Brd4-Independent Transcriptional Repression Function of the Papillomavirus E2 Proteins

Michal-Ruth Schweiger,† Matthias Ottinger,† Jianxin You, and Peter M. Howley*

Department of Pathology, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, Massachusetts 02115

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The papillomavirus E2 protein is a critical viral regulatory protein with transcription, DNA replication, and genome maintenance functions. We have previously identified the cellular bromodomain protein Brd4 as a major E2-interacting protein and established that it participates in tethering bovine papillomavirus type 1 E2 and viral genomes to host cell mitotic chromosomes. We have also shown that Brd4 mediates E2-dependent transcriptional activation, which is strongly inhibited by the disruption of E2/Brd4 binding as well as by short hairpin RNA (shRNA) knockdown of Brd4 expression levels. Since several mutants harboring single amino acid substitutions within the E2 transactivation domain that are defective for both transcriptional transactivation and Brd4 binding are also defective for transcriptional repression, we examined the role of Brd4 in E2 repression of the human papillomavirus E6/E7 promoter. Surprisingly, in a variety of in vivo assays, including transcription reporter assays, HeLa cell proliferation and colony reduction assays, and Northern blot analyses, neither blocking of the binding of E2 to Brd4 nor shRNA knockdown of Brd4 affected the E2 repression function. Our study provides evidence for a Brd4-independent mechanism of E2-mediated repression and suggests that different cellular factors must be involved in E2-mediated transcriptional activation and repression functions.

Human papillomaviruses (HPVs) are associated with papillomas, warts, and certain human cancers. The genital tract papillomaviruses (PVs) are classified as low risk (HPV6 and HPV11) or high risk (HPV16 and HPV18) depending upon the potential for malignant progression of the pathological lesions which they cause. DNA from a high-risk HPV type can be found in 98% of cervical cancer cases, suggesting that HPV is the causative agent for these malignancies. Cervical cancer is the second most common cancer among women worldwide, with nearly 500,000 new cases each year (54, 80).

The papillomavirus E2 protein is a multifunctional regulatory protein essential for the viral life cycle (30). Together with the viral E1 protein, it initiates origin-specific viral DNA replication. In addition, it is required for viral genome maintenance and the regulation of viral transcription. The structure of the E2 protein resembles that of a prototypic transcription factor, with an amino-terminal transcriptional activation (TA) domain and a carboxy-terminal DNA-binding and dimerization domain, separated by a variable hinge region (46). Using a proteomic approach, we have previously identified the bromodomain-containing protein Brd4 as a major E2-interacting protein and have shown that it functions to tether the bovine papillomavirus type 1 (BPV1) E2 protein and viral DNA to mitotic chromosomes (78). Through this tethering process, the Brd4/E2 interaction participates in the stable maintenance of viral genomes in dividing cells (1, 9, 33, 42, 56, 66, 78, 79).

Brd4 is a member of the BET (bromodomain and extrater-

* Corresponding author. Mailing address: Harvard Medical School, Department of Pathology, 77 Avenue Louis Pasteur, Room 950, Boston, MA 02115. Phone: (617) 432-2884. Fax: (617) 432-2882. E-mail: peter_howley@hms.harvard.edu.
† These authors contributed equally to this work.
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the N-terminal transactivation domain of E2 and may therefore be recruited to E2-responsive promoters in an E2 transcription factor-dependent manner (Fig. 1). This requirement of Brd4 for transcriptional activation of E2 is conserved among different PV groups and suggests an essential function in the viral life cycle (47). It should be noted that the viral E2 protein has been shown to interact with a number of cellular factors in addition to Brd4 that involve basic transcription machinery and chromatin-targeting/remodeling complexes, including TFIIIB, TATA box binding protein, AMF-1 (activation domain-modulating factor 1), p/CAF, p300/CBP, and NAP-1 (nucleosome assembly protein) (5, 10, 40, 41, 57, 58, 77). However, of the cellular factors, only Brd4 has correlated well with the transcriptional activation function of E2 (50, 69, 71). However, the E1-binding domain localizes to a different surface of E2 than the Brd4-binding domain (64). The region of the E2 N-terminal TA domain involved in transcriptional repression is identical to that required for transcriptional activation and Brd4 binding. Specific deletion and amino acid substitution mutations in the TA domain of E2 affect the transcriptional activation as well as the transcriptional repression and growth repression functions of HPV16 E2 (4, 18, 23, 26, 52). In contrast to those of its transcriptional activation function, the mechanisms by which E2 functions as a transcriptional repressor are less well known. Understanding the mechanism of PV E2 transcriptional repression is important for papillomavirus oncogenesis because it plays an important role in regulating high-risk HPV oncogene E6 and E7 expression (6, 59, 70). In high-risk HPV cervical cancers, the viral DNA is generally integrated into the cellular DNA in a manner that disrupts the viral E2 gene (2, 19, 63). The loss of E2 expression leads to the derepression of viral E6 and E7 expression (6, 59, 70). The resulting elevated E6 and E7 protein levels in turn induce ubiquitylation and degradation of p53 and functional inactivation of the retinoblastoma protein pRb, respectively (20, 25, 62). The reintroduction of E2 into HPV-positive cervical cancer cells represses E6/E7 expression and causes cell growth arrest and senescence (18, 23, 26, 70, 72).

The transcriptional activation function of the PV E2 protein on authentic viral promoters has been best studied for BPV1. Using the C-terminal domain of Brd4 (Brd4 CTD) to disrupt the binding between E2 and the endogenous Brd4 full-length protein as well as short hairpin RNA (shRNA) knockdown of Brd4, we were able to establish that Brd4 is required for the transcriptional function of BPV1 E2 on the authentic BPV1 long control region (LCR) P\textsubscript{op} promoter. We have further explored the role of Brd4 in the regulation of the HPV LCR promoters; in particular, we determined whether Brd4 has a role in mediating E2-dependent transcriptional repression. In contrast to its role in mediating the PV E2 transcriptional activation function, Brd4 did not have any effect on E2-dependent transcriptional repression, and we concluded that the papillomavirus E2 protein has Brd4-independent repression functions.

MATERIALS AND METHODS

Plasmids and antibodies. The expression vectors for HPV11 E2 (p2090), HPV16 E2 (p3662), BPV1 E2 (p2450), and HPV18 E6 (p3035) have been described previously (12, 31, 60, 75). The plasmid containing the C-terminal domain of Brd4, pcDNA4C-SV40NLS-Brd4-CTD (p9498), has been described by You et al. (78). LCR18F-Luc (p5190) was derived from pCATb-LCR18F (p3726) by subcloning the HPV18 LCR (nucleotides [nt] 7000 to 110) in the SacI-BglII sites of pGL3 (52). For pGL4-LCRBPV1 (p5191), pGL4-LCRHPV11 (p5192), and pGL4-LCRHPV18 (p5194), the BPV1 (nt 6958 to 91), HPV11 (nt 7072 to 102), and HPV18 (nt 6943 to 105) LCRs were cloned in the SacI-BglII sites of pGL4.20 (Promega), respectively. The reporter plasmids CAT-LCRBPV1 (p1066) and p2x2xE2BS (p3973) have been described previously (39, 67), pEGFP, pSV\textsubscript{b}-GAL, and the neomycin selection plasmid pLNCX were purchased from BD Biosciences, pcDNA4C was purchased from Invitrogen, and the puromycin selection plasmid pBABE-puro was obtained from Stratagene.

RNA interference. For Brd4 shRNA knockdown experiments 19-nt sequences were cloned in the pSUPER.retro.puro vector system (OligoEngine). The shRNA-Brd4(NT) (p5197) and shRNA-Brd4(CT) (p5188) targeting sequences were 5'-GACACTATGGAAACACCAG-3' and 5'-GGCGGAGGAGCGAAGA-3', respectively (64). The control scrambled shRNA (shRNA-scr, p5195) contained the sequence 5'-AGACACAGTACCTAGAA-3'. For transient transfection experiments, cells were left untransfected or transfected with pLm518 (p1067), pcDNA4C-SV40NLS-Brd4-CTD, pcDNA4C-SV40NLS-Brd4(NT) or shRNA-scr was used. Cells were grown under puromycin selection 3.2 g/ml puromycin and harvested 72 h after transfection. For pEGFP N1 transfection experiments, cells were transfected with pEGFP N1 plasmid DNA (Invitrogen) 24 h after transfection.

Cell lines and transfections. The human cervical cancer cell lines HeLa (HPV18 positive), SiHa (HPV16 positive), and C33A (HPV negative) were purchased from American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal calf serum (HyClone). Plasmid DNAs were introduced into cells by FuGENE 6 transfection reagent (Roche).

TA and transcriptional repression reporter assays. Approximately 2 x 10^6 C33A cells in 6-cm dishes were cotransfected with 0.8 \mu g reporter plasmid and 1.6 \mu g of the E2 expression plasmid by using FuGENE 6 (Roche). For experiments with the Brd4 CTD, 0.8 \mu g of the Brd4 CTD expression plasmid was included in the transfection and cells were harvested 48 h after transfection. For Brd4 knockdown experiments 3.2 \mu g of shRNA expression plasmid shRNA-Brd4(NT) or shRNA-scr was used. Cells were grown under puromycin selection with 0.625 \mu g/ml puromycin and harvested 72 h after transfection. To determine and normalize transfection efficiencies, 0.1 \mu g pSV\textsubscript{b}-GAL and 0.3 \mu g pEGFP were cotransfected. The total amounts of DNA per transfection were kept constant by using the same empty vector DNA. Cells were lysed with reporter lysis buffer (Promega), and luciferase activities were measured according to the manufacturer's protocol (Luciferase assay system; Promega). Luciferase activities were normalized to the \beta-galactosidase activity (luminescent \beta-galactosidase detection kit II; BD Biosciences). Chloromphenicol acetyltransferase...
(CAT) activities were measured with a colorimetric CAT enzyme-linked immuno-
usororbent assay (Roche). The results were normalized to the protein concentra-
tion in the lysates. For the titration experiments whose results are shown in
Fig. 3B and C, 2 × 10^6 C33A cells in 6-cm dishes were transfected with 200 ng
pGL4.20 LCRHPV18 (p5194) luciferase reporter plasmid plus the same amount
of simian virus 40 (SV40) β-galactosidase reporter. In addition, pCMV HPV16
E2 and pDNA4C-Brd4-CTD were transfected as indicated in the legend to
Fig. 3. The total amounts of DNA were kept constant at 6.4 μg DNA per dish,
using the respective empty vectors. For the experiments whose results are de-
picted in Fig. 8, approximately 10^5 C33A cells were seeded per well in 12-well
plates. Twenty-four hours later, cells were transfected with plasmids in the
quantities noted in the legend to Fig. 8. At 4 h posttransfection, sodium butyrate
was added to a final concentration of 5 mM where indicated. At 48 h posttrans-
fec tion, cells were lysed and processed as described above. Luciferase activities
were normalized for transfection efficiency separately for samples with or without
sodium butyrate by using β-galactosidase activities.

Northern blot analysis of endogenous E6/E7 expression. Approximately 2 × 10^6
HeLa cells were cotransfected with 2 μg of the E2 expression plasmid, 2 μg
of the Brd4 CTD expression plasmids, or empty vector. For Brd4 shRNA knock-
down experiments, HeLa cells were cotransfected with 4 μg of the shRNA
eexpression plasmids and cells were put under puromycin selection for 72 h. Total
RNA was prepared from 10^6 cells by using TRIzol reagent (Invitrogen) accord-
ing to the manufacturer’s instructions. RNA samples (10 μg) were resolved on
1% agarose gels containing formaldehyde and transferred to a charged nylon
membrane (Hybond N*; Amersham). Filters were hybridized overnight at 42°C
using probes specific for HPV18 E6/E7 (p3035) or GAPDH (glyceraldehyde-3-
phosphate dehydrogenase) labeled with [32P]dCTP by using a random prime labeling kit
(Stratagene). Blots were washed twice at room temperature and once at
45°C with wash buffer (2 × SSC [1 × SSC is 0.15 M NaCl plus 0.015 sodium citrate])
and 0.1% sodium dodecyl sulfate) and exposed to Kodak MS autoradiography film.

Whole-cell extract preparation and Western blot analysis. Whole-cell
cellular lysates were prepared using lysis buffer (10 mM HEPES, pH 7.4, 10 mM NaCl, 3 mM
MgCl\(_2\), 1 mM dithiothreitol, protease inhibitors [Roche]) (16). Cells were scraped
and transferred to Eppendorf tubes, and incubated on ice for 10 min before they
were passed through a 21-gauge needle 10 times. NaCl was added to a final concentra-
tion of 300 mM, and lysates were rotated at 4°C for 20 min. The protein extracts
were then centrifuged for 5 min at 2,000 × g, and supernatants were transferred to a new
Eppendorf tube. Insoluble proteins were incubated with lysis containing 300
mM NaCl, 20 mM MgCl\(_2\), and DNase I for 60 min at 37°C. Supernatants were
combined with the soluble protein fractions, and 20 to 30 μg of protein extracts was
separated on 4 to 12% bis-Tris gradient gels. Proteins were transferred to a polyvi-
ynylidiene difluoride membrane (PerkinElmer), and immunoblot analyses were
performed. Affinity-purified rabbit polyclonal Brd4 antibodies have been described
previously (64). The mouse monoclonal antiactin was purchased from
Chemicon (1501), the mouse monoclonal antibody anti-p53 from Santa Cruz (sc-
126), and the anti-Xpress-tag antibody from Invitrogen.

Cellular growth repression assay. Approximately 2 × 10^6 HeLa cells
were cotransfected with combinations of 0.6 μg pBABE-puro, 0.4
μg pEGFP, 2 μg of the E2 expression plasmid, and 1.5 μg pDNA4C-Brd4-CTD.
Twenty-four hours after transfection, cells were split into approximately 3 × 10^6
cells per 35-mm petri dish and put under puromycin selection (0.5 μg/ml). Cells from three
independent plates were counted each day.

 Colony reduction assay. A total of approximately 2 × 10^6 HeLa cells were
cotransfected with 2.4 μg of the E2 expression plasmid, 1.5 μg pDNA4C-hBrd4-
CTD, 0.2 μg pEGFP, and 0.4 μg pLNCX. At 24 h posttransfection, cells were
split and cultured in growth medium supplemented with G418 (900
mg/ml) for 21 days. Cells were washed with phosphate-buffered saline, fixed with methanol,
and stained with methylene blue. The total number of neomycin-resistant colonies
was determined.

RESULTS

Brd4 is required for the E2-dependent activation of the
BPV1 LCR P\(_{50}\) promoter. The cellular bromodomain protein
Brd4 binds E2 on the surface of the N-terminal transcriptional
activation domain of E2 (34, 64, 65). In our previous study that
established a role for Brd4 in E2-mediated transcriptional ac-
tivation, we employed reporter plasmids that used a heterolo-
gous promoter driven either by multiple E2-binding sites or by
the BPV1 LCR in an enhancer configuration upstream of a

basal SV40 early promoter (p407-1) (64). To extend these
studies and to address whether Brd4 is involved in the E2
activation of an authentic BPV1 LCR, the cellular bromodomain protein
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Brd4 CTD alone had little effect on the basal activity of this reporter plasmid (Fig. 2A, lane 4). Similar results were obtained by shRNA knockdown of Brd4. In the presence of shRNA-ser, E2 induced CAT expression approximately 36-fold (Fig. 2B, lane 2). This activation was reduced significantly by shRNA knockdown of Brd4 protein levels (Fig. 2B, lane 4). The effectiveness of the shRNA in the knockdown of Brd4 protein levels has previously been shown (64) and was confirmed as a part of this experiment (Fig. 2C). These results demonstrate that Brd4 is required for E2-mediated transcriptional activation of an authentic papillomavirus promoter.

Disruption of the HPV16 E2 interaction with Brd4 does not affect the transcriptional repression function of E2. Functional analyses of single amino acid substitution mutants in the N-terminal TA domain of E2 have indicated that the transcriptional activation and repression functions of E2 are mediated by the same face on the E2 TA domain and that this area is identical to that of the Brd4-binding domain. In addition, a recent study based largely on biochemical analyses has implicated Brd4 in E2-mediated transcriptional silencing (73). We therefore addressed the potential role of Brd4 in E2-mediated repression by using a variety of cell-based assays. First, we employed a reporter assay using a plasmid containing the HPV18 P105 promoter-driven luciferase to assess E2 transcriptional repression (Fig. 3A). The HPV18 LCR reporter by itself exhibited activity approximately 20-fold over that of the promoterless empty vector in C33A cells (not shown).

To investigate the possibility of dose-dependent effects and to define the linear range for our assays, we first carefully titrated HPV16 E2 in the LCR repression assay. As expected, we observed moderate activation of the HPV18 LCR at very low doses of E2 (10 to 100 ng HPV16 E2 plasmid), but at higher doses of E2 (1 to 5 µg HPV16 E2 plasmid), the HPV18 LCR was repressed in a dose-dependent manner to a level of about 25% of its initial activity (Fig. 3B). Cotransfection of 0.8 µg of the Brd4 CTD plasmid, the amount that is effective in disrupting the ability of E2 to transactivate (Fig. 3D), resulted in a moderate reduction of LCR activity in the absence of E2 (Fig. 3B). The Brd4 CTD also reduced the ability of E2 to activate the LCR. At higher E2 doses, the Brd4 CTD increased the ability of E2 to repress the LCR (Fig. 3B). Based on these results, we used doses of E2 throughout this study that were in the range for a linear response. We also carefully titrated the measured using a heterologous promoter reporter construct (0.8 µg p2x2xE2BS-Luc) containing four E2-binding sites (p3973). Cotransfection of 1.6 µg E2 plasmid (p3662) activates this reporter plasmid approximately 16-fold (lane 2). This activation is inhibited by cotransfection of 0.5 µg Brd4 CTD plasmid, the amount that is effective in disrupting the ability of E2 to transactivate (Fig. 3D), resulting in a moderate reduction of LCR activity in the absence of E2 (Fig. 3B). The Brd4 CTD also reduced the ability of E2 to activate the LCR. At higher E2 doses, the Brd4 CTD increased the ability of E2 to repress the LCR (Fig. 3B). based on these results, we used doses of E2 throughout this study that were in the range for a linear response. We also carefully titrated the

FIG. 3. The transcriptional repression function of HPV16 E2 is independent of Brd4. (A) Schematic of the E2-dependent transcriptional repression LCR18 plasmid (p5190). The LCR of HPV18 is cloned upstream of the luciferase reporter gene. The four E2-binding sites, the origin of replication (ori), an Sp1 site, and a TATA box in the proximity of the P105 promoter are shown. (B) Titration of HPV16 E2 on the HPV18 LCR in the absence and the presence of the dominant-negative Brd4 CTD. C33A cells were cotransfected with 0.2 µg pGL4.20-LCR-HPV18 (p5194) luciferase reporter, 0.2 µg SV40 β-galactosidase plasmid, 0.8 µg of pcDNA4C-Brd4-CTD, or the empty vector together with increasing amounts of pCMV HPV16 E2 expression plasmid as indicated. At 48 h posttransfection, cell lysates were prepared and analyzed. (C) Titration of the Brd4 CTD. C33A cells were transfected with reporter constructs as for panel B together with the indicated amounts of pCMV HPV16 E2 and Brd4 CTD plasmids. (D) The Brd4 CTD strongly inhibits HPV16 E2-mediated transcriptional activation. HPV16 E2-dependent transcriptional activation was
amount of the Brd4 CTD (Fig. 3C). HPV16 E2 alone repressed the LCR activity to approximately 60%. Increasing amounts of the Brd4 CTD further increased the E2-dependent transcriptional repression function but did not inhibit this repression (Fig. 3C). This HPV18 activity (Fig. 3E, lane 1) was repressed by HPV16 E2 by approximately 40% (Fig. 3E, lane 2).

Surprisingly, the Brd4 CTD did not rescue repression by E2; instead, the repression was further enhanced (Fig. 3E, compare lanes 2 and 3). Thus, in contrast to the inhibitory effect of the Brd4 CTD on E2-dependent transcriptional activation functions (Fig. 3D), the Brd4 CTD did not inhibit the ability of E2 to repress the HPV18 P105 promoter (Fig. 3E). Transcriptional activation and repression assays were performed at the same time, under comparable conditions, by expression of the same amounts of E2 and the Brd4 CTD and by using the same amount of reporter plasmids. The comparable levels of CTD expression were confirmed by Western blot analysis (data not shown). Since in this experiment the Brd4 CTD induced a strong inhibition of E2 transcriptional activation (Fig. 3D) but had no negative effect on E2 transcriptional repression (Fig. 3E), the data suggest that disruption of the Brd4/E2 interaction by the Brd4 CTD does not play an inhibitory role in the repression of the HPV18 P105 promoter under these conditions.

Taken together, none of the concentrations of E2 or the Brd4 CTD reversed the E2-mediated repression of the HPV18 LCR. These data provide evidence that E2-mediated repression of the LCR in cells does not require Brd4.

The Brd4 CTD does not rescue E2-induced growth repression of HeLa cells or reduction in colony formation. In HeLa cells, the HPV18 genome is integrated into the host cellular DNA in a manner that disrupts the E2 open reading frame (ORF). Reexpression of E2 results in cellular growth inhibition and a reduction in colony formation due to E2-dependent repression of the HPV18 P105 promoter, which controls E6 and E7 expression levels (18, 23, 32, 70). We therefore investigated the role of Brd4 in E2-mediated repression of the integrated HPV18 promoter in HeLa cells by using the Brd4 CTD to disrupt the binding of E2 to cellular Brd4. For growth repression assays, an HPV16 E2 expression plasmid or empty vector, with or without an expression plasmid for the Brd4 CTD, was cotransfected into HeLa cells, which were split and placed under puromycin selection. The normal doubling time of HeLa cells (approximately 24 h) was not affected by the expression of the Brd4 CTD (Fig. 4A). The expression of HPV16 E2, however, induced a marked growth arrest.

This growth inhibition was not rescued by the expression of the Brd4 CTD, indicating that the transcriptional repression function of E2 does not depend upon the interaction of E2 with the cellular Brd4 protein. Similar results were obtained with a colony reduction assay (Fig. 4B) in which HeLa cells were cotransfected with an E2 expression plasmid or vector alone, with or without the Brd4 CTD expression plasmid, together with a plasmid conferring neomycin resistance. Cells were split and maintained under neomycin selection for 21 days. In the absence of E2, HeLa cells efficiently formed neomycin-resistant colonies. Colony formation was markedly inhibited by either HPV16 E2 or BPV1 E2 and was unaffected by the coexpression of the Brd4 CTD, indicating that the ability of E2 to suppress HeLa cell growth does not depend upon an interaction with Brd4 (Fig. 4B). To directly assess the effect of the Brd4 CTD on E2 transcriptional repression of the HPV18 E6/E7 promoter, we performed Northern blot analyses examining HPV18 RNA levels in transfected cells (Fig. 5A).

HPV18 transcription is normally robust in HeLa cells and, as has previously been shown, is repressed by E2 (14, 18, 26). This E2 repression of HPV18 E6/E7 mRNA was unaffected by the coexpression of the Brd4 CTD and E2 (Fig. 5A), indicating that the disruption of E2/Brd4 binding does not influence the
transcriptional repression function of E2. Quantification and normalization of the Northern blot showed that the E2 protein repressed the basal promoter activity to approximately 59% and that the coexpression of the Brd4 CTD further enhanced the transcriptional repression to approximately 66%. These results are in good agreement with those of the transcriptional repression reporter assays (Fig. 3). We further confirmed the lack of an effect of the Brd4 CTD on this repression phenotype by examining p53 protein levels. E6 induces the ubiquitylation and degradation of p53 (62), and E2 repression of the HPV18 P105 promoter leads to a reduction in E6 levels, resulting in the stabilization of p53 (Fig. 5B). We therefore examined and quantified the p53 levels as a biologically relevant readout for HPV oncoprotein expression, we found no evidence that shRNA knockdown could relieve the stabilization of p53 by E2 (Fig. 6D, lanes 1 and 4). However, Brd4 knockdown alone reduced p53 protein levels by approximately 20% (Fig. 6D, lanes 1 and 3). We extended this analysis to SiHa cells, which are HPV16-positive cervical carcinoma cells. Similar to its function in HeLa cells, E2 represses the integrated viral promoter in SiHa cells, resulting in a decrease in viral E6 and E7 levels (data not shown) and in the stabilization of p53. This effect was not reversed by shRNA knockdown of endogenous Brd4 (Fig. 6E). These results further confirm that the HPV16 E2 transcriptional repression functions in cervical carcinoma cells are not dependent upon Brd4.

The low-risk HPV11 E2 transcriptional repression function is also independent of Brd4. In contrast to our results presented here, a recent study has implicated a role for Brd4 in the transcriptional silencing function of HPV11 E2 (73). Similar to HPV16 E2 and HPV18 E2, the low-risk HPV11 E2 protein also has transactivation properties and can repress the E6/E7 promoter in the HPV11 LCR (11, 28). We therefore examined the possibility that Brd4 might specifically be involved in the E2 repression functions of HPV11. Using a luciferase reporter assay with the HPV11 LCR positioned upstream of the luciferase gene, we found that HPV11 E2 repressed the basal activity of the reporter construct by approximately 60% in the presence of control shRNA-scr (Fig. 7B, lanes 1 and 2) and that the Brd4 knockdown had no significant effect on the repression capacity of HPV11 E2 (Fig. 7B, lanes 3 and 4). Simultaneously performed Brd4 shRNA knockdown experiments resulted in a strong inhibition of BPV1 E2 transactivation functions, indicating that the knockdown was sufficient to inhibit E2 functions (Fig. 7A). In addition, we examined the effect of the Brd4 CTD on the HPV11 E2 transcriptional repression function (Fig. 7C). We observed that HPV11 E2 repressed the HPV11 LCR promoter (Fig. 7C, lanes 1 and 2) and that the coexpression of the Brd4 CTD did not rescue the repression of the E6/E7 promoter (Fig. 7C, lanes 3 and 4). This result is consistent with observations for the HPV18 LCR. The slight increase of E2-dependent transcriptional repression in the presence of the Brd4 CTD is likely due to the effect of the Brd4 CTD on stabilization of the E2 protein (34). Thus, even though Brd4 is required for the physiologic transcriptional activation function of BPV1 E2 (Fig. 7A), it is not required for the transcriptional repression func-

FIG. 5. Repression of HPV18 transcription and stabilization of p53 protein levels by HPV16 E2 in HeLa cells is not rescued by Brd4 CTD expression. (A) Northern blot analysis of the HPV18 E6 mRNAs. HeLa cells were transfected with empty vector (lane 1) or with plasmids expressing HPV16 E2 (2 μg; lane 2 and 3) or HPV16 E2 plus the Brd4 CTD (2 μg; lane 3). Forty-eight hours after transfection, 10 μg of total cell RNA from HeLa cells was separated on a 1% formaldehyde gel, transferred to a Hybond N nylon membrane, and hybridized with a probe to the HPV18 E6 ORF. The loading control for 18S rRNA is shown. (B) Immunoblot analysis of p53. Similar to what was carried out for the Northern blot analyses, HeLa cells were transfected with an empty vector alone (lane 1) or with plasmids expressing HPV16 E2 (2 μg; lane 2 and 3) or HPV16 E2 plus the Brd4 CTD (2 μg; lane 3). Cells were harvested 48 h after transfection, and 30 μg of total cell extracts was subjected to immunoblot analysis.

FIG. 6. E2 represses the integrated viral promoter in SiHa cells, resulting in a decrease in viral E6 and E7 levels (data not shown) and in the stabilization of p53. This effect was not reversed by shRNA knockdown of endogenous Brd4 (Fig. 6E). These results further confirm that the HPV16 E2 transcriptional repression functions in cervical carcinoma cells are not dependent upon Brd4.

transcriptional repression function of E2. Quantification and normalization of the Northern blot showed that the E2 protein repressed the basal promoter activity to approximately 59% and that the coexpression of the Brd4 CTD further enhanced the transcriptional repression to approximately 66%. These results are in good agreement with those of the transcriptional repression reporter assays (Fig. 3). We further confirmed the lack of an effect of the Brd4 CTD on this repression phenotype by examining p53 protein levels. E6 induces the ubiquitylation and degradation of p53 (62), and E2 repression of the HPV18 P105 promoter leads to a reduction in E6 levels, resulting in the stabilization of p53 (Fig. 5B). We therefore examined and quantified the p53 levels as a biologically relevant readout for HPV oncoprotein expression levels and found that the expression of the Brd4 CTD did not significantly alter the amount of p53 seen in E2-expressing HeLa cells (Fig. 5B). This experiment provides further evidence that the interaction of E2 with Brd4 is not necessary for E2-mediated repression in vivo in HPV-positive cervical carcinoma cells with the integrated HPV18 genome.

E2 functions as a transcriptional repressor even in the absence of Brd4. The above-described experiments using the Brd4 CTD to compete with the binding of E2 to the endogenous Brd4 protein established that the full-length Brd4 protein is not required for E2 transcriptional repression functions. However, it remained possible that the Brd4 CTD might contain a repressive activity on its own (Fig. 3B and C, two left-most lanes). We therefore further investigated the effect of shRNA Brd4 knockdown on the E2 repression function. In the presence of control shRNA-scr, the basal activity of the HPV18 LCR promoter was efficiently repressed by HPV16 E2 (Fig. 6B, lanes 1 and 2). Knockdown of Brd4 protein levels with shRNA-Brd4(NT) had no effect on E2 repression and resulted in similar levels of luciferase expression as in control experiments (Fig. 6B, lanes 3 and 4). In a control experiment performed simultaneously, the Brd4 knockdown nearly completely abolished the transcriptional activation function of BPV1 E2 (Fig. 6A). Western blot analysis of Brd4 protein levels confirmed a significant knockdown of Brd4 protein levels. Thus, in the HPV18 LCR luciferase reporter assay, Brd4 was not required for E2-mediated repression.

We next examined the effect of Brd4 knockdown on HPV18 E6/E7 transcriptional repression by E2 directly in HeLa cells by Northern blot analysis (Fig. 6C). HPV18 mRNA was strongly reduced by E2 in the presence of a control shRNA (Fig. 6C, compare lanes 1 and 2). shRNA knockdown of Brd4 did not relieve this repression (Fig. 6C, compare lanes 2, 4, and 6). Furthermore, using p53 protein levels as a marker of E6 expression, we found no evidence that shRNA knockdown could relieve the stabilization of p53 by E2 (Fig. 6D, lanes 2 and 4). However, Brd4 knockdown alone reduced p53 protein levels by approximately 20% (Fig. 6D, lanes 1 and 3). We extended this analysis to SiHa cells, which are HPV16-positive cervical carcinoma cells. Similar to its function in HeLa cells, E2 represses the integrated viral promoter in SiHa cells, resulting in a decrease in viral E6 and E7 levels (data not shown) and in the stabilization of p53. This effect was not reversed by shRNA knockdown of endogenous Brd4 (Fig. 6E). These results further confirm that the HPV16 E2 transcriptional repression functions in cervical carcinoma cells are not dependent upon Brd4.

The low-risk HPV11 E2 transcriptional repression function is also independent of Brd4. In contrast to our results presented here, a recent study has implicated a role for Brd4 in the transcriptional silencing function of HPV11 E2 (73). Similar to HPV16 E2 and HPV18 E2, the low-risk HPV11 E2 protein also has transactivation properties and can repress the E6/E7 promoter in the HPV11 LCR (11, 28). We therefore examined the possibility that Brd4 might specifically be involved in the E2 repression functions of HPV11. Using a luciferase reporter assay with the HPV11 LCR positioned upstream of the luciferase gene, we found that HPV11 E2 repressed the basal activity of the reporter construct by approximately 60% in the presence of control shRNA-scr (Fig. 7B, lanes 1 and 2) and that the Brd4 knockdown had no significant effect on the repression capacity of HPV11 E2 (Fig. 7B, lanes 3 and 4). Simultaneously performed Brd4 shRNA knockdown experiments resulted in a strong inhibition of BPV1 E2 transactivation functions, indicating that the knockdown was sufficient to inhibit E2 functions (Fig. 7A). In addition, we examined the effect of the Brd4 CTD on the HPV11 E2 transcriptional repression function (Fig. 7C). We observed that HPV11 E2 repressed the HPV11 LCR promoter (Fig. 7C, lanes 1 and 2) and that the coexpression of the Brd4 CTD did not rescue the repression of the E6/E7 promoter (Fig. 7C, lanes 3 and 4). This result is consistent with observations for the HPV18 LCR. The slight increase of E2-dependent transcriptional repression in the presence of the Brd4 CTD is likely due to the effect of the Brd4 CTD on stabilization of the E2 protein (34). Thus, even though Brd4 is required for the physiologic transcriptional activation function of BPV1 E2 (Fig. 7A), it is not required for the transcriptional repression func-
down of Brd4. C33A cells were cotransfected with 0.8 μg/shRNA-expressing plasmid, either the control shRNA-scr (lanes 1 and 2) or shRNA-Brd4(NT) (lanes 3 and 4), together with the empty vector (lanes 1 and 3) or 1.6 μg plasmid expressing HPV16 E2 (lanes 2 and 4). Cells were grown under puromycin selection and harvested 72 h after transfection for immunoblot and luciferase assays. The luciferase activities were normalized for transfection efficiencies by using β-galactosidase activities. (B) shRNA knockdown of Brd4 does not affect the E2-dependent transcriptional repression function. C33A cells cotransfected with 0.8 μg HPV18 LCR promoter luciferase reporter plasmid (lanes 1 to 4), empty vector (lanes 1 and 3), or 1.6 μg plasmid expressing HPV16 E2 (lanes 2 and 4) and 3.2 μg vectors expressing control shRNA-scr (lanes 1 and 2) or shRNA-Brd4(NT) (lanes 3 and 4) were grown under puromycin selection for 72 h. Cells were then harvested for immunoblot analyses. RLU, relative light units.

FIG. 6. shRNA knockdown of Brd4 abolishes E2-mediated transcriptional repression. (A) Inhibition of E2 transcriptional activation by knockdown of Brd4. C33A cells were cotransfected with 0.8 μg E2 luciferase reporter plasmid (p2x2x2E2BS-Luc) and 3.2 μg shRNA-expressing plasmid, either the control shRNA-scr (lanes 1 and 2) or shRNA-Brd4(NT) (lanes 3 and 4), together with the empty vector (lanes 1 and 3) or 1.6 μg plasmid expressing HPV16 E2 (lanes 2 and 4). Cells were grown under puromycin selection and harvested 72 h after transfection for immunoblot and luciferase assays. The luciferase activities were normalized for transfection efficiencies by using β-galactosidase activities. (B) shRNA knockdown of Brd4 does not affect the E2-dependent transcriptional repression function. C33A cells cotransfected with 0.8 μg HPV18 LCR promoter luciferase reporter plasmid (lanes 1 to 4), empty vector (lanes 1 and 3), or 1.6 μg plasmid expressing HPV16 E2 (lanes 2 and 4) and 3.2 μg vectors expressing control shRNA-scr (lanes 1 and 2) or shRNA-Brd4(NT) (lanes 3 and 4) were grown under puromycin selection for 72 h. Cells were then harvested for immunoblot analyses. RLU, relative light units.

DISCUSSION

The papillomavirus E2 protein is important for viral DNA replication, viral genome maintenance, and viral transcriptional regulation (30). Of these functions, its role in facilitating viral DNA replication may be best understood (68). E2 helps to recruit the viral E1 helicase to the origin of replication in order to initiate viral DNA replication. The roles of E2 in genome maintenance and viral transcriptional regulation are less well understood; however, the recent identification of Brd4 as a major E2-interacting protein has provided new insights into these functions (78). The transcriptional activation function was the first activity of E2 to be described as a classical transcriptional activation of either the low-risk HPV11 E2 protein or the high-risk HPV16 E2 protein.

Ther repression of the HPV11 LCR by the HPV11 E2 protein is independent of Brd4 in the absence or presence of the histone deacetylase inhibitor sodium butyrate. Brd4 has been implicated in promoting RNP-driven transcriptional elongation through its interaction with pTEFb (37, 78). Many specific and general transcription factors dissociate from hypoacetylated chromosomes. Since Brd4 binds to chromatin via acetylated histones in an acetylation-dependent manner (16), we asked whether the role of Brd4 in transcriptional regulation might depend on the acetylation status of cellular factors. We therefore studied the repression function of the HPV11 E2 protein in the HPV11 LCR in C33A cells in either the absence or the presence of the histone deacetylase inhibitor sodium butyrate 48 h or 24 h (data not shown) after transfection. As depicted in Fig. 8, HPV11 E2 repressed the HPV11 LCR both in the absence and in the presence of sodium butyrate (Fig. 8A to D, compare two leftmost columns). The coexpression of the Brd4 CTD further enhanced the repression by HPV11 E2, without itself acting as a significant repressor (Fig. 8A and B, two rightmost columns), independent of the addition of sodium butyrate. Furthermore, the knockdown of Brd4 did not significantly rescue the HPV11 E2 repression function (Fig. 8C and D) either in the absence or in the presence of sodium butyrate. In parallel experiments, we observed a strong inhibition of BPV1 E2 transactivation of the BPV1 LCR in both the absence and the presence of sodium butyrate (Fig. 8E and F).
enhancer protein for the BPV1 P89 promoter (27, 67). Structural and functional studies with E2 have shown a correlation of Brd4 binding with its transactivation function and have shown that the transcriptional activation function of E2 is in fact mediated through Brd4 (Fig. 2) (34, 47, 64, 65). In line with this, recent studies have established a direct role for Brd4 in transcriptional regulation. Brd4 interacts with cyclin T1 and Cdk9, both components of the positive transcriptional elongation factor P-TEFb, as well as with several components of the Mediator complex, TRAP80, TRAP100, TRAP170, TRAP230, and TRAP220 (29, 35, 38, 76). Brd4 can displace an inhibitory subunit (7SK snRNA and MAQ1/HEXIM1) from the P-TEFb complex, thereby transforming P-TEFb into its transcriptionally active form. Brd4 might therefore provide a functional link between E2 and the active P-TEFb and components of Mediator complexes and thereby connect E2 to the general transcription machinery (Fig. 1).

The transcriptional repression functions of E2 have been...
best studied for the genital tract HPVs, the low-risk HPV11 as well as the high-risk HPV16 and HPV18 types. Transcriptional modulation of the E6/E7 promoter of high-risk HPV16 and HPV18 types is likely important for regulating the expression of the E6 and E7 viral oncoproteins as part of the normal virus-host cell interaction, and its dysregulation contributes to the pathogenesis of cervical cancer. By binding to E2-binding sites located within the LCR, E2 represses transcription of the HPV16 P\textsubscript{\gamma} and HPV18 P\textsubscript{105} viral promoters, which direct the expression of E6 and E7 (17, 59, 69, 70). This repression occurs through E2-binding sites located in close proximity to essential promoter elements, such as an Sp1-binding site and the TATA box (13). However, the mechanism by which E2 mediates the repression of these promoters has yet to be mechanistically elucidated.

The E2-dependent transcriptional repression function cannot simply be achieved by the displacement of cellular factors on the E6/E7 promoter, since the C-terminal DNA-binding domain by itself and certain N-terminal mutants of E2 which can still bind the promoter region are unable to repress these promoters (4, 18, 23, 26, 52). Interestingly, alanine substitution mutants localized the transcriptional repression and transcriptional activation domains to exactly the same amino acids on the N terminus of E2 (4, 26, 52). Therefore, since Brd4 binds to the same region that mediates the transcriptional activation function of E2, it seemed likely that Brd4 might also be important for transcriptional repression. However, transcriptional reporter assays revealed that E2-mediated repression is Brd4 independent (Fig. 3 and 6 to 8). Disruption of the Brd4/E2 interaction by the Brd4 CTD or shRNA knockdown of Brd4 did not rescue the transcriptional repression of E2 in vivo (Fig. 3 to 8). In fact, we observed a slight enhancement of transcriptional repression by the Brd4 CTD, which is likely due to a stabilization of E2 by the Brd4 CTD (34; data not shown). Thus, Brd4 mediates E2 transcriptional activation functions but not repression functions. These results were further verified by examining the effect of Brd4 CTD expression or Brd4 knockdown on E2-dependent transcriptional repression of the endogenous E6/E7 promoter in HPV-positive cervical carcinoma cell lines (Fig. 4 to 6).

Our data are in contrast to those of a recent study that implicated Brd4 in HPV11 E2-dependent transcriptional silencing (73). That study focused on the low-risk HPV11 E2 protein and relied upon in vitro assays. The differences between the results of our study and the results of Wu et al. might have been due to the different E2 proteins used. The E2 proteins from high-risk HPVs are stronger transcriptional activators than their low-risk counterparts, and the strength of activation is intrinsic to the N-terminal domain of E2 (39). To address the question of whether low-risk and high-risk PV E2 proteins differ in their requirements for Brd4 for their transcriptional repression function, we also performed HPV11 E2-dependent transcriptional reporter assays using an authentic HPV11 LCR promoter construct (Fig. 7 and 8). However, neither disruption of the Brd4/E2 interaction by using the Brd4 CTD nor the knockdown of Brd4 alleviated HPV11 E2 repression. Our results for the low-risk HPV11 E2 and the HPV11 LCR mirror our observations with the high-risk HPV16 E2. Thus, the discrepancies between these two studies do not appear to be intrinsic differences between the E2 proteins used and might therefore be due to the different kinds of assays employed. Wu et al. used primarily biochemical in vitro transcription assays (73), whereas we concentrated on cell-based in vivo experiments. In addition, the cotransfection experiments using a luciferase reporter assay presented by Wu et al. were performed in the presence of 5 mM sodium butyrate, a histone deacetylase inhibitor. Treatment of cells with the histone deacetylase inhibitor trichostatin A has been reported to increase the association of Brd4 with chromosomes (15, 48) and might therefore affect E2 transcription functions. However, we explored a potential connection between protein acetylation and the E2 repression function by examining E2 repression in cells that had been treated with sodium butyrate. While sodium butyrate treatment enhanced the overall levels of expression of the reporter plasmids, it did not reveal any Brd4-dependent repression activity for E2 in experiments using either the Brd4 CTD or knockdown of Brd4 (Fig. 8).

As discussed above, the binding of Brd4 to P-TEFb displaces an inhibitory subunit from the P-TEFb complex and activates transcription (Fig. 1) (35, 76). It is tempting to extend this mechanism of transcriptional regulation to the PV E2 proteins. E2 might recruit the P-TEFb complex to the promoter through Brd4. Our studies show that there is a Brd4-independent mechanism of transcriptional repression for E2. In the absence of Brd4, the inhibitory subunit could remain bound to P-TEFb and transcriptional elongation is paused. The analysis of alanine-scanning mutants of the N-terminal region of E2 might suggest that a cellular factor might bind to E2 through the same face of E2 involved in binding to Brd4 (64). If so, it should be noted that the Brd4 CTD that binds to this face of E2 would not be able to compete with the binding to this putative cellular factor since the Brd4 CTD did not rescue the E2 repression function. The mechanisms involved in E2-mediated repression are unknown, and it is not clear whether the P-TEFb complex is involved in the process in a Brd4-independent manner. Regardless, our data indicate that there is an E2-dependent transcriptional repression function that is independent of Brd4.

There are also other transcription factors engaged in both transcriptional activation and transcriptional repression. Interestingly, Runx1 and the glucocorticoid receptor (GR) also mediate their transcriptional regulation functions through P-TEFb. Runx1 binds cyclin T1 and thereby blocks P-TEFb function and arrests RNAPII (37). For transcriptional repression by the GR, the GR competes with P-TEFb for binding to the RelA subunit of NF-\kappaB and displaces P-TEFb from the interleukin-8 promoter (43). The P-TEFb nonresponsive Isk kinase \alpha promoter is not repressed by the GR.

Another transcriptional regulation mechanism resembling the one described for E2 is that of the cellular CCAAT/enhancer binding protein \beta (C/EBP\beta) (7, 49). C/EBP\beta engages Mediator complexes involved in transcriptional activation and repression (8, 51). C/EBP\beta acts as a repressor by recruiting the Cdk8 module-containing Mediator complex. The Cdk8 module is a variable part of the Mediator, composed of Med12/Sub8, Med13/Srb9, Cdk8/Srb10, and CycC/Srb11. By binding to the Mediator, the Cdk8 module sterically blocks Mediator interactions with RNAPII and transcription is inhibited (21). However, the active C/EBP\beta form recruits the Mediator complex devoid of the Cdk8 module. RNAPII can then be bound and
transcriptionally activated. Interestingly, the exchange of Mediator complexes is regulated by the Ras/mitogen-activated protein kinase pathway. Phosphorylation of C/EBPβ leads to a preferential binding of the active Mediator complex to C/EBPβ.

Additional examples of transcription factors that can function as activators or repressors are heat shock factor 1 (HSF1), peroxisome proliferator-activated receptor γ (PPARγ), and myocyte enhancer factor 2 (MEF2), which use a phospho-sumoyl switch to control their transcriptional activities (74). Phosphorylation of a conserved ϕKXEXXX site followed by sumoylation inhibits transcription. Sumoylation can lead to transcriptional repression by recruiting histone deacetylases, other transcriptional cofactors, or by changing the subcellular localization of proteins (24). Although some phosphorylation sites of BPV1 E2 have been found and characterized (45), future work will need to be done to determine whether the HPV E2 proteins are also posttranscriptionally modified and whether these modifications play a role in Brd4 binding and/or transcriptional regulation. In conclusion, this study finds that the papillomavirus E2 proteins have a Brd4-independent repression function that is important for the high-risk HPV types associated with human cervical cancer.

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