The transcriptional regulation of the human telomerase reverse transcriptase (hTERT) gene is a critical step in transformation and differentiation. Human papillomavirus E2 protein inhibits cell growth in HPV-infected cells and triggers apoptosis in HeLa cells. Because E2 induces cell growth suppression and senescence, we hypothesize that the protein may modulate cellular gene expression related to these processes. In this report, we demonstrate that E2 inhibits the hTERT promoter. The mapping of the E2-responsive region of hTERT reveals that Sp1 is important for E2-mediated repression of this promoter in 293T cells. Site-directed mutagenesis data on the hTERT promoter show that E2 does not abolish E-Box-mediated transcription and represses promoter activity via the Sp1 binding site. Furthermore, chromatin immunoprecipitation assays indicate that E2 is actively recruited to the hTERT promoter region. Our findings provide novel insights into the biological function of human papillomavirus E2.

Papillomavirus (PV), which infects mammalian cells, encodes two transcription/replication regulatory proteins (E1 and E2) in its ~7900-bp genome (1). Both E1 and E2 associate with host cellular proteins involved in the transcriptional regulation and DNA replication, making PV a useful model for the study of these cellular processes in mammals (2–5). The bovine PV E1 protein functions as an ATPase, DNA helicase, and a replication origin-specific binding protein initiating viral-specific DNA replication in concert with viral E2 protein (6–11). The protein encoded by the E2 open reading frame is a regulatory factor that controls the transcription of the viral genome by host RNA polymerase II (12). PV E2 binds as a dimer to 12-bp inverted repeats (ACCCGGT) present in the PV upstream regulatory region and consequently activates or represses transcription (13–15). E2 contains an NH₂-terminal transcription activation domain and a COOH-terminal DNA-binding domain separated by a flexible hinge (16). Various regulatory proteins interact with human PV E2 to modulate E2-mediated transcription and viral replication (17–21). Recent reports (22–24) suggest that the E2 transcriptional activation function is required for the E6/E7 promoter repression. The point mutations in specific conserved sites within the bovine or human PV E2 transactivation domain that result in the loss of transcriptional activation also eliminate E6/E7 promoter repression. E2 induces senescence in HPV-positive cells via pRb- and p21-dependent pathways (25). Because E2 is associated with growth suppression and senescence, it is possible that the protein modulates cellular gene expression related to these processes.

Telomeres form the ends of eukaryotic chromosomes composed of tandem arrays of telomeric repeats (5'-TTAGGG-3' in human) (reviewed in Ref. 26). Telomerase, the ribonucleoprotein enzyme responsible for maintaining the ends of chromosomes, extends chromosomes with telomeric repeat sequences (reviewed in Ref. 27). Telomeric DNA in yeast and human ends in a 3' single-stranded overhang. The lengths of the overhangs are cell cycle-regulated and increase in the S phase (28). Although most normal somatic cells show no detectable telomerase activity, the activation of telomerase is a critical step in cellular transformation (reviewed in Ref. 29). Telomerase activity is mainly regulated by human telomerase reverse transcriptase (hTERT) gene expression (30–33). The hTERT promoter region lacks a canonical TATA or CAAT box and possesses a relatively high density of CpG dinucleotides and Sp1 sites (reviewed in Ref. 29). Additionally, the promoter contains a number of putative transcription factor binding sites such as Sp1, c-Myc, MZF-2, WT1, and possibly MAZ (34, 29). Earlier results indicate negative or positive regulation of hTERT gene expression by trans-acting factors including MZF-1 (35), p53 (36), Mad1 (37), KSHV LANA (38), and HPV-16 E6 (39–41). The observation that the introduction of viral E2 into HPV-infected cells inhibits telomerase activity raises the possibility of a link between hTERT expression and the E2 protein. In this study, we demonstrate that E2 represses the hTERT promoter. Moreover, Sp1 sites within the hTERT promoter are important for negative regulation of the HPV E2 protein.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—The pGL3B-hTERT luciferase reporter and pCG18-E2 constructs were described previously (34, 18). The pCDNA3–15E2, pCDNA3–16E2, pCDNA3–11E2, and pCDNA3–6B2E2 constructs were prepared by subcloning various E2 open reading frames into the EcoRI-XhoI sites of pCDNA3 (Invitrogen) using PCR amplifi-
FIG. 1. **E2 down-regulates the hTERT promoter in various cell lines.** A, C33A, COS-1, 293T, and HeLa cells were co-transfected with 1 μg of full-length hTERT promoter reporter vector (pBT-3970) and 3 μg of E2 expression vector. The extracts were prepared, and relative luciferase activities were normalized for transfection efficiency by the corresponding Renilla luciferase values. The experiments were performed at least three times. B, analysis of telomerase activity in 293T cells expressing E2. Lane 1, heat treatment of extracts before primer elongation; lane 2, RNase treatment of extracts before primer elongation; lane 3, no addition of extracts; lanes 4–6, indicated amounts of E2 expression vector-transfected extract samples. Relative activities were obtained by PhosphorImager (Fuji BAS1500).
culation. The E2 mutant expression vectors were described previously (18). The c-Myc expression vector was a generous gift from Dr. Martin Eilers. For producing glutathione S-transferase fusion proteins in bacteria, each gene was digested with EcoRI-XhoI and inserted into the corresponding restriction sites of pGEX4T-1 (Amersham Biosciences). The proteins were expressed in Escherichia coli JM109 and purified as described by the manufacturer (Amersham Biosciences).

**Cells, Transfections, and Reporter Assay—**293T, HeLa, COS-1, and C33A cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. At 24 h before transfection, 3 × 10^5 293T or C33A cells were plated in 6-cm dishes. The transfections were performed by the calcium phosphate precipitation method (42). DNA was prepared using the manufacturer’s protocol (Qiagen), and the total amount of transfected DNA was adjusted by the addition of blank plasmid DNA. In some experiments, pRL-tk was co-transfected and the total amount of transfected DNA was adjusted by the addition of blank plasmid DNA. In some experiments, pRL-tk was co-transfected for normalization of transfection efficiency. Equal amounts (5 × 10^5 cells) of cell lysates were used for the detection of luciferase activity (Promega, Madison WI). Renilla luciferase activity was measured with the Dual luciferase kit (Promega).

**Telomeric Repeat Amplification Protocol Assays—**Telomerase activity was analyzed using modified telomeric repeat amplification protocol assay (43). Crude cell extracts from 293T cells were prepared by the CHAPS lysis method (44). After a 30-min incubation for telomerase-mediated primer extension, the mixtures were heated to 90 °C for 30 min to inactivate telomerase activity. During this treatment, we added RP primer and RPC3g primer, and the samples were subjected to 29 cycles of PCR amplification. We used MTS primer (5'-AGCAGTCGAGCAGAGTGTT-3’) for elongation with telomerase and RP primer (5'-TAGAGCACAGCTGCGTGCTGTTG-3’) and RPC3g (5'-TAGAGCACAGCTGCGTGCTGTTGCTGCTGTTG(CTAACC),GG-3’) for PCR amplification.

**Western Blot—**Cells transfected with E2 expression vectors were pelleted, boiled for 5 min, separated on a SDS-PAGE, and electroblotted onto nitrocellulose membranes. The blots were blocked and hybridized with anti-E2 antibody. The proteins were detected by chemiluminescence (ECL, Amersham Biosciences).

**Mutagenesis—**The pBTDel-204-Sp1-mt and pBTDel-266-Myc- mt plasmids were constructed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) using manufacturer’s instructions. 10 ng of plasmid template and 125 ng of each primer were incubated in a final volume of 50 μl. Each construct was sequenced to confirm the incorporated mutations.

**Chromatin Immunoprecipitation (ChIP) assays—**ChIP assays were performed essentially as described in the Upstate Biotechnology (Waltham, MA) protocol. 50% confluent 293T cells in a 10-cm dish were transfected with 1 μg of hTERTp-Luc reporter plasmid and 2 μg of pCG-18E2 plasmid. Twenty-four hours after transfection, cells were cross-linked and treated as protocol. Approximately 1 μg of each indicated antibody was used. Precipitated DNA was resuspended in 50 μl of water. To detect the precipitated plasmid sequence, 1–5 μl of DNA in a total volume of 100 μl was subjected to 22–30 cycles of PCR with the following primers for luciferase. Luc5 (5’-ATGGAGACGCGACAAAACAT-3’) and Luc3 (5’-CATAGCTCTCTGCCAACGCCGAA-3’).

**RESULTS**

**HPV-18 E2 Down-regulates the Full-length hTERT Promoter in Various Cell Lines—**The cell growth suppression activity and down-regulation of telomerase activity by E2 suggests a role for the protein in hTERT promoter regulation (45). Because the catalytic subunit of telomerase is a primary target for activity and its gene expression is mainly regulated at the transcriptional level, we examined whether hTERT promoter activity was regulated by E2. The C33A cells were co-transfected with E2 and the full-length hTERT reporter construct (pBTDel-3970). The pBTDel-3970 reporter was repressed upon co-transfection with E2 expression plasmids (pCG-18E2). Similar assays were performed with other cell lines including COS-1, 293T, and HeLa. As expected, E2 down-regulated the hTERT promoter in all of the cell lines (Fig. 1A). To examine the effect of E2 on endogenous telomerase activity, we performed telomeric repeat amplification protocol assay using transient transfection of E2. Consistent with Fig. 1A, cells expressing E2 showed low levels of telomerase activity in a dose-dependent manner (Fig. 1B). As a control experiment, the preheated or RNase-treated samples before extension reaction exhibited no telomerase activity. We also
performed reverse transcription-PCR analysis using the same extracts, and we observed the down-regulation of hTERT expression by E2 (data not shown). These data suggested that E2 down-regulates telomerase activity through inhibiting hTERT expression.

The relationship between hTERT transcription and HPV-18 E2 expression was further characterized by comparing E2 with p53 and HPV-16 E6 proteins. p53 down-regulates while HPV-16 E6 up-regulates hTERT expression (41, 36). As shown in Fig. 2, E2 decreases hTERT promoter activity in a dose-dependent manner similar to p53, whereas HPV-16 E6 dose-dependently increases hTERT promoter activity. These results

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**Fig. 4. Deletion analysis of hTERT promoter repression by E2.** A, schematic representation of various hTERT promoter constructs used in this assay. B, pBT-3970, pBT-310, and pBT-266 are down-regulated by E2. Luciferase reporter vectors with serial deletions of the hTERT promoter were prepared. Following transfection of these reporters into 293T cells, luciferase assays were performed. The luciferase activities of pGL3 basic vector were normalized to 100, and the relative luciferase activity is shown. Experiments were performed at least three times. C, pBTdel-463, pBTdel-263, pBTdel-204, and pBTdel-185 are down-regulated by E2.
HPV E2 Down-regulates the hTERT Promoter

To determine the cis-element responsible for hTERT down-regulation by E2, we examined a series of hTERT promoter deletion mutants (Fig. 4A). The pBT-3970, pBT-310, and pBT-266 were down-regulated by E2. 3’-E-box-deleted hTERT promoter mutants (pBTdel-463, pBTdel-263, pBTdel-204, and pBTdel-185) were also repressed by E2 (Fig. 4, A and B), whereas the pGL3 basic promoter (a negative control) remained unaffected. Our data indicate that E2 does not influence E-box elements. Previous studies show that bovine papillomavirus E2 binds the Sp1 transactivation domain and superactivates GAL4-Sp1 in vivo. We speculate that E2 may activate or repress Sp1-related transcription in a context-dependent manner. To confirm our results and examine the importance of Sp1 binding sites in the hTERT promoter, we performed transient transfection assays using c-Myc and various hTERT promoters. As expected (Fig. 6C), c-Myc activated the hTERT promoter (pBT-266) and single c-Myc binding site promoter constructs. The results indicate that c-Myc binding sites are not involved in E2-mediated repression of the hTERT promoter. To further confirm that c-Myc does not affect the double mutant hTERT promoter (pBT-266-Myc-mt21), we performed transient transfection assays using c-Myc and various hTERT promoters. As expected (Fig. 6C), c-Myc activated the hTERT promoter (pBT-266) and single c-Myc binding site promoter constructs. However, no activation of the double mutant hTERT promoter (pBT-266-Myc-mt21) was observed. These data correlate with previous results and confirm that c-Myc binding sites are not required for E2-mediated down-regulation of the hTERT promoter.
pCG-18E2 or a control vector. The presence of a particular promoter in chromatin immunoprecipitates was detected by semi-quantitative PCR using specific pairs of primers spanning the luciferase sequence region. The antibodies to FLAG and E2 precipitated the wild-type reporter sequence (pBTdel-204) but failed to precipitate significant amounts of reporter DNA from pBTdel-204-Sp1mt-54321 (Fig. 8). Therefore, the results from the ChIP assay confirm that E2 binds the hTERT promoter region via Sp1 in vivo.

**DISCUSSION**

The HPV E2 protein is essential for viral DNA replication and transcription. In HPV-positive cells, the overexpression of full-length E2 protein results in growth inhibition and apopto-

**Fig. 6. Mutational analyses of c-Myc binding sites in the minimum hTERT promoter.** A, schematic diagram of the minimum hTERT promoter region containing c-Myc binding sites. Crossed-out boxes indicate the mutated sites for c-Myc as shown above figure. B, 293T cells were co-transfected with pBTdel-204, mutant reporter vectors, and E2 expression vector. The luciferase activities of pBT-266 vector were normalized to 100, and the relative luciferase activity is shown. The experiments were performed a minimum of three times. C, c-Myc up-regulates the hTERT promoter. However, hTERT mutated at the c-Myc binding site is not up-regulated by c-Myc. 293T cells were co-transfected with pBTdel-204, its mutant reporter vectors, and c-Myc expression vector.
The continuous expression of E6 and E7 genes from integrated HPV DNA is a hallmark in the majority of HPV-positive carcinoma cell lines. E6 and E7 play central roles in the progression and maintenance of cancers. Several recent reports show that E6 stimulates the hTERT promoter and activates telomerase activity (39–41). HPV E2 suppresses the expression of E6 and E7 in HeLa cells, which in turn leads to cell growth arrest (22, 25). Intact E2 transactivation function is

**FIG. 7. Mutational analyses of Sp1 binding sites in the minimum hTERT promoter.** A, schematic diagram of the minimum hTERT promoter region containing Sp1 binding sites. Crossed-out ovals indicate the mutated sites for Sp1 depicted above the figure. B, 293T cells were transfected with a combination of pBTdel-204, its mutant reporter vectors, and E2 expression vector. The luciferase activities of pGL3 basic vector were normalized to 100, and the relative luciferase activity is shown. The experiments were performed a minimum of three times. C, mutations in Sp1 sites of hTERT promoter abrogates E2-mediated repressional activity.
required for the binding of this protein to the integrated E6/E7 promoter to suppress promoter activity in HeLa cells. However, only E6/E7 suppression by ectopic expression of E2 is associated with E2-mediated senescence and cell growth suppression, because the overexpression of Rb or p53 in HeLa cells does not result in senescence (25). It is possible that E2 has other functions that contribute to cell growth suppression. Here we demonstrate that the overexpression of E2 results in the down-regulation of the hTERT promoter. Because E2-mediated suppression of the hTERT promoter is observed in p53 and pRb-negative C33A cells, this inhibition may not be necessary for the activity of these two proteins. Although we have not determined the possibility of the physical interactions between E2 and telomerase, the identification of differentially expressed subunits of telomerase indicates major control points at the level of transcription. Accordingly, we speculate that E2 may control telomerase activity primarily at the transcriptional level. Our data indicate that protein-protein interactions between E2 and Sp1 are significant for E2-mediated down-regulation of the hTERT promoter. Through competition with co-activators, E2 down-regulates the transactivation function of Sp1-mediated hTERT promoter activity. However, our ChIP assay indicates that E2 is recruited to the hTERT promoter in a Sp1-dependent manner, suggesting the existence of another mechanism. Alternatively, E2 may recruit the co-repressor complex via its DNA-binding domain. The hTERT promoter is affected by trichostatin A, a histone deacetylase inhibitor. Sp1 plays an important role in the enhancement of the hTERT promoter by trichostatin A (47, 48). These data suggest that the hTERT promoter is regulated by the status of acetylation within the promoter region. Moreover, Sp1 recruits either the histone deacetylase or histone acetylase complex to the hTERT promoter. NFκB is a good example of a factor that uses co-repressors and co-activators to regulate gene expression. The activation of transcription by NFκB requires a number of co-activators, whereas repression requires histone deacetylase co-repressors (49). It is not yet known whether E2-mediated repression of the hTERT promoter is derived from the recruitment of the histone deacetylase complex. We are currently attempting to determine the mechanism of E2-mediated repression of the hTERT promoter activity by E2.

HPV DNA is maintained as an episome in precancerous lesions, and E2 is important for episomal DNA replication (1, 9, 13). The E2 function is almost lost during carcinogenic progression as a result of viral DNA integration into the cellular genome.
genome and concomitant disruption of the E2 open reading frame. The loss of E2 increases the level of E6/E7 whose continuous expression is necessary to maintain the transformed state of cells (reviewed in Ref. 1). These phenomena indicate that the loss of E2 protein expression contributes to carcinogenesis. The observed down-regulation of the hTERT promoter by E2 suggests that this protein has another role in host cell growth control. Moreover, loss of E2 protein expression may be contributed to E2-mediated cell growth suppression. Although E2 represses E6/E7 expression in HPV-infected cells, the protein may contribute to cell growth suppression in other ways (25). We believe that E2-mediated repression of the hTERT promoter is not sufficient to suppress cell growth. However, our data suggest that E2 itself has a direct role in senescence induction, independent of the down-regulation of E6/E7.

In this report, we clearly demonstrate that E2 down-regulates the hTERT promoter in various cell lines. Our findings elucidate the biological activity of E2 in cell growth suppression and reveal the cellular target genes modulated by this protein.

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