Acetylation of the foamy virus transactivator Tas by PCAF augments promoter-binding affinity and virus transcription

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It was shown recently that retrovirus transactivators interact with transcriptional coactivators, such as histone acetyltransferases (HATs). Foamy viruses (FVs) direct gene expression from the long terminal repeat and from an internal promoter. The activity of both promoters is strictly dependent on the DNA-binding transactivator Tas. Recently, it was shown that Tas interacts with the HATs p300 and PCAF. Based on these findings, it is demonstrated here that PCAF has the ability to acetylate Tas in vitro and in vivo. Tas acetylation resulted in enhanced DNA binding to the virus promoters. In vitro transcription reactions on non-chromatinized template showed that only acetylated Tas enhanced transcription significantly. These results demonstrate that acetylation of the FV transactivator Tas may be an effective means to regulate virus transcription.

Virus transcription is often regulated by the interaction of the cellular transcription machinery with viral components. Retroviruses have developed multiple ways to activate virus transcription. Integrated retrovirus genomes are packaged into chromatin, which inhibits transcription at the initiation and elongation steps. For transcription to proceed, viruses must remodel the chromatin structure. Thus, viruses must recruit cellular proteins to alleviate nucleosome-mediated transcriptional repression. Besides ATP-dependent remodelling complexes, which facilitate transcription by altering the nucleosome position, transcriptional coactivators with histone acetyltransferase (HAT) activity influence promoter activity (Becker & Horz, 2002; Struhl, 1998). HATs alter histone–DNA interactions and nucleosome-stability functions through acetylation of N-terminal lysines of the core histones (Muth et al., 2001). In addition, complex retroviruses encode regulatory proteins, which influence transcription of virus templates (Cullen, 1991, 1998; Jordan et al., 2001). It was shown that virus transactivators of deltaretroviruses and lentiviruses recruit HATs to their respective promoters. Deltaretroviruses encode the transcriptional transactivator Tax, a CREB/ATF-binding protein. Tax–ATF complexes bind to Tax-responsive elements located in the long terminal repeat (LTR) upstream of the transcription start site and thereby recruit p300 to the promoter region (Bex & Gaynor, 1998). CBP/p300 recruitment leads to acetylation of histones, resulting in chromatin remodelling. Furthermore, CBP/p300 interacts with the RNA polymerase II holoenzyme and subsequently activates LTR promoter-directed transcription (Bex & Gaynor, 1998). Lentiviruses encode Tat, an RNA-binding protein required to recruit cellular elongation complexes (Zhu et al., 1997). This is necessary for phosphorylation of the Pol II C-terminal domain, resulting in the successful elongation of primary transcripts. During transcription, Tat itself is acetylated by p300 and recruits the p300/CBP-associated factor (PCAF) to the promoter region (Bres et al., 2002; Kaehlcke et al., 2003; Kiernan et al., 1999). Functional consequences of the p300–PCAF interaction for chromatin structure are still to be discovered, but Tat-associated factors seem to alter the nucleosome structure of the lentivirus promoter (Jordan et al., 2001).

Foamy viruses (FVs) direct gene expression from the LTR promoter like all other retroviruses but, in addition, possess an internal promoter (IP) located in the env region. The LTR promoter directs gene expression of the structural proteins and virus enzymes, whereas regulatory genes are expressed almost exclusively from the IP (Bodem et al., 1998a; Holzscheu et al., 1998; Löchelt et al., 1993). The activity of both promoters is strictly dependent on the FV Tas transactivator protein. It was shown for the prototype primate FV (PFV) and for Feline foamy virus (FFV) that Tas is a direct DNA-binding protein, containing at least two functional domains, the N-terminal DNA-binding domain and the C-terminal activation domain (Bodem et al., 2004; Garrett et al., 1993; He et al., 1996; Kang et al., 1998). The herpesvirus VP16 activation domain can functionally replace the TAS acidic activation domain, suggesting that both domains are functional homologues (Garrett et al., 1993). In both promoters, Tas binds directly to target DNA sequences located upstream of the transcription start site and thereby activates promoter function. It was reported that the IP contains a high-affinity Tas-binding site and is
therefore the first to become active (Kang et al., 1998). The promoter-binding sites for Tas are virus species-specific and adapted to the corresponding Tas proteins (Bodem et al., 2004).

The molecular mechanisms of transcriptional activation by Tas remain unclear. It was shown that the PFV and FFV Tas proteins are fully functional in yeast (Kang et al., 1998; J. Bodem, unpublished observation), indicating that all required cellular factors are conserved in eukaryotes. In addition, Bannert et al. (2004) reported that PFV Tas interacts with the cellular HATs p300 and PCAF. Overexpression of both HATs was shown to increase transactivation by Tas. Whether this effect was based on the changed histone–DNA interaction or on the changed transactivation properties remained open. In this report, the functional consequences of the PCAF–Tas interaction on FV transcription were analysed.

To demonstrate the interaction of Tas with PCAF, 293T cells were co-transfected with FLAG-tagged PCAF and pCMV–PFV Tas (Muth et al., 2001; Bodem et al., 1998b). In control reactions, PFV Tas was replaced by complete FFV Tas or FFV Tas lacking the last 20 aa (Δ20C–FFV Tas). Neither protein is recognized by PFV Tas antiserum. Cells were lysed in immunoprecipitation buffer [5 mM MgCl₂, 350 mM NaCl, 20 mM Tris/HCl (pH 7.9)] by sonication 2 days post-transfection and Tas complexes were precipitated by using PFV Tas antiserum coupled to protein G–agarose. Beads were washed intensively with the incubation buffer. Immunoprecipitates were analysed for co-precipitated PCAF by immunoblotting with anti-FLAG (M2) antibody as described by the manufacturer (Sigma-Aldrich). As shown in Fig. 1(a) (lane 6), PFV Tas co-precipitated FLAG–PCAF. In control reactions with beads alone (lane 5), lysates from untransfected cells (lane 2), cells transfected with FFV Tas or Δ20C–FFV Tas (lanes 3 and 4), no FLAG–PCAF was detected. These experiments show that Tas interacts specifically with complexes containing PCAF in mammalian cells and corroborate previous findings (Bannert et al., 2004). To analyse whether PCAF interacts directly with Tas, pull-down assays were performed. Equal amounts of immobilized maltose-binding protein (MBP), MBP–PFV Tas or MBP–FFV Tas fusion protein were incubated with glutathione S-transferase (GST)–PCAF for 2 h at 4 °C in immunoprecipitation buffer. Beads were washed extensively and bound proteins were analysed by Western blotting with anti-GST antibody (Fig. 1b). Recombinant PCAF interacted specifically with both Tas proteins. We conclude that the PCAF–Tas interaction is a conserved mechanism.

To analyse whether the interaction of Tas with cellular HATs results in Tas acetylation, in vitro acetylation assays were performed. Highly purified recombinant MBP–FFV Tas immobilized on amylose beads was incubated with 0.5 mg transcription-competent nuclear extract from HeLa cells and acetyl-CoA at 30 °C for 30 min. Beads were washed in order to remove unincorporated acetyl-CoA and cellular proteins, and immobilized MBP–FFV Tas was analysed by

![Fig. 1. Interaction of FV Tas proteins with PCAF.](image)

**Fig. 1.** Interaction of FV Tas proteins with PCAF. (a) Co-precipitation of PCAF with PFV antibodies. Cells were co-transfected with FLAG-tagged PCAF and FFV Tas proteins were precipitated with anti-PFV Tas antibodies and immobilized on protein G–Sepharose. Co-precipitated PCAF was visualized by immunoblotting using anti-M2 antibody. Lanes 3 and 4 refer to extracts of cells transfected with constructs of FFV Tas, which does not cross-react with the PFV Tas antiserum. (b) Direct interaction of PCAF with FFV Tas was analysed by pull-down assays using immobilized MBP–Tas proteins. (c) Tas is acetylated by nuclear extracts. Recombinant MBP or MBP–FFV Tas was incubated with acetyl-CoA and nuclear extracts. Acetylation was determined by immunoblotting with anti-FLAG antibody. (d) In vitro acetylation of FV Tas. Immobilized MBP–FFV Tas was incubated with [³H]acetyl-CoA with recombinant GST, GST–PCAF, GST–PCAF–BX, a PCAF variant containing the catalytic domain (Muth et al., 2001), and a HAT-domain deletion mutant, GST–ΔHAT–PCAF. (e) Acetylation of PFV Tas in vivo. Cells were co-transfected with constructs for PCAF and PFV Tas or FFV Tas. Tas was precipitated by using PFV Tas antiserum and analysed by immunoblotting with Tas (lanes 1–3) or anti-acetyllysine antiserum (lanes 4–6). (f) In vivo acetylation of FFV Tas. 293T cells were transfected with two independent clones (cl1 and cl2) specifying HA-tagged FFV Tas. FFV Tas was precipitated by anti-HA mAb and acetylation was determined by anti-acetyllysine immunoblot analysis (lanes 2 and 3). Control lanes in (a), (e) and (f) represent mock-transfected cells.
immunoblotting using anti-acetyl-lysine serum (Muth et al., 2001). The antibody reacted strongly with MBP–FFV Tas, but not with MBP alone (Fig. 1c). This showed that Tas is acetylated by a cellular activity present in the extracts.

After confirmation of the Tas–PCAF interaction, we investigated whether the latter catalyses the observed acetylation of Tas. To analyse this, in vitro protein acetyltransferase assays were carried out as described by Muth et al. (2001) using recombinant MBP–FFV Tas protein as substrate. Bead-bound MBP–FFV Tas was incubated at 30°C with PCAF and [3H]acetyl-CoA in 150 mM KCl, 20 mM Tris/HCl (pH 7.9), 5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol for 30 min. Protein beads were washed with buffer containing 300 mM KCl to remove unincorporated acetyl-CoA and the acetylated proteins were visualized by fluorography. As shown in Fig. 1(d), FFV Tas was acetylated in vitro by PCAF (lanes 2 and 3). In the control reactions, neither GST alone nor ΔHAT–PCAF acetylated Tas (Fig. 1d, lanes 1 and 4). These results demonstrate in vitro acetylation of FV Tas by PCAF.

To investigate the in vivo acetylation of FV Tas, 293T cells were transfected with pCMV–PFV Tas or pCMV–FFV Tas. The cells were washed 2 days post-transfection with PBS and lysed by sonication in immunoprecipitation buffer (Muth et al., 2001). PFV Tas was precipitated with the specific antiserum. The precipitates were analysed by immunoblotting using anti-acetyl-lysine (Sigma-Aldrich) (Fig. 1e, lanes 1–3) antibodies. Precipitated Tas protein was found to be acetylated in pCMV–PFV Tas-transfected cells (Fig. 1e, lane 5). The specificity of the reaction was demonstrated by using empty protein G beads or by precipitation from cells expressing FFV Tas that is unreactive with the rabbit serum used (Fig. 1e, lanes 1, 3, 4 and 6).

To show that acetylation of FV Tas proteins is a general feature, we co-transfected 293T cells with FLAG-tagged PCAF and HA-tagged FFV Tas. The cells were lysed in AM350 buffer (350 mM KCl) by sonication, and cellular debris was removed by centrifugation 2 days post-transfection (Muth et al., 2001). FFV Tas was precipitated with anti-HA tag mAb immobilized on protein G-Sepharose. Acetylation of FFV Tas bound to the beads was subsequently analysed by immunoblot with anti-acetyl-lysine serum. As shown in Fig. 1(f) (lanes 2 and 3), acetylated FFV Tas protein was precipitated with HA antibodies. The observation that Tas acetylation is conserved between primate and feline FVs suggests that it plays an important role in transcriptional activation.

As the FV Tas proteins bind directly upstream of the transcription start site and for other transcription factors, such as TAF68, it was shown that acetylation influences their DNA-binding properties (Muth et al., 2001), we performed gel-retardation assays (electrophoretic mobility-shift assay; EMSA) with an FFV LTR fragment and purified recombinant MBP–FFV Tas protein. FFV Tas was shown to bind specifically to a fragment encompassing nt −70 to −50 relative to the start of transcription. Retarded complexes were shown to be supershifted by anti-MBP antibodies and could be outcompeted by an oligonucleotide encompassing this region (Bodem et al., 2004). To determine the binding conditions of MBP–FFV Tas to the LTR promoter, a fragment from nt −80 to +22 was used in EMSA with titrated Tas from 7.5 to 180 ng. MBP–FFV Tas and the promoter fragment were incubated in binding buffer at 25°C for 30 min (Bodem et al., 2004). DNA–protein complexes were dissociated during PAGE and visualized by autoradiography (Fig. 2a). The equivalent amount of free and shifted probe in this experiment was reached with 30 ng Tas (Fig. 2a, lane 5). To analyse whether the DNA-binding affinity was raised upon Tas acetylation, an EMSA with lower protein concentrations (from 0.2 to 7.5 ng) was performed (Fig. 2b). In the case of an elevated DNA-binding affinity of acetylated Tas, a shifted complex should be detectable at lower protein concentrations than upon

Fig. 2. Tas acetylation enhances promoter-binding affinity. (a) Increasing amounts of FFV Tas were titrated in EMSAs using a 32P-labelled FFV LTR probe encompassing nt −80 to +22 to determine Tas–DNA affinities. Amounts of Tas (in ng) are indicated on top of the lanes. Reactions were analysed by native PAGE. (b) To analyse changes in DNA affinity upon Tas acetylation, EMSAs using the LTR promoter probe and MBP–FFV Tas, preincubated at 30°C with either GST–PCAF or GST for 1 h, were performed. Increasing amounts of the preincubated MBP–FFV Tas protein (0.2, 0.4, 0.8, 1.7, 3.5 and 7.5 ng) were incubated with the promoter probe. DNA–protein complexes were analysed by native PAGE followed by exposure to X-ray film.
incubation with unacetylated Tas. MBP–FFV Tas was preincubated with either purified recombinant GST–PCAF and acetyl-CoA (Fig. 2b, lanes 2–7) or GST and acetyl-CoA (Fig. 2b, lanes 8–13) at 30 °C before allowing for complex transformation with the LTR fragment that encompasses the FFV Tas-binding site. After incubation, protein–DNA complexes were analysed by native PAGE followed by autoradiography. Fig. 2 demonstrates that lower concentrations of acetylated Tas were sufficient to shift the LTR probe than was possible with unacetylated Tas (Fig. 2a, b). Therefore, acetylated Tas has a higher DNA-binding affinity than the unmodified protein.

To analyse the effects of Tas acetylation on transcription, an in vitro transcription system was established with nuclear extracts from exponentially growing (8 × 10⁵ cells ml⁻¹) HeLa cells and an FFV IP fragment encompassing nt −300 to +1 relative to the promoter start site, driving a luciferase gene as template.

Purified MBP–FFV Tas was acetylated with recombinant PCAF and acetyl-CoA at 30 °C for 30 min. For a control, the same amount of recombinant MBP–FFV Tas was incubated with GST or PCAF–ΔHAT (data not shown). Template (100 ng), reaction mix (Muth et al., 2001) and Tas protein were preincubated for 10 min to allow protein–DNA complex formation. After this preincubation, 35 µg HeLa nuclear extract and NTPs [0.66 mM each of ATP, GTP and CTP, 0.01 mM UTP, and 0.5 µCi (18.5 kBq) [α-32P]UTP (5000 Ci mmol⁻¹)] were added and in vitro transcriptions were allowed to proceed at 30 °C for 1 h. RNA was purified and analysed by denaturing PAGE. In this system, the template DNA is in a non-chromatinized form and side effects of histone acetylation should therefore be eliminated. MBP–FFV Tas alone activated transcription slightly from template DNA is in a non-chromatinized form and side effects of histone acetylation should therefore be eliminated. MBP–FFV Tas alone activated transcription slightly from an FFV IP fragment encompassing nt −300 to +1 relative to the promoter start site, driving a luciferase gene as template.

In this report, we show that Tas interacts with PCAF and is subsequently acetylated. This recruitment of HAT activity was described previously for the lentivirus Tat protein (Kaehlcke et al., 2003). Tat was shown to interact with HATs and to be acetylated by p300. This acetylation step was found to be essential for successful elongation. FV Tas acetylation enhances DNA-binding affinity. This finding diverges from that for the Tat protein, for which it was shown that nucleic acid (RNA)-binding activity was not influenced by acetylation (Kaehlcke et al., 2003). By modifying the binding affinity of its transactivator, FV adapts a strategy that was shown for several cellular transcription factors, such as TIF2IB, p53 and GATA-I (Boyes et al., 1998; Gu & Roeder, 1997; Muth et al., 2001). Tas acetylation leads to augmented FV transcription on a non-chromatinized template. This observation is in line with the model of a stabilized initiation complex, which results in transcriptional activation of the FV promoters or an enhanced elongation rate by acetylated Tas. The in vitro transcription system used in this study reflects modulation of the initiation and elongation step and therefore supports both mechanisms. However, we cannot exclude the possibility that acetylation also changes the Tas transactivation domain. The consequence would probably be a modification of the activity of both protein functions in a cooperative way.

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References


