JOURNAL OF VIROLOGY, June 2008, p. 5127–5136 Vol. 82, No. 11
0022-538X/08/$08.00+0 doi:10.1128/JVI.02647-07

Inhibition of Transcription and DNA Replication by the Papillomavirus E8^E2C Protein Is Mediated by Interaction with Corepressor Molecules[▽]

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Received 13 December 2007/Accepted 10 March 2008

Papillomavirus genomes replicate as nuclear plasmids at a low copy number in undifferentiated keratinocytes. Papillomaviruses encode the E1 and E2 proteins that bind to the origin of replication and are required for the activation of replication. In addition to E2, several papillomaviruses express an E8^E2C protein, which is generated by alternative splicing and functions as a transcriptional repressor and inhibitor of the E1/E2-dependent replication of the viral origin. Previous analyses suggested that the E8 domain functions as a transferable repression domain. In this report we present evidence that the E8 domain is responsible for the interaction with cellular corepressor molecules such as histone deacetylases, the histone methyltransferase SETDB1, and the TRIM28/KAP-1/TIF1β/KRIP-1 protein. Whereas the interaction with histone deacetylases is involved only in transcriptional repression, the interaction with TRIM28/KAP-1/TIF1β/KRIP-1 contributes to the inhibition of E1/E2-dependent replication. The corepressor TRIM28/KAP-1/TIF1β/KRIP-1 has been described to be part of multicomponent complexes involved in transcriptional regulation and functions as a scaffold protein. Since neither histone deacetylases nor the histone methyltransferase SETDB1 appears to be involved in the inhibition of E1/E2-dependent replication, most likely the modification of non-histone proteins contributes to the replication repression activity of E8^E2C.

Persistent infections with certain human papillomavirus (HPV) types are a necessary risk factor for the development of cervical cancer (6). Papillomavirus genomes replicate as nuclear plasmids with ~100 copies per cell in undifferentiated keratinocytes, and viral copy number increases substantially upon induction of differentiation of the host cell (38). Viral proteins derived from the E1 and E2 genes function as sequence-specific DNA binding proteins and are involved in the initiation of DNA replication, control of viral transcription, and segregation of viral genomes (20, 21, 27, 35). The E1 protein represents the viral replication initiator protein and acts as a replicative hexameric helicase (35). The viral E2 protein is a sequence-specific DNA binding protein that recruits the E1 protein to the viral replication origin by protein-protein and protein-DNA interactions (35). In addition, E2 is a transcriptional modulator with opposing activities: E2 represses the viral E6 promoter from promoter-proximal E2 binding sites (E2BS) but can also strongly activate synthetic promoters from distal sites (21). Transactivation activity is mediated by the interaction of the E2 amino terminal domain with cellular proteins such as AMF1/Gps1, p300/CBP, Brd4, and cNAP1 (2, 12, 17, 23, 28, 32).

In addition to E2, several papillomaviruses express a second protein derived from the E2 gene, named E8^E2C, in which the E8 gene replaces the E2 activation domain that is responsible for transcriptional control and the activation of DNA replication (5, 8, 13, 26, 34, 37). The E2C domain common to E2 and E8^E2C mediates dimerization and the interaction with E2BS in the viral regulatory region (21). The E8 domain encoded by HPV31 consists of only 12 residues and bears no recognizable sequence homology to the amino terminal domain of E2. HPV31 mutant genomes that do not express E8^E2C display an overreplication phenotype, suggesting that E8^E2C restricts HPV31 copy number in undifferentiated keratinocytes (37). In contrast to E2, E8^E2C acts as a transcriptional repressor from both promoter-proximal and promoter-distal E2BS (40). Repression from promoter-distal E2BS is dependent upon the E8 domain (40). HPV31 genomes carrying mutations within the E8 gene that abolish transcriptional repression from promoter-proximal E2BS replicate at a high copy number, suggesting a critical role for the E8 domain in limiting viral genome replication by E8^E2C (46). The analysis of E8-Gal4 DNA binding domain fusion proteins revealed that the E8 domain is a transferable transcriptional repression domain (46). Importantly, E8-Gal4 fusion proteins not only inhibit transcription but also interfere with the E1/E2-dependent replication of the viral origin (46). Transcriptional repression by E8^E2C occurs independently from other viral gene products in all human cells tested so far (37, 39, 40, 46). In contrast to E2, no interaction partners for E8^E2C proteins have been described so far that may account for the repression activities.

Active transcriptional repression by sequence-specific DNA binding proteins can be achieved through the recruitment of histone deacetylases (HDACs) (41). HDACs remove acetyl groups from the amino termini of histones, and this is regarded as an important step for the generation of condensed chromatin that is inaccessible to transcription activators (41). HDACs

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▽ Published ahead of print on 19 March 2008.

can bind directly to site-specific repressors or via adaptor proteins, which are termed corepressors, and can be found as parts of large multisubunit complexes such as the SIN3, Mi2/NuRD, and CoRest complexes (14, 16, 25, 41). In addition to HDACs, corepressor complexes may recruit other histone modifying enzymes such as histone methyltransferases, DNA methyltransferases, and heterochromatin 1 (HP1) proteins to achieve transcriptional repression (14, 16, 25, 41).

In addition to a role in transcriptional repression, the mSin3B protein, which is a central component of SIN3 complexes, has been demonstrated to interfere with viral DNA replication when targeted to the polyomavirus origin of replication (44). The inhibition of T-antigen (T-Ag)-dependent replication by mSin3B is independent of HDAC activity and the presence of HDACs at the origin but requires the interaction with nuclear corepressor (N-CoR)/silencing mediator of retinoid and thyroid receptor proteins (44). This suggests that corepressor complexes have the ability to restrict DNA virus replication but that this activity is independent from histone modifications.

MATERIALS AND METHODS

Plasmids. The luciferase reporter plasmid pC18-Sp1-4xGal4-luc and the replication reporter plasmid pGL31URR-4XGal4 have been previously described (46). Plasmid p31ori is derived from pGL31URR-4xGal4 and was generated by removing the BglII/BamHI fragment encompassing the multimerized Gal binding sites and the coding sequence for the luciferase gene. Expression plasmids for HPV31 replication proteins pSGE8ˆE2C, pSXE2, and pSGE1 have been previously described (9, 36, 37). Expression constructs pSG-E8ˆE2C(1-37)-Gal4, pSG-E8ˆE2C(1-37) KWK-Gal4, and pSG-Gal4 have been described previously (46). The Kruppel-associated box (Krab) domain in the construct expressing Gal4-Krab is derived from KOX1 and was a kind gift of R. Slany (Erlangen, Germany). The constructs expressing Gal4-TRIM28 (pG4M polyII-mTIF1β; TRIM28 is (tripartite motif-containing 28 [GeneID 10155]), Gal4-HP1β (pG4M polyII-mHP1β), and Gal4-HP1γ (pG4M polyII-mHP1γ) were a kind gift of R. Losson, Strasbourg, France (24). Plasmids pGALO Gal4-mSin3B, pGal4-HDAC1, and pGal4-HDAC2 were kind gifts from W. Folk (44). Plasmid pM-hsHP1a was constructed by inserting the PCR-amplified HP1α coding sequence using IMAGE clone 3448801 (RZPD, Germany) into pM (Clontech, Heidelberg, Germany). Plasmids for the in vitro transcription and translation of HDAC1, -2, and -3 were provided by T. Stamminger and E. Verdin (4). Plasmid pGal4-HDAC3 was constructed by inserting the PCR-amplified HDAC3 coding sequence between the EcoRI and BamHI sites of pM (Clontech). Plasmid pBluescript2 SetDB1 (KIAA0067) encompassing the coding sequence for SETDB1 was kindly provided by the Kazusa DNA Research Institute, Japan. The SETDB1 coding sequence was PCR amplified with oligonucleotides adding SalI restriction sites and then cloned into the SalI site of pM, giving rise to pGal4-SETDB1. Plasmid pSG5 Strep-HDAC3 was constructed by inserting the HDAC3 coding sequence derived from pGal4-HDAC3 between the EcoRI and BamHI sites of pSG5 and then inserting an oligonucleotide encoding a streptavidin tag epitope (5′-GATCAAGCGCTTGGAGCCACCCGCAGTTCGAGA AAGGTGGAGGTTCCGGAGGTGGATCGGGAGGTGGATCGTGGAGC CACCCGCAGTTCGAAAAATAAG-3′) into the BamHI site. To construct pZOME E8ˆE2C and pZOME E8ˆE2C d3-12 (with a deletion of residues 3 to 12), the coding sequences of E8ˆE2C and the E8ˆE2C d3-12 deletion mutant were amplified by PCR to introduce BamHI restriction sites and to remove the stop codon, and then the fragment was cloned into the BamHI site of pZome 1C (Cellzome, Heidelberg, Germany). All PCR-amplified sequences were verified by sequencing of the respective recombinant plasmids.

Luciferase assays. Human keratinocytes immortalized with the HPV16E6/E7 oncogenes (11) were grown in supplemented keratinocyte serum-free medium (Invitrogen, Karlsruhe, Germany), and SCC13 cells were grown in E-medium/5% (vol/vol) fetal bovine serum on mitomycin-treated NIH 3T3-J2 feeder cells. Cells (1×10^5) were seeded into 24-well plates the day before transfection and cotransfected with Lipofectamine (Invitrogen, Karlsruhe, Germany) and 100 ng of reporter plasmid pC18-Sp1-4xGal4-luc and 20 ng of the respective expression plasmids, according to the manufacturer's instructions. After 30 h, HDAC inhibitors or dimethyl sulfoxide (DMSO) was added for 14 h; cells were then harvested, and luciferase activity was determined. MS275 (ALX-270-378; Alexis Biochemicals, Lausen, Switzerland) and *Helminthosporium carbonum* toxin ([HC toxin] ALX-630-102; Alexis Biochemicals, Lausen, Switzerland) were dissolved in DMSO.

Transient replication assays. SCC13 cells (7.5×10^5) were cotransfected with Lipofectamine (Invitrogen) and an equimolar mixture of the replication reporter plasmids pGL31URR-4xGal4 (250 ng) and p31ori (150 ng) and expression vectors for E1 (1,000 ng), E2 (100 ng), and the respective Gal4 fusion proteins (300 ng). For HDAC inhibition experiments, HC toxin was added 5 h after transfection to the medium, and low-molecular-weight DNA was isolated from the cells 14 h later. Replication of the reporter plasmids was determined by digestion with the methylation-sensitive enzymes DpnI and XhoI and Southern blotting using a ^{32}P-labeled HPV31 origin fragment. After exposure of the membrane to phosphorimager screens, replicated DNA was quantitated using the AIDA software package (Raytest, Berlin, Germany).

GST pull-down experiments. Glutathione *S*-transferase (GST) expression plasmids for E8ˆE2C, E8ˆE2C d3-12, and HP1 are based upon plasmid pET42b (Merck KGaA, Darmstadt, Germany). *Escherichia coli* BL21(DE3) pLysS cells carrying the respective plasmids were grown to an optical density at 600 nm of ~0.7. Isopropyl-β-D-thiogalactopyranoside was added to a concentration of 0.4 mM, and cells were incubated for an additional 2 h at 25°C. Soluble extracts were obtained by sonification of pelleted bacteria in phosphate-buffered saline supplemented with protease inhibitors (complete mini EDTA free; Roche Diagnostics, Mannheim, Germany). After the addition of Triton X-100 to a final concentration of 1%, the supernatant was clarified by centrifugation. GST fusion proteins were purified by incubation with glutathione-agarose (GE Healthcare Europe GmbH, Munich, Germany), followed by extensive washing with 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Igepal 630, and protease inhibitors. Similar amounts of GST or GST fusion proteins, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), were incubated for 2 h at 4°C with reticulocyte lysate containing in vitro translated ^{35}S-labeled target proteins or whole-cell lysates. Beads were pelleted by centrifugation and washed seven times with 1 ml of buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Igepal 630, and protease inhibitors). Bound proteins were eluted with 4× SDS gel loading buffer (Carl Roth, Karlsruhe, Germany), heated to 95°C, and then separated by 12% SDS-PAGE. ^{35}S-labeled proteins were detected by exposure of dried gels to phosphorimager screens and AIDA software. Unlabeled proteins were detected by immunoblotting with the respective antibodies. All pull-down experiments were repeated at least once with different GST protein preparations to ensure reproducibility.

Coimmunoprecipitation. Phoenix cells derived from 293 cells were transfected by the calcium coprecipitation method with 10 μg of the plasmid constructs indicated in the figures per 3×10^6 cells. Whole-cell lysates were prepared 48 h posttransfection by incubation in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% [vol/vol] Igepal 630, and protease inhibitors). Supernatants were cleared by centrifugation (10 min at 20,000 × *g* and 4°C) and then incubated with preequilibrated immunoglobulin G (IgG)-Sepharose (GE Healthcare Europe GmbH, Munich, Germany) or Strep-Tactin matrix (IBA GmbH, Germany) at 4°C for 2 h. Beads were pelleted by centrifugation and washed eight times with 1 ml of buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Igepal 630, and protease inhibitors). Bound proteins were eluted by adding 20 μl of 4× Roti-Load 1 protein loading buffer (Carl Roth, Karlsruhe, Germany), heated to 95°C, and then separated by SDS-PAGE and analyzed by immunoblotting.

Immunoblotting. Separated proteins were transferred in 10 mM CAPS [3-(cyclohexylamino)-1-propanesulfonic acid; pH 10.3] buffer on a nitrocellulose membrane (Protran, Whatman, Dassel, Germany). Membranes were blocked by incubation in Tris-buffered saline–0.1% Tween 20–5% nonfat dry milk for 1 h and then incubated with the following diluted primary antibodies: anti-tubulin, 1:1,500 (Oncogene CP06); anti-HDAC3, 1:500 (Santa Cruz sc-17795); anti-acetyl-histone H3, 1:3,000 (Upstate 06-599); anti-Gal4, 1:500 (Santa Cruz sc-577); anti-KRIP1 (TRIM28), 1:1,000 (Transduction Laboratories K57620); and anti-SETDB1, 1:1,000 (Upstate 07-378). Bound antibodies and protein A fusion proteins were detected with anti-rabbit (polyclonal swine anti-rabbit Ig-horseradish peroxidase [HRP]; Dako, Hamburg, Germany) or mouse antibodies conjugated to HRP (polyclonal rabbit anti-mouse Ig-HRP; Dako, Hamburg, Germany) and SuperSignal West Pico reagent (Perbio Science, Bonn, Germany). Chemiluminescent signals were recorded with a FluorSMax Imaging system (Bio-Rad, Munich, Germany).

RESULTS

Transcriptional repression by E8ˆE2C is mediated by interaction with HDACs. E8ˆE2C inhibits in an E8-dependent way

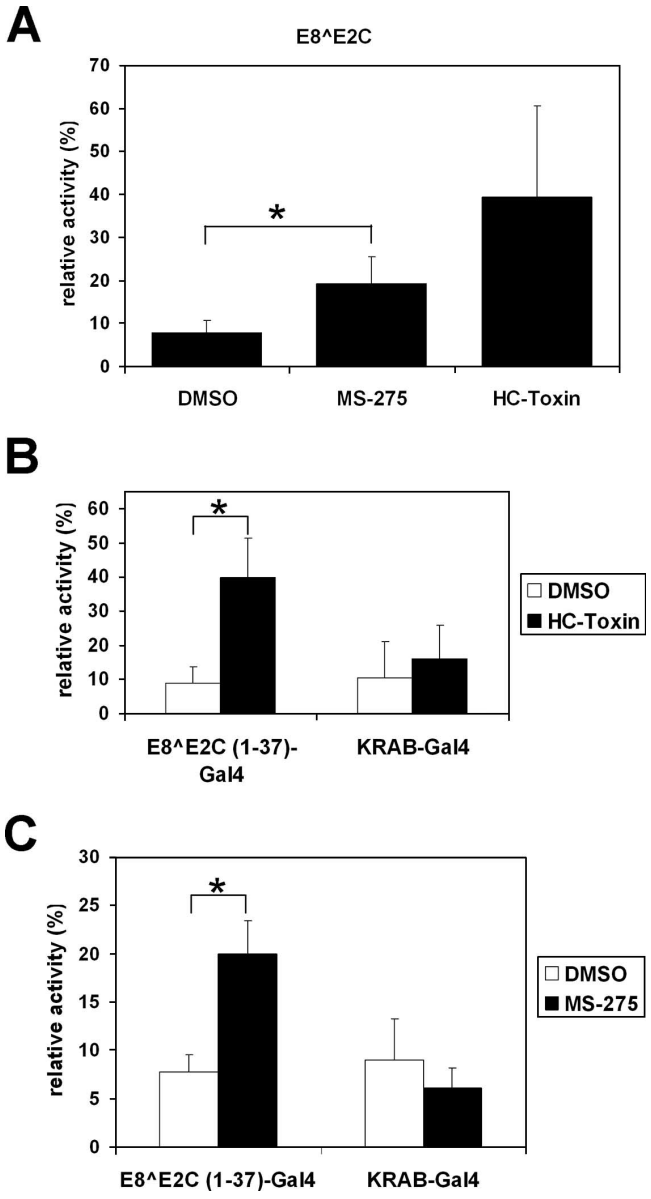

FIG. 1. HDAC inhibitors counteract E8^E2C-mediated reporter gene inhibition. (A) E6/E7-expressing keratinocytes were cotransfected with the pC18-Sp1-4xGal4-luc plasmid and the pSG5 E8^E2C expression plasmid or the empty vector pSG5. HDAC inhibitor MS-275 or HC toxin was added at 10 or 3 μM, respectively, to the cells 14 h before reporter gene expression was determined. Control transfections received the appropriate concentration of DMSO to account for solvent effects. Data are presented relative to empty vector-transfected samples. Error bars indicate standard deviations derived from several independent experiments. The difference between the control (DMSO) and MS-275-treated samples is statistically significant ($P = 0.018$) as determined by a Student's t test. (B) E6/E7-expressing keratinocytes were cotransfected with the pC18-Sp1-4xGal4-luc plasmid and pSG-Gal4 (Gal4), pSG5 E8^E2C(1–37)-Gal4, or pSG-Gal4-Krab expression plasmids. The HDAC inhibitor HC toxin was added at a concentration of 3 μM to the cells 14 h before reporter gene expression was determined. Data are presented relative to empty vector-transfected samples. Error bars indicate standard deviations derived from several independent experiments. The difference between E8^E2C(1–37)-Gal4-transfected cells treated with DMSO and HC toxin is statistically significant ($P = 0.0002$) as determined by a Student's t test. (C) E6/E7-expressing keratinocytes were cotransfected with the pC18-Sp1-4xGal4-luc plasmid and pSG-Gal4 (Gal4), pSG5 E8^E2C(1–37)-Gal4, or pSG-Gal4-Krab expression plasmids. The HDAC inhibitor MS-275 was added at 10 μM to the cells 14 h before reporter gene expression was determined. Data are presented relative to empty vector-transfected samples. Error bars indicate standard deviations derived from at least three independent experiments. The difference between E8^E2C(1–37)-Gal4-transfected cells treated with DMSO and MS-275 is statistically significant ($P = 0.005$) as determined by a Student's t test.

both transcription and DNA replication (39, 40, 46). Since transcriptional repressors often recruit HDACs to inhibit gene transcription (14, 16, 41), we tested whether HDAC activity contributes to the E8-dependent transcriptional inhibition of reporter gene expression. Immortalized keratinocytes were transiently cotransfected with the pC18-SP1-4xGal4-luc reporter plasmid, which harbors four E2 and four Gal4 binding sites and an E8^E2C expression plasmid. To inhibit HDAC activity, the two structurally unrelated inhibitors MS-275 and HC toxin were used (3, 30). MS-275 or HC toxin was added at 10 or 3 μM, respectively, to the cells 14 h before reporter gene expression was determined (Fig. 1A). Titration experiments revealed that cell viability is not influenced at these concentrations (data not shown). Results indicated that repression activity by E8^E2C was reduced three- to fivefold in the presence of either HDAC inhibitor (Fig. 1A). To further confirm these findings, experiments were carried out with a fusion protein consisting of the repression domain derived from E8^E2C (residues 1 to 37) fused to Gal4 [pSG E8^E2C(1–37)-Gal4]. In addition, an expression vector for a Gal4-Krab fusion protein was used that is a potent transcriptional repressor and is independent of HDAC activity (18, 19). The addition of MS-275 or HC toxin reduced repression by E8^E2C(1–37)-Gal4 three- to fivefold, comparable to the effects observed with the full-length E8^E2C protein (Fig. 1B and C), suggesting that the HDAC inhibitors target the E8 repression activity. In contrast, no effect on the inhibition by Gal4-Krab was observed by either HDAC inhibitor (Fig. 1B and C), indicating that the effect is specific for E8^E2C. This suggested that the impaired transcriptional repression by E8^E2C was due to the inhibition of HDACs.

To investigate whether the E8^E2C protein interacts with HDACs, GST pull-down experiments were performed with the major class I HDACs 1, 2, and 3 (14). Soluble GST-E8^E2C and the mutant GST-E8^E2C d3-12 protein, which lacks most of the E8 domain and has no repressive activity from promoter-distal binding sites (40), were purified from bacteria by affinity chromatography (Fig. 2A). Identical amounts of GST fusion proteins or only the GST moiety were incubated with in vitro translated ^{35}S-labeled HDAC1, -2, or -3. After samples were washed extensively, the amounts of retained HDACs were analyzed by SDS-PAGE. As can be seen in Fig. 2B and C, approximately 10% of the input of HDAC1, -2, or -3 was bound to GST-E8^E2C whereas less than 0.2% of the input was bound to GST. This interaction was dependent upon the E8 domain as binding of HDAC1, -2, and -3 to GST-E8^E2C d3-12 was reduced four- to fivefold (Fig. 2C). To further confirm the interaction of E8^E2C with HDACs, cells were cotransfected with an epitope-tagged version of HDAC3 (Strep-HDAC3) and pSG-Gal4, pSGE8^E2C(1–37)-Gal4 or pSGE8^E2C(1–37)-KWK-

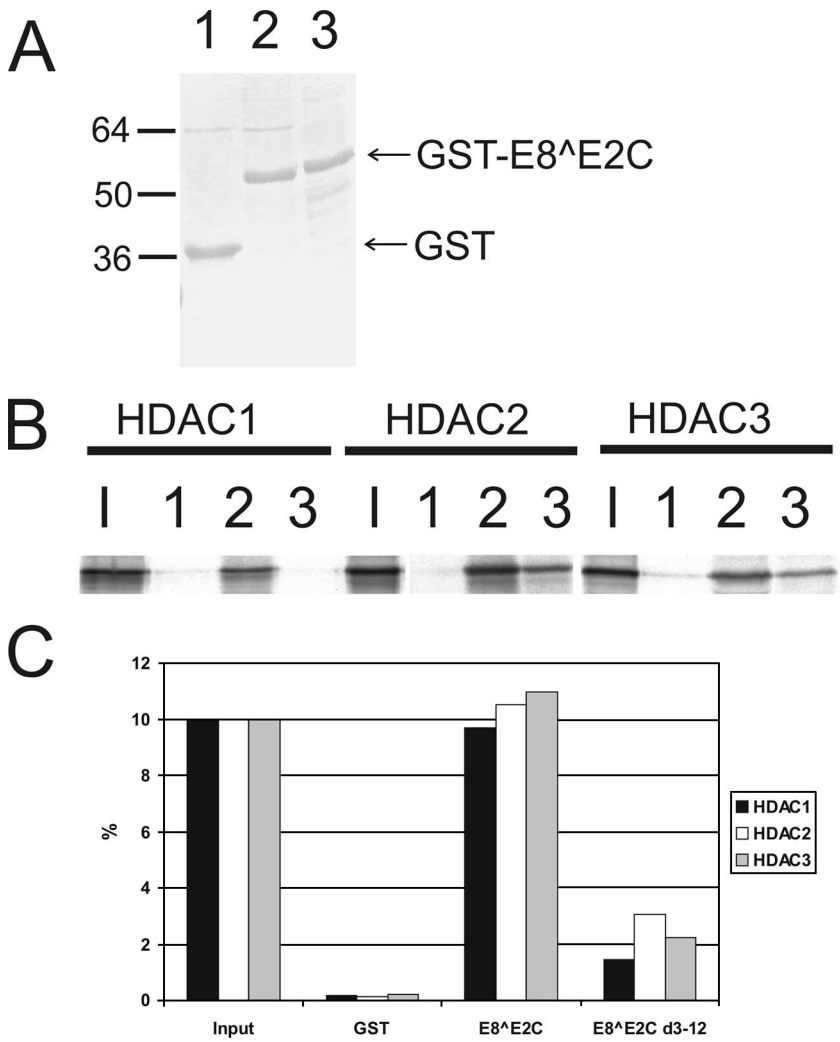

FIG. 2. E8ˆE2C interacts with class I HDACs. (A) Coomassie-stained gel of affinity-purified GST (lane 1), GST-E8ˆE2C d3-12 (lane 2), and GST-E8ˆE2C (lane 3) proteins. The positions of GST and GST-E8ˆE2C proteins are indicated by arrows. On the left a molecular size marker (in kDa) is shown. (B) Similar amounts of GST (lane 1), GST-E8ˆE2C (lane 2), or GST-E8ˆE2C d3-12 (lane 3) proteins were incubated with in vitro translated ^{35}S-labeled HDAC1, HDAC2, or HDAC3; samples were washed extensively, and then the retained proteins were separated by SDS-PAGE and exposed to phosphorimager screens. In lane 1, 10% of the input in vitro translation reaction is shown. (C) Quantitation of retained HDAC proteins shown in panel B. Data are presented relative to the input.

Gal4, which represents a repression-negative mutant protein (46). Cell extracts were prepared from transfected cells, and immunoblotting with a Gal4 antibody and an antibody directed against HDAC3 confirmed that the Gal4 fusion proteins and the transfected streptavidin-tagged HDAC3 were present at similar levels (Fig. 3). When a streptavidin-Sepharose matrix was used to immunoprecipitate HDAC3 (Fig. 3), immunoblotting using a Gal4 antibody revealed that only E8ˆE2C(1–37)-Gal4 coprecipitated with HDAC3 whereas the repression-negative mutant E8ˆE2C(1–37)KWK-Gal4 did not (Fig. 3). This suggested that HDAC3 binds to the E8 repression domain of E8ˆE2C and that this interaction contributes to the transcriptional repression activity.

HDACs do not contribute to the replication repression activity by E8ˆE2C. To investigate whether the inhibition of E1/E2-dependent replication of the HPV31 replication origin by E8ˆE2C(1–37)-Gal4 also requires HDAC activity, transient replication assays were performed. First, the influence of the HDAC inhibitor HC toxin was evaluated by reporter expression assays in SCC13 cells, which were previously used for the characterization of the replication repression activities of E8ˆE2C (37, 40, 46). As can be seen in Fig. 4A, the addition of 3 μM HC toxin almost completely prevented the E8ˆE2C(1–37)-Gal4-dependent repression of the reporter plasmid, suggesting that the effects are even more pronounced in SCC13 cells than in E6/E7-immortalized keratinocytes (Fig. 1). Immunoblot analyses of cell extracts derived from transfected SCC13 cells treated with HC toxin or DMSO alone revealed that the levels of acetylated histone H3 were greatly increased by HC toxin treatment in contrast to anti-tubulin, confirming the inhibition of HDAC activity (Fig. 4B). Also the levels of E8ˆE2C(1–37)-Gal4 were increased by the HC toxin treatment, indicating that the loss of transcriptional repression is not due to reduced protein levels (Fig. 4B). SCC13 cells were

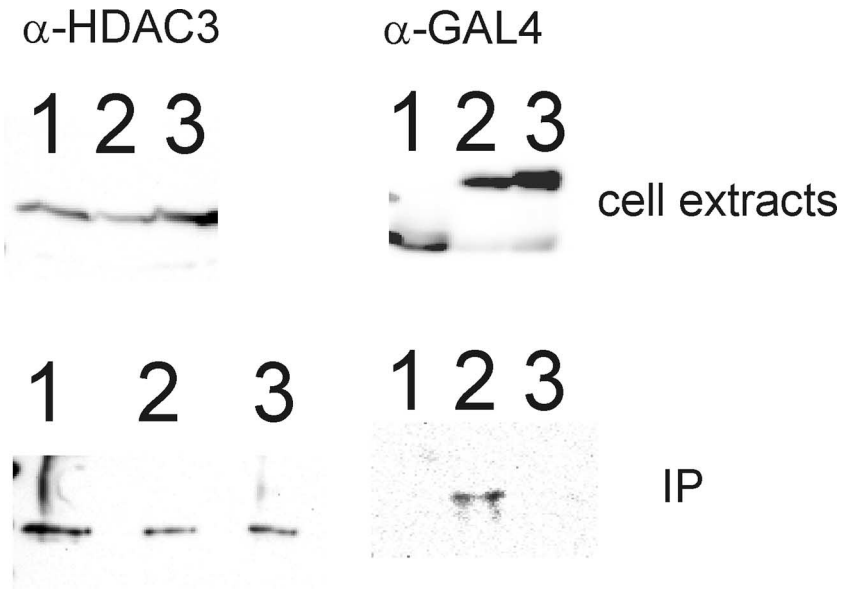

FIG. 3. The E8 domain interacts with HDAC3 in vivo. An expression vector for Strep-HDAC3 was cotransfected with pSG-Gal4 (lane 1), pSG-E8^E2C(1–37)-Gal4 (lane 2), or pSG-E8^E2C(1–37) KWK-Gal4 (lane 3) into Phoenix cells, and 48 h posttransfection soluble lysates were prepared. Cell extracts were analyzed by immunoblotting for the presence of HDAC3 (upper left panel) or for the presence of Gal4 proteins (upper right panel) with specific monoclonal antibodies. Lysates were incubated with Strep-Tactin matrix, and retained proteins were analyzed by immunoblotting for the presence of HDAC3 (lower left panel) or for the presence of Gal4 proteins (lower right panel). IP, immunoprecipitation; α, anti.

then transfected with a mixture of two replication reporter plasmids: pGL31URR-4xGal4, which contains the HPV31 origin of replication and multimerized Gal4 binding sites (46), and p31ori, which contains the HPV31 origin but is devoid of Gal4 binding sites. The origin reporter plasmids were cotransfected with expression vectors for HPV31 E1, HPV31 E2, and Gal4 or E8^E2C(1–37)-Gal4. Cells were then treated with 3 μM HC toxin for 14 h. Low-molecular-weight DNA was isolated and analyzed by Southern blotting for newly replicated (DpnI resistant) plasmids. The expression of E8^E2C(1–37)-Gal4 inhibited replication of the pGL31URR-4xGal4 plasmid ~10-fold whereas replication of p31ori was only slightly diminished, indicating a binding site-dependent inhibition of replication, as has been described for its transcriptional repression activity (Fig. 4C) (40). The addition of HC toxin did not result in relief from the inhibition of pGL31URR-4xGal4 replication in the presence of E8^E2C(1–37)-Gal4 (Fig. 4C), suggesting that HDAC activity does not contribute to the repression of origin replication by E8^E2C. However, we noted that replication of pGL31URR-4xGal4 was reduced approximately threefold in the presence of the HDAC inhibitor in the control reaction receiving pSG-Gal4, indicating a surprising requirement for HDAC activity for replication (Fig. 4C). Since it was possible that HDACs inhibit replication by a mechanism that does not require HDAC activity, we performed transient replication assays with constructs expressing fusion proteins consisting of HDACs 1, 2, or 3 and Gal4 to specifically target HDACs to the replication origin. In contrast to E8^E2C(1–37)-Gal4 (Fig. 4c), the fusion proteins were unable to inhibit the E1/E2-dependent replication of the pGL31URR-4xGal4 plasmid (Fig. 4D). This indicated that recruitment of HDACs by E8^E2C does not contribute to the inhibition of replication.

E8^E2C interacts with the TRIM28 corepressor protein. However, we observed that the coexpression of a Gal4-Krab protein inhibited replication of the pGL31URR-4xGal4 plasmid as efficiently as E8^E2C(1–37)-Gal4 (Fig. 4C). The Krab domain represses transcription independently from HDAC activity (41) (Fig. 1B and C) through recruiting the corepressor molecule TIF1β (transcription intermediary factor 1 beta)/KAP-1 (Krab-associated protein-1)/KRIP1 (Krab-A interacting protein 1) (10, 15, 22). This gene is referred to as TRIM28 in the GenBank database.

We next tested whether TRIM28 is able to bind to E8^E2C in GST pull-down experiments. Whole-cell extracts derived from Phoenix cells or E6/E7-expressing keratinocytes were incubated with GST or a GST-E8^E2C, GST-E8^E2C d3-12, or GST-HP1α fusion protein as a positive control for TRIM28 interaction. Bound proteins were eluted and analyzed by immunoblotting with a TRIM28 antibody. Only GST-HP1α and GST-E8^E2C precipitated the endogenous TRIM28 protein (Fig. 5A and C). This interaction was dependent upon the presence of the E8 repression domain as no signal was obtained when the GST-E8^E2C d3-12 protein was used (Fig. 5A and C). To confirm this interaction, transfection experiments were performed. Expression vectors encoding E8^E2C-protein A and E8^E2C d3-12-protein A fusion proteins or protein A alone were transfected into Phoenix cells, and cell extracts were immunoprecipitated with IgG-Sepharose. Precipitates were then analyzed by immunoblotting for the presence of TRIM28 protein. Only the E8^E2C-protein A fusion precipitated TRIM28 and not protein A alone or the E8^E2C d3-12-protein A fusion (Fig. 5B). This indicated that the E8 repression domain interacts with TRIM28 protein in vivo. TRIM28 has been reported to interact with the histone methyltrans-

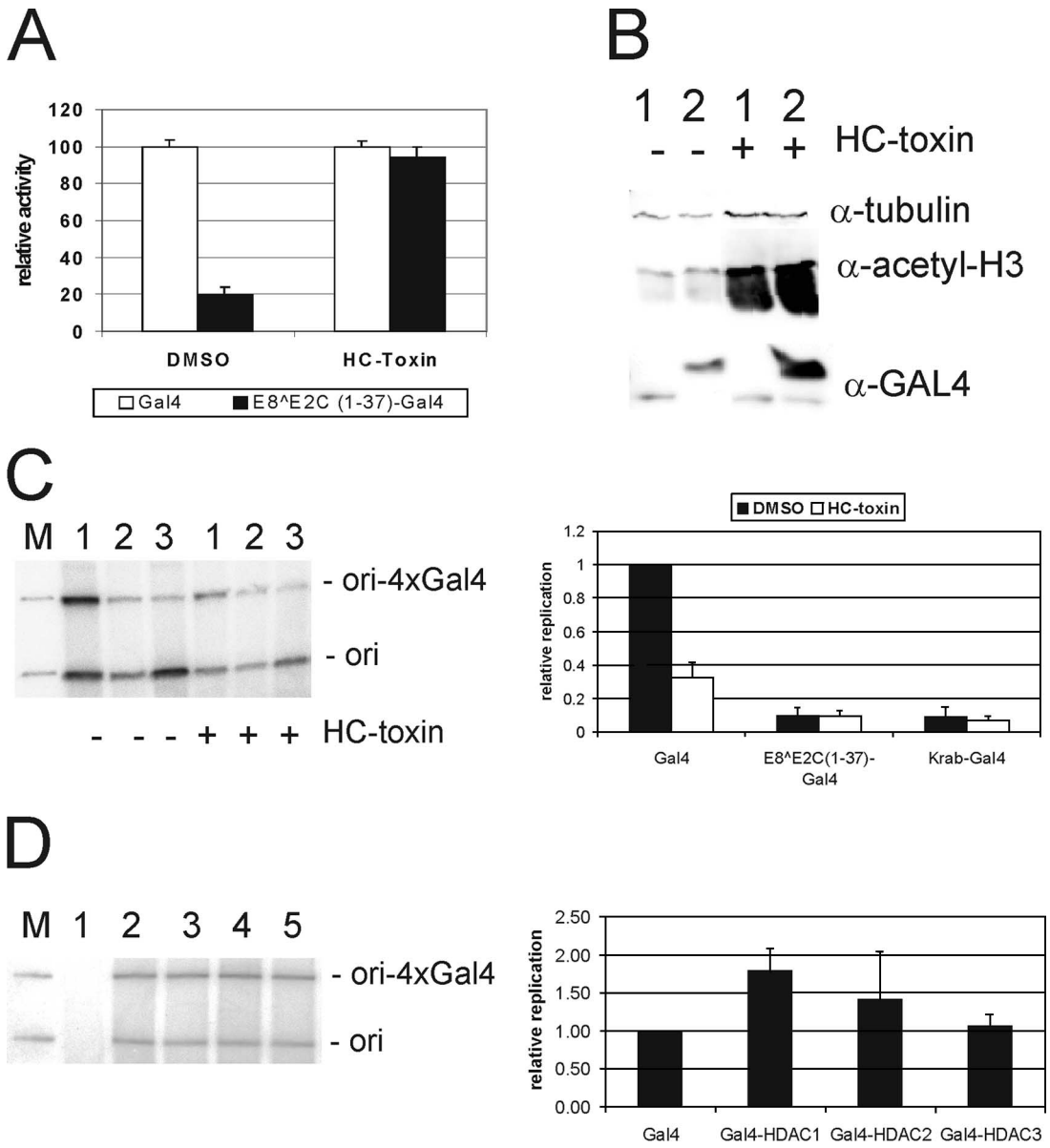

FIG. 4. HDACs are not involved in the inhibition of the E1/E2-dependent replication of the HPV31 origin plasmid. (A) SCC13 cells were cotransfected with the pC18-Sp1-4xGal4-luc plasmid and pSG-Gal4 (Gal4) or pSG5 E8ˆE2C(1–37)-Gal4 expression plasmids. The HDAC inhibitor HC toxin dissolved in DMSO or DMSO only was added at 3 μM to the cells 14 h before reporter gene expression was determined. Data are presented relative to empty vector-transfected samples. Error bars indicate standard deviations derived from several independent experiments. (B) SCC13 cells transfected with pSG-Gal4 (lane 1) or pSG5 E8ˆE2C(1–37)-Gal4 (lane 2) were incubated with DMSO or HC toxin as described in panel A, and total cell extracts were analyzed by immunoblotting for the presence of anti-tubulin, acetyl-histone 3 (acetyl-H3), or Gal4. (C) SCC13 cells were cotransfected with a mixture of replication reporter plasmids pGL31URR-4xGal4 (ori-4xGal4) and p31ori (ori) and expression vectors for HPV31 E1 and E2. In addition, expression vectors for Gal4 (lane 1), E8ˆE2C(1–37)-Gal4 (lane 2), or Gal4-Krab (lane 3) were added. Cells were incubated in the absence or presence of HC toxin (3 μM), and the levels of newly replicated DNA in the low-molecular-weight fraction were determined by resistance to digestion with DpnI followed by Southern blotting (left panel). Lane M received 100 pg of each of the linearized reporter plasmids. Replication levels of pGL31URR-4xGal4 were quantitated by phosphorimaging analyses and are presented relative to the levels of pGL31URR-4xGal4 in the presence of Gal4 expression plasmid and DMSO (right panel). Error bars indicate standard deviations derived from three independent experiments. (D) Cells were transfected as described in panel C but received either only the expression vector control plasmid (lane 1) or expression vectors for E1 and E2 (lanes 2 to 5) and expression vectors for Gal4 (lane 2), Gal4-HDAC1 (lane 3), Gal4-HDAC2 (lane 4), or Gal4-HDAC3 (lane 5). The levels of newly replicated DNA in the low-molecular-weight fraction were determined by resistance to digestion with DpnI, followed by Southern blotting (right panel). Lane M received 100 pg of each of the linearized reporter plasmids. Quantitation of the data was by phosphorimager analysis (left panel). Signal intensities obtained for replicated pGL31URR-4xGal4 were corrected for the replication levels of p31ori and are presented relative to replication levels in the presence of Gal4 alone. Data are derived from three independent experiments, and error bars indicate the standard deviations. α, anti.

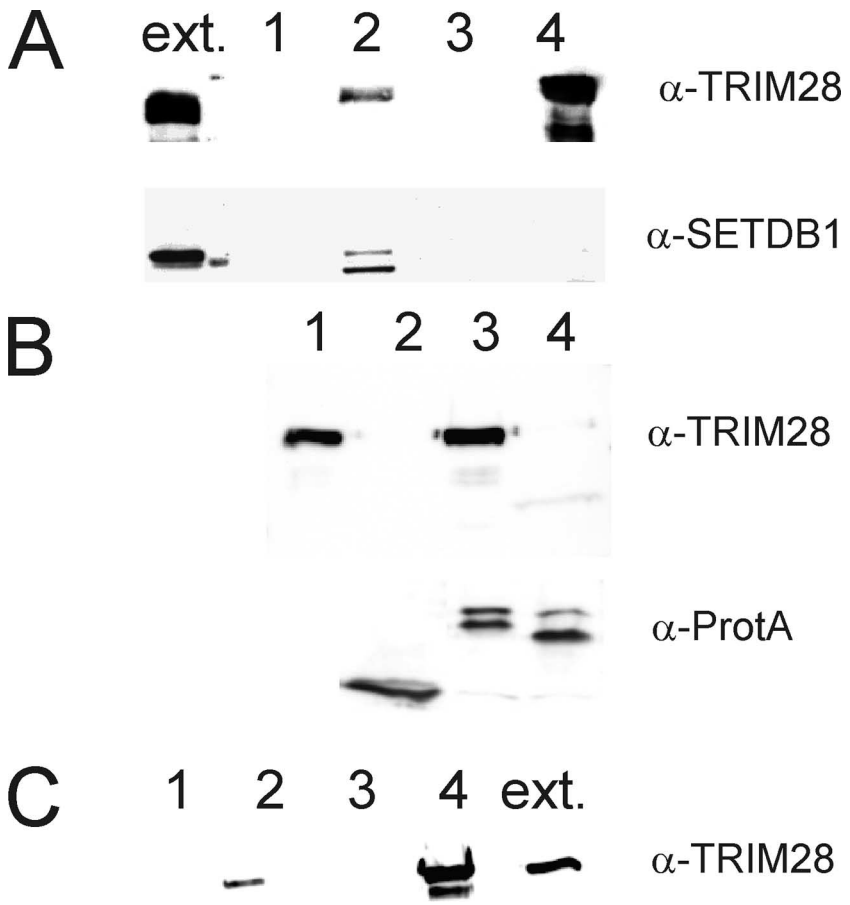

FIG. 5. E8^E2C interacts with the corepressors TRIM28 and SETDB1. (A) Purified GST (lane 1), GST-E8^E2C (lane 2), GST-E8^E2C d3-12 (lane 3), or GST-HP1α proteins were incubated with whole-cell extracts derived from Phoenix cells, and retained proteins were analyzed by immunoblotting for the presence of TRIM28 (upper panel) or SETDB1 (lower panel). In lane ext, an aliquot of the whole-cell extract is shown. (B) Phoenix cells were transfected with expression vector pZOME 1C (lane 2) or expression vectors encoding E8^E2C-protein A (lane 3) or E8^E2C d3-12-protein A (lane 4). Whole-cell extracts were prepared 48 h posttransfection, and protein A (Prot A) fusions were precipitated by incubation with IgG-Sepharose. Precipitates were analyzed by immunoblotting for the presence of TRIM28 (upper panel) or protein A fusions (lower panel). In lane 1 an aliquot of the whole-cell extract was analyzed. (C) Purified GST (lane 1), GST-E8^E2C (lane 2), GST-E8^E2C d3-12 (lane 3), or GST-HP1α protein was incubated with whole-cell extracts derived from E6/E7-expressing keratinocytes, and retained proteins were analyzed by immunoblotting for the presence of TRIM28. In lane ext, an aliquot of the whole-cell extract was used. α, anti.

ferase SETDB1 (31). We therefore tested whether E8^E2C interacted with SETDB1 in GST pull-down assays. Whole-cell lysates were prepared and incubated with GST, GST-E8^E2C, GST-E8^E2C d3-12, or GST-HP1α. After extensive washing, bound proteins were analyzed for the presence of SETDB1 by immunoblotting. As can be seen in Fig. 5A, only GST-E8^E2C precipitated SETDB1 but not GST alone, GST-E8^E2C d3-12, or GST-HP1α. This suggested that E8^E2C interacts not only with class I HDACs and TRIM28 but also with SETDB1. Since interactions between TRIM28 and SETDB1, SETDB1 and class I HDACs, and between TRIM28 and HDAC3 have been described previously (31, 42, 45), it is likely that E8^E2C recruits corepressor complexes to inhibit transcription and replication.

Recruitment of TRIM28 inhibits E1/E2-dependent replication of the HPV31 origin. To test whether TRIM28 is able to inhibit the E1/E2-dependent replication of the HPV31 origin, transient replication assays were carried out. SCC13 cells were transfected with a mixture of pGL31URR-4xGal4 and p31ori alone or together with expression vectors for HPV31 E1 and E2 and for Gal4, Gal4-Krab, Gal4-TRIM28, or Gal4-HP1α since TRIM28 can also be recruited to promoters by interaction with members of the HP1 family (24, 29). Low-molecular-weight DNA was isolated and analyzed by Southern blotting for newly replicated (DpnI resistant) plasmids. Both Gal4-Krab and Gal4-TRIM28 inhibited the replication of pGL31URR-4xGal4 approximately eightfold but did not inhibit replication of p31ori (Fig. 6). In addition, the recruitment of all three isoforms of HP1 (α, β, and γ) to the origin also specifically inhibited the replication of pGL31URR-4xGal4 to a similar extent as Gal4-TRIM28 (Fig. 6). This suggested that the recruitment of TRIM28 by protein-protein interactions to the origin results in the inhibition of replication. Since we observed that SETDB1 also interacted with E8^E2C (Fig. 5A), we tested whether a Gal4-SETDB1 fusion protein repressed origin activity. In

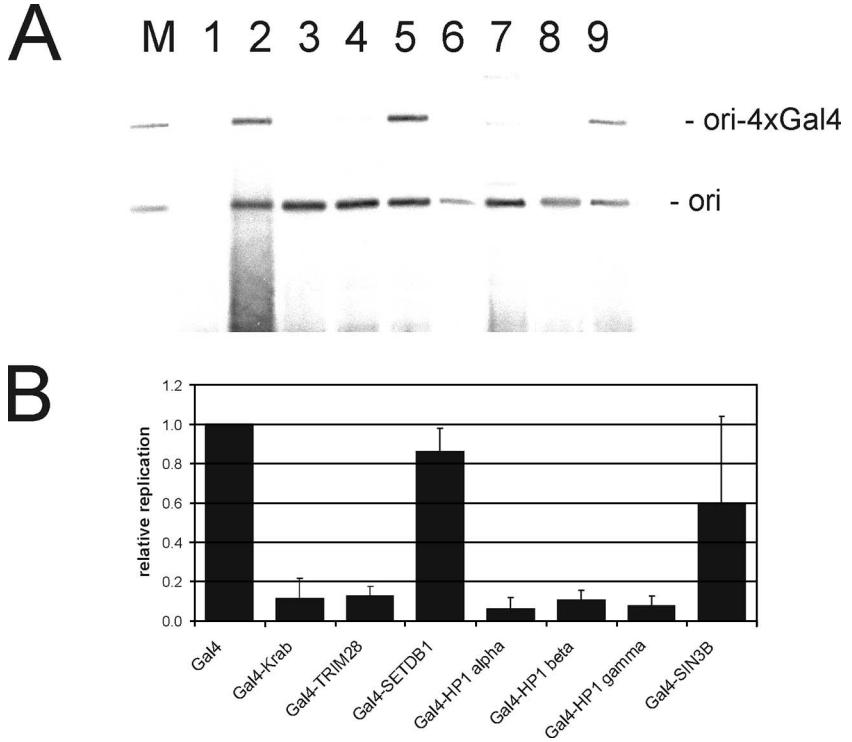

FIG. 6. Recruitment of TRIM28 to the HPV31 origin prevents E1/E2-dependent replication. (A) SCC13 cells were cotransfected with a mixture of replication reporter plasmids pGL31URR-4xGal4 (ori-4xGal4) and p31ori (ori) and either the expression vector control plasmid (lane 1) or expression vectors for E1 and E2 (lanes 2 to 9) and expression vectors for Gal4 (lane 2), Gal4-Krab (lane 3), Gal4-TRIM28 (lane 4), Gal4-SETDB1 (lane 5), Gal4-HP1α (lane 6), Gal4-HP1β (lane 7), Gal4-HP1γ (lane 8), or Gal4-SIN3B (lane 9). The levels of newly replicated DNA in the low-molecular-weight fraction were determined by resistance to digestion with DpnI followed by Southern blotting. Lane M received 100 pg of each of the linearized reporter plasmids. (B) Quantitation of the data shown in panel A by phosphorimager analysis. Signal intensities obtained for replicated pGL31URR-4xGal4 were corrected for the replication levels of p31ori and are presented relative to replication levels in the presence of Gal4 alone. Data are derived from three independent experiments, and the standard deviation is indicated by error bars.

contrast to the Gal4-Krab, -TRIM28, and -HP1 fusions, no significant inhibition of the replication of pGL31URR-4xGal4 could be observed with Gal4-SETDB1 (Fig. 6). We also tested in the transient replication assay a Gal4-mSin3B fusion protein that has been shown to inhibit the T-Ag-dependent replication of the polyomavirus origin (44). SIN3 proteins are central parts of multiprotein complexes involved in transcription repression and associate both with class I HDACs and SETDB1 but have so far not been linked to TRIM28 (18, 33). In contrast to Gal4-TRIM28, the Gal4-mSin3B fusion protein only moderately inhibited replication of pGL31URR-4xGal4 (Fig. 6). This suggested that SIN3B is not a major repressor of papillomavirus replication and confirmed our findings that recruitment of class I HDACs does not contribute to repression (Fig. 4C and D). Furthermore, it also suggests that the recruitment of SETDB1 does not have a major impact on replication repression, which suggests that other activities aside from histone modifying enzymes are involved.

DISCUSSION

Several animal and human papillomaviruses express two proteins from the viral E2 gene. E2 represents the replication activator protein that also can repress and activate transcription. Both replication and transcription activities require the interaction with the viral E1 helicase and host cell proteins (2, 12, 17, 23, 28, 32, 35). On the other hand, E8^E2C acts only as a repressor of transcription and replication (1, 5, 37, 39, 40, 46). Due to the common E2C part, both proteins interact with E2BS within the viral origin of replication (1, 40). This led to the model that E8^E2C prevents the E2 activator protein from binding to E2BS via binding site competition. Therefore, E8^E2C was regarded as a dominant-negative E2 protein acting as a passive repressor molecule. However, we have previously demonstrated that the amino-terminal 37 residues of the E8^E2C protein represent a transferable repression domain and that this activity is important for controlling the replication of the HPV31 genome (46). This strongly suggested that E8^E2C acts as an active repressor molecule in addition to competing with E2. Similar to the transcription activation and repression functions of E2, E8^E2C's repression function is independent from the presence of additional papillomavirus proteins and works in a large number of both normal and immortalized human cells (37, 39, 40, 46). We therefore reasoned that E8^E2C interacts with host cell proteins to inhibit transcription and replication.

We now provide evidence that E8^E2C interacts with ubiquitously expressed cellular transcriptional corepressors such as

class I HDACs, the histone methyltransferase SETDB1, and the TRIM28 corepressor protein. E8^E2C interacts with different class I HDACs, and HDAC inhibitors relieve a reporter gene from repression by E8^E2C, strongly suggesting that the recruitment of HDACs by E8^E2C contributes to transcriptional repression. In contrast to transcriptional repression activity, the addition of HDAC inhibitors did not counteract the inhibition of the E1/E2-dependent replication of an origin plasmid (Fig. 4C). Also the recruitment of HDACs 1, 2, or 3 to the HPV31 origin via the Gal4 DNA binding domain did not influence replication activity (Fig. 4D). In line with this, the addition of HDAC inhibitors to cells with replicating HPV31 genomes did not influence viral copy number (7). Furthermore, we found that the inhibition of the replication of HPV31 origin by a Gal4-Krab protein was also independent from HDAC activity. Comparable to these observations, a Gal4-mSin3B fusion protein repressed T-Ag-dependent replication of the polyomavirus origin also independently from HDAC activity (44).

However, HDACs are often part of large multiprotein complexes that include multiple activities in order to control transcription. Therefore, the interaction of E8^E2C with HDACs might not be important to modulate protein acetylation but may actually serve to recruit other components that are important for the activities of E8^E2C. In line with this, we were able to demonstrate that the E8 domain interacts with TRIM28/TIF1β/KAP-1/KRIP1. The direct and indirect recruitment of TRIM28 to the viral origin prevents E1/E2-dependent DNA replication, suggesting that TRIM28 is a replication inhibitor. TRIM28 is not only the corepressor for Krab domain-containing proteins (10, 15, 22) but might also be part of the Mi2/NuRD HDAC complex through interaction with Mi-2α; and TRIM28 can be found in a 2-MDa complex that consists of N-CoR1, HDAC3, and several other proteins (42), which would explain E8^E2C's interaction with both TRIM28 and HDACs. TRIM28 also interacts directly with the histone methyltransferase SETDB1 and HP1 (24, 29, 31). We have also observed that SETDB1 interacts with E8^E2C, but this does not appear to contribute to the inhibition of replication since neither Gal4-SETDB1 nor Gal4-SIN3B, which has been reported to interact with SETDB1, was able to repress replication of the HPV31 origin (45). However, we cannot exclude the possibility that the fusion of the Gal4 domain to SETDB1 results in a protein that fails to form proper protein complexes and therefore does not inhibit replication. Since the replication repression of E8^E2C is also independent from HDAC activity, this suggests that mechanisms aside from histone modification account for replication repression. Currently, there is no evidence that TRIM28 has intrinsic enzymatic activities that may account for the repression of replication. But aside from being part of multisubunit repressor complexes that have multiple enzymatic activities, TRIM28 has also recently been demonstrated to interact with MDM2, which represents a major E3 ubiquitin ligase for p53 (43). As a consequence of this interaction ubiquitylation levels of p53 increase, and the acetylation levels of p53 are reduced (43). This suggests that TRIM28 can also inactivate a DNA-binding transcriptional activator by modulating its posttranslational modification levels. Thus, it might be possible that the E8^E2C-mediated recruitment of TRIM28 inactivates viral and/or cellular proteins that are required for the replication of the HPV31 origin by changing their posttranslational modifications.

ACKNOWLEDGMENTS

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Stu 218/3-1) to F.S. and by funding under the Sixth Research Framework Programme of the European Union, Project INCA (LSHC-CT-2005-018704).

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