knockdown experiments were transfected with SMARTpool PML siRNA (Dharmacon), again using a Nucleofector device with Nucleofector solution V and program D-37. The transfections were performed according to the manufacturer’s instructions (Amaxa).

**RANTES ELISA assay.** Two experiments each were performed on peripheral blood mononuclear cells (PBMCs) extracted from two different persons, yielding a total of four measurements. Cells were infected with HHV-6B (320 TCID50) for 24 h, after which the amount of RANTES in the supernatant was measured by using a Quantikine Human RANTES immunoassay (R&D Systems). Spectroscopic measurements were performed on a Multiscan EX ELISA reader.

**Luciferase measurements.** HCT116 cells were transfected with plasmids pcDNA3/FLAG-U19, pcDNA3/FLAG-U19A1–194, pcDNA3/FLAG-U19A44–200, pcDNA3/FLAG-U19A205–302 or pcDNA3/FLAG-U19A306–389, together with pGL2-RANTES-luc, pGL2-RANTES-mutCRE-luc, pGL2-RANTES-mutNF-kB1-luc, pGL2-RANTES-mutSRE-luc or pGL2-RANTES-mutNF-il6-luc, by using the Amaxa transfection system [the pGL2-RANTES-luc constructs were kindly provided by A. Casola (Casola et al., 2001)]. Luciferase activity was measured 48 h post-transfection with the Luciferase 1000 assay system (Promega) on an Ascent Luminoskan. When measuring luciferase activity during infection, HCT116 cells were transfected with one of the pGL2-RANTES-luc constructs and incubated for 24 h, after which the cells were infected with HHV-6B and incubated for an additional 24 h before measuring the luciferase activity. Luciferase measurements during PML knockdown were performed by co-transfecting one of the pGL2-RANTES-luc constructs together with PML siRNA (SMARTpool siRNA; Dharmacon) into an HCT116 cell line stably expressing U19. Cells were incubated for 48 h before measuring luciferase activity.

**Confocal microscopy.** Cells were transfected with pcDNA3/FLAG-U19, pcDNA3/FLAG-U19A1–194, pcDNA3/FLAG-U19A44–200, pcDNA3/FLAG-U19A205–302 or pcDNA3/FLAG-U19A306–389 and incubated for 24 h. Cells were then transferred to poly-l-lysine coated slides and incubated for an additional 24 h, followed by ﬁxation in 4% formalin/PBS (pH 7.5). Cells were washed twice in PBS, blocked in 5% BSA/PBS and permeabilized in 0.2% Triton X-100/PBS. HHV-6B infection was visualized by using a monoclonal antibody (mAb) against HHV-6B p41 (1:200). PML was visualized by using a mAb directed against the N-terminal part of the protein (1:200). Secondary antibodies were either an F(ab’)2, antibody conjugated with Alexa Fluor 488 (Invitrogen) (1:500) or an F(ab’)2, antibody conjugated with Alexa Fluor 546 (Invitrogen) (1:500). Flag-tagged U19 protein was visualized by using an FITC-conjugated polyclonal antibody against the DDDDK Flag tag (1:200). The nucleus was visualized by using the DNA dye TO-PRO-3 iodide 642/661 (1:1000). Imaging was done by using the 488 nm line of the multiline argon laser, the 543 nm line of the green helium–neon laser and the 633 nm line of the helium–neon laser on an upright LEICA TCS SL confocal microscope with a ×40 oil-immersion objective.

Quantification of ND10 domains was done by counting the total number of PML bodies in uninfected cells and at different time points during HHV-6B infection. The total cell number analysed was 52 wild-type (wt) cells, 59 cells at 16 h post-infection (p.i.), 27 cells at 24 h p.i. and 33 cells at 48 h p.i. The relative amount of PML was measured by marking the nucleus of cells and measuring the mean intensity in the PML channel, after which the channel was switched to p41 and cells were identiﬁed as either p41-positive or p41-negative. The total number of analysed cells was 22 p41-positive and 22 p41-negative cells at 24 h p.i., and 16 p41-positive and 16 p41-negative cells at 48 h p.i.

**RESULTS**

**Analysis of the U19 transcript**

PCR performed with U19-speciﬁc end primers on cDNA from HHV-6B (strain PL-1)-infected MOLT3 cells yielded a single band on agarose gels. This is consistent with an unspliced 1180 bp mRNA product corresponding to the predicted U19 ORF (Fig. 1a). Sequencing of the ampliﬁed PCR product conﬁrmed that this was indeed the entire unspliced U19 ORF. PCR ampliﬁcation with primers speciﬁc for a previously reported splice site in U19 (Mirandola et al., 1998) (Supplementary Table S1) gave no detectable bands (data not shown). Semi-quantitative PCR performed on cDNA 2 h after HHV-6B infection of MOLT3 cells revealed induction of U19 mRNA (data not shown).

The complete U19 ORF was cloned into the pcDNA3/FLAG vector, which is translated into an N-terminal Flag-tagged U19 protein (Fig. 1b). Western blotting analysis of whole-cell lysates from pcDNA3/FLAG-U19-transfected HCT116 cells probed with a Flag tag-speciﬁc antibody revealed a distinct protein band of approximately 47 kDa, corresponding to the predicted size of U19 (Fig. 1c).

**U19 transactivates the RANTES promoter**

HHV-6B primarily infects T cells and monocytes, but is also capable of infecting other cell types (Lusso et al., 1991, 1993; He et al., 1996; Luppi et al., 1999). We have shown previously that the human colon carcinoma cell line HCT116 can be infected by HHV-6B (Øster et al., 2006). The U19 protein has not been examined previously, but is predicted to be a transactivator based on homology between the U19 and the HCMV UL38 genes. To demonstrate transactivating ability on a complex promoter directly, we chose a RANTES full-length promoter construct and an HIV-LTR promoter construct, both containing a luc reporter gene.

As HHV-6A infection leads to RANTES production by the infected cells (Caruso et al., 2003; Csoma et al., 2006), we first established whether HHV-6B was able to induce RANTES production. Infection by HHV-6B led to an increased level of RANTES in the supernatant (Fig. 2a). To establish whether this was due to increased transcription of
the RANTES promoter, we examined whether HHV-6B was able to induce RANTES-driven luciferase activity. Infection by HHV-6B activated the RANTES promoter and induced luciferase activity to a level approximately fourfold higher than that in mock-infected cells (Fig. 2b).

To examine whether HHV-6B was dependent on a known specific promoter sequence or, conversely, led to overall enhanced transcription, we examined RANTES promoters mutated in the CRE, NF-κB, ISRE and NF-IL6 sites. Although mutation of the NF-κB site impaired the production of luciferase significantly, it also impaired basal luciferase induction. In contrast, mutated ISRE and perhaps NF-IL6 sites led to increased transcription (Fig. 2b). Thus, when comparing fold induction, none of the mutants were deleterious to HHV-6B-induced luciferase activity.

We next examined whether U19 was able to transactivate the RANTES promoter. HCT116 cells transfected with U19 demonstrated a fivefold increase in RANTES-driven luciferase activity compared with mock-transfected cells (Fig. 2c). Similar to HHV-6B infection, U19 transactivation of the RANTES promoter was not critically dependent on any of the tested promoter sites (CRE, NF-κB, ISRE or NF-IL6). There was, however, a tendency toward increased transcription following mutation of the NF-IL6 site (Fig. 2c). To eliminate the possibility that the observed differences were due to different U19 expression levels after co-transfections, we co-transfected HCT116 cells with U19 together with either RANTES or one of the four mutated RANTES constructs, and analysed the cells by Western blotting. This demonstrated that expression of U19 was comparable between the different co-transfections (Fig. 2d).

In conclusion, both HHV-6B infection and overexpression of U19 led to increased transcription from the RANTES promoter. Transactivation of the promoter was not prevented by mutation of the CRE, NF-κB, ISRE or NF-IL6 sites in the promoter.

We next wanted to establish whether the transactivating ability of U19 is specific for the RANTES promoter. We tested the transactivating ability of both transient and stably integrated U19 on the HIV-LTR promoter, a promoter known to be activated by several HHV-6B transactivators. U19-expressing HCT116 cells showed a 12-fold increase in luciferase activity compared with mock-transfected cells (Fig. 2e).

**U19 localizes to ND10**

In order to determine the subcellular localization of U19, we transfected HCT116 cells with U19-Flag or mock plasmid. This allowed us to analyse the localization of U19 by confocal microscopy using an antibody specific for the Flag tag. As shown in Fig. 3(a), U19 localized to the nucleus in a speckled pattern resembling ND10 structures. ND10 are large protein complexes with varying protein content depending on cellular status, and have been implicated in a number of cellular pathways, including the responses to DNA damage and cellular stress (Dellaire & Bazett-Jones, 2004; Everett, 2006). One of the major structural proteins in ND10 is the PML protein, and PML−/− mouse primary embryonic fibroblasts show disruption of the ND10 structures (Ishov et al., 1999). Analysis of PML localization in U19-transfected HCT116 cells showed co-localization between U19 and PML, demonstrating that U19 was indeed recruited to ND10 (Fig. 3a).

The centre of ND10 domains contains DNA and chromatin proteins, including heterochromatin protein 1 (HP1) (Luciani et al., 2006). Immunofluorescence analysis of HP1 localization shows a diffuse distribution in the nucleus with increased staining in ND10 areas (Bartova et al., 2007).
We co-stained U19-transfected cells with antibodies against U19–Flag and HP1 to further strengthen the identification of the U19 staining pattern as ND10. Anti-HP1 antibodies gave rise to a diffuse staining in the nucleus, with HP1 present in areas positive for U19 (Fig. 3b). A single ND10 domain contains a number of different protein layers; the PML protein localizes to the outer layers, giving rise to a doughnut shape at high magnification by confocal microscopy (Luciani et al., 2006). Scanning a single ND10 domain at high magnification revealed U19 staining within the doughnut-shaped PML staining (Fig. 3a, insert). This indicated that U19 did not bind PML directly, but rather interacted with another protein partner.

In order to determine which domain in U19 is responsible for its localization to ND10, we generated four truncated versions of Flag-tagged U19 (Fig. 4a). Full-length and truncated U19 constructs were expressed transiently in HCT116 cells, which were subsequently stained with antibodies specific for the Flag tag and PML. The N-terminally truncated U19 protein (U19D1–94) failed to enter the nucleus and thus could not co-localize with PML. The N-terminal domain may therefore contain either a nuclear localization signal (NLS) or a region responsible for binding to a shuttle protein (Fig. 4b). The U19D94–200 protein was transported to the nucleus, but failed to co-localize with PML or ND10 structures (Fig. 4b). The U19D205–302 protein gave a diffuse nuclear staining, but also localized to the ND10 structures with PML co-localization. In addition, U19D205–302 also gave a diffuse cytoplasmic staining (Fig. 4b). The C-terminal truncation (U19A306–389) allowed localization to the nucleus in a manner similar to that seen in cells expressing the full-length U19 protein, with PML co-localization in ND10-like dots (Fig. 4b).
**Fig. 3.** Distribution of endogenous PML and U19–Flag within the nuclei of HCT116 cells. Confocal microscopy images of representative individual cells transfected with U19-Flag. (a) Cells were stained with antibodies against the Flag tag (FITC) and against PML (Alexa Fluor 546). The insert shows a closer scan of a single ND10 domain (white box). (b) Cells were stained with antibodies against the Flag tag (Alexa Fluor 546) and against HP1 (Alexa Fluor 488). Arrows indicate areas with intense HP1 staining and co-localization between U19 and HP1. The cells were stained with TO-PRO-3 to visualize nuclei. Bar, 1 μm.

**Fig. 4.** Localization and transactivating function of truncated U19. (a) Schematic representation of the truncated U19 proteins. (b) Distribution of the four truncated U19 proteins in transfected HCT116 cells. Confocal microscopy images of representative cells are shown. Flag-tagged proteins were visualized with a specific polyclonal antibody (FITC), PML was visualized with an N-terminal-specific mAb (Alexa Fluor 546) and nuclei were visualized with TO-PRO-3. (c) Luciferase activity (RLU) in HCT116 cells co-transfected with the RANTES-luc construct together with U19 or one of the four truncated U19 constructs. All measurements are means of two independent experiments performed with 10 measurements in each experiment; SD is indicated on top of each bar.
We next tested the transactivating ability of the four truncated U19 constructs by co-transfecting these plasmids into HCT116 cells together with the full-length RANTES-luc construct. None of the U19-truncated mutants were able to transactivate the RANTES promoter in the same manner as full-length U19, although the Δ94–200, Δ205–302 and Δ306–389 mutants appeared to retain very modest transactivating ability, if any (Fig. 4c). We cannot exclude the possibility that the N-terminal part of U19 contains the transactivating domain, as U19Δ1–94 failed to enter the nucleus. Alternatively, the absence of a sequence within the C-terminal half of the protein abolished transactivation, although U19Δ205–302 and U19Δ306–389 could localize to ND10 structures.

**The amount of PML protein increases and the number of ND10 bodies decreases during infection**

ND10 is known to be a dynamic structure that may change during HHV-6B infection. To identify HHV-6B-infected HCT116 cells, we used an antibody against the HHV-6B polymerase processivity factor p41 encoded by U27. Upon HHV-6B infection, the p41 protein appears as a number of nuclear dots, some of which are associated loosely with PML (Fig. 5a).

We quantified the number of ND10 bodies during infection by counting ND10 bodies in a number of randomly selected cells, which were subsequently identified as infected or uninfected based on p41 staining. The number of ND10 bodies decreased steadily during HHV-6B infection, from a mean of 12 in mock-infected cells to a mean of 6 at 48 h p.i. (Fig. 5b). It was not possible to quantify ND10 bodies in cells 72 h p.i., because of syncytium formation. The PML protein was still found in a distinct, punctate pattern after 72 h p.i. and stayed in close proximity to p41, as seen at earlier time points during infection (Fig. 5a). The intensity of PML staining of the ND10 structures appeared to increase during infection (Fig. 5a). We measured the PML fluorescence intensity of a number of cells and subsequently stratified this according to the presence or absence of p41 staining (i.e.
HHV-6B infection). The mean PML intensity increased by more than twofold in infected cells, despite the reduced number of ND10 structures (data not shown). This indicated an increased amount of PML protein in HHV-6B-infected cells. To establish whether there was increased transcription of the PML gene in infected cells, we analysed cDNA from infected and uninfected cells by semiquantitative PCR with primers specific for the N-terminal part of the PML gene, ensuring identification of all isoforms. The amount of PML mRNA increased significantly during infection, corresponding to the observed increase in fluorescence intensity (Fig. 5c).

U19 can localize to nuclear foci in the absence of PML

Many proteins, such as Sp100 and Daxx, fail to localize to ND10 domains in the absence of PML, indicating a major structural role of PML in recruiting other proteins to ND10 (Tavalai et al., 2006). We therefore wanted to examine the importance of PML for the U19-localization pattern. HCT116 cells were co-transfected with U19-Flag and PML siRNA and analysed by confocal microscopy. Whilst PML was knocked down and was not present in ND10, U19 continued to locate in speckled nuclear foci (Fig. 6). This demonstrated that U19 was capable of localizing to nuclear foci in the absence of PML.

PML inhibits U19 transactivation of RANTES

We wanted to determine the effect of PML knockdown upon the transactivating ability of U19 on the RANTES promoter. We first generated a HCT116 clone that stably expressed U19. This clone was co-transfected with the RANTES-luc construct or PML siRNA, or both, and luciferase intensity was measured and compared with transfections in wt HCT116 cells. The clone stably expressing U19 transfected with RANTES-luc and PML siRNA showed an activity increased by more than threefold compared with that of the clone transfected with RANTES-luc alone, and increased by more than 10-fold compared with that of wt cells transfected with RANTES-luc only (Fig. 7a). This indicated that PML exerted a negative-regulatory role on U19-induced transcriptional activation. In order to eliminate the possibility that the increased transcription during PML knockdown was due to increased U19 protein levels after siRNA treatment, we analysed HCT116 cells transfected with U19 with or without PML siRNA by Western blotting. There was no alteration in U19 protein levels during PML knockdown (Fig. 7b).

DISCUSSION

Similar to HHV-6A, the HHV-6B genome contains two distinct IE gene loci: the IE-A locus (ORF U86–U92) and the IE-B locus (ORF U16–U19) (Gompels et al., 1995; Dominguez et al., 1999; Isegawa et al., 1999). The IE-A locus encodes at least two proteins, IE-1 and IE-2 (Takeda et al., 1996; Papanikolaou et al., 2002), of which IE-1 has been shown to localize to ND10 domains during infection and to be involved in transcriptional regulation (Gravel et al., 2002). Whilst the IE-A locus genes and their protein products have been studied, little is known about the genes residing in the IE-B locus. In this report, we cloned the entire U19 gene and partly mapped the domain structure of U19, which is a nuclear protein of approximately 47 kDa.

Infection by HHV-6A stimulates production of RANTES in infected cells (Csoma et al., 2006). Similarly, HHV-6B also induces RANTES production through activation of the RANTES promoter. The U19 protein was capable of transactivating RANTES and could transactivate the CRE-, NF-κB-, ISRE- and NF-IL6-mutated promoters. U19 activity was reduced on all four mutated promoters, but when compared with the basal activity seen in wt cells, U19 induced responses that were four- to sixfold higher, even...
on the strongly inhibited NF-κB-mutated promoter. This suggests that U19 does not activate transcription directly, but rather that it enhances normal cellular transcription. U19 transactivates RANTES in a manner similar to that seen during HHV-6B infection and could thus be responsible for increased RANTES production in infected cells, although other viral factors might play a role. Furthermore, U19 transactivated the HIV-LTR promoter, demonstrating a more general transactivating ability.

U19 localized to the nucleus in an ND10-like pattern and co-localized with PML, although U19 was distributed all over the ND10 domain, whereas PML was present in the outer layers. The core of ND10 domains contains DNA and it is possible that U19 interacts with DNA either directly, or indirectly through a DNA-binding protein that is also present in the core of ND10 domains. ND10 domains are small, nuclear structures involved in gene transcription and the DNA-damage response (Dellaire & Bazett-Jones, 2004). Many of the proteins that are recruited to ND10 are SUMO-modified, and it appears that the SUMOylation is essential for correct ND10 targeting (Shen et al., 2006). It has been demonstrated that the HCMV proteins IE-1 and IE-2 and the HHV-6B protein IE-1, which all localize to ND10, are SUMO-1-modified (Gravel et al., 2002; Sadanari et al., 2005). The HHV-6B IE-2 protein, however, is not SUMO-1-modified, although it does associate with ND10 domains (Tomoiu et al., 2006). This led us to investigate whether U19 was SUMO-1-modified. Co-immunoprecipitation analysis of U19 and SUMO-1 gave no indications of SUMO-1 modification (data not shown). This is consistent with the notion that SUMO-1 modification is not essential for ND10 targeting of all viral ND10-interacting proteins.

Transactivating ability was reduced significantly in all U19-truncated proteins. When the first 94 aa were deleted, U19 lost the ability to enter the nucleus and thus had no transactivating function. Although no known NLS is present in the N-terminal domain of U19, these data suggested strongly that this domain was responsible for nuclear entrance. The U19A94–200 mutant was able to enter the nucleus, but did not associate with ND10 and failed to transactivate the RANTES promoter. The U19A205–302 protein gave a diffuse nuclear staining, which included the ND10 structures. In contrast, the U19A306–389 mutant did localize to ND10, but had significantly reduced transcriptional activity compared with full-length U19. This suggests that a sequence within the N-terminal half of U19 is responsible for ND10 binding, whereas the C-terminal domain is required for full transcriptional activity. Additional mutations of U19 will be needed to map these functions further.

It has been noted previously that ND10 domains appear to accumulate into fewer subnuclear bodies during infection by HHV-6B (Gravel et al., 2002). In this report, we quantified the mean number of ND10 bodies in a single cell at different time points during HHV-6B and mock infections. During HHV-6B infection, the ND10 domains accumulated into approximately half the number of bodies at 48 h p.i. Despite the accumulation into fewer ND10 bodies, the PML protein was upregulated, either through increased transcription or through increased stability of the PML mRNA. The PML protein localized in close proximity to HHV-6B protein p41 early during infection, but the two proteins became associated more loosely later during infection, when the number of p41 dots increased and the number of PML dots decreased.

Knockdown of PML in cells transiently expressing the U19 protein did not disrupt U19 nuclear foci. This supports the notion that U19 may bind in the DNA core of ND10, as PML knockdown has been shown to disrupt the speckled staining pattern of the Daxx and Sp100 proteins, which both reside in the outer layers of ND10 (Ishov et al., 1999; Everett et al., 2006; Luciani et al., 2006). When PML was knocked down in cells stably expressing the U19 protein and transiently expressing a RANTES-luc construct, U19-induced transcription was enhanced, indicating that U19 was inhibited by PML. This corresponds well with earlier studies that ascribed PML a repressor role on both viral and cellular transcription factors (Alcalay et al., 1998; Vallian et al., 1998; Tavalai et al., 2006).

**Fig. 7.** PML negatively regulates U19 transactivation of the RANTES promoter. (a) HCT116 cells stably expressing U19 were mock-transfected or transfected with a RANTES-luc construct, PML siRNA or both. Luciferase activity was measured 48 h after transfection with PML siRNA. All measurements are means of two independent experiments performed with 10 measurements in each experiment; SD is indicated on top of each bar. (b) Western blotting analysis of lysates from HCT116 cells transfected with U19-Flag with or without PML siRNA, immunoblotted with antibodies against Flag tag, PML and GAPDH.
PML inhibits infection by a number of different viruses, including HCMV, HSV-1 and poliovirus (Everett, 2006; Everett et al., 2006; Pampin et al., 2006; Tavalai et al., 2006). One of the ways in which PML might inhibit viral infection is through repression of viral transcription factors, such as U19 in HHV-6B. PML is involved in regulation of transcription through chromatin remodelling and is recruited to specific loci, rather than just sites of high transcriptional activity (Kumar et al., 2007). The co-localization of ND10 and the genomes from HCMV or HSV-1 early in infection may therefore be an antiviral defence aimed at inhibiting viral IE transcription factors, rather than an exploitation of the transcription machinery by the virus. Thus, HSV-1 and HCMV infection tends to eliminate ND10 structures. In contrast, the fusion of ND10 during HHV-6B infection may succeed in inhibiting viral transcription. Indeed, HHV-6B infection is known to be much slower than infection by HCMV and particularly HSV-1.

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