Human papillomaviruses (HPVs) are small DNA tumor viruses that infect cutaneous or mucosal epithelial cells. Approximately 30 HPV types are sexually transmitted and can be further categorized according to their associations with malignancies (65). Low-risk HPV types (e.g., types 6 and 11) are associated with benign lesions, while DNAs of high-risk HPV types (e.g., types 16, 18, 33, and 39) are generally expressed in HPV-positive cervical cancers, strongly suggesting that HPV is a causative agent of this disease (65). The only two viral proteins that are linked to carcinogenesis. For example, HPV E6-dependent and proteasome-mediated degradation has been reported for several PDZ domain-containing proteins, including Dlg (13), MAGI (16, 55), MUPP1 (36), and Scribble (43). A C-terminal PDZ binding domain, present within all cancer-associated HPV E6 proteins but not present in non-cancer-associated HPV E6 proteins, mediates interactions with this set of proteins. The integrity of the C-terminal PDZ binding domain has been linked to the ability of E6 to induce epithelial hyperplasia in mice in vivo (44). While Scribble ubiquitination was dependent on E6AP in vitro (43), it has been reported that E6AP does not mediate the degradation of Dlg in vitro (47), suggesting that E6 may target proteins for degradation by an alternative mechanism. E6 has also been reported to target Myc (19), E6TP1 (11, 12), and the proapoptotic Bak protein (26, 54) for degradation. The involvement of E6AP in Bak and Myc degradation has not been determined, while E6TP1 degradation was shown to be E6AP dependent in vitro and in vivo (10).
activity of the HPV E6 protein which is linked to cellular immortalization is its ability to induce transcriptional activation of the TERT gene (14, 45, 57, 58), which encodes the catalytic subunit of telomerase. TERT activation does not appear to be a downstream effect of p53 inactivation, since a mutant form of E6 (SAT 

produced telomerase activation is also dependent on E6AP. Gewin et al. showed that the ability of HPV16 E6 mutants to interact with E6AP correlated positively with the ability to activate telomerase (14), and they very recently identified a telomerase repressor (NFX1-91) that interacts with HPV E6/E6AP, suggesting that this is a novel target of E6/E6AP (15). E6-interacting proteins that are not thought to be targeted for degradation include paxillin (56), E6-BP (6), CBP/p300 (46, 64), and Tyk2 (37).

Therefore, while the cancer-associated HPV E6 proteins appear to be multifunctional, E6AP has been shown to mediate only a subset of these functions (e.g., targeting of p53 and E6TP1). Evidence for E6AP involvement in other functions is either conflicting (e.g., targeting of PDZ domain-containing target proteins), lacking (e.g., targeting of Myc and Bak), or suggestive (telomerase activation). In addition, it is unclear whether all of the reported E6 interactions are biologically meaningful and, if they are meaningful, at which point in the viral life cycle or immortalization-transformation process they are most relevant. Rather than determining whether E6AP is involved in each of the individual functions attributed to E6, we have addressed the question of E6AP involvement in E6 functions globally by examining alterations in the transcriptional profiles of HPV-positive cervical carcinoma cell lines upon small interfering RNA (siRNA)-mediated knockdown of either E6 or E6AP. We predicted that if all HPV E6 functions were dependent on E6AP, then the set of transcriptional alterations observed upon knockdown of E6AP would nearly entirely within the set of transcriptional alterations observed upon knockdown of E6. In contrast, if only a subset of E6 functions were mediated by E6AP, then the set of transcriptional alterations upon E6 knockdown would only partially overlap with the alterations observed upon E6AP knockdown. In both scenarios, the overlapping set of genes would be expected to include those that are transcriptionally induced, either directly or indirectly, by p53. While not all functions of E6 are likely to be affected by alterations in the transcriptional profile, a previous microarray-based study showed that E6 induces many p53-independent transcriptional alterations in human keratinocytes (8).

Overall, our results indicate that essentially all HPV E6 functions that impact on the global transcriptional program of HPV-positive cell lines are mediated by E6AP. In addition, a direct analysis of telomerase activity indicated that E6-induced telomerase activation is also dependent on E6AP.

**MATERIALS AND METHODS**

**siRNAs.** siRNAs were designed based on the suggestions of Tuschl and coworkers (9). All siRNAs were obtained from Dharmaco as purified, annealed duplexes or as single-stranded RNA oligonucleotides which were annealed according to the manufacturer’s instructions. To minimize possible off-targeting effects, we synthesized siRNAs corresponding to two different regions of each of the target genes and confirmed the knockouts by Western blotting and/or reverse transcription-PCR (RT-PCR). An siRNA against green fluorescent protein (GFP) was used as a control for changes in gene expression as a result of siRNA transfection. The siRNA target sequences were as follows: E6AP-A, 5′-CAAC TCTGGCTCTGAGATTA; E6AP-B, 5′-GATGTGACTTACCTAACAAG; E6AP-A, 5′-GGGTTTTGATTTGTCAT; E6-B, 5′-CTAATCAACTGGTGTAT; E6A-A, 5′-GGGTTTATGTAGTCTTCT (27); E6E6-B, 5′-ACCTGTTGGT TTGTTGA; and GFP, 5′-GGCTACCTCGAGGAGGCCAC.

**Tissue culture, RNA isolation, and Western blotting.** HPV type 18 (HPV18)-positive HeLa cervical carcinoma cells and HPV16-positive SiHa and Caski cervical carcinoma cells (American Type Culture Collection) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cells were plated 1 day prior to transfection to obtain 40 to 50% confluence at the time of transfection. Cells were transfected in 15-cm-diameter plates without antibiotics by the use of 2 to 3 μl of Oligofectamine (Invitrogen) per ml and a final siRNA concentration of 20 nM. HeLa cells were transfected in the presence of 10% fetal bovine serum. A Cy3 fluorescently labeled siRNA (Ambion) was used to estimate the siRNA transfection efficiency, which was determined to be nearly 100% for each cell line. Cells were harvested for RNA and proteins at 48 h posttransfection. siRNAs were isolated by use of a FastTrack 2.0 kit (Invitrogen). The integrity and purity of the siRNAs were determined by gel electrophoresis and UV absorbance measurements. A portion of the cells were lysed in NP-40 lysis buffer (100 mM Tris [pH 7.5], 100 mM NaCl, 1% NP-40, 1 mM dithiothreitol), and the total protein concentration was determined by use of the Bradford assay (Bio-Rad). Equal amounts of proteins were boiled in sample buffer and resolved by sodium dodecyl sulfate–8% polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes (Millipore), blocked in 5% nonfat milk, probed with a p53 antibody (Ab-6; Oncogene), an E6AP antibody, or a pRb antibody (IF8; Santa Cruz Biotechnology) according to standard procedures, and analyzed by enhanced chemiluminescence (Super Signal West Pico enhanced chemiluminescent substrate; Pierce).

**RT-PCR and real-time quantitative PCR.** mRNAs (250 to 500 ng) were reverse transcribed by the use of Superscript II (Invitrogen) and either an oligo(dT) primer, random primers (Invitrogen), or a gene-specific primer, as suggested by the manufacturer. Semiquantitative RT-PCRs for 18E6 and E6AP were performed by using 1 μl of a 1:20 dilution of cDNA that had been synthesized with random primers. The cycling parameters were 95°C for 2 min and then 35 cycles of 95°C for 15 s, 55°C for 20 s, and 75°C for 40 s, with a final extension step of 75°C for 2 min. The bands were quantified with Quantity One software (Bio-Rad). Real-time quantitative PCRs were performed in a 96-well plate with 20-μl reaction volumes containing 1 μl of cDNA template that had been synthesized with an oligo(dT) primer or a gene-specific primer by use of a Dynamo RT-PCR and real-time quantitative PCR.

**Microarray hybridization and analyses.** For microarray analysis, mRNAs were isolated from mock-transfected or siRNA-transfected cells. mRNAs (250 to 500 ng) were reverse transcribed in the presence of amino-allyl deoxyuridine triphosphates. Cy3 and Cy5 fluorescent dyes (Amersham Biosciences) were then coupled to the amino-allyl groups. Cy3 (green) was coupled to cDNAs from mock-transfected cells, and Cy5 (red) was coupled to cDNAs from siRNA-transfected cells. The two cDNA probes were combined and simultaneously hybridized to spotted cDNA microarrays provided by V. Iyer (University of Texas). Each microarray contained ~49,000 elements, including ~31,000 discrete Unigene clusters (61). The microarrays were scanned with a GenePix 4000 scanner (Axon Instruments), and the accompanying GenePix Pro 4.2 software package was used to locate and extract data from individual spots on the microarrays. Data filtering was performed by use of the Longhorn Array Database, a relational microarray database and analysis tool set (29). The elements (genes) chosen for further analysis were selected based on several data quality standards, including minimum intensity and pixel consistency. Genes were considered significant if the log2[Red] was ≥±0.85, where R is the red/green normalized ratio. Only genes whose expression was similarly altered for both siRNAs against a single target gene and was not altered in controls were considered significant.

**Telomerase assays.** Telomerase activities were assayed by a telomerase repeat amplification protocol (TRAP) as previously described (35), with slight modifications. Briefly, cells were washed with phosphate-buffered saline, pelleted, washed in a buffer containing 10 mM Tris-HCl (pH 7.5), 1.5 mM MgCl2, 10 mM KCl, and 1 mM dithiothreitol, pelleted again, and lysed in lysis buffer (10 mM Tris-HCl [pH 7.5], 1 mM MgCl2, 1 mM EGTA, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol, and 0.1 mM phenylmethylsulfonyl fluoride) at 4°C for 30 min. Cell debris was pelleted by centrifugation at 12,000 × g, and the
protein concentrations in the supernatants were determined by use of the Bradford assay (Bio-Rad). TRAP assays were performed in 0.2-ml PCR tubes containing 50 μl of a reaction mixture composed of TRAP buffer (20 mM Tris-HCl [pH 8.3], 1.5 mM MgCl₂, 63 mM KCl, 0.005% Tween 20, and 1 mM EGTA), a 0.2 mM concentration of each deoxynucleoside triphosphate, 0.1 mg of bovine serum albumin/ml, 0.002 μg of TS primer (5’-AATCGCGGAAGCAGTT-3’)/μl, 2 U of Taq DNA polymerase (Invitrogen), 0.3 μl of [α-32P]dGTP (10 μCi/μl; Perkin-Elmer), and 0.1 or 0.02 μg of protein. After 20 min at room temperature, 0.1 μg of CX primer (5’-CCCTACCCTACCCTAATTG3’) was added. PCRs were performed for 27 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C. Ten microliters of each reaction mixture was subjected to electrophoresis in 12% nondenaturing polyacrylamide gels (1× Tris-borate-EDTA). The gels were dried and subjected to autoradiography.

RESULTS

Synthetic double-stranded siRNAs were designed to target the mRNAs encoding human E6AP and the HPV type 16 and 18 E6 proteins. Two functional siRNAs were identified for each target. The siRNAs against E6AP, designated E6AP-A and E6AP-B, were targeted to two 21-bp regions within the first 320 bases of the E6AP open reading frame (ORF). Both siRNAs were predicted to target mRNAs encoding all of the known isoforms of E6AP (31, 62). siRNAs against HPV16 and HPV18 E6-encoding mRNAs were designed to avoid a simultaneous knockdown of HPV E7, as E6 and E7 are translated from an alternatively spliced bicistronic mRNA (7, 50). E6 is expressed from the unspliced transcript, which includes the complete E6 and E7 ORFs, while E7 is translated from a transcript that has been spliced to remove an intron that lies entirely within the E6 ORF. siRNAs were therefore targeted to the intron region to specifically knockdown the unspliced E6 and E7 transcripts. This strategy has been shown previously to knockdown E6 expression without affecting E7 expression (5, 27).

Transfection of either the E6AP-A or -B siRNA into three HPV-positive cell lines (HeLa, Caski, and SiHa) resulted in a decrease in E6AP protein levels by 24 h posttransfection, with a maximal decrease between 48 and 72 h (data for the 48-h time point are shown in Fig. 1A to C). As expected, the p53 protein was stabilized over the same time course. Although both siRNAs against E6AP resulted in a significant decrease in the amount of E6AP protein and an accumulation of the p53 protein, the E6AP-A siRNA consistently resulted in lower levels of E6AP and higher levels of p53 than did E6AP-B. Both HPV18 siRNAs (18E6-A and -B) resulted in the stabilization of p53 in the HPV18-containing cell line (HeLa), while the HPV16 siRNAs (16E6-A and -B) resulted in the stabilization of p53 in the HPV16-containing cell lines (Caski and SiHa). As a further control for the specificity of the E6 siRNAs, we determined that the HPV18 E6 siRNAs did not stabilize p53 in the HPV16-containing cell lines and that the HPV16 E6 siRNAs did not stabilize p53 in HeLa cells (data not shown). Immunoblots demonstrating the knockdown of E6 proteins are shown because of the difficulty in detecting the low levels of E6 protein in these cell lines with available antibodies; however, a semiquantitative RT-PCR analysis showed that E6-expressing transcripts were reduced approximately threefold by the E6-A and E6-B siRNAs (Fig. 1D). E6AP transcript levels were reduced four- to sixfold by the E6AP-A and E6AP-B siRNAs. E6AP siRNAs did not affect E6 transcript levels, and vice versa (Fig. 1D).

FIG. 1. siRNAs against E6AP and HPV E6 stabilize p53. Western blots of extracts from HeLa (A), Caski (B), and SiHa (C) cells after transfection with siRNAs (20 nM) against E6AP and HPV E6 are shown. The cells were harvested at 48 h posttransfection, and proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted by use of a rabbit polyclonal antibody against E6AP (upper panels) or a mouse monoclonal antibody against p53 (lower panels). (D) Semiquantitative RT-PCRs confirmed the knockdown of E6AP (upper panel) and HPV E6 (middle panel) transcripts. GAPDH was used as a control (lower panel). (E) Western blot of HeLa cell extracts with a mouse monoclonal antibody against the retinoblastoma protein (pRb) showing no change in protein levels following the knockdown of HPV 18E6 or E6AP. pRb-p, phosphorylated pRb.
Although the expression of E6 and E7 has been shown to be required for the continual growth of HPV-positive cervical carcinoma cell lines (18, 59), we did not observe any morphological indications of apoptosis or a loss of viability of cells transfected with either E6 or E6AP siRNAs. In addition, p53 levels returned to normal by ~7 days posttransfection, and the viability of cells at late time points was similar to that of cells transfected with a control siRNA against GFP. All of the experiments described here used a final concentration of 20 nM siRNA, which has been shown to be within the optimal range for observing siRNA silencing while minimizing off-targeting events and nonspecific effects (51). Transfection with a higher concentration (200 nM) of E6 or E6AP siRNAs, but not GFP siRNA, did result in cell death at later time points (5 to 7 days), in agreement with previous reports (5). Cell death was likely due to a more efficient and prolonged inactivation of E6- and E6AP-dependent degradation of p53 at the higher siRNA concentration.

We examined the effects of E6AP and E6 siRNAs on the global transcriptional profiles of the HeLa, Caski, and SiHa cell lines 48 h after siRNA transfection. Fluorescently labeled (Cy5; red) cDNAs from siRNA-transfected cells were mixed with fluorescently labeled (Cy3; green) cDNAs from mock-transfected cells and hybridized to spotted cDNA microarrays. Microarray analyses of siRNA experiments were repeated at least four times for each cell line. The results discussed below are from duplicate transfections hybridized with spotted cDNA microarrays from a single printing of the arrays for consistency in data analysis. Data were filtered for spot intensity and uniformity, and genes that were induced or repressed ≥1.8-fold were considered significant. In addition, only those genes that were similarly affected by both siRNAs against a single target gene were considered significant in our analyses. Cells transfected with the GFP siRNA were used to control for artifacts due to siRNA transfection and/or microarray handling. A small number of mRNAs (~10) were reproducibly altered ≥1.8-fold after GFP siRNA transfection into the three cell lines, although none of these genes were affected by the E6 or E6AP siRNAs.

To validate the general approach of combining siRNA transfection with microarray analyses, we initially examined microarray elements (i.e., specific genes) for predicted effects of E6AP or E6 siRNAs. For example, E6AP mRNA levels were confirmed to be lower (green) in cells that were treated with E6AP siRNAs, but not in cells treated with E6 or GFP siRNAs (Fig. 2A). In addition, while p53 protein levels were induced by both E6 and E6AP siRNAs (Fig. 1), p53 transcript levels were not expected to change, since the stabilization of p53 occurs posttranslationally, and this was also confirmed by semi-quantitative RT-PCR (data not shown). In contrast, genes that are known to be directly transcriptionally activated by p53 were expected to be induced after the transfection of either E6 or E6AP siRNAs. As shown in Fig. 2A, the genes encoding p21, HDM2, and p53-inducible nuclear protein 1 (p53INP1) were induced after transfection with either E6 or E6AP siRNAs. A real-time quantitative PCR analysis confirmed the up-regulation of transcript levels for HDM2 and p21 (Fig. 2B). We also examined genes predicted to be affected by changes in the expression of HPV E7 in order to indirectly confirm that our E6 siRNAs did not also alter E7 functions. HPV E7 interacts with pRb, p107, and p130, activating E2F transcription factors and increasing the transcription of E2F-responsive genes, such as those encoding cdc6, PCNA, thymidine kinase, minichromosome maintenance proteins, cyclin A, cyclin E1, cdc25A, E2F2, E2F3, and dihydrofolate reductase (reviewed in reference 52). Therefore, if our E6 siRNAs also reduced E7 expression, we would expect this set of E2F target genes to be down-regulated. No E2F target genes were significantly up- or down-regulated by either the E6 or E6AP siRNAs (the expression of cdc6 and dihydrofolate reductase is shown in Fig. 2A). Additionally, E7 has been reported to target pRb for proteasomal degradation (4, 17, 60), although a candidate ubiquitin ligase has not been identified. The fact that E6AP siRNAs did not affect E2F-regulated genes or pRb protein levels (Fig. 1E) indicates that E6AP does not mediate the E7-dependent degradation of pRb. This is consistent with in vitro experiments that have also failed to show an involvement of E6AP in E7-dependent pRb ubiquitination (unpublished results).

The overall expression profiles after siRNA transfection were first analyzed separately for each of the three HPV-
positive cell lines. For effects to be considered significant, individual genes had to be either induced or repressed ≥1.8-fold by both siRNAs against either E6 or E6AP in at least three of the four experiments shown in Fig. 3. The total number of genes affected in each cell line was 75 for HeLa, 38 for Caski, and 17 for SiHa. Together, these represented a total of 99 genes, as the sets were partially overlapping (discussed below). Figure 3 illustrates the relative degrees of alteration in mRNA levels for the 99 genes across all three cell lines, and Table 1 lists the complete set of 99 genes by accession number, gene name, and symbol, indicates in which cell line(s) they were affected, and shows whether they are known p53-responsive genes. Table S1 in the supplemental material shows the same information along with the functions of the gene products (if known) and the relative induction/repression ratio for each gene in all of the experiments shown in Fig. 3. Interestingly, all of the genes that met the criteria for being significantly affected were up-regulated (i.e., their relative expression is represented in red in Fig. 3). A dye switch control experiment resulted in the same set of genes being up-regulated but appearing green (not shown). The most striking result was that for each cell line, the set of genes affected by the E6 siRNAs was, with only two exceptions, completely concordant with the set of genes affected by the E6AP siRNAs. That is, there were almost no genes that were specifically affected by the E6 siRNAs that were not also affected by the E6AP siRNAs, and vice versa. The two exceptions were the genes for ENTPD7 (ectonucleoside triphosphate diphosphohydrolase 7) and interleukin-11, which were up-regulated by both 18E6 siRNAs, but not by the E6AP siRNAs, in HeLa cells. Overall, with the exception of these two genes in HeLa cells, these results suggest that nearly the complete spectrum of HPV E6 protein functions that are reflected in the transcriptional program of HPV-positive cell lines are mediated by E6AP. Furthermore, the sets of genes affected by the E6 and E6AP siRNAs were essentially identical, indicating that no natural (E6-independent) functions of E6AP are reflected in the transcriptional profiles of these cell lines.

Figure 4 illustrates the overlap among the total set of 99 genes affected by E6 and/or E6AP siRNAs in the three cell lines. Twenty-three of the 99 genes have been previously shown to be induced, either directly or indirectly, by wild-type p53. Fifty-six genes were induced specifically in HeLa cells that were not significantly induced in either the Caski or SiHa cell line, and nine of these were known p53-responsive genes. Nineteen genes, two of which were known p53-induced genes, were specific to Caski cells, and five genes, two of which were known p53-induced genes, were specific to SiHa cells. A set of

**FIG. 3.** Cluster image showing expression profiles of HeLa, Caski, and SiHa cells after transfection with siRNAs against HPV E6 and E6AP. Columns, rows, and colors are the same as those described in the legend to Fig. 2. Gray represents inadequate or missing data (data that did not pass the filter criteria). The numbers assigned to each gene correspond to those used in Table 1 for gene descriptions. The identities of some of the specific genes discussed in the text are indicated to the left. M (top) represents a control experiment in which cDNAs from mock-transfected cells were labeled with Cy5 and hybridized with the same cDNAs labeled with Cy3.
<table>
<thead>
<tr>
<th>Cell line or gene no.</th>
<th>Accession no.</th>
<th>Protein encoded by gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>N75719</td>
<td>Serine (or cysteine) proteinase inhibitor E1 (SERPINE1)</td>
</tr>
<tr>
<td>26</td>
<td>A1989728</td>
<td>Serine (or cysteine) proteinase inhibitor B5 (mapsin) (SERPINB5)</td>
</tr>
<tr>
<td>28</td>
<td>A1540460</td>
<td>Prostaglandin E synthase (PTGES)</td>
</tr>
<tr>
<td>39</td>
<td>W654651</td>
<td>Dual specificity phosphatase 5 (DUSP5)</td>
</tr>
<tr>
<td>44</td>
<td>H72027</td>
<td>Gelsolin (Finnish type) (GSN)</td>
</tr>
<tr>
<td>48</td>
<td>A1982657</td>
<td>Growth differentiation factor 15 (MIC-1) (GDF15)</td>
</tr>
<tr>
<td>51</td>
<td>AA480815</td>
<td>Immediate early response 3 (IER3)</td>
</tr>
<tr>
<td>53</td>
<td>H84481</td>
<td>EphA2 (EPHA2)</td>
</tr>
<tr>
<td>55</td>
<td>AA453410</td>
<td>Tumor necrosis factor receptor superfamily 10B (TNFRSF10B)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Caski cells</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>75</td>
<td>AA405449</td>
<td>Damage-specific DNA binding protein 2, 48kDa (DDB2)</td>
</tr>
<tr>
<td>83</td>
<td>AA903201</td>
<td>Notch 1 (NOTCH1)</td>
</tr>
<tr>
<td>83</td>
<td>AA504624</td>
<td>Inositol polyphosphate-5-phosphatase (INPP50)</td>
</tr>
<tr>
<td>21</td>
<td>W84711</td>
<td>Fibronectin 1 (FN1)</td>
</tr>
<tr>
<td>22</td>
<td>H09620</td>
<td>FLJ31869</td>
</tr>
<tr>
<td>23</td>
<td>A6578132</td>
<td>Hairy and enhancer of split homolog 2 (HES2)</td>
</tr>
<tr>
<td>24</td>
<td>A123041</td>
<td>Serine (or cysteine) proteinase inhibitor B7 (megsin) (SERPINB7)</td>
</tr>
<tr>
<td>78</td>
<td>AA600587</td>
<td>Pyoglutamy peptide 1 (PGP1)</td>
</tr>
<tr>
<td>88</td>
<td>AA496957</td>
<td>FLJ21245</td>
</tr>
<tr>
<td>87</td>
<td>H90930</td>
<td>N-Acylphosphoglycerol amidohydrolase 3-like (ASAH3L)</td>
</tr>
<tr>
<td>88</td>
<td>AA497002</td>
<td>Metastasis-associated protein 4B (MAT2A)</td>
</tr>
<tr>
<td>89</td>
<td>AA521292</td>
<td>ATP-binding cassette A1 (ABCA1)</td>
</tr>
<tr>
<td>90</td>
<td>AA001432</td>
<td>Laminin, alpha 3 (LAMA3)</td>
</tr>
<tr>
<td>94</td>
<td>AA453754</td>
<td>Serine/threonine kinase 17a (STK17A)</td>
</tr>
</tbody>
</table>
12 genes was up-regulated in all three cell lines, and 7 of the these genes were known p53-responsive genes. The other four genes in this set were uncharacterized expressed sequence tags or encoded proteins of unknown function.

The TERT gene, encoding the catalytic subunit of telomerase, was expected to be down-regulated following E6 knockdown, since it has been shown that E6 induces the transcription of TERT (57). However, we could not analyze TERT expression alterations on the array because the intensity of the hybridization signal at the TERT microarray element was consistently low and the spot was filtered out during the selection of high-quality data. We therefore directly assayed the telomerase catalytic activity in extracts from HeLa cells that had been transfected with E6 or E6AP siRNAs. Using a TRAP assay, we found that the transfection of either of the siRNAs against HPV18 E6 resulted in a significant decrease in telomerase activity compared to that in mock-transfected cells (Fig. 5). As a control for specificity, transfection with HPV16 E6 siRNAs did not affect telomerase activity in HeLa cells (an HPV18 cell line). Additionally, the transfection of either the E6AP-A or -B siRNA resulted in a decrease in telomerase activity equivalent to that seen with the HPV18 E6 siRNAs. The down-regulation of hTERT transcript levels by both E6 and E6AP siRNAs was verified by real-time quantitative PCR (Fig. 2B). These results indicate that E6AP mediates the ability of E6 to activate telomerase, a process that was shown previously to be p53 independent (14, 32).

DISCUSSION

The goal of this study was to determine if a broad spectrum of functions of the high-risk HPV E6 proteins was dependent on the E6AP ubiquitin ligase. This was addressed by comparing gene expression profiles of >30,000 genes after siRNA-mediated silencing of E6 or E6AP expression in HPV-positive cell lines. This experimental approach had the advantages of (i) being unbiased with respect to the significance of reported E6 functions, (ii) revealing potential effects due to as yet uncharacterized functions of E6, and (iii) having, as an internal
tein levels were not posttranslationally affected by the silencing of E6 protein expression and possibly to the use of poor immunoblotting conditions. However, until protein array technology becomes available that allows for the surveillance of a significant fraction of the proteome (63), gene expression analysis provides the most comprehensive and unbiased method for analyzing the response of HPV-positive cells to the depletion of E6 or E6AP.

Strikingly, our overall finding was that in all three HPV-positive cell lines examined, the alterations in gene expression following E6 silencing were nearly identical to the alterations observed following E6AP silencing. This indicates (i) that the spectrum of E6 functions that are reflected in the transcriptional program is dependent on E6AP and (ii) that essentially no normal (i.e., E6-independent) functions of E6AP are reflected in the transcriptional profiles of the cell lines examined here. Of the set of 99 genes that were affected in HeLa and/or SiHa and Caski cells, 23 were either known direct targets of the transcriptional activation function of p53 (e.g., p21, HDM2, TP53INP1, and Maspin) or known to be induced indirectly after p53 activation (e.g., Notch1 and gelsolin).

Our experimental design was based on the presumption that E6 siRNAs would not affect E6AP mRNA or protein levels, and vice versa. We showed that this was true at the mRNA level by examining E6 and E6AP transcripts by RT-PCR, and we showed by immunoblotting that E6 siRNAs did not affect E6AP protein levels. The difficulty of detecting the E6 protein in HPV-positive cervical carcinomas due to the low level of protein expression and possibly to the use of poor immunologic reagents prevented us from demonstrating that E6 protein levels were not posttranslationally affected by the silencing of E6AP. Our microarray results are not consistent with a scenario in which E6 is stabilized by the silencing of E6AP, since the transcriptional effects of the E6AP knockdown were nearly identical to the effects of silencing E6. Conversely, if the silencing of E6AP led to a concomitant decrease in E6 protein levels, we would have expected our microarray results to be similar to those observed, and while our overall conclusion—that all E6 functions are dependent on E6AP—would have been the same, the underlying mechanism would have been quite distinct. Importantly, however, there are no reports in the literature to suggest that E6AP affects E6 protein expression levels, and a previous study has shown that steady-state levels of functional, epitope-tagged E6 proteins are not influenced, either positively or negatively, by their ability to interact with E6AP (28). It therefore seems unlikely that the silencing of E6AP would have affected E6 protein levels in our experiments.

While we did not see overt signs of apoptosis when we transfected cells with E6 or E6AP siRNAs at a concentration of 20 nM, many of the induced genes encode apoptosis-related proteins, including Notch1, gelsolin, and STK17A. At the same time, a small number of antiapoptotic proteins were also induced (TRAF1, GAS6, and PEA15). It was not possible to directly link the induction of the apparent p53-independent genes to other targets or binding partners of E6, such as PDZ domain-containing proteins, E6TP1, Bak, Myc, or CBP/p300. It should be noted, however, that neither Myc nor Bak was stabilized after the E6 or E6AP siRNA knockout and that neither protein was ubiquitinated in the presence of E6 or the E6/E6AP complex in vitro under conditions in which p53 was efficiently recognized (S. Beaudenon, M. L. Kelley, and J. M. Huibregtse, unpublished data). The basis of differences in observations with Myc (19) and Bak (53) relative to published reports is unknown.

All cervical cancer-associated HPV E6 proteins, but not other human and animal papillomavirus E6 proteins, contain a PDZ domain binding epitope consisting of a T-X-V/L sequence at their C termini. The high-risk HPV E6 proteins have been shown to interact with several PDZ domain-containing proteins and to target them for degradation, including Dlg (13), Scribble (43), MUPP1 (36), and MAGI (16, 55). Scribble ubiquitination has been shown to be E6AP dependent (43), although Dlg degradation has been reported to be E6AP independent (47). While work from our lab indicates that Dlg degradation is also E6AP dependent (Beaudenon et al., unpublished data), it is not clear how any of the observed expression changes that we observed upon E6 or E6AP silencing might be linked to the stabilization of any of the known PDZ domain-containing target proteins. Scribble and Dlg have been linked to cell growth and differentiation decisions through their influence on epithelial cell polarity in Drosophila (3). Potential gene expression alterations due to Scribble or Dlg stabilization may be a reflection of the role that cell adhesion and junctional complexes can have on signaling pathways in epithelial cells (25). It is perhaps significant in this regard that several of the genes that were up-regulated after an E6 or E6AP siRNA treatment were involved in cell adhesion, motility, or extracellular matrix interactions (fibronectin, MCAM, tenascin C, and Jagged 1).

Although there was an overlap of affected genes after the
knockdown of HPV E6 and E6AP among these three cell lines, with the majority of these genes known to be p53 responsive, many of the observed effects appear to be cell line specific. HPV E6 and E6AP silencing appears to have a greater impact on the transcriptional program of HeLa cells than on that of either Caski or SiHa cells. These differences among cell lines are not explained by differences in the transfection efficiencies of the cell lines, as these were determined to be nearly 100% for all three lines. While we cannot offer an obvious explanation for differences in the transcriptional program responses, it is likely that the inherent genetic differences among these cell lines are the main contributors. In addition, the tissue types from which the original tumors arose were not the same. HeLa cells were derived from an adenocarcinoma, the SiHa cell line was derived from a squamous cell carcinoma, and Caski was derived from a metastasis of a cervical carcinoma (American Type Culture Collection [www.atcc.org]).

The stabilization of telomeres or chromosome ends, often by the activation of telomerase activity, is a hallmark of cancer cells (20). It was previously shown that the high-risk HPV E6 proteins stimulate telomerase activity and that this stimulation is independent of the effects of E6 on p53 (14, 32). While induction occurs at the level of transcriptional activation of the TERT gene (14, 45, 57, 58), TERT gene expression was not clearly altered in our microarray experiments after E6 or E6AP silencing. However, the strength of the hybridization signal at the TERT element on the array was reproducibly very low. This was consistent with an earlier microarray-based study that also failed to detect E6-dependent up-regulation of TERT on the array, even though telomerase was in fact induced in those experiments (8). This prompted us to directly assay the telomerase activity to determine the effects of E6AP on this function. We found that both E6 and E6AP siRNAs led to a significant reduction in telomerase catalytic activity. The existence of an E6/E6AP target involved in the regulation of hTERT transcription was previously suggested based on correlational data showing that HPV16 E6 mutants that retained the ability to interact with E6AP also retained the ability to activate telomerase, while mutants that did not interact with E6AP did not activate telomerase (14). In agreement with these results, it was recently reported that the transduction of HFK cells with a siRNA targeting E6AP, with or without HPV16 E6 expression, also decreased telomerase activity (15). This previous report identified an E6/E6AP-interacting protein that is a known repressor of telomerase transcription, NFX1-91, and showed that the expression of E6/E6AP stimulated the ubiquitination and degradation of NFX1-91, suggesting that NFX1-91 is a substrate of E6/E6AP (15). Interestingly, E6 has been shown to be present at the TERT promoter by the use of chromatin immunoprecipitation assays (58), and Schlegel and coworkers have also found that E6AP is present at the TERT promoter in E6-expressing cells (37a). There is accumulating evidence that the ubiquitination of transcriptional activators or core components of the transcription machinery can serve to “license” their activities (42), and it is interesting to speculate that the E6/E6AP complex may provide such a function at the TERT promoter.

While E6AP was discovered during the characterization of E6-mediated p53 inactivation, it is now known that mutations in the gene encoding E6AP (UBE3A) are the cause of Angelman syndrome (AS), a severe form of mental retardation (30, 39). Both alleles of UBE3A are expressed in virtually all tissues and cell types, with the exception of subregions of the brain where only the maternal allele is expressed (1). AS patients lack expression of a wild-type maternal UBE3A allele and therefore do not express E6AP in these brain subregions, which include the hippocampal neurons and Purkinje cells. The lack of ubiquitination of one or more substrates of E6AP in the brain is hypothesized to be the cause of AS. The identity of the putative critical AS-related substrate(s) of E6AP in the brain is unknown, and only a few natural (HPV E6-independent) targets of E6AP are known for any cell type (33, 34). We were therefore hopeful that siRNA silencing of E6AP in conjunction with microarray expression analysis might reveal pathways that are normally controlled or affected by E6AP independently of E6. However, at least with the cell types examined here, we did not observe any gene expression alterations after E6AP knockdown that were not also seen after E6 knockdown. In other words, none of the normal functions of E6AP appear to be reflected in the transcriptional profile. The transfection of E6AP siRNAs into an HPV-negative cervical carcinoma cell line (C33-A) also did not reveal any significant alterations in the gene expression profile of these cells (not shown). The general approach of utilizing transcriptional profiles to reveal pathways affected by E6AP may yet prove useful if it is applied to cells of a neuronal origin.

ACKNOWLEDGMENTS

This work was supported by a grant from the National Cancer Institute (CA72943 [J.M.H.]) and by a National Institutes of Health National Research Service Award (M.L.K.). Microarray setup was supported by a grant from the National Institute of Alcohol Abuse and Alcoholism (grant AA13518 of the INIA Project).

We thank Vishwanath Iyer, Jonathan Davies, and Patrick Kilion for providing cDNA arrays and assistance with computer analysis, Sylvie Beaudenon for a critical reading of the manuscript, and Richard Schlegel for valuable discussions and communications of unpublished results.

REFERENCES


38. Lee, C. J., E. J. Suh, H. T. Kang, J. S. Im, S. J. Um, J. S. Park, and E. S. Hwang. 2002. Induction of senescence-like state and suppression of telomer-


