The Cellular Protein Daxx Interacts with Avian Sarcoma Virus Integrase and Viral DNA To Repress Viral Transcription

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The cellular protein Daxx was identified as an interactor with avian sarcoma virus (ASV) integrase (IN) in a yeast two-hybrid screen. After infection, Daxx-IN interactions were detected by coimmunoprecipitation. An association between Daxx and viral DNA, likely mediated by IN, was also detected by chromatin immunoprecipitation. Daxx was not required for early events in ASV replication, including integration, as Daxx-null cells were transduced as efficiently as Daxxexpressing cells. However, viral reporter gene expression from ASV-based vectors was substantially higher in the Daxx-null cells than in Daxx-complemented cells. Consistent with this observation, histone deacetylases (HDACs) were found to associate with viral DNA in Daxx-complemented cells but not in Daxx-null cells. Furthermore, Daxx protein was induced in an interferon-like manner upon ASV infection. We conclude that Daxx interacts with an IN-viral DNA complex early after infection and may mediate the repression of viral gene expression via the recruitment of HDACs. Our findings provide a novel example of cellular immunity against viral replication in which viral transcription is repressed via the recruitment of antiviral proteins to the viral DNA.

The integration of retroviral DNA into the host genome is essential for the efficient and stable production of progeny virus. After the entry and uncoating of the virus, its RNA genome is reverse transcribed by the virally encoded reverse transcriptase. The newly synthesized double-stranded viral DNA associates with several viral proteins, as well as host proteins, to form a nucleoprotein structure known as the preintegration complex. To gain access to host chromatin, the preintegration complex must be transported from the cytoplasm, the site of reverse transcription, to the nucleus. There, the virally encoded enzyme, integrase (IN), catalyzes the joining of the viral ends to the cellular DNA. After the integration process is completed, viral DNA is stably propagated with the host DNA during cellular DNA replication and division.

These early events in viral replication are facilitated by various host cell factors. Host proteins likely mediate the trafficking of the preintegration complex to the nucleus, entry of the preintegration complex into the nucleus via the nuclear pore or during nuclear membrane breakdown, docking with and accessing the host chromatin, and repair of the integration intermediate. Because IN associates with viral DNA soon after its synthesis and remains associated with the viral DNA until integration is complete, it is a prime candidate for recruitment of the host factors required to complete these events.

Several host proteins have been found to copurify with retroviral preintegration complexes, including HMGa1 (14) and BAF (30), but none have been reported to act directly with a viral protein to provide a function that is essential for viral replication. Yeast two-hybrid and coimmunoprecipitation experiments have identified Ini-1 (28) and LEDGF/75 (5) as human immunodeficiency virus type 1 (HIV-1) IN interactors. While a role for Ini-1 in virion particle production has been established (45), no direct role in integration in vivo has yet been demonstrated. However, Ini-1 can stimulate integration in vitro (28) and appears to translocate from the nucleus to the cytoplasm in response to infection (43). LEDGF/75, in addition to stimulating integration in vitro (5), is required for the nuclear localization of ectopically expressed HIV-1 IN (5). Importin-7 has also been found to promote the nuclear import of HIV-1 reverse transcription complexes (15). DNA-dependent protein kinase and other components of the nonhomologous end joining repair pathway (7, 8), the ataxia-telangiectasia-mutated kinase (ATR) (6), and the polyADP-ribose polymerase (PARP) (20) have all been implicated in the repair of the retroviral integration intermediate.

In contrast to proteins that support retroviral replication, other host proteins may be engaged in an antiviral response against the incoming virus. APOBEC3G, a cytidine deaminase, becomes incorporated into murine leukemia virus and HIV-1 virions and mediates the degradation of viral DNA during the infection of the next cell (reviewed in reference 17). Vif, an HIV-1 accessory protein, counteracts the effects of APOBEC3G (38). Other host proteins have been demonstrated to restrict the replication of murine leukemia virus and HIV-1. TRIM5α, from the rhesus monkey, has been identified as a restriction factor against HIV-1 infection in primate cells (40). A related TRIM protein, promyelocytic leukemia (PML) protein, confers resistance to various viruses, including the spumavirus foamy virus (35). PML is also a member of the interferon-inducible gene family that includes many antiviral proteins.

In this study, we conducted a yeast two-hybrid screen with avian sarcoma virus (ASV) IN as bait to identify HeLa cell
interacting clones were selected for growth on medium without leucine and further screened by using the secondary yeast two-hybrid β-galactosidase screen. No β-galactosidase activity in controls with biocid, c-raf, or cmyc, using the IN as bait, was observed.

**Purification of GST-Daxx (466-740).** The EcoRI/XhoI fragment of HeLa 50, corresponding to amino acids 466 to 740 in Daxx (Fig. 1A), was moved into a GEX vector (a gift from E. Golemis). The generated glutathione S-transferase (GST) fusion protein GST-Daxx (466 to 470) was introduced into MCI061 bacterial cells, which were then grown overnight at 29°C. Expression of the fusion protein was induced with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 2 h at 29°C. After bacterial cells were pelleted, they were resuspended in phosphate-buffered saline (PBS) with 1 mM AEBSF ([4-(2-aminoethyl)-benzenesulfonyl fluoride] and 10 mg of lysozyme. Cells were sonicated on ice with a Vibra cell sonicator (Sonics & Materials, Inc.). Triton X-100 was added to a 1% final concentration. The bacterial lysate was mixed with glutathione Sepharose 4B (Sigma) overnight with gentle agitation. Beads were washed four times with PBS. Beads were resuspended in GST buffer (50 mM Tris, pH 8.0, 0.5 M NaCl, and 10 mM CHAPS (3-(3-cholamidopropyl)-dimethylammonio)-1-propanesulfonate)) with 1% bovine serum albumin. The amount of GST fusion protein bound to beads was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

**In vitro pull-down assay.** Five hundred nanograms of purified GST-Daxx (466-740) GST fusion protein was mixed with 100 ng of ASV integrase in GST buffer in the presence of 1 mM EDTA and 1 mM (4-(2-aminoethyl)benzenesulfonyl fluoride. Beads were washed twice. Samples were resuspended in protein loading buffer (50 mM Tris, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 10% 2-mercaptoethanol), subject to electrophoresis in SDS-polyacrylamide gels, transferred to a nitrocellulose filter and immunoblotted with rabbit α-IN antibody and antibody to GST.

**Infections.** All infections were performed with filtered (0.45-μm-pore-size) virus-containing cell supernatants in the presence of 10 μg of DEAE-dextran/ml. Virus-containing media were typically removed after 2 to 4 h of incubation with target cells. Viral titers were determined by the transduction of HeLa cells. Green fluorescence protein (GFP) transduction was quantified 2 to 3 days post-infection by flow cytometry as previously described (29). Cells were treated with 100 ng of trichostatin A/ml for 24 h prior to infection.

**Immunoprecipitation.** HeLa cells (5 × 10⁶) were infected with the ASVA-CMV GFP vector at a multiplicity of infection (MOI) of 0.5 to 3.0 at 37°C in the presence of 10 μg of DEAE-dextran/ml. At indicated times post-infection, cells were rinsed with PBS and lysed with cold IP-lysis buffer (50 mM Tris [pH 7.6], 150 mM NaCl, 1 mM EDTA, 1% Igepal C-630/NP-40, 0.5% sodium deoxycholate) supplemented with 10 mM NaCl, 25 mM EDTA, and 1% SDS. Cross-links were reversed by heating, tates were collected, and DNA was eluted from the beads with 50 mM Tris, 250 mM NaCl, Tris, pH 8.0, 1% SDS, 1.0% NP-40, 0.5% sodium deoxycholate supplemented with 10 μg of aprotinin/ml, 10 μg of leupeptin/ml, 0.1 mM sodium orthovanadate, 1 μM β-glycerophosphate, and 1 mM sodium fluoride) on ice. The chromatin was fragmented by sonication to an average length of 1 kb. Cellular debris was removed and the lysate was cleared with salmon sperm DNA-coated protein A agarose (Upstate). Lysates were incubated overnight in the presence of 5 μg of antibody and salmon sperm DNA-coated protein A agarose. Immunoprecipitates were extracted with protein loading buffer, run on SDS-polyacrylamide gels, transferred to Immobilon-P transfer membranes (Millipore), immunoblotted with rabbit α-Daxx antibody, and developed with chemiluminescence (Pierce).

**Chromatin immunoprecipitation.** HeLa or Daxx cells (5 × 10⁶) were infected with the ASV vector. At indicated times, cells were fixed with a final concentration of 1% formaldehyde for 30 min at room temperature. Crude nuclear fractions were prepared by treating the collected cells with nuclei extraction buffer (5 mM PIPES [piperazine-N,N′-bis(2-ethanesulfonic acid)], 85 mM KCl, 0.5% Igepal C-630/NP-40) on ice and then pelletted at 5,000 × g. The nuclei were then lysed with IP-lysis buffer (rat α-Daxx) or RIPA buffer (150 mM NaCl, Tris, pH 8.0, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate) supplemented with 10 μg of aprotinin/ml, 10 μg of leupeptin/ml, 0.1 mM sodium orthovanadate, 1 μM β-glycerophosphate, and 1 mM sodium fluoride) on ice. The chromatin was fragmented by sonication to an average length of 1 kb. Cellular debris was removed and the lysate was cleared with salmon sperm DNA-coated protein A agarose (Upstate). Lysates were incubated overnight in the presence of 5 μg of antibody and salmon sperm DNA-coated protein A agarose. Immunoprecipitates were collected, and DNA was eluted from the beads with 50 mM Tris, 250 mM NaCl, 25 mM EDTA, and 1% SDS. Cross-links were reversed by heating, and the samples were incubated with proteinase K (Roche). DNA was extracted with phenol-chloroform and precipitated with ethanol in the presence of 10 μg of glycyogen (Roche). Dried pellets were resuspended in water. Twenty-four PCR cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s were performed with indicated primers. Nested pol primers correspond to first round primers 5′-GTG ACT GAC TCT GCG TTT G-3′ and 5′-ATG GCC TTT GGT CTT CCC and second round primers 5′-GGT GTC CAC AGT AAC AGC-3′ and 5′-GCG GAG GAG TCA TCT GGA AGT-3′. Nested PCR products were detected by ethidium

**Fig. 1. Interaction between ASV IN and human Daxx.** (A) Yeast two-hybrid analysis was conducted using ASV IN as bait and a human cDNA library in pJG4-5 as prey. The overlapping open reading frames isolated that correspond to Daxx are shown in relationship to a schematic representation of the full-length protein. Two putative nuclear localization signals (NLSs), a putative coiled-coil domain, and acidic residues were depicted. The number of times that each clone was isolated is indicated in parentheses. (B) GST-Daxx (466-740), GST, or beads alone were incubated with (+) or without (–) purified ASV IN and collected on glutathione beads. Samples were analyzed by Western blotting with rabbit α-IN antibody and 5 ng of purified ASV IN as a marker.

**Materials and Methods**

**Cells.** The DF-1 chicken embryo fibroblast line (37) was a gift from D. Foster. Daxx cell lines were described previously (25).

**Antibodies.** Rabbit α-ASV IN and murine monoclonal α-IN A1 have been described previously (32). Rabbit α-Daxx was a gift from A. Hollenbach. Rabbit α-HDAC1, α-HDAC2, and α-hemagglutinin tag were used in ChIP from Upstate. Murine monoclonal α-actin (Sigma) was used as a gel loading control. The Fox Chase Cancer Center Laboratory Animal Facility generated rat α-Daxx from His-tagged clones for HeLa 2 and HeLa 18 (Fig. 1). Antibody was purified on a Murine monoclonal Daxx cell lines were described previously (25).

**IN-α-Daxx IN antibody and 5 ng of purified ASV IN as a marker.**
bromide staining in agarose gels. For Southern blots, long terminal repeat (LTR) primers 5'-AAA AGC ACC GTG CAT GC-3' and 5'-AGG TGC ACA ATG TGG TG-3' were used. Amplified DNA was detected by Southern hybridization with a random-labeled LTR probe (Prime-It II; Stratagene) generated from the PCR primers 5'-GAT TGG TGG AAG TAA GGT GG-3' and 5'-GAA ATG GCG TTT ATT GTA TCG-3' by following the manufacturer's instructions. Control primers 5'-TTT CC ACCT TTC ACC ATT CC-3' and 5'-GGC AGA TCA CAT ACC CTG TT-3' corresponding to a region of the p21 promoter, were used with Southern blotting as a DNA-negative control. Products were quantified with a bio-imaging analyzer (Fuji).

Quantitative real-time PCR. Immunoprecipitated viral DNA was also quantified by real-time PCR using an amplicon designed near the ASV LTR. The primers 5'-TGGA GAT GGC CGG-3' and 5'-TCG TTA CCA TGT TGC AAG ACT-3' were used with a probe, (FAM) CCG TTG ATT CCC TGA CTA CGA GCA (BHQ), that was synthesized with the fluorescent dye 6-carboxyfluorescein (FAM) and the nonfluoresgenic Black Hole Quencher, BHQ-1 (Biotech Solutions Technologies). Each reaction contained 1 × reaction buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl), 0.25 mM concentrations of each dNTP, 2.5 mM MgCl2, 400 nM concentrations of primers, 200 nM probe, and 2.5 U Platinum Taq polymerase (Invitrogen). Results were obtained with calculations using a standard curve generated from a serial dilution of a viral plasmid.

Integration quantitation by B2 PCR analysis. Quantitation for integrated DNA was performed as described previously (20) with some modifications. Daxx cell lines were infected with the ASVA-CMVGFP vector at an MOI of 0.1 for 4 h in the presence of 10 μg of DEAE-dextran/ml. DNA was isolated 48 h postinfection with the DNAeasy tissue kit (Qiagen) according to the manufacturer's instructions. Fifty, 100, and 200 ng of chromosomal DNA were used in the first-round PCR with B2 primer 5'-TTT TTC ACCT TTC ACC ATT CC-3' and ASV viral primer 5'-GGC TTC TGT TGT ACG CGG TTA GGA GT-3'. Samples were denatured for 2 min at 94°C and then subjected to 20 PCR cycles of 94°C for 40 s, 65°C for 40 s, and 72°C for 1 min 30 s. Products were diluted (1:1,000) in water to reduce the unintegrated DNA background such that the product was B2 primer dependent, subjected to a second round of PCR using the LTR primers, and the products were analyzed by the Southern blotting procedure used in ChIP.

Detection of Daxx protein. HeLa cells (106) were infected at an MOI of 1.0 with filtered ASVA-CMVEGFP or mock infected with filtered DF-1 supernatants. At the indicated times, the cells were lysed in protein loading buffer. Lysates were loaded on a polyacrylamide gel. Proteins were transferred to Immobilon-P transfer membrane (Millipore), blotted with appropriate antibodies, and developed with chemiluminescence (Pierce).

RESULTS

Yeast two-hybrid screen with ASV integrase. ASV normally replicates in avian cells, but with the proper pseudotyping, it can transduce mammalian cells. Transduction, as measured by reporter gene expression, requires completion of all early events in virus replication, including integration. In order to identify host proteins that may be involved in integration, a yeast two-hybrid screen of a human cDNA library was conducted with ASV IN as bait. A screening of 4.5 × 106 cDNAs from a HeLa cell library resulted in the isolation of nine overlapping clones corresponding to four open reading frames. These clones were subsequently identified as derivatives of the gene that encodes a 740-amino-acid cellular protein, Daxx (Fig. 1A). To verify that the interaction between Daxx and IN occurs in vitro, the Daxx fragment from the largest isolated clone, containing amino acids 466 to 740, was fused to GST. When mixed with purified integrase, this GST-Daxx (466-740) fusion could specifically pull down approximately 5% of the input IN protein under the most stringent conditions used, while no detectable IN was bound to GST alone (Fig. 1B). This test confirmed the yeast two-hybrid interaction between ASV IN and Daxx in vitro and suggested that Daxx and ASV IN interact after infection.

Daxx was originally isolated as a potentiatior of Fas-mediated apoptosis (44). Subsequent investigations have demonstrated a nuclear role for Daxx as a component of ND10 (24, 42) and as a modulator of transcriptional activity in development (4, 23). These nuclear functions and DNA binding capabilities of Daxx initially suggested that it might play a role in accessing or docking the ASV preintegration complex to the host chromatin. Our first efforts therefore focused on testing this hypothesis.

Detection of the association of ASV IN and Daxx after infection. Because Daxx and ASV IN interacted in vitro, we first looked for coimmunoprecipitation of Daxx and IN after infection with an ASV vector. This ASV vector, ASVA-CMVEGFP, expresses a GFP reporter gene from the CMV immediate early promoter and can infect mammalian cells, as it encodes the murine amphototropic env gene (29). Coimmunoprecipitation of Daxx with IN antibodies was detected after infection of the HeLa cells with this ASV vector at an MOI of 1 to 2 transducing virions per cell (Fig. 2A). To follow this interaction temporally, samples were collected at various times after infection and immunoprecipitated with anti-IN antibodies. This analysis revealed Daxx-IN interaction between 4 and 24 h postinfection (Fig. 2B). Three forms of Daxx were detected in the lysates, a 70-kDa form and two forms of 98 and 120 kDa that are modified by phosphorylation (23) and SUMOylation (27). Unmodified Daxx was the major form present in the coimmunoprecipitate. The significance of the unmodified form of Daxx binding to ASV IN is unclear, as the function of the different forms of Daxx is unknown. The amount of Daxx immunoprecipitated with IN was approximately 1% of the total in the lysate. At the low MOI (1 to 3) used in these experiments, only a few nanograms of IN would be transmitted to the infected cells. With this limited quantity of IN, we would expect to immunoprecipitate only a small percentage of the much more abundant cellular Daxx, as is consistent with our observations.

Association of Daxx with viral DNA as detected by chromatin immunoprecipitation. To determine if Daxx associates with the preintegration complex, chromatin immunoprecipitation (ChIP) experiments were performed. As the quantity of total viral DNA increases during the first several hours after infection, both Daxx association and total viral DNA were monitored. ChIP analysis of ASV-infected HeLa cells demonstrated that Daxx was associated with viral DNA, and no association was observed after ChIP, using rat preimmune serum (Fig. 2C). These experiments also showed the expected IN-viral DNA association as a positive control. The rat α-human Daxx antibody used in these studies cross-reacted with proteins of sizes similar to those of mammalian Daxx in avian cells (70 and 120 kDa; data not shown), and we found that this antibody also precipitated viral DNA from ASV-infected avian DF-1 cells early after infection (Fig. 2C). The association of Daxx with viral DNA does not imply a direct interaction but one likely to be mediated by IN.

A temporal analysis of the association of Daxx and IN with viral DNA (Fig. 2D) showed that the IN association reaches a maximum earlier than Daxx. Similar results were obtained with real-time PCR analysis (not shown). This result is expected, as the IN domain is also present in the β subunit of ASV reverse transcriptase. Because Daxx is primarily a nuclear protein, it might be expected to associate with IN only after the preinte-
The preintegration complex enters the nucleus. These results are consistent with the coimmunoprecipitation data (Fig. 2B), which show maximum association between 8 and 12 h postinfection. These findings are also consistent with the hypothesis that Daxx is recruited to the preintegration complex via its association with IN. Neither the IN nor Daxx antibodies precipitated DNA corresponding to the cellular p21 promoter (DNA negative control; data not shown).

**Requirements for Daxx association with viral DNA.** Although the PCR primers used for ChIP analysis were more than 1 kb away from the CMV promoter and the GFP reporter gene in the ASVA-CMVEGFP vector, it seemed possible that Daxx might be recruited specifically to these nonviral sequences. To verify that the interaction between Daxx and the preintegration complex was independent of these elements, a different ASV vector was used. ASVA-LTRNEO contains a neomycin reporter gene under the control of the ASV LTR promoter (8). ChIP analysis of cells infected with this vector also showed a specific association of Daxx with viral DNA (Fig. 2E). Therefore, the CMV promoter and the GFP reporter gene sequences do not account for the Daxx association with viral DNA documented in Fig. 2D.

To determine if integration is required for Daxx to be associated with viral DNA, an ASVA-LTRNEO vector (8) encoding a catalytically inactive integrase (D64E IN) was used. This vector is competent for viral DNA synthesis but is severely defective in transduction. To monitor integration, we measured the association of viral DNA with HDAC4 by ChIP. HDAC4 is a histone deaceteylase (HDAC) that has been found to form foci in response to DNA damage (6). Consistent with...
other evidence that retroviral DNA integration is detected as DNA damage by the cells, HDAC4 foci are formed after infection of HeLa cells with the integrase competent vector but not the integration-deficient cell line, in which expression was ca. 50% lower. The percentage of GFP-positive cells did not change after 6 days in culture (Fig. 3A). Because reporter gene transduction should require both integration and expression of the vector DNA, the B2 PCR technique (20) was used to measure integration directly (Fig. 3B). This method detects integrations that occur within several kilobases of the high-copy-number B2 sequences in the mouse genome. Quantification of the B2 PCR products in the null and complemented lines showed no significant difference in the relative efficiencies of integration, with the exception of the slight decrease observed for +C-complemented cell lines (Fig. 3C). This result is consistent with a direct correlation between integration of this ASV vector and reporter gene expression. Because detection by B2 PCR requires that all early events occur, including entry, uncoating, reverse transcription, nuclear entry, and integration, are completed, we conclude that all of these early events proceed similarly in the Daxx-null and -complemented cell lines. These results suggest that Daxx is not required for integration. However, as it is clear that Daxx binds to ASV IN, and associates with the ASV viral DNA within the preintegration complex, we considered alternative roles for Daxx in the preintegration complex.

**Amount of Daxx protein increases after ASV infection.** While conducting the coinmunoprecipitation experiments, we observed that the amount of Daxx protein in the cell lysates appeared to increase after ASV infection. Because Daxx is an interferon-inducible protein, it seemed likely that this increase

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**FIG. 4. Increase in the amount of Daxx protein after ASV infection.** HeLa cells were lysed at indicated times after infection with ASVA-CMVEGFP or mock infection with control, virus-free (DF-1) medium. Daxx and β-actin were detected by immunoblotting with rabbit α-Daxx and murine α-β-actin antibody after SDS-polyacrylamide gel electrophoresis analysis. A representative Western blot is shown. Arrows indicate the expected mass in kilodaltons of different forms of Daxx. Lines mark the positions of molecular mass standards.
amount of Daxx protein, compared to a medium from uninfected avian cells). As shown in Fig. 4A, the increasing times after infection or mock infection (conditioned was the result of an antiviral or stress response to the incoming virus. This rapid, virus-induced increase in Daxx expression under these conditions, indicating that the increase is not due to a stress response or specific effects of factors produced by avian cells. This rapid, virus-specific increase in Daxx protein may represent either the induction of Daxx transcription or a stabilization of the Daxx protein. HeLa cells treated with alpha interferon respond similarly, with a robust increase in Daxx protein (data not shown). As shown in Fig. 5B, the mean GFP intensity in the Daxx-null cells was ca. 25 to 40% higher than that in the complemented cells, confirming the results from microscopy. This difference in GFP expression in positive cells was independent of the MOI in the range of 0.01 to 10 (data not shown).

To examine the possibility that this difference was related to GFP expression from the CMV promoter, a similar analysis was carried out with ASVA-LTREGFP. This vector is identical to ASVA-LTRNEO, described above, except that the GFP reporter is substituted for the neomycin resistance gene (29). The results showed that the intensity of expression of GFP from the ASV LTR promoter in this vector is significantly lower (almost twofold) than that from the CMV promoter, as expected. However, as shown in Fig. 5C, there were similar differences in expression of GFP in the Daxx-null cell line and the reconstituted cell lines. These data indicate that the reduction of reporter expression in the Daxx-complemented lines is independent of the CMV promoter, and they are consistent with the hypothesis that Daxx associates with a complex that includes viral DNA.

Because Daxx protein expression is heterogeneous in the reconstituted cell lines and subject to gene silencing effects, the cell lines were treated with trichostatin A (an HDAC inhibitor) to increase the expression of the Daxx constructs (41). Such treatment resulted in a twofold increase in the number of cells with detectable Daxx expression in the complemented cell lines and, by immunofluorescence, appeared to increase slightly the Daxx intensity (data not shown). As shown in Fig. 5B, treatment of the Daxx cell lines with trichostatin A prior to infection resulted in a significant decrease in GFP reporter intensity in +WT-complemented cells compared to untreated cells, while only a slight reduction was seen for Daxx-null cells. This result supports the notion that increased Daxx expression leads to decreased GFP reporter expression. We also observed that after trichostatin A treatment, GFP intensity increased in +N-complemented cells whereas the intensity did not change significantly in the +C-complemented cells. Similar results were obtained with the ASVA-LTREGFP vector (Fig. 5C).

Daxx-dependent recruitment of HDACs to ASV DNA. In considering possible mechanisms for the reduction of transduced gene expression in Daxx-expressing cells, we asked if Daxx might recruit cellular repressive factors to viral DNA. It is generally assumed that decreases in gene expression are correlated with reduced histone acetylation via the action of histone deacetylases at promoter regions in chromatin (reviewed in reference 10). HDAC2 has been found in a large complex with Daxx (22) and has been suggested to play a role in Daxx-mediated repression. HDAC1 has also been found to interact with Daxx (4). We therefore used ChIP to determine if HDAC1 or HDAC2 was associated with viral DNA in ASVA-LTRNEO-infected Daxx-expressing cells. As shown in Fig. 6, both HDAC1 and HDAC2 associate with viral DNA in the +WT-complemented cell line, whereas no association was detected for the Daxx-null cell line. In contrast, HDAC4 is

FIG. 5. Decreased reporter expression in Daxx-complemented cells. (A) GFP expression from Daxx-null and -complemented cell lines 2 days after infection with ASVA-CMVEGFP at an MOI of 1.0. Representative fields of confluent monolayers are shown. (B) Mean GFP intensities of GFP positive cells after infection with ASVA-CMVEGFP at MOI, 0.1, not treated (filled bars) or treated with 100 ng of trichostatin A/ml (open bars). Shown are mean fluorescent intensities and standard deviations (error bars), determined by flow cytometry, from three samples. (C) The same cells as in panel B but infected with ASVA-LTREGFP at an MOI of 0.2.

was the result of an antiviral or stress response to the incoming virus. To test this possibility, cell lysates were collected at increasing times after infection or mock infection (conditioned medium from uninfected avian cells). As shown in Fig. 4A, the amount of Daxx protein, compared to a β-actin control, rapidly increased (Fig. 4B) after infection of HeLa cells, before peaking at 8 to 10 h postinfection. Mock-infected cells showed little increase in Daxx expression under these conditions, indicating that the increase is not due to a stress response or specific effects of factors produced by avian cells. This rapid, virus-specific increase in Daxx protein may represent either the induction of Daxx transcription or a stabilization of the Daxx protein. HeLa cells treated with alpha interferon respond similarly, with a robust increase in Daxx protein (data not shown) (31).

The role of Daxx in retroviral gene expression. As described above, using B2 PCR, we observed no significant differences in the integration efficiencies of the Daxx-null cell line and the complemented lines. However, when GFP expression from the Daxx cell lines was examined, substantial differences were observed. In the Daxx-null cells, the amount of GFP expressed appeared to be substantially higher than that in the Daxx-complemented cell lines (Fig. 5A). The effect of complementation is likely underestimated because, as noted above, only a subpopulation of complemented cells expresses Daxx. To verify that GFP reporter expression is lower in the complemented cells, the mean GFP fluorescence intensity in the cells that scored positive for GFP expression was examined by flow cytometry. As shown in Fig. 5B, the mean GFP intensity in the Daxx-null cells was ca. 25 to 40% higher than that in the complemented cells, confirming the results from microscopy. This difference in GFP expression in positive cells was independent of the MOI in the range of 0.01 to 10 (data not shown).

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the deacetylation of histones. Daxx binds to ASV IN, associates with viral DNA, and recruits HDAC1 and HDAC2 to viral DNA in both cell lines. These results suggest a potential mechanism for Daxx-mediated repression whereby Daxx binds to ASV IN, associates with viral DNA, and recruits HDAC1 and HDAC2 to viral DNA to repress transcription via the deacetylation of histones.

**DISCUSSION**

Here we report the identification of Daxx as an interactor of ASV IN. IN was chosen as the bait protein because it associates with viral DNA during reverse transcription and remains associated with the viral DNA until it is integrated. Throughout the early events in the retroviral replication cycle, IN may interact with host proteins necessary for trafficking or completion of the integration process. We note that IN interactors could also play a role in the assembly and packaging steps during the late events of the retroviral life cycle.

The interaction between Daxx and ASV IN was discovered via a yeast two-hybrid screen and verified in vitro by coimmunoprecipitation. Coimmunoprecipitation and ChIP demonstrated that this interaction also occurs after infection of cells with ASV and is therefore likely to be physiologically relevant. The association of Daxx with viral DNA in human and avian cells suggests that Daxx is a conserved ASV IN binding protein. ChIP analysis of cells infected with an integration-defective [IN(D64E)] vector demonstrated that Daxx can associate with unintegrated DNA. Time course experiments suggest that Daxx is recruited to an IN-viral DNA complex at 8 to 10 h postinfection but Daxx may remain associated with the viral DNA through other interactions at later times.

Several methods were used to determine if Daxx plays a role in retrovirus-mediated transduction, i.e., stable viral DNA integration and efficient reporter gene expression. Knockdown of Daxx protein with small interfering RNA resulted in reduced ASV transduction, but this treatment also reduced the cellular growth rate and increased apoptotic cell death (data not shown), both of which could account for the reduced transduction. Daxx-null cells derived from a knockout mouse, used in subsequent experiments, did not show growth defects compared to the +WT-complemented line. Because similar amounts of integrated DNA were detected in the Daxx-null and complemented cell lines, we conclude that Daxx is not required for the early steps in ASV infection, including all events necessary for stable integration. The C terminus of Daxx, which interacts with IN, as determined by the yeast two-hybrid assay, had a slight inhibitory effect on integration, possibly by affecting the preintegration complex in a dominant-negative manner or by hindering the attachment of other IN-interacting proteins.

Further examination of the role of Daxx in retroviral transduction revealed a 25 to 40% increase in the GFP reporter intensity from the ASV-based vector in the Daxx-null cells compared to complemented cells. This observation suggested that the presence of Daxx leads to the repression of viral gene expression. This phenomenon was observed when the GFP reporter was expressed from either a CMV promoter or an ASV LTR promoter. Expression of the N-terminal or C-terminal portions of Daxx in the null cells also decreased reporter gene expression (Fig. 5A). As these Daxx derivatives retain the ability to repress transcription (4, 22, 42), this observation supports a model whereby Daxx mediates repression of viral gene expression. However, the overexpression of the N-terminal fragment of Daxx may deplete available HDACs, as this fragment can bind to HDACs but localizes to heterochromatin instead of ND10 (26). This relocalization might reduce the pool of HDACs available to associate with Daxx and viral DNA. Such a reduction is predicted to lead to an increase in GFP expression when these cells are treated with trichostatin A as observed in Fig. 5B and C.

**Interaction of Daxx with other viral proteins.** Daxx has been reported to interact with a variety of viral proteins, including HIV-1 p6 (2) and hantavirus nucleocapsid protein (12). Daxx is required for the localization of pp71, a human cytomegalovirus-transactivating tegument protein, to ND10 and modulates pp71 function (25). Adenovirus E1B binds to and sequesters Daxx from the ND10 (46). Daxx also interacts with human papillomavirus L2 to retain this protein in ND10 (1, 16). It is possible that Daxx recognizes a viral motif or a protein modification that allows it to interact specifically with a wide array of unrelated proteins. Such recognition may allow Daxx to repress expression from different viral genomes.

**Role of Daxx in transcriptional repression.** A role for Daxx in repression of transcription has been elucidated through the study of its interactions with developmental transcription factors, including PAX3 (23) and ETS (4). Daxx was hypothesized to recruit repressive components, such as HDACs, to promoters that are controlled by these transcription factors. Daxx was found to form a stable complex with HDAC2 and the chromatin-associated protein DEK (22). Daxx has also been shown to interact with HDAC1 constitutively (4). Daxx may therefore recruit HDAC1 and HDAC2 to cellular promoters, as well as to DNA of the incoming retroviral vector. Consistent with this hypothesis, results from ChIP experiments demonstrated that HDAC1 and HDAC2 associate with ASV DNA in the Daxx-complemented cell line but not in the Daxx-null cell line. HDAC4 was associated with the integrated viral DNA regardless of Daxx expression, consistent with its possible role in
postintegration repair. The ChIP results also indicate that Daxx associates with the viral DNA, most likely via its interaction with IN, but at later times after infection Daxx may bind DNA directly. The association of HADC1 and HADC2 with the viral DNA is also likely to be indirect, mediated by Daxx. This model is consistent with other proposals for Daxx-mediated repression in which Daxx binds transcription factors and recruits HADCs to repress transcription.

Role of HADC1 and HADC2 in transcriptional repression.
Recruitment of HADC1 and HADC2 to viral DNA is a widespread phenomenon. The transcription factor YY1 binds to the HIV LTR (36) and recruits HDAC1 to the viral DNA, thereby contributing to the repression of HIV LTR-mediated transcription in latent proviruses (12). The transactivation functions of human T-cell leukemia virus type 1 (HTLV-1) Tax (12) and Kaposi’s sarcoma-associated herpesvirus viral transactivator protein ORF50 (19) are also affected by HADC1. Indeed, the repression of viral gene transcription by the activities of HADC1 and HADC2 appears to be a general mechanism for limiting the replication of certain viruses. However, the mechanisms for initiation of this repression have not been revealed.

Daxx and PML as interferon-induced repressors of viral gene expression. Treatment of HeLa cells with alpha interferon induces Daxx expression (our unpublished data). As the amount of Daxx protein increases after infection (Fig. 4), it is possible that Daxx is a component of the interferon-mediated response against incoming ASV. Expression of PML, the protein responsible for retaining Daxx at ND10, is also interferon stimulated (21). The overexpression of PML is associated with resistance to influenza (3), vesicular stomatitis virus (3), foamy virus (35), and lymphocytic choriomeningitis virus (11). Three distinct pathways, the double-stranded RNA-dependent protein kinase R, the 2’-5’ oligoadenylate synthetase/RNaseL, and the Mx proteins, mediate the known interferon-inducible antiviral response (reviewed in reference 39). However, cells from mice deficient in all of these pathways retain some interferon-induced antiviral activity (47). This observation suggests that other interferon-inducible proteins also play a role in the antiviral response. PML and other ND10-associated proteins, such as Daxx, are candidates for this alternative interferon-mediated pathway.

In addition, ND10 is the replication site of a variety of DNA viruses, including herpes simplex virus (HSV), human cytomegalovirus, and adenovirus, all of which require the disruption of ND10 for a productive infection (13). It is unclear if these requirements for replication at and disruption of ND10 represent a need for ND10-associated protein function for replication or a need to overcome the antiviral activity mediated by ND10-associated proteins. Deletion of ICP0, the HSV early gene product responsible for PML degradation, delays the expression of HSV gene products (34) in a manner that can be overcome by treatment with trichostatin A (33). This result suggests a role for ICP0 in counteracting HSV gene repression that is mediated by ND10-associated proteins. Murine cytomegalovirus does not cause the disruption of ND10, but this virus encodes a protein that competes with the interaction of Daxx and HADC2, presumably to prevent the repressive action of HADC2 (41). Therefore, both Daxx and PML in ND10 recruit repressive factors to viral DNA to restrict viral transcription as part of an interferon-mediated antiviral response. This fact is consistent with our model in which Daxx binds to ASV IN and recruits HADC1 and HADC2, which then participate in the repression of viral transcription. We note that ASV infection did not lead to detectable dissociation of Daxx from ND10 in HeLa cells and ASV integration, as detected by the marker γ-H2AX (9), did not occur at Daxx foci (data not shown).

Here we show that the cellular protein Daxx interacts with ASV IN. This interaction allows Daxx access to viral DNA. Daxx IS not required for integration and expression of an ASV vector. However, the presence of Daxx leads to a significant repression of ASV reporter gene expression, most likely via the recruitment of HADC1 and HADC2 to the viral DNA. These observations, combined with the increase in the amount of Daxx protein after ASV infection, suggest that Daxx is part of an innate interferon-induced stress response designed to repress viral and foreign DNA expression.

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REFERENCES


