The E6AP Ubiquitin Ligase Is Required for Transactivation of the hTERT Promoter by the Human Papillomavirus E6 Oncoprotein*

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Most human cancer cells display increased telomerase activity that appears to be critical for continued cell proliferation and tumor formation. The E6 protein of malignancy-associated human papillomaviruses increases cellular telomerase in primary human keratinocytes at least partly via transcriptional activation of the telomerase catalytic subunit, hTERT. In the present study, we investigated whether E6AP, a ubiquitin ligase well known for binding and mediating some of the activities of the E6 oncoprotein, participated in the transactivation of the hTERT promoter. Our results demonstrate that E6 mutants that fail to bind E6AP are also defective for increasing telomerase activity and transactivating the hTERT promoter. More importantly, E6AP knock-out mouse cells and small interfering RNA techniques demonstrated that E6AP was required for hTERT promoter transactivation in both mouse and human cells. Neither E6 nor E6AP bound to the hTERT promoter or activated the promoter in the absence of the partner protein. With all transactivation-competent E6 proteins, induction of the hTERT promoter was dependent upon E box elements in the core promoter. It appears, therefore, that E6-mediated activation of the hTERT promoter requires a complex of E6-E6AP to engage the hTERT promoter and that activation is dependent upon Myc binding sites in the promoter. The recruitment of a cellular ubiquitin ligase to the hTERT promoter during E6-mediated transcriptional activation suggests a role for the local ubiquitination (and potential degradation) of promoter-associated regulatory proteins, including the Myc protein.

The high risk human papillomaviruses (e.g. HPV-161 and HPV-18) have been identified as causative agents of more than 95% of cervical cancers and are etiologically linked to additional human cancers, including those of anogenital, oral, and epidermal origin (1). The main transforming genes of the high risk HPVs are E6 and E7. Both are required and sufficient for the efficient immortalization of primary keratinocytes (2). HPV E7 is capable of binding and inactivating the retinoblastoma tumor suppressor protein and retinoblastoma homologs (3) as well as several other cellular targets (4). Although the best known function of HPV E6 is the targeting and ubiquitin-dependent degradation of the p53 tumor suppressor protein (5–7), E6 also binds to additional cellular proteins and has functions that are independent of p53 degradation (8). The growing list of E6 target proteins includes E6-BP (9), paxillin (10), hDlg (the human homologue of the Drosophila disc large tumor suppressor protein) (11, 12), Mcm7 (minichromosome maintenance protein 7) (13, 14), IRF-3 (IFN regulatory factor 3) (15), Myc (16, 17), Bak (Bcl-2-homologous antagonist/killer) (18, 19), E6TP-1 (E6-targeting protein 1) (20, 21), CREB-binding protein/p300 (22, 23), Tyk2 (protein-tyrosine kinase 2) (24), hScrib (the human homologue of the Drosophila Scribble protein (29), PKN (a novel protein kinase with a catalytic domain homologous to that of protein kinase C) (26), MUPP1 (multi-PDZ domain protein 1) (27), MAGI-1 (membrane-associated guanylate kinase protein) (28), Gps2 (G-protein pathway suppressor 2) (29), ADA3 (30, 31), and tuberin (32).

In order for E6 to bind and degrade p53, it must first form a complex with the cellular ubiquitin ligase, E6AP. Although the precise orientation of the E6-E6AP-p53 complex is unresolved, it appears that there is a small helical domain within E6AP (L2G2) that allows E6 to bind and then associate with p53 (33). Not all E6 target proteins use this domain for binding; however. For example, E6 binding to paxillin is dependent upon a PDZ domain (11, 12, 34), whereas E6 binding to p300 involves three noncontiguous regions unrelated to PDZ (22). The E6-p300 binding is also independent of E6AP and does not result in protein degradation. Thus, E6 binding to target proteins can proceed via either PDZ domains, PDZ domains, or new uncharacterized sequences.

Several studies also suggest that E6 has p53-independent activities that may yet be dependent on E6AP. For example, the bovine papillomavirus type 1 E6 protein, which does not directly degrade the p53 protein, still interacts with E6AP, and this interaction correlates with its transforming activity (35, 36). Similarly, the HPV-16 E6 mutant, E6–8S9A10T, which cannot degrade p53, still binds E6AP and induces telomerase (37). Indeed, many of the growth-promoting effects of E6 cannot be mimicked by p53 degradation. Transgenic mice expressing HPV-16 E6 in the basal layer of the skin display cellular hyperproliferation and epidermal hyperplasia and develop ma-
lignant skin cancers, but p53-null mice do not (38). E6 also confers resistance to serum- and calcium-induced differentiation of human foreskin keratinocytes (HFKs) (39), but the analysis of E6 mutants, Mdm-2 overexpression, and dominant negative p53 constructs indicates that p53 degradation is insufficient to induce these cellular alterations (40). Evidently, there are additional E6 targets that are critical for inducing abnormal cell proliferation, and it is presumed that some of these targets require E6-E6AP interactions.

E6AP belongs to a class of ubiquitin-protein ligases called HECT E3 proteins, which directly transfer ubiquitin to their substrates (41, 42). E6AP function is critical for normal development, and the disruption of E6AP expression in the hippocampal and Purkinje neurons of the brain induces a severe mental retardation and coordination disorder known as the Angelman syndrome (43, 44). The catalytic domain of HECT proteins is a conserved 350-amino acid region defined by its homology to the E6AP carboxyl terminus (HECT). The HECT domain binds to specific E2 enzymes and contains an active site cysteine residue that forms a thiolester bond with ubiquitin (41, 45). Interestingly, E6AP also appears to function independently as a coactivator for the nuclear hormone receptors (46).

HPV-16 E6 protein increases telomerase activity in primary keratinocytes, and this induction is also independent of its ability to degrade p53 (47). Telomerase is a specialized reverse transcriptase that synthesizes repeat DNA sequences at the ends of chromosomes termed telomeres (48). The absence of telomerase activity in most normal human cells results in the progressive shortening of telomeres with each cell division (49, 50), which ultimately leads to chromosomal instability and cellular replicative senescence or growth arrest (49, 51). Telomerase activation is considered a critical event in the process of cell immortalization. The telomerase enzyme is a ribonucleoprotein complex composed of two core subunits, a template RNA subunit (hTERT) (52), and a catalytic protein subunit (hTERT) (53, 54), as well as subunits important for telomere maintenance and stability (48, 55). Whereas the hTERT template subunit is ubiquitously and equivalently expressed in both normal and tumor tissues (56), the hTERT subunit is selectively expressed in a small subset of normal cells (possibly transient amplifying cells), tumor tissues, and tumor-derived cell lines (53, 54, 57, 58), indicating that hTERT is the rate-limiting component of telomerase activity. Indeed, ectopic expression of hTERT alone in telomerase-negative cells is sufficient to restore telomerase activity and to induce the immortalization of several primary human cell types (50, 59, 60). In addition, the introduction of a dominant-negative hTERT into cancer cells inhibits telomerase activity and limits their growth (54). More recent studies have shown that E6 induces the hTERT promoter (17, 37, 61, 62) and that this induction depends upon E6 binding to the E6AP E3 ubiquitin ligase (37). Myc protein binding sites (E boxes) in the hTERT promoter require both the E6 and E6AP proteins, that the E6 and E6AP proteins are physically and functionally associated with this promoter, and that the E6 and E6AP proteins appear to form a complex prior to promoter engagement. It is possible that requisite recruitment of E6AP to the hTERT promoter by E6 reflects a requirement for the local ubiquitination of promoter-associated proteins such as Myc.

MATERIALS AND METHODS

Cell Culture and Plasmids—Primary HFKs were cultured from neonatal foreskins as described (64) and maintained in keratinocyte growth media (Invitrogen), supplemented with gentamycin (50 μg/ml). Primary HFKs P8 (passage 8) were infected with amphotropic LXSX retroviruses expressing AU1-tagged HPV-16 WT E6 (E6A), E6A/ SS9A10T, E6A/Δ9–13, or 6bE6A, as described elsewhere (37, 45). Retrovirus-infected cells were selected in G418 (100 μg/ml) for 5 days. Resistant colonies were pooled and passaged every 3–4 days (1:4 ratio). HeLa cells, NIH 3T3 (mouse fibroblasts), and E6AP knock-out cells (mouse fibroblasts) (66, 67) were maintained in Dulbecco’s modified Eagle’s medium.

Reverse Transcription (RT)-PCR—Total cellular RNA was isolated with TRIZol reagent (Invitrogen), and DNA was removed using the DNA-free kit (Ambion) according to the manufacturer’s instructions. Total RNA was treated with RNase-free DNase (Epicentre) and DNase-free cDNA was synthesized with some modifications using 1 μg of total cellular RNA following the instructions of the Superscript first strand synthesis system for RT-PCR (Invitrogen). Five percent of the RNase H-treated cDNA products were subjected to PCR in a total volume of 50 μl containing 10 μM sense primer and 10 μM antisense primer as described in the manufacturer’s protocol. Initial denaturation for 3 min at 94 °C was followed by 30 cycles of PCR amplification (94 °C for 30 s, 52 °C for 30 s, 72 °C for 60 s). PCR products were analyzed for E6 mRNA with an Agilent 2100 Bioanalyzer (Agilent Technology) and analyzed for E6AP and GAPDH mRNA with 1.5% agarose gel. The primers used for RT-PCR were as follows: 5′-ATGGTTCAGCAGCAACAAGAAGAGAATTTG-3′ and 5′-CACGCTGGTCTTCTCAGTGTTTACAGGAGAATTTG-3′ for E6 (size of PCR product, full-length E6: 453 bp for WT and SS9A10T, 438 bp for Δ9–13; E6*I: 270 bp for WT and SS9A10T, 255 bp for Δ9–13; E6*II: 153 bp for WT and SS9A10T, 149 bp for Δ9–13) (41, 42); 5′-TCTACACAGGCTGTAAATTGGGCATATAAAAGAGATTG-3′ and 5′-AAATTCTACTTATATGAAGCATGCTGTTTGTTGTCAATAAAGGATTG-3′ for E6AP primers (413 bp); 5′-TCTAGACACCATTGGGGAGGATTGGAG-3′ and 5′-ATGAGTCTAGGCTGTGTTGGCATC-3′ for GAPDH (458 bp).

Chromatin Immunoprecipitation (ChIP) Assay—HeLa cells were transfected with HPV-E6A constructs. E6AP knock-out cells were co-transfected with the pGL3B-255 hTERT promoter plasmid plus HPV-16 E6A and HA-tagged E6AP expression vectors using Lipofectamine 2000 (Invitrogen). ChIP was performed as described previously (17). Normal rabbit, goat, or mouse IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit anti-Myc polyclonal antibody (N-282; Santa Cruz Biotechnology), goat anti-E6 polyclonal antibody (C-19; Santa Cruz), mouse anti-hTERT (N-14 and C-18; Santa Cruz Biotechnology), mouse anti-AU1 (MMS-130; Covance), and anti-HA monoclonal antibodies (F-7; Santa Cruz Biotechnology) were applied to appropriate immunoprecipitation. PCR products were separated on a 1.8% agarose gel and visualized by ethidium bromide stain.

Immunoprecipitation and Western Blot—Immunoprecipitation and Western blot were performed as described previously (68, 69). E6, E6AP, and β-actin were detected with monoclonal AU1 (MMS-130; Covance), polyclonal anti-E6AP (25), and monoclonal anti-β-actin (A-541; Sigma), respectively.

siRNA Transfection—HeLa cells at 50% confluence or HFKs transfected with HPV-16 E6A at 60–70% confluence were transfected with a siRNA transfection reagent (Dharmacon) and Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. siRNA duplexes plus pGL3B-255 hTERT and the Renilla reniformis luciferase gene were co-transfected with siRNA duplexes plus pGL3B-255 hTERT and the Renilla reniformis luciferase gene.

Transfection and Luciferase Assay—1 × 10^5 telomerase-negative HFK cells were seeded onto 24-well plates and grown overnight. Transient transfections were performed using Lipofectamine 2000 reagent (Invitrogen) according to the protocol provided by the manufacturer. Cotransfections were performed using 0.5 μg of a core hTERT reporter plasmid (pGL3B-255) and 20 ng of each expression vector as indicated (AU1-tagged HPV-16 E6 or mutants, HA-tagged E6AP in pCMV4 from
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Peter Howley) or empty vectors as control for basal promoter activity. Cells were also cotransfected with 2 μg of the pRL-CMV plasmid (Promega), which contains the R. reniformis luciferase gene as a transfection control. Firefly and Renilla luciferase activities were measured 24 h after transfection using the dual luciferase reporter assay system (Promega).

TRAP and Real Time Quantitative TRAP (70, 71)—HFKs transfected with LXSIN, E6A, E6A/SS9A10T, or 6bE6A were grown in 100-mm tissue culture dishes to 80% confluence, harvested by trypsinization, washed in cold PBS, and transferred to a microcentrifuge tube. Cell pellets were lysed for 30 min on ice in 400 μl of TRAP buffer (0.5% Chaps, 10 mM Tris, pH 7.5, 1 mM MgCl2, 1 mM EGTA, 5 μM β-mercaptoethanol, 10% glycerol, 0.1 mM 4-(2-aminophenyl)benzenesulfonyl fluoride hydrochloride). Lysates were centrifuged at 14,000 × g for 5 min at 4 °C, the supernatant was transferred to a new tube, and protein concentration was determined (Bio-Rad). A TRAP assay was performed on 1 μg of protein lysates as described, with some modifications. Lysates were incubated for 30 min at room temperature in a 50-μl reaction volume containing 1× PCR buffer (20 mM Tris, pH 8.4, 50 mM KCl), 100 ng of telomerase substrate primer 5′-AACCCGTCGGACGAGTGT-3′, 50 μmol/liter each deoxynucleoside triphosphate (dATP, dTTP, dGTP, and dCTP), 1.5 mM MgCl2, and 0.5 μg of T4 gene protein (Roche Applied Science). After initial denaturation for 4 min at 94 °C, 100 ng of downstream primer 5′-CCCTTACCCTTACCCTTACCCTAAACATCTCGG-3′ and 0.2 μg of Taq DNA polymerase were added, followed by 31 cycles of PCR amplification (94 °C for 30 s, 50 °C for 30 s, 72 °C for 45 s). Twenty percent of the PCR products were separated on 10% nondenaturing polyacrylamide gels and visualized using the Gelcode color silver staining kit (Pierce).

For the quantitative TRAP assay, the first step of the assay was essentially the same as the above conventional protocol. In detail, each 40-μl reaction contained 2.0 μg of cell lysate diluted into 26 μl of 0.1 mg/ml bovine serum albumin, 1× TRAP reaction buffer (20 mM Tris-HCl, pH 8.3, 1.5 mM MgCl2, 10 mM EGTA, 63 mM KCl), 50 μM of each deoxynucleotide triphosphate, 0.1 μg of telomerase substrate primer, and 0.2 μg of T4 gene protein (Amersham Biosciences). The reaction mixture was incubated at room temperature for 20 min, and then telomerase was inactivated by heating at 95 °C for 10 min. In the second step of the assay, real time quantitative PCR was used to quantify the number of substrate molecules to which telomeric repeats had been added. SYBR Green real time quantitative PCR assays were carried out. Each 25-μl reaction contained 300 nm telomerase substrate and ACX (5′-GCCCGCGTACCTACCTACCTACCTACC-3′) primers and 1.0 μl of the product from the first step of the assay. A standard curve was produced for the real time quantitative TRAP assay using serially diluted 293 cell extracts. All samples were run in triplicate. This assay is linear over at least a 500-fold range (0.008–4 μg of 293 cell protein input).

RESULTS

Generation of Keratinocyte Cell Strains Expressing WT and Mutant E6 Proteins—To determine whether E6AP might be involved in telomerase activation, we analyzed several E6 mutants that exhibited varying abilities to bind this ubiquitin ligase. HFKs were transfected with retroviruses to stably express each of the following AU1-tagged E6 proteins: wild-type HPV-16 E6 (binds E6AP and degrades p53), HPV-16 E6/SS9A10T (binds E6AP but cannot degrade p53), HPV-16 E6/Δ9–13 (cannot bind E6AP or degrade p53), or HPV-6b E6 (cannot bind E6AP or degrade p53). We then characterized these cells for expression of their respective E6 mRNAs and proteins (Fig. 1). The keratinocytes transfected with WT E6, E6A/SS9A10T, and E6/Δ9–13 vectors express full-length E6, E6*I, and E6*II mRNAs of predicted sizes, with full-length mRNAs being predominant in each cell strain (Fig. 1A). Immunoprecipitation/immunoblotting techniques confirmed that wild-type and mutant AU1-tagged E6 proteins were expressed at similar levels in these cells, including the low risk HPV-6b E6 protein (Fig. 1B). Although the HPV-6 and HPV-16 E6 proteins (and the various mutants) vary in their amino acid sequence, the presence of the AU1 tag on each of these constructs allows the quantitative comparison of their expression levels.

E6 Proteins Defective for Binding E6AP Do Not Induce Cellular Telomerase Activity—To determine the ability of the E6 mutants to induce endogenous hTERT activity, we performed a standard TRAP (Fig. 1C) as well as a quantitative TRAP assay (Fig. 1D). As anticipated, the WT 16E6 protein induced telomerase activity in the keratinocytes (17, 37, 47, 61, 62). In addition, the HPV-16 E6/SS9A10T mutant also induced telomerase activity, confirming previous studies that p53 degradation is not required for induction (37, 47, 72). We did note in the quantitative TRAP assay that this mutant was about half as active as the WT E6 construct (Fig. 1D). More importantly, the HPV-16 E6/Δ9–13 and HPV-6b E6 constructs failed to increase cellular telomerase activity, demonstrating the critical role of the E6AP binding domain on E6. Either E6AP is required for this activation or the E6AP binding site on E6 is a critical region for alternative E6 functions.

E6 Mutants Defective for E6AP Binding Do Not Transactivate the hTERT Promoter—To determine whether the above differences in E6-induced telomerase activity correlated with the ability of the E6 mutants to transactivate the hTERT promoter, we transfected a luciferase minimal hTERT promoter construct pGL3B-255 into the stably transfected keratinocytes and measured relative promoter activity 24 h after transfection (Fig. 2A). The WT E6 or E6A/SS9A10T cells showed a 3–5-fold increase in hTERT promoter activity compared with control cells (Fig. 2A), although, as observed with the telomerase assay, the HPV-16 E6/SS9A10T mutant was somewhat less effective than the WT E6. Transcriptional activation of the hTERT promoter, similar to telomerase induction, was dependent upon the ability of E6 to bind E6AP, since the E6/Δ9–13 mutant was unable to transactivate the promoter. The HPV-6b E6 protein was also greatly reduced in activity (data not shown). Finally, we also co-transfected primary, nontransduced keratinocytes with the pBL3B-255 core hTERT promoter construct plus the indicated E6 expression vectors and have obtained similar results (Fig. 2B), suggesting that stable and transient conditions of E6 expression produce analogous results. Thus, whereas p53 degradation is dispensable for promoter activation, it appears that either E6AP or the E6AP binding site on E6 is necessary.

E6AP Augments E6-mediated Induction of Exogenous hTERT Promoter—As described above, the ability of E6 to increase cellular telomerase and to transactivate the hTERT promoter correlates with its ability to bind E6AP. We were therefore curious whether E6AP protein levels might be a rate-limiting event for E6-mediated transactivation. To address this question, we transfected HFKs with plasmid DNAs encoding the pGL3B-255 hTERT promoter construct, WT E6, and the HA-tagged E6AP (Fig. 2). Wild type E6 alone increased hTERT promoter activity 3–5-fold. When E6AP was added, there was a further increase up to 5–7-fold, indicating that E6AP protein levels are not completely saturating in keratinocytes for our assay. However, it is clear that E6AP does not appear to be a major limitation to promoter activation by E6. These experiments also demonstrated that E6AP could not induce the hTERT promoter by itself, suggesting that the observed augmentation of E6 activation was not an additive event as observed between E6 and c-Myc (17). E6AP-augmented promoter activity was also observed with the E6/SS9A10T mutant that is able to interact with E6AP, but not with the E6/Δ9–13 mutant that cannot bind E6AP, further substantiating the necessary role of E6-E6AP binding for promoter activation.

E6-E6AP-mediated Transactivation of the hTERT Promoter Is Dependent upon E Box Elements—Previous studies have shown that activation of the hTERT promoter by c-Myc (73, 74) or E6 (17, 37, 61, 63) is dependent on intact E box elements in the core promoter region of hTERT. To investigate whether the E boxes are required for transactivation by the E6-E6AP com-
plex, we performed transient reporter assays in telomerase-negative HFKs using a promoter construct that was mutated in both of the E boxes (Fig. 2C). Again, E6 alone led to 3–5-fold induction of wild-type core hTERT promoter activity in HFKs, whereas E6 plus E6AP led to a 5–8-fold induction. However, the promoter containing the E box mutations was unresponsive to E6 or E6 plus E6AP, verifying a critical role for these elements in mediating transactivation of the core hTERT promoter. As observed above, E6AP alone did not transactivate the hTERT promoter.
E6AP Binds to the hTERT Promoter Only in the Presence of E6—In a previous study, we demonstrated that the E6 and Myc proteins were associated in vivo and that they both bound and activated endogenous and exogenous hTERT promoters (17). In this study, we have shown so far that the E6AP binding site on E6 is required for transactivation of the hTERT promoter. The simplest hypothesis is that E6 is actually binding and recruiting E6AP to the hTERT promoter, potentially leading to the ubiquitination of target proteins. To address this possibility, we performed ChIP assays to determine if E6AP was bound to the hTERT promoter in the presence of E6. Primary HFKs were infected with an E6 retrovirus or control

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**Fig. 2.** E6AP augments E6-mediated induction of the hTERT promoter in an E box-dependent manner. **A,** E6AP increases E6-mediated hTERT promoter activity in stable keratinocyte cell lines. The E6-transduced keratinocyte strains generated in Fig. 1 were co-transfected with the minimal hTERT promoter luciferase construct (pGL3B-255 vector) and either E6AP or control vector. The pHL-CMV R. reniformis reporter plasmid was also transfected into the cells to standardize for transfection efficiency. Luciferase activity was measured 24 h after transfection using the dual luciferase reporter assay system (Promega). Relative -fold activation reflects the normalized luciferase activity induced by E6-E6AP compared with the normalized activity of vector control. The error bars show the S.D. value for at least three independent experiments. **B,** E6AP increases hTERT promoter activity in transiently transfected HFKs. Keratinocytes were transfected with the hTERT promoter vector in addition to the indicated E6 and E6AP vectors described in **A.** Luciferase activity was assayed 24 h later. Transfection efficiency was again standardized with the R. reniformis reporter plasmid. **C,** induction of hTERT promoter activity by E6-E6AP is dependent on E boxes. Primary HFKs were transfected with vectors encoding the minimal hTERT promoter with either intact (WT) or mutated E boxes as well as the indicated E6 and E6AP vectors.
retrovirus, selected in G418, and then prepared for ChIP analysis by formaldehyde cross-linking and sonication as described under "Materials and Methods." Protein-DNA complexes were precipitated with goat anti-E6 antibody (Santa Cruz Biotechnology), anti-E6AP antibody (Santa Cruz Biotechnology), or c-Myc antibody (Santa Cruz Biotechnology). The immunoprecipitated chromatin was then subjected to PCR amplification using sequence specific primers for the endogenous hTERT promoter, producing a 257-bp PCR product as demonstrated by the positive control hTERT plasmid and input DNA lanes. As previously described (17), immunoprecipitation with anti-E6 antibody generated a positive PCR signal from E6-transduced cells but not from vector-transduced cells (Fig. 3). We also obtained a positive signal using Myc antibodies in either the presence or absence of E6, suggesting that Myc is present on this promoter whether or not it is transcriptionally active. More importantly, E6AP antibodies precipitated the hTERT promoter in cells expressing E6 but not in vector-transduced cells, demonstrating that this ubiquitin ligase is recruited to the hTERT promoter by E6 during transactivation.

E6 Transactivates the hTERT Promoter Only in the Presence of E6AP—The preceding experiments indicate that E6AP does not associate with the hTERT promoter in the absence of E6. Whereas this initially suggests that E6 binds to Myc on the hTERT promoter and then recruits E6AP, it is also possible that E6 and E6AP need to form a complex prior to engaging the hTERT promoter. To distinguish between these possibilities, we expressed E6AP in immortalized mouse embryo fibroblasts with targeted deletions of both UBE3A (E6AP) alleles (66, 67) and determined whether E6 binding to the hTERT promoter was affected. E6AP knock-out cells were transfected with the E6 and the hTERT promoter or with the E6, E6AP, and hTERT promoter. In these experiments, the E6 protein was tagged with the AU1 epitope, and the E6AP protein was tagged with the HA epitope, and ChIP assays were performed with the corresponding antibodies. As shown in Fig. 4A, when transfected together, both E6 and E6AP associated with the hTERT promoter. In contrast, when E6AP was not present, E6 failed to associate with the promoter. Coupled with the data in Fig. 3, it is evident that neither E6 nor E6AP can associate with the promoter alone, suggesting that an E6-E6AP complex is required for E6 promoter engagement.

E6 Transactivates the hTERT Promoter Only in the Presence of E6AP—To verify that the above alterations in promoter association were reflected in promoter activation, we performed the luciferase assays in the same E6AP knock-out cells (Fig. 4B). Normal (NIH3T3) and E6AP knock-out mouse cells were transfected with E6, E6AP, or both constructs together. hTERT promoter activation was quantified via luciferase assays. The data indicate that E6 reproducibly induced the hTERT promoter in NIH3T3 cells, although the level of transactivation was less than that observed in human keratinocytes, reflecting differences in the response of mouse and human cells as well as epithelial and fibroblast cells. We observed the same cooperative effect of E6 and E6AP in the mouse cells as we previously showed in HFKs (see increase of promoter activity when E6 and E6AP are co-transfected). Most importantly, E6 could not induce the hTERT promoter in E6AP knock-out cells unless it was accompanied by E6AP. By itself, E6AP was unable to transactivate the promoter in either cell type. Thus, E6AP is also required for hTERT promoter activation, consistent with our E6 mutant analysis in Fig. 2.

Knockdown of E6AP with siRNA Inhibits hTERT Promoter Activation and Telomerase Activity in HeLa Cells—We also verified the critical need for E6AP in hTERT promoter activation in the human cervical cancer cell line, HeLa, which expresses the HPV-18 E6 protein. HeLa cells were transfected with an E6AP-specific siRNA duplex and then assayed for the expression of E6AP mRNA by RT-PCR as described. Fig. 5A demonstrates that the siRNA duplex reduced the level of E6AP mRNA in HeLa cells at 24 h post-transfection but did not affect the expression of GAPDH. Parallel to the changes in mRNA, the level of E6AP protein was also reduced in the siE6AP-transfected cells, whereas actin protein was unaltered (Fig. 5B). We also obtained the same results at 48 h posttransfection with siRNAs (data not shown). When hTERT promoter activity was assayed in these cells (Fig. 5C), there was a corresponding decrease in promoter activity in cells with E6AP siRNA. This inhibition was not observed in HeLa cells transfected with a control siRNA duplex. We also observed promoter inhibition by E6AP siRNA in nontumorigenic, nonimmortalized keratinocytes transfected with the HPV-16 E6 only (Fig. 5D). Evidently, the hTERT promoter is similarly sensitive to E6AP knockdown in both nonimmortal and tumorigenic cells, suggesting a continued dependence upon E6. As anticipated, it is also clear that E6AP is required for promoter activation by both HPV-16 (E6-transduced HFKs) and HPV-18 (HeLa) E6 proteins. The reduction in exogenous hTERT promoter activity by E6AP siRNA is paralleled by a reduction in the endogenous telomerase activity of HeLa cells (Fig. 5E). HeLa cells treated with E6AP siRNA have a significantly reduced TRAP assay activity.
Our previous studies demonstrated that the HPV-16 E6 and Myc proteins are physically associated in cells (17) and that both proteins bind to and transactivate the hTERT promoter, with the consequent induction of cellular telomerase activity. In the current study, we have shown that E6 binding to the hTERT promoter requires E6AP. Reciprocally, E6AP binding to the hTERT promoter requires E6. The combined binding of E6 and E6AP to the promoter correlates directly with transactivation, and it appears from our data that E6 and E6AP need to form a complex prior to associating with the hTERT promoter. Once associated with the promoter, we presume that the E6-E6AP proteins bind to Myc to form a trimeric complex of E6-E6AP-Myc. It is likely that additional cellular factors are also components of the complex.

Our hypothesis that E6-E6AP are interacting with Myc to facilitate hTERT transcription is reinforced by a previous in vitro study demonstrating that E6-Myc binding requires the presence of E6AP (16). The in vitro binding data is entirely consistent with our ChIP analysis and TRAP assays. In addition, our findings on the critical role of E6AP in modulating E6-induced hTERT transcription are supported by recent findings from the Huibregtse laboratory, which has found that the knockdown of either E6 or E6AP results in the inhibition of hTERT expression as well as the inhibition of other E6-responsive genes (data not shown).

The precise role of E6AP at the hTERT promoter is unknown, although it seems most likely that it directs the ubiquitination of specific regulatory proteins. Ubiquitination (with or without subsequent protein degradation) plays a critical role in the control of transcription factor activity (75, 76), and we postulate that proteins at or nearby the proximal E box are potential targets. For example, the BRCA1 tumor suppressor protein has been shown to bind at the hTERT E box and to suppress expression (77), and E6 is capable of binding BRCA1 and relieving this repression. It is possible that this relief of suppression is mediated by the ubiquitination/degradation of BRCA1. Myc is another potential target for ubiquitination and degradation by E6AP. Indeed, the stability and transcriptional activity of Myc is already known to be regulated by ubiquitination via the Skp2 and/or Fbw7 ubiquitin ligases (78–82). Similar to many other transcription factors, Myc contains a transactivation domain (at the N terminus) that also targets the protein for ubiquitination and degradation (78, 82). This overlap between transcription domains and ubiquitination/degradation domains (degrons) has been postulated to be important for tightly regulating gene transcription (76, 79, 82). Interestingly, in the middle of the Myc activation/degradation domain, there is a consensus L2G domain that can potentially regulate E6-E6AP binding. (We have adopted the term “L2G box” from the study of E6AP interactions with Mcm7 protein (13, 34), E6-E6AP interactions (83, 84), and sequences that were identified in peptides that bound to HPV E6 (85).) It is possible, therefore, that E6-E6AP might bind to this L2G site.

**DISCUSSION**

Our previous studies demonstrated that the HPV-16 E6 and Myc proteins are physically associated in cells (17) and that both proteins bind to and transactivate the hTERT promoter, with the consequent induction of cellular telomerase activity. In the current study, we have shown that E6 binding to the hTERT promoter requires E6AP. Reciprocally, E6AP binding to the hTERT promoter requires E6. The combined binding of E6 and E6AP to the promoter correlates directly with transactivation, and it appears from our data that E6 and E6AP need to form a complex prior to associating with the hTERT promoter. Once associated with the promoter, we presume that the E6-E6AP proteins bind to Myc to form a trimeric complex of E6-E6AP-Myc. It is likely that additional cellular factors are also components of the complex.

Our hypothesis that E6-E6AP are interacting with Myc to facilitate hTERT transcription is reinforced by a previous in vitro study demonstrating that E6-Myc binding requires the presence of E6AP (16). The in vitro binding data is entirely consistent with our ChIP analysis and TRAP assays. In addition, our findings on the critical role of E6AP in modulating E6-induced hTERT transcription are supported by recent findings from the Huibregtse laboratory, which has found that the knockdown of either E6 or E6AP results in the inhibition of hTERT expression as well as the inhibition of other E6-responsive genes (data not shown).

The precise role of E6AP at the hTERT promoter is unknown, although it seems most likely that it directs the ubiquitination of specific regulatory proteins. Ubiquitination (with or without subsequent protein degradation) plays a critical role in the control of transcription factor activity (75, 76), and we postulate that proteins at or nearby the proximal E box are potential targets. For example, the BRCA1 tumor suppressor protein has been shown to bind at the hTERT E box and to suppress expression (77), and E6 is capable of binding BRCA1 and relieving this repression. It is possible that this relief of suppression is mediated by the ubiquitination/degradation of BRCA1. Myc is another potential target for ubiquitination and degradation by E6AP. Indeed, the stability and transcriptional activity of Myc is already known to be regulated by ubiquitination via the Skp2 and/or Fbw7 ubiquitin ligases (78–82). Similar to many other transcription factors, Myc contains a transactivation domain (at the N terminus) that also targets the protein for ubiquitination and degradation (78, 82). This overlap between transcription domains and ubiquitination/degradation domains (degrons) has been postulated to be important for tightly regulating gene transcription (76, 79, 82). Interestingly, in the middle of the Myc activation/degradation domain, there is a consensus L2G domain that can potentially regulate E6-E6AP binding. (We have adopted the term “L2G box” from the study of E6AP interactions with Mcm7 protein (13, 34), E6-E6AP interactions (83, 84), and sequences that were identified in peptides that bound to HPV E6 (85).) It is possible, therefore, that E6-E6AP might bind to this L2G site.

**FIG. 4.** A, E6 binds to the hTERT promoter in vivo only when E6AP is present. ChIP analysis was used to evaluate the binding of E6 to the hTERT promoter in E6AP knockout mouse cells (mouse embryo fibroblasts with targeted deletions of both EBE3A alleles). The hTERT promoter construct (pGL3B-255) and AU1-tagged E6 vector were cotransfected into E6AP knockout cells in the presence or absence of HA-tagged E6AP vector. ChIP analysis was performed using AU1 and HA monoclonal antibodies to immunoprecipitate (IP) the E6 and E6AP proteins, respectively. Mouse IgG was used as a control, and PCR detection of precipitated hTERT construct was performed as described under “Materials and Methods.” Input, PCR signal generated from DNA of transfected mouse cells. E6 and E6AP antibodies immunoprecipitated the hTERT promoter only when both respective proteins were expressed. B, both E6 and E6AP are required to activate the hTERT promoter in E6AP knockout mouse cells. The above E6AP knockout mouse cells and control NIH3T3 cells (E6AP-positive) were transfected with the E6 expression vector, the hTERT promoter luciferase construct, with or without the E6AP vector. Mouse IgG was used as a negative control. Luciferase activity was assayed 24 h later as described previously. hTERT was transactivated in NIH3T3 cells by either E6 or E6 plus E6AP, whereas hTERT was only induced in E6AP knockout cells when both E6 and E6AP were expressed (E6 + E6AP).
and potentiate the turnover of Myc. However, we believe that this increased turnover might have an additional function other than preventing excessive gene transcription (i.e. the hTERT promoter has a unique placement of the proximal E box; the Myc binding site is downstream of the transcription start site). Whereas it is clear that Myc binding to the proximal E box is essential for gene transcription, it is also obvious that Myc must dissociate from this site (or be degraded) so that

**FIG. 5.** Knockdown of E6AP inhibits transactivation of the hTERT promoter by E6 in human cells. E6AP-specific duplex siRNA (or control siRNA) was transfected into HeLa cells or E6-transduced keratinocytes as described under “Materials and Methods.” The cells were analyzed by RT-PCR and Western blotting 24 h later. A, RT-PCR detection of E6AP mRNA (upper panel) and GAPDH mRNA (bottom panel) in transfected HeLa cells. The PCR signal for E6AP mRNA was reduced in HeLa cells treated with siRNA for E6AP, whereas the expression of GAPDH mRNA was unaffected. The relative level of mRNA expression was quantified by densitometry and is indicated below the respective lanes. B, Western blot detection of E6AP protein (upper panel) and β-actin (bottom panel) in E6AP-siRNA-treated HeLa cells. HeLa cells were treated identically with siRNA for E6AP as described in A but were then analyzed for E6AP protein expression by Western blot. In parallel to the results with mRNA, E6AP protein expression was reduced in the siRNA-treated cells, whereas actin protein expression was not. Again, densitometric quantification of mRNA expression is indicated below the respective gel lanes. C, hTERT promoter activity in siRNA-transfected HeLa cells. HeLa cells were cotransfected with control or E6AP-specific siRNA duplexes and the hTERT promoter reporter construct. Luciferase activity was assayed 24 h later. The E6AP-siRNA-treated HeLa cells showed an 80% inhibition of promoter activity. D, hTERT promoter activity in siRNA-treated E6-transduced HFKs. Luciferase assays were performed identical to those in C except that E6-transduced keratinocytes were used instead of HeLa cells. There was a similar inhibition (70%) of the hTERT promoter in the presence of the E6AP siRNA. E, Inhibition of telomerase activity in HeLa cells treated with E6AP siRNA. HeLa cells were treated as in C but were then assayed by TRAP assay. The cells treated with E6AP siRNA exhibited reduced telomerase activity, and densitometry techniques were used to quantify the inhibition as previously described (88).
transcription can proceed past this point. Thus, the activity of the hTERT promoter might be regulated by the complex kinetics of Myc engaging and disengaging the proximal E box, a process that could be enhanced by E6-E6AP. If this model were valid, we anticipate that there might be additional cellular promoters regulated by Myc (and with similar positioning of E boxes) such that they are also transactivated by E6.

Although p53 degradation is an important function of the E6 protein, the analysis of E6 mutants indicates that other activities are required for its full transforming activity (3, 4). Our results indicate that E6-induced telomerase activity is independent of p53 degradation and affirm findings from other laboratories (37, 72), although some studies have shown that p53 is an inhibitor of telomerase (86, 87). Using four E6 proteins, only one of which can degrade p53 (WT E6) and only two of which can associate with E6AP (WT E6 and E6/8S9A10T), we were able to show that association with E6AP is essential for the activation of both endogenous and exogenous hTERT (Figs. 1, C and D, and 2, A and B). The two E6 proteins (E6/Δ9–13 and 6b E6) unable to bind E6AP were also unable to activate telomerase. As anticipated, the exogenous expression of E6AP had no effect with these E6 constructs. Interestingly, telomerase activity in HeLa cells was inhibited by E6AP-specific siRNA duplexes. This suggests that, even in the tumorigenic HPV-positive cells, the high levels of telomerase activity depend upon continued E6-E6AP interactions.

The study of promoter regulation by ubiquitination is a new and rapidly developing field, and the association of the HPV E6 protein with E6AP on the hTERT promoter is an excellent model for dissecting the intricate roles of viral and cellular proteins in regulating gene expression. In addition, the ability of E6-E6AP to increase Myc transcriptional activity also has implications for the basis of HPV-induced neoplasia.

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