VIROLOGY 239, 132–149 (1997)
ARTICLE NO. VY978860

A Comparative Analysis of the Interactions of the E6 Proteins from Cutaneous

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Received June 17, 1997; returned to author for revision July 9, 1997; accepted September 24, 1997

A common necessity for all papillomaviruses is to induce DNA synthesis in quiescent cells. This is commonly achieved by the E7 gene product, which interferes with the function of members of the retinoblastoma family controlling transition from the G1-phase to the S-phase of the cell cycle. Uncontrolled entry into S-phase activates, however, negative growth control signals which have to be bypassed to achieve production of progeny viruses. In addition to inherent activities of the E7 protein, high risk genital types encode an E6 protein that overcomes p53-mediated G1-arrest and apoptosis in concert with the cellular factor E6AP by targeting p53 for the enhanced ubiquitin-dependent degradation. The key question, which of these functions of genital E6 and E7 proteins is responsible for the carcinogenic phenotype, is still not completely answered. In contrast to high risk genital types no immortalizing or transforming activities have been found for the E7 proteins of the high risk cutaneous HPV8 and 47. On the other hand the ability of the E6 protein to transform established rodent fibroblasts seems to be a property shared by high risk genital and cutaneous types. To examine the existence of a common E6-mediated transforming pathway for both virus groups we compared the properties of the cutaneous E6 proteins with already known functions of E6 proteins of genital viruses. For this we analyzed the E6 proteins of low risk and high risk cutaneous and genital papillomaviruses with respect to cell transformation, to their abilities to bind, degradate, and influence the activity of human p53, and to bind E6AP. The results of our study demonstrate a clear lack of interaction between the transforming E6 proteins of HPV1 and HPV8 and both cellular proteins p53 and E6AP. In contrast, we found E6AP-independent binding of HPV16 E6 and HPV6 E6 to p53, although both proteins were different in their transforming potential. Of all four proteins investigated, only HPV16 E6 was able to bind to p53 and E6AP and to induce degradation of the p53 protein in the reticulocyte system. When we investigated in frame deletion mutants of the E6 protein of HPV16 for their abilities to bind to p53 or E6AP, degradate, and inhibit the transactivation function of p53 and to transform rodent fibroblasts, no correlation between the different activities could be found. Mutants still able to bind p53 and E6AP lacked transforming ability and other mutants that were transformation-competent were deficient in p53 and E6AP binding.

INTRODUCTION

The carcinogenic potential of papillomaviruses was detected in 1935, when Rous and Beard (Rous and Beard, 1935) showed that inoculation of cottontail rabbit papillomavirus (CRPV) into rabbits was sufficient to induce skin carcinomas in these animals. The first human papillomaviruses (HPV) shown to be involved in the development of malignancies represent a subgroup of cutaneous HPV types specifically associated with the rare disease epidermodysplasia verruciformis (EV). Patients with EV have an increased susceptibility to infections with viruses of this subgroup. Certain high risk EV-types, like HPV5 and HPV8, are frequently detected in skin cancers that develop from multiple flat warts in sun-exposed areas of the body in 30–60% of all EV patients (Pfister, 1992; Orth, 1987). More recent data suggest that EV-related papillomaviruses also play a role in the origin of skin cancers of immunosuppressed patients (Berkhout *et al.,* 1995; Shamanin *et al.,* 1996). Renal allograft recipients that are at highly increased risk of developing squamous cell carcinomas share surprising similarities with EV-patients; e.g., in the stepwise progression from cutaneous warts through squamous epithelial atypia into invasive cancer, in the role of UV light as environmental cofactor, and in the presence of papillomavirus sequences in cancer biopsies (Pfister, 1992; Barr *et al.,* 1989; Hoxtell *et al.,* 1977; Berkhout *et al.,* 1995).

Another large group of human papillomaviruses associated with human malignancies consists of more than 24 genital HPV types that are associated with lesions in the anogenital tract (de Villiers, 1989). Distinct high risk types of this group are frequently detected in malignant tumors, like HPV16 or HPV18, which are associated with 70% of all cervical carcinomas. The malignant phenotype of genital high risk types seems to depend on the properties of the gene products of both viral genes E6 and E7.

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0042-6822/97 $25.00

This was first evident from studies with cervical carcinomas in which almost all tumors revealed a continuous expression of the E6/E7 region (zur Hausen, 1994; Smotkin and Wettstein, 1986; Schwarz *et al.,* 1985). In addition antisense RNA experiments with cultured cervical carcinoma cells showed that the expression of high risk E6 and E7 was necessary for growth potential and tumorigenicity of the carcinoma cells (von Knebel Doeberitz *et al.,* 1992; Crook *et al.,* 1989; von Knebel Doeberitz *et al.,* 1988). From a number of studies it soon became apparent that the properties of E6 and E7 of high risk and low risk genital viruses in tissue culture correlated well with the carcinogenic potential of the respective genital HPV type (Huibregtse and Scheffner, 1994). The E7 gene of the high risk genital HPV16 was found to be sufficient to immortalize primary foreskin keratinocytes (Halbert *et al.,* 1991; Hudson *et al.,* 1990), whereas the E7 protein of low risk type HPV6, which is predominantely found in benign genital warts revealed no immortalizing activity on its own (Halbert *et al.,* 1992). High risk HPV16 E6 protein induces anchorage-independent growth of C127 and NIH 3T3 cells, whereas the E6 proteins of the low risk genital HPV types express no comparable cell-transforming activities (Barbosa *et al.,* 1991).

Many other abilities of the E6 and E7 proteins of high risk genital types have been described till today, but a key feature is certainly their ability to inactivate tumor suppressor proteins. All papillomaviruses share the need to induce DNA synthesis in quiescent host cells for the replication of the viral DNA. This is commonly achieved by the interaction of the E7 protein with members of the retinoblastoma tumor suppressor family, like p105Rb, p107, and p130 (Schmitt *et al.,* 1994; Gage *et al.,* 1990; Davies *et al.,* 1993; Dyson *et al.,* 1989). Such unscheduled DNA synthesis, however, leads to the induction of growth arrest or apoptosis in the infected cell. Additional properties of the E6 and E7 proteins are therefore required to allow cells to proliferate even in the presence of negative growth signals. In the case of HPV16, it was shown that E7 alone could bypass growth arrest signals mediated by different pathways. An elegant mutational analysis revealed that yet unidentified functions in the E7 protein besides Rb binding are necessary for the ability to abrogate growth arrest. This property, however, correlated well with the transforming potential of the different mutants in rodent fibroblasts (Demers *et al.,* 1996). One well-characterized pathway for the induction of growth arrest and apoptosis is by the activation of the p53 tumor suppressor protein (Ko and Prives, 1996). In order to abrogate this cellular response some E6 proteins bind to p53 in concert with another cellular factor, E6AP, and target p53 for the enhanced ubiquitin-dependent degradation by the proteasome (Huibregtse and Scheffner, 1994).

Differences in the abilities of the oncoproteins from distinct genital types to target cell cycle regulators correlate with the capacities of their E6 and E7 proteins to induce cell proliferation (Huibregtse and Scheffner, 1994; Vousden, 1991). The high risk genital HPV E7 proteins were found to have a much higher affinity to the Rb protein than the low risk HPV E7 proteins (Heck *et al.,* 1992), and their transforming capacity parallels the Rb binding activity. The E6 proteins of high risk HPV16 and 18 were shown to efficiently cause degradation of p53, whereas the ability of low risk HPV6 and 11 E6 proteins to bind p53 *in vitro* is controversial, although they clearly do not mediate degradation of p53 (Huibregtse and Scheffner, 1994; Lechner and Laimins, 1994; Crook *et al.,* 1991). As with Rb, the mechanism of induction of growth arrest or apoptosis by wild-type p53 seems to be related to events at the transcriptional level (Jansen Dürr, 1996). The p53 protein can bind directly to its recognition sequence on the DNA resulting in transcriptional activation of a nearby located promotor (Barak *et al.,* 1993; el Deiry *et al.,* 1993). In addition p53 can negatively regulate certain promoters in a sequence-independent way (Lechner *et al.,* 1992, and references therein).

All these data indicate that a number of different properties of the E6 and E7 proteins seem to be required for the transformation of cells. Whereas the sole function of E6 in the immortalization of human keratinocytes could be to inactivate p53, this function may not be sufficient for the transformation of rodent fibroblasts. To identify new functions of the E6 protein besides the ability to degradate p53, other cellular ligands were searched for. One newly identified ligand of E6, termed E6BP, is a putative calcium binding protein (ERC-55) and interacted with the E6 proteins of high risk genital types and with BPV1 E6 (Chen *et al.,* 1995), but not with nontransforming genital E6 proteins or with the transforming E6 protein of CRPV (Harry and Wettstein, 1996). Recently the focal adhesion protein paxillin was found to interact specifically only with transforming mutants of the BPV1 E6 protein and with the E6 proteins of high risk genital HPV16 but not with the low risk types 6 and 11. In the case of BPV1 E6 this interaction correlated with the disruption of the actin cytoskeleton in transformed rodent cells pointing to a role for this interaction for cell transformation by fibropapillomaviruses (Tong and Howley, 1997).

As compared to the genital papillomaviruses, only a small number of investigations has been performed on the transforming potential of the E6 and E7 proteins of cutaneous types. One interesting common feature of all cutaneous high risk HPV types is that their oncogenic potential mainly seems to rely on the activity of the E6 protein, but not on E7. Transforming potential could be assigned only to the gene product of the E6 ORF, which revealed transforming potential in rodent fibroblasts but no immortalizing activities in human keratinocytes (Iftner *et al.,* 1988; Kiyono *et al.,* 1989, 1992; Schmitt *et al.,* 1994; Pfister, 1992). Furthermore with the help of the rabbit

model system we recently could show that increased expression of CRPV E6 in the proliferating epithelial cell layers of domestic rabbits as compared to cottontail rabbits correlates with an enhanced progression of the skin tumors (Zeltner *et al.,* 1994). The E7 gene product of the high risk EV-associated HPV8 and HPV47 did not exhibit detectable transforming function and showed only reduced Rb binding activity (Iftner *et al.,* 1988, 1990; Schmitt *et al.,* 1994; Kiyono *et al.,* 1989). In contrast, the E7 protein of the low risk cutaneous HPV1 exhibited strong transforming and high Rb binding activities, which makes a correlation between the Rb binding affinity of the E7 proteins of cutaneous viruses and the carcinogenic potential of the parental type highly unlikely (Schmitt *et al.,* 1994). These data show that the only transforming protein of high risk EV-associated papillomaviruses known to date is the E6 protein. The mechanism how E6 of cutaneous types transforms rodent cells remains unknown. So far no interaction between cutaneous E6 proteins and the p53 protein has been described, although one potential problem of the only two studies suggesting a lack of interaction between CRPV E6 and HPV8 E6 with p53 was the usage of extracts from murine F9 cells as source for the p53 protein (Steger and Pfister, 1992; Harry and Wettstein, 1996). Species-specific differences between p53 proteins from mice or man may influence the binding ability of different E6 proteins to p53, as demonstrated by the inability of HPV18 E6 to bind to murine p53 (Scheffner, 1997). In addition both groups checked for neither degradation of p53 nor for binding of the E6 proteins to E6AP. Although another study reported an inhibition of p53-mediated transcriptional transactivation by HPV1 E6 under highly artificial conditions, the authors failed to demonstrate an interaction between HPV1 E6 and p53 till today (Kiyono *et al.,* 1994). In view of this conflicting data, we were interested in directly comparing the properties of the E6 proteins of low risk and high risk cutaneous and genital papillomaviruses by using different experimental systems with respect to cell transformation, to their abilities to bind, degradate, and influence the activity of human p53 and to bind E6AP. The results of our study demonstrate a clear lack of any interaction between the transforming E6 proteins of HPV1 and HPV8 and both cellular proteins p53 and E6AP. In contrast, we found E6AP-independent binding of HPV16 E6 and HPV6 E6 to p53, although both proteins differed in their transforming potential. Of all four proteins investigated, only HPV16 E6 was able to bind to p53 and E6AP and to induce degradation of the p53 protein in the reticulocyte system. When we investigated in frame deletion mutants of the E6 protein of HPV16 for their abilities to bind p53 or E6AP, degradate, and inhibit the transactivation function of p53 and to transform rodent fibroblasts, no correlation between the different activities could be found. Mutants still able to bind p53 and E6AP lacked transforming ability and other mutants that were transformation-competent were deficient in p53 and E6AP binding.

MATERIALS AND METHODS

Plasmid constructions

Plasmids encoding fusion proteins between the DNA binding domain (DBD) of GAL4 (amino acids 1–147) and E6 were constructed by PCR amplification of the coding sequences for E6 from plasmids pBR322/HPV1 (nt 101–524) (Danos *et al.,* 1983), pBR322/HPV16 (nt 104–557) (Seedorf *et al.,* 1985), pSVL86/7 (nt 206–660) (Iftner *et al.,* 1990), and pHPV6a(wg) (nt 103–555) (de Villiers *et al.,* 1981). Expression vectors encoding fusion proteins between the activation domain (AD) of GAL4 (amino acids 768–881) and p53 or E6AP, respectively, were constructed by PCR amplification of the coding sequences for p53 (nt 1–1179 of the wild-type p53 sequence) from plasmids BSKSp53 (Zakut Houri *et al.,* 1985) and pGEM4/p53(135) (Chiba *et al.,* 1990), and for E6AP (nt 638–2596 of the E6AP sequence) from plasmid pGEM1/E6AP (Huibregtse *et al.,* 1993a). To construct the N-terminal deletion mutant of p53, an ATG initiation codon was introduced in the 5′ oligonucleotide that starts at amino acid 101. To create the C-terminal deletion mutant of p53 the PCR reaction was carried out with a 3′ oligonucleotide that contained a TAA stop codon immediately after amino acid 326. All cloned amplification products were sequenced following a standard protocol (Ausubel *et al.,* 1990). All oligonucleotide primers used for cloning contained additional restriction sites. PCR-amplified products were cleaved with appropriate restriction enzymes and in the case of E6 inserted into the multiple cloning site of the vector pAS2 (Harper *et al.,* 1993) or in the case of p53 and E6AP inserted in the multiple cloning site of pGAD 424 (Bartel *et al.,* 1993). The resulting plasmids encode the GAL4 DNA binding domain fused in frame with the E6 proteins and the GAL4 activation domain fused in frame with the proteins p53 and E6AP, respectively. Constructs based on pAS2 contain additionally a short nucleotide stretch of 27 nucleotides encoding the hemagglutinin epitope in frame between the coding sequences of GAL4 and E6. For COS7 expression HPV16 E6wt-HA and 142/149-HA were subcloned into pEFcx (Mizushima and Nagata, 1990). Constructs for *in vitro* transcription/translation were based on the pBS+ expression vector (Vector cloning systems).

PCR mutagenesis

In order to generate a DNA template usable for the PCR mutagenesis the plasmid pGEM HPV16 E6/E7 (Bøhm *et al.,* 1993) was amplified with the oligonucleotides 16 E6-*Bam*HI-1: 5′-$_{90}$AA-AAG-GGA-TCC-GCA-ATG-TTT-CAG-GAC-CCA-CAG$_{121}$-3′ and 16 E6-*Bam*HI-tag-2:

3′$_{537}$GT-TCT-TGT-GCA-TCT-CTT-TGG-GTC-GAC$_{556}$-ATG-GGT-ATG-CTA-CAA-GGT-CTA-ATG-CGA-ATC-TCC-TAG-GGG-CAC-5′, using an annealing temperature of 50°C. By doing this the stop codon of the ORF encoding 16 E6 was removed and the sequence for the hemagglutinin epitope was added to the 3′ end. In order to construct five in frame deletion mutants of HPV16 E6, we chose the method of site-directed mutagenesis with four consecutive rounds of PCR amplification. Two *Bgl*II sites were introduced in frame with the coding sequence of HPV16 E6, cloned into pLXSN (Halbert *et al.,* 1992), digested with *Bgl*II and religated to create each individual mutant. All in frame deletion mutants were completely sequenced in both directions with the help of an ABI sequencer. Unless otherwise indicated in the oligonucleotide sequence, the bold and underlined nucleotides represent the sequence for the *Bgl*II restriction enzyme. Numbers at the 5′ and 3′ end of each oligonucleotide refer to the nucleotide position in the HPV16 E6 wt sequence. The construction of the in frame deletion mutants is exemplarily described for 18/25. To create the first *Bgl*II-site HPV16 E6wt-HA was used as a template in two parallel PCR reactions in which 16 E6-*Bam*HI-1 and aa 18 *Bgl*II-3: 3′-$_{140}$GGT-GTC-AAT-ACG-GTC-TAG-AAG-GTT-TGT-TGA-TAT-G$_{173}$-5′ were used as oligonucleotides in one reaction and aa 18 *Bgl*II-4: 5′-$_{140}$ CCA-CAG-TTA-TGC-CAG-ATC-TTC-CAA-ACA-ACT-ATA-C$_{173}$-3′ and 16 E6-*Bam*HI-tag-2 were used in the other reaction. The resulting PCR products were gel purified and used as templates in equal amounts for the second PCR amplification where 16 E6-*Bam*HI-1 and 16 E6-*Bam*HI-tag-2 were used as primer. The resulting DNA-fragment was used as a template for the introduction of a second *Bgl*II-site using the oligonucleotides 16 E6-*Bam*HI-1 and aa 25 *Bgl*II-3: 3′-$_{165}$GT-TGA-TAT-GTA-TTC-TAG-AAT-AAT-CTT-ACA-CAC-ATG-ACG$_{202}$-5′ as well as aa 25 *Bgl*II-4: 5′$_{165}$CA-ACT-ATA-CAT-AAG-ATC-TTA-TTA-GAA-TGT-GTG-TAC-TGC$_{202}$-3′ and 16 E6-*Bam*HI-tag-2 in two parallel PCR reactions. The PCR products were gel purified and used as templates in equal amounts for the fourth PCR amplification step, where the same primer pair as in step two was used. The resulting PCR product was cloned into the eukaryotic expression vector pLXSN via the two *Bam*HI restriction sites incorporated in the outer primer pair 16 E6-*Bam*HI-1 and 16 E6-*Bam*HI-tag-2. The construct was digested with the restriction enzyme *Bgl*II and religated to obtain the in frame deletion mutant 18/25. Four more in frame deletion mutants were made as described above with the help of the following oligonucleotides: for 35/56 first *Bgl*II-site: 16 E6-*Bam*HI-1 and aa 35 *Bgl*II-3: 3′$_{192}$CA-CAC-ATG-ACG-TTC-TAG-AAC-AAT-GAC-GCT-GC$_{222}$-5′; aa 35 *Bgl*II-4: 5′-$_{192}$GT-GTG-TAC-TGC-AAG-ATC-TTG-TTA-CTG-CGA-CG$_{222}$-3′ and 16 E6-*Bam*HI-tag-2, second *Bgl*II-site: 16 E6-*Bam*HI-1 and aa 56 *Bgl*II-3: 3′-$_{255}$CG-TAT-CAT-ATA-TCT-CTC-TAG-AAA-GGT-ATA-CGA-C$_{287}$-5′; aa 56 *Bgl*II-4: 5′-$_{255}$GC-ATA-GTA-TAT-AGA-GAG-ATC-TTT-CCA-TAT-GCT-G$_{287}$-3′ and 16 E6-*Bam*HI-tag-2. For 71/90 first *Bgl*II-site: 16 E6-*Bam*HI-1 and aa 71 *Bgl*II-3: 3′$_{291}$CA-CTA-TTT-ACA-AAT-TTC-AAA-GTC-TAG-AAT-TAA-TCA-CTC-ATA-TC$_{333}$-5′; aa 71 *Bgl*II-4: 5′-$_{291}$GT-GAT-AAA-TGT-TTA-AAG-TTT-CAG-ATC-TTA-ATT-AGT-GAG-TAT-AG$_{333}$-3′ and 16 E6-*Bam*HI-tag-2, second *Bgl*II-site: 16 E6-*Bam*HI-1 and aa 90 *Bgl*II-3: 3′-$_{356}$CCT-TGT-TGT-AAT-CTT-CTC-TAG-ATC-TTG-TTT-GGC-AAC$_{391}$-5′; aa 90 *Bgl*II-4: 5′$_{356}$GGA-ACA-ACA-TTA-GAA-GAG-ATC-TAC-AAC-AAA-CCG-TTG$_{391}$-3′ and 16 E6-*Bam*HI-tag-2. For 108/126 first *Bgl*II-site: 16 E6-*Bam*HI-1 and aa 108 *Bgl*II-3: 3′$_{408}$CC-ACA-TAA-TTG-ACA-TTC-TAG-AGT-GAC-ACA-GGA-C$_{440}$-5′; aa 108 *Bgl*II-4: 5′$_{408}$GG-TGT-ATT-AAC-TGT-AAG-ATC-TCA-CTG-TGT-CCT-G$_{440}$-3′ and 16 E6-*Bam*HI-tag-2, second *Bgl*II-site: 16 E6-*Bam*HI-1 and aa 126 *Bgl*II-3: 3′-$_{460}$C-CTG-TTT-TTC-GTT-TCT-TTC-TAG-AGA-TAT-TCC-CCA-GC$_{495}$-5′; aa 126 *Bgl*II-4: 5′$_{460}$-G-GAC-AAA-AAG-CAA-AGA-AAG-ATC-TCT-ATA-AGG-GGT-CG$_{495}$-3′ and 16 E6-*Bam*HI-tag-2. For 142/149 first *Bgl*II-site: 16 E6-*Bam*HI-1 and aa 142 *Bgl*II-3: 3′$_{514}$C-AGA-ACA-ACG-CTC-TAG-AGT-TCT-TGT-GCA-TC$_{543}$-5′; aa 142 *Bgl*II-4: 5′$_{514}$G-TCT-TGT-TGC-GAG-ATCTCA-AGA-ACA-CGT-AG$_{543}$-3′ and *Bam*HI-p2-pLXSN: 3′CTC-CTA-GGC-CGA-CAC-CTT-ACA-C-5′, second *Bgl*II site: 16 E6-*Bam*HI-1 and aa 149 *Bgl*II-3: 3′$_{537}$GT-GCA-TCT-CTT-GTC-TAG-AAC-ATG-GGT-ATG$_{565}$-5′; aa 149 *Bgl*II-4: 5′$_{537}$CA-CGT-AGA-GAA-CAG-ATC-TTG-TAC-CCA-TAC$_{565}$-3′ and *Bam*HI-p2-pLXSN.

Softagar assays

Transfection of NIH 3T3 rodent fibroblasts, selection procedure by G418 and softagar assays were performed as described previously (Iftner *et al.,* 1988; Schmitt *et al.,* 1994).

Expression of the GAL4 fusion proteins in yeast

Yeast strain Y190 (His$^-$, Leu$^-$, and Trp$^-$; Harper *et al.,* 1993) was transfected simultaneously with 2 μg of a pAS2-derived plasmid (DBD of GAL4) as well as 2 μg of a pGAD424-based plasmid (AD of GAL4) by a modified lithium-acetate method as described previously (Soni *et al.,* 1993). Transfectants were allowed to grow on synthetic media (SD), lacking the amino acids histidine, leucine, and tryptophan for 4 days (Sherman *et al.,* 1986), but containing 15 mM 3-amino-1,2,4-triazole (Sigma, München) to suppress leaky expression of the *HIS3* gene (Durfee *et al.,* 1993; Harper *et al.,* 1993). For blue white screening Leu$^+$, Trp$^+$ transfectants were soaked in chloroform, dryed, and overlaid with 1% low melting agarose (FMC Bioproducts) containing 1 mg/ml X-Gal (Roth, Germany), 100 mM KPO_4, and incubated at 30°C for 24 h. Colonies which turned blue due to β-galactosidase expression were picked and transferred to a fresh SD-Leu$^-$-

Trp$^-$-plate. Liquid β-galactosidase assays were performed with mid-log phase cultures by measuring the release of *o*-nitrophenol from the substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG, Sigma, München). The results are expressed as units defined by Miller (1972). At least five independent transfectants were analyzed for each pair of constructs and each assay was performed several times.

Western blot and immunoprecipitation

For Western blotting yeast clones cotransfected with GAL4-DBD-E6 and GAL4-AD-p53 were grown in 5 ml SD medium and harvested at an $OD_{600} = 0.7$. Cells were quickly chilled on ice, centrifugated, and resuspended in 500 μl of cold IP-buffer (1% Triton-X 100; 0.2% SDS; 0.3% desoxycholate; 50 mM NaCl; 5 mM EDTA; 50 mM Tris–HCl at pH = 8.0), containing 1 mM of the protease inhibitors PMSF and leupeptin, aprotinin, chymostatin, and pepstatin at a concentration of 2 μg/ml. Cells were lysed with glass beads and six pulses of vortexing for 30 s separated by 30s incubations on ice. An additional 500-μl aliquot of IP-buffer was added and the extract was clarified by centrifugation. Then 100 μl of the protein extract was mixed with an equal amount of standard sample buffer (Maniatis *et al.,* 1989) and proteins were separated by 15% SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Western blotting was performed by standard technique as described previously (Coligan *et al.,* 1994). For detection of the GAL4-DBD-E6 fusion proteins a monoclonal antibody specific for hemagglutinin (HISS Diagnostics, Freiburg) was used. Fusion proteins containing p53 were detected with the monoclonal antibodies PAb 421 (Dianova, Hamburg, Germany) and PAb 1801 (St. Cruz, Munich, Germany). Proteins were visualized by ECL Western blotting System (Amersham). For immunoprecipitation, the pellet of 10 ml of a liquid yeast culture ($OD_{600} = 0.5$) was resuspended in 300 μl of SD medium lacking methionine. Then 500 μCi [^{35}S]-methionine/cysteine translabel (Amersham) was added for 5 min at 30°C. Labeled yeast cells were chased with 200 μl methionine (4 mg/ml). Cell extracts were prepared as described above. Immunoprecipitations were performed with the same antibodies as described for Western blotting, using a protocol which was described previously (Iftner *et al.,* 1990), with the inclusion of two further washing steps of the beads in 15 μl 1% SDS (in IP-buffer) to reduce the background. Metabolic labeling and immunoprecipitations of transfected COS7 cells were performed as described previously (Iftner *et al.,* 1990; Gage *et al.,* 1990). Quantification of signals was done by using a phosphoimager (Bas 2000, Fuji).

Cell culture, transfections, and CAT assays

COS7 cells (CRL 1651; ATCC, MD) were grown and transfected as described previously (Iftner *et al.,* 1990). All other transient transfection experiments were performed in the HPV negative cervical carcinoma cell line C33A (ATCC, MD). Then 1 μg CAT reporter (PG13CAT) (Kern *et al.,* 1992), 10 ng p53 wild-type expression construct (pC53SN3) (Baker *et al.,* 1990), and expression vectors for the E6 proteins of HPV1, 6, 8, or 16 (Schmitt *et al.,* 1994) in various concentrations were cotransfected in C33A cells (Iftner *et al.,* 1988). Always 10 μg of the expression constructs for the mutant 16 E6 proteins were transfected. The concentration of DNA in each transfection was kept constant by the addition of parental expression vector DNA to a total of 20 μg. CAT assays were performed for 3 h according to a standard protocol (Ausubel *et al.,* 1990) with 150 μg of protein extract to keep the chloramphenicol-conversion rate within the linear range. Acetylated and nonacetylated products were separated by thin-layer chromatography. The percentage of acetylation was determined by quantification of acetylated and nonacetylated [^{14}C]chloramphenicol with the help of a phosphoimager (Bas 2000, Fuji).

In vitro transcription/translation and degradation assays

For the expression of human wild-type p53 and HA-tagged HPV E6 proteins, we used T3 or T7 RNA polymerase and a combined *in vitro* transcription/translation system (TNT rabbit reticulocyte lysate or TNT wheat germ extract, Promega). To generate radioactively labeled proteins, translations were performed in the presence of [^{35}S]methionine/cysteine translabel (Amersham). Degradation assays were performed as described previously (Scheffner *et al.,* 1990). For ubiquitination assays up to 2 mM nonhydrolyzable ATPγS was added to the reaction mixture. Proteins were separated on 15% SDS–PAGE and visualized by fluorography.

RESULTS

Transforming properties of genital and cutaneous HPV E6 proteins

To directly compare the transforming abilities of low and high risk cutaneous versus genital papillomaviruses we investigated the transforming potential of the E6 proteins of HPV1, 6, 8, and 16 in NIH 3T3 rodent fibroblasts with the help of a semiquantitative softagar assay. Two weeks after transfection G418-resistant colonies were pooled and seeded in 0.3% agarose. Growing clones consisting of more than 16 cells were identified and counted after 2 to 3 weeks. The E6 proteins of cutaneous high risk type 8 and low risk type 1 were found to be almost equally efficient in inducing anchorage-independent growth of NIH 3T3 cells as the E6 protein of genital high risk type 16. In contrast the number of colonies found with low risk HPV6 E6 protein was not significantly

different from G418-resistant clones established by transfection with the parental pZipNeoSV(X)-1 (Cepko *et al.,* 1984) expression vector (Fig. 1A). Therefore a significant difference between high versus low risk cutaneous E6 proteins in terms of transforming potential, as it is known from the genital group, does not seem to exist.

Cutaneous E6 proteins do not induce ubiquitination or degradation of p53

To abrogate one negative growth signal after unscheduled induction of DNA synthesis the E6 protein of HPV16 targets p53 and leads to its enhanced degradation (Scheffner *et al.,* 1990; Huibregtse *et al.,* 1993a). To investigate such a direct effect on the p53 protein, we first performed *in vitro* ubiquitination and degradation assays with the cutaneous E6 proteins. For this, p53 was translated in the reticulocyte lysate system in the presence of [^{35}S]methionine/cysteine and incubated with *in vitro*-translated E6 proteins of HPV1, 6, 8, and 16 at 25°C. Increasing amounts of nonhydrolyzeable ATPγS, which blocks the energy-dependent degradation of p53 (Scheffner *et al.,* 1990), were added and the reactions were stopped after 1 h. The total reaction mixtures were analyzed on SDS–PAGE and the p53 proteins were visualized by fluorography. Even in the presence of 2 mM ATPγS neither HPV1 E6 nor HPV8 E6 mediated ubiquitination of p53 in contrast to the positive control HPV16 E6 (Fig. 1B). As a negative control we used p53 in the presence or absence of 2 mM ATPγS. Since we detected no influence of the cutaneous E6 proteins on the stability of p53 within 60 min of incubation time we next performed a time course experiment to determine if longer incubation periods would allow degradation of the tumor suppressor protein. As shown in Fig. 1C in the absence of E6 protein p53 is stable at least for 180 min. When *in vitro*-translated high risk HPV16 E6 protein is added, a time-dependent degradation of p53 could be observed, with 96% of the input p53 protein degradated after 60 min. As described earlier by other groups no degradation could be observed with HPV6 E6 (Crook *et al.,* 1991, and data not shown). Similarly, incubation with the E6 proteins of HPV1 or 8 (Fig. 1C) caused no degradation, thereby indicating that the cutaneous E6 proteins do not mediate ubiquitin-dependent degradation of p53.

Binding of the E6 proteins to p53 in the yeast two-hybrid system

Although it is clear from the current data that the E6 proteins of low risk genital HPV6 and 11 do not mediate the degradation of p53, some groups reported binding of the low risk E6 proteins to the tumor suppressor molecule (Crook *et al.,* 1991; Lechner and Laimins, 1994). In addition binding of HPV11 E6 to p53 was shown to influence the transrepressor function and sequence specific

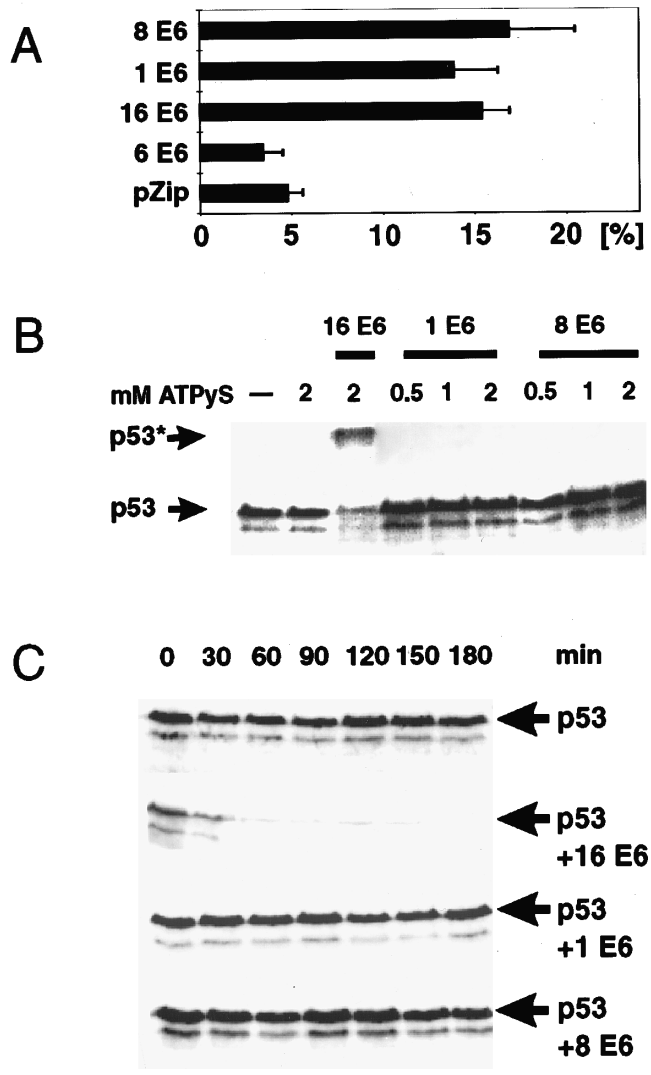


FIG. 1. The transforming E6 proteins of the cutaneous HPV types 1 and 8 do not degradate p53 *in vitro.* (A) Anchorage-independent growth of NIH 3T3 cells transfected with different E6 expression vectors. The cloning efficiency is given as the percentage [%] of seeded cells that formed colonies of more than 16 cells after 2 to 3 weeks. Each bar represents the average value of six individual experiments, while each experiment consisted of two independent transfections. Standard deviations are depicted as black lines. (B) p53 and various E6 proteins were translated *in vitro* in rabbit reticulocyte lysate in the presence of [^{35}S]methionine/cysteine. Ubiquitination reactions were performed in the presence of increasing amounts of the nonhydrolyzable ATP analog ATPγS, as indicated. p53 and the high molecular weight ubiquitinated forms of p53 (p53*) are marked on the left. (C) Time course experiment of E6 induced degradation of p53. p53 was incubated in the absence or in the presence of equal amounts of either HPV16 E6, HPV1 E6, or HPV8 E6 at 25°C as described under Materials and Methods. The reactions were stopped at different time points and analyzed by SDS–PAGE followed by fluorography. The arrows at the right indicate the position of p53.

DNA binding ability of p53 (Lechner and Laimins, 1994; Lechner *et al.,* 1992). As far as cutaneous E6 proteins are concerned the results of our experiments did not

indicate any interaction with p53. However, our *in vitro* assays only allowed the detection of prominent activities and would not allow the identification of weak protein–protein interactions, which still could be of biological significance. To be able to investigate such possible weak interactions of the cutaneous E6 proteins with p53 we used the yeast two-hybrid system (Fields and Song, 1989). Yeast cells do not contain gene products similar to the human E6AP protein (Goffeau *et al.,* 1996), which was shown in reticulocyte lysates to mediate the interaction between HPV16 E6 and p53. Therefore any interaction between E6 and p53 found with the yeast two-hybrid system should represent an E6AP-independent event.

First the E6 genes of HPV1, HPV8, HPV6, and HPV16 were cloned in frame into the vector pAS2 (Harper *et al.,* 1993) to produce fusion proteins with the aminoterminal DNA binding domain of the GAL4 transcription factor (DBD; aa 1–147). As counterpart the coding sequence of human wild-type p53 protein (p53wt) was cloned in frame into the vector pGAD424 (Bartel *et al.,* 1993) to produce a fusion protein with the GAL4 carboxyterminal transactivation domain (AD; aa 768–881). To be able to select for transfected yeast cells, the vector pAS2 contains the nutritional selection marker tryptophan and pGAD424 encodes the marker leucine. Any interaction between the fusion proteins produced by these vectors can be directly measured by the activation of the two GAL4-dependent reporter genes HIS3 and lacZ present in the yeast strain Y190 (Harper *et al.,* 1993). As negative controls we transfected Y190 cells with GAL4-DBD-1 E6, -8 E6, -6 E6, -16 E6, and GAL4-AD or cotransfected GAL4-DBD with GAL4-AD-p53 to prove that activation of the reporter genes was only due to the interaction of fusion proteins and not to the activity of a single compound.

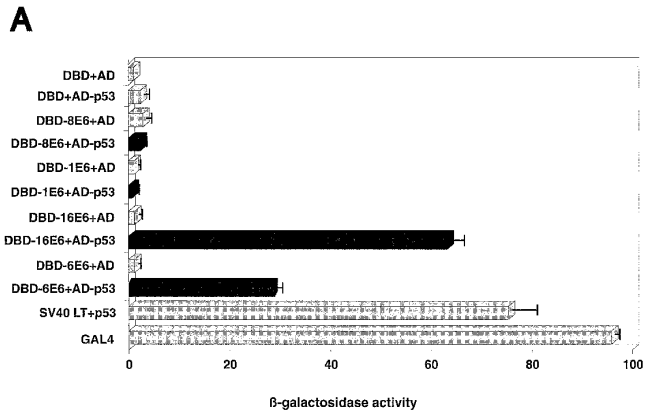
Cotransfection of yeast cells of strain Y190 with the expression vectors GAL4-DBD-16 E6 or GAL4-DBD-6 E6 together with GAL4-AD-p53 resulted in an activation of the reporter gene HIS3, which could be detected by growth of the yeast colonies on synthetic media lacking histidine. Cotransfectants with GAL4-DBD-1 E6 or -8 E6 and GAL4-AD-p53 did not result in colony formation on media lacking histidine (data not shown). In a next step all cotransfectants were tested for the expression of the second independent reporter gene lacZ. For this transfected Y190 cells were grown on synthetic media supplied with histidine but lacking the two selection markers leucine and tryptophan. Five individual clones from each transfection experiment were tested for β-galactosidase activity. A quantitative test was performed to measure the enzyme activity in units as defined by Miller (1972). The values given in Fig. 2A were referred to the activity of Y190 cells cotransfected with the parental vectors, encoding the DBD and AD of GAL4, which was set to the value "1."

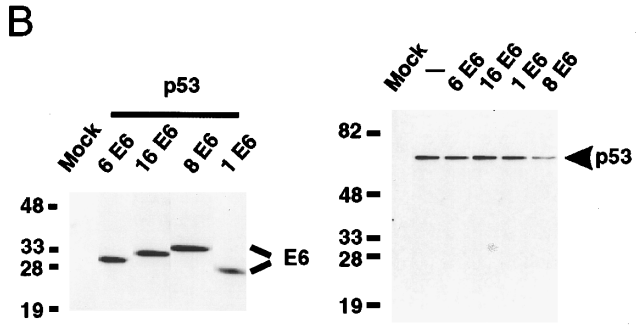
Cotransfectants containing GAL4-DBD-6 E6 or -16 E6 together with GAL4-AD-p53 showed a clear activation of the reporter gene lacZ, but the 16 E6 protein was twofold more efficient. As far as the cutaneous E6 proteins of HPV1 and HPV8 are concerned there was no activation of the reportergene lacZ when p53 was coexpressed (Fig. 2A). These data show that the yeast two-hybrid system allows measurement of direct interactions between the E6 proteins of HPV6 or 16 and p53 even in the absence of E6AP.

Detection of E6 and p53 proteins in transfected yeast cells

Since we did not detect any interaction between the E6 proteins of HPV1 or HPV8 and human p53 when fused to the functional domains of GAL4, we performed Western blotting analysis to confirm the presence and stability of the proteins in the transfected yeast clones. Protein extracts from yeast clones cotransfected with GAL4-DBD-E6 and GAL4-AD-p53 were run on SDS–polyacrylamide gels, transferred to nitrocellulose filters, and incubated with a monoclonal antibody directed against the hemagglutinin (HA) epitope that is present in all GAL4-DBD-E6 fusion proteins (Fig. 2B, left panel). As negative control we used extracts of mock-transfected Y190 cells (Fig. 2B, mock). The GAL4-DBD-E6 proteins were visualized using the ECL detection system (Amersham). This analysis demonstrated the presence of all E6 fusion proteins in the protein extracts from yeast clones cotransfected with human p53. In parallel, the GAL4-AD-p53 protein was visualized by using the p53-specific monoclonal antibody PAb 421 (Fig. 2B, right panel). In Fig. 2B (last lane) the amount of GAL4-AD-p53 protein in cells containing GAL4-DBD-8 E6 tends to be somewhat reduced; however, this effect was not reproducible. In summary these data clearly show that the inability of cutaneous E6 proteins to interact with p53 is not simply due to insufficient protein amounts.

To verify the interactions in yeast cells found by indirect reporter assays between p53 and E6 we next performed coimmunoprecipitation experiments with protein extracts from yeast clones cotransfected with GAL4-AD-p53 and the different GAL4-DBD-E6 constructs. For this, the respective Y190 clones were labeled with [^{35}S]-methionine/cysteine and protein extracts were prepared using IP-buffer. When we performed immunoprecipitations using the p53-specific monoclonal antibody PAb 421 we obtained a clear coprecipitation of the GAL4-DBD-E6 protein only with cell extracts from Y190 cells cotransfected with GAL4-DBD-16 E6 (Fig. 2C, lane 1). Extracts from Y190 cells cotransfected with the expression vectors GAL4-DBD-1 E6 and -8 E6 failed to coprecipitate the E6 proteins together with GAL4-AD-p53 (Fig. 2C, lanes 3 and 4). Vice versa, when we used an HA-epitope-specific antibody we observed coprecipitation of GAL4-





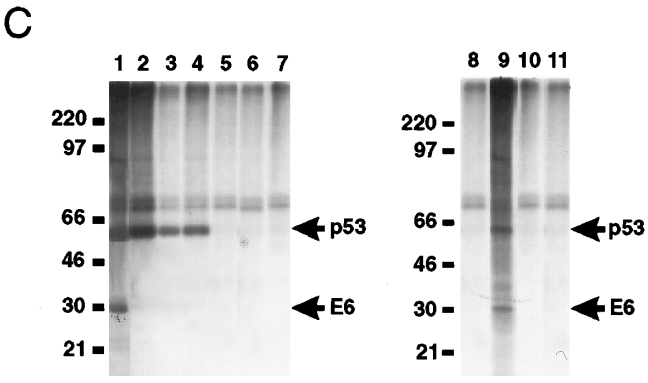


FIG. 2. Interaction between HPV E6 and p53 in yeast cells. (A) Y190 cells were cotransfected with GAL4-DBD-E6 fusion protein expression vectors and with an expression vector for the GAL4-AD fused to human p53 (dark bars). Transfected yeast clones were grown on synthetic media supplied with histidine but lacking the two selection markers leucine and tryptophan. As negative controls served cotransfections of the respective expression vectors with either parental vector construct (GAL4-DBD + GAL4-AD-p53, GAL4-DBD-8 E6 + GAL4-AD, GAL4-DBD-1 E6 + GAL4-AD, GAL4-DBD-16 E6 + GAL4-AD, and GAL4-DBD-6 E6 + GAL4-AD). The interaction between SV40 large T and p53 as well as the activity of the full-length GAL4 transcription factor served as positive controls. All values were referred to the β-galactosidase activity of Y190 cells cotransfected with the parental expression vectors (GAL4-DBD + GAL4-AD) whose values were set to "1." Standard deviations are depicted as black lines. (B) For Western blotting extracts of cotransfected yeast cells were separated on 15% SDS–PAGE and blotted onto nitrocellulose filter membrane. Left panel: Fusion proteins consisting of the GAL4-DBD, a hemagglutinin epitope of 9 aa length and the different E6 proteins were detected with a monoclonal antibody against hemagglutinin. Right panel: Fusion proteins consisting of the GAL4-AD and p53 were detected with the p53 specific antibody PAb 421. The second lane (−) represents yeast cells transfected with GAL4-AD-p53 alone. The mock-transfected control cells harbor the GAL4 transcription factor. The positions of the molecular weight markers (in kDa) are shown on the left. (C) Y190 cells were grown in the presence of [^{35}S]methionine/cysteine before protein extracts were prepared. Yeast cells were transfected with GAL4-AD-p53 (lane 2) and DBD-1E6 (lane 3), DBD-8E6 (lane 4), or DBD-16E6 (lane 1). The p53-specific antibody PAb 421 was used and the position of GAL4-AD-p53 and GAL4-DBD-16 E6 are indicated with an arrow. In lane 9 the same extract as in lane 1 was used, but incubated with an HA-specific antibody. Negative controls are precipitations of transfected cells incubated with unrelated antibodies (lane 5 and 8) or extracts of untransfected (lane 6 and 10) as well as mock-transfected yeast cells (lane 7 and 11) precipitated with specific antibodies.

AD-p53 in Y190 cells cotransfected with GAL4-DBD-16 E6 (Fig. 2C, lane 9).

Characterization of the p53 protein domain responsible for E6 interaction in yeast cells

While some groups already reported a direct E6AP-independent interaction between genital E6 proteins and p53 *in vitro* (Lechner and Laimins, 1994; Li and Coffino, 1996), we were interested which domain of p53 mediates interaction with HPV6 and 16 E6 in the more physiological milieu of yeast cells. For this we constructed different expression vectors that allow the expression of GAL4-AD-p53 fusion proteins with deletions at the aminoterminal (p53ATG101) and carboxyterminal (p53Stop326) ends of p53 or with an amino acid exchange within the p53 core region at aa position 135 (Cys-135-Tyr) (Chiba *et al.,* 1990). Y190 cells were cotransfected with the p53 mutant constructs and either expression vectors for GAL4-DBD-E6 or -16 E6 and grown in selective media. Five positive colonies were next tested for expression of the second independent reporter gene lacZ in a quantitative β-galactosidase assay (Fig. 3). Again a clear interaction could be found between the GAL4-DBD-E6 fusion proteins of HPV6 and 16 with the wild-type GAL4-AD-p53 protein. While GAL4-DBD-16 E6, showing a three- to fourfold stronger binding to GAL4-AD-p53 wild type, was still able to interact with a C-terminal deletion mutant of p53 (GAL4-AD-p53Stop326); the GAL4-DBD-E6 protein of HPV6 lacked this ability. The N-terminal deletion mutant (GAL4-DBD-p53ATG101) and the core mutant of GAL4-AD-p53 (Cys-135-Tyr) did not interact with each of the E6 proteins. This clearly shows that the aminoterminus and the core region of p53 are essential for E6 binding. In addition the results obtained with the carboxyterminal mutant of p53 suggest an influence of this domain on the strength of the protein–protein interaction. However, it is clear that none of the p53 domains by themselves were able to mediate interaction with E6. The difference

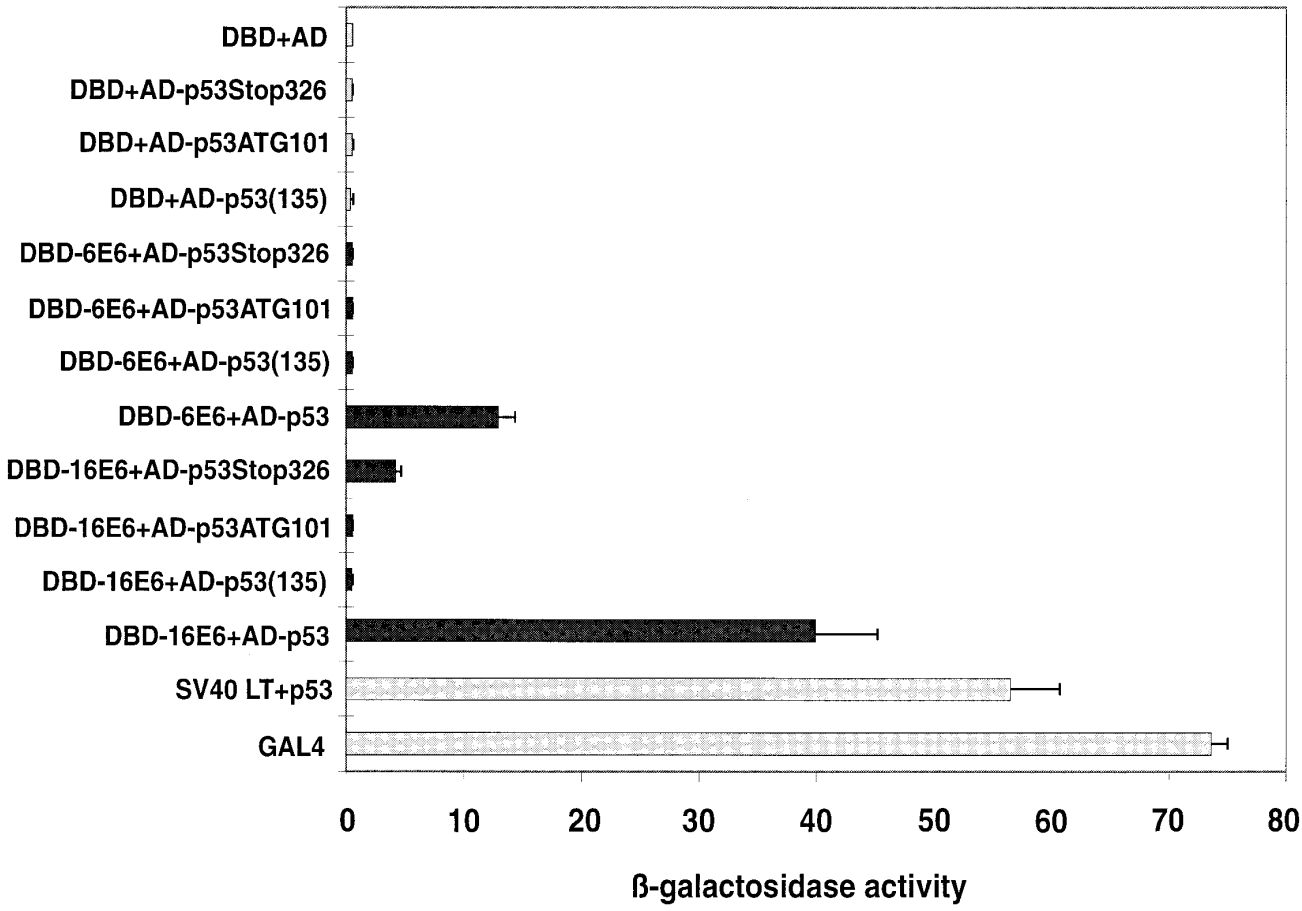


FIG. 3. Interaction between genital HPV E6 proteins and mutant p53 proteins in yeast cells. Y190 cells were cotransfected with GAL4-DBD-E6 and various GAL4-AD-p53 mutants (dark bars). Positive and negative controls are represented by grey bars. For other details see legend to Fig. 2A.

in the binding ability of low risk HPV6 E6 and high risk HPV16 E6 to the carboxyterminal deletion mutant of p53 may reflect distinct properties between both E6 proteins or simply results from the already lower affinity of HPV6 E6 to p53 in the yeast cells. To ensure a sufficient expression of all GAL4-AD-p53 and GAL4-DBD-E6 proteins involved, Western blot analysis of cotransfectants were performed, which revealed that all proteins were equally stable in yeast cells (data not shown).

Binding of the E6 proteins to E6AP in the yeast two-hybrid system

It has been described that the E6-associated protein (E6AP) is necessary for the E6-mediated degradation of p53 in reticulocyte lysates. The role of E6AP in the normal ubiquitin-dependent degradation of p53 in uninfected mammalian cells remains, however, unclear (Maki *et al.,* 1996). In a recent report Chen *et al.* (1995) showed an interesting phenotype for the transforming E6 protein of BPV1. While BPV1 E6 did not bind to or degradated p53, however, it bound to E6AP. In addition it was shown by others that high risk genital E6 proteins are able to interact with E6AP *in vitro* in the absence of p53 (Huibregtse *et al.,* 1991, 1993b), and in this report we observed an interaction between the nontransforming E6 protein of HPV6 with p53 although it cannot bind to E6AP *in vitro* (Chen *et al.,* 1995). If the interaction of E6AP with E6 proteins may also be relevant with respect to transformation is to date unknown. To address this question for the cutaneous transforming E6 proteins we investigated whether GAL4-DBD-1 E6 and -8 E6 are able to interact with E6AP in the yeast system. As controls we used GAL4-DBD-6 E6 and 16 E6. To do so we fused the coding sequence of E6AP (Huibregtse *et al.,* 1993a) to the activation domain of GAL4 and performed β-galactosidase assays of cotransfected yeast cells, which contained each of the GAL4-DBD-E6 fusion proteins. No measurable interaction between the GAL4-DBD-E6 proteins of the cutaneous types 1 and 8 or of HPV6 E6 and the GAL4-AD-E6AP protein could be detected (Fig. 4). In contrast, GAL4-DBD-16 E6 interacted with GAL4-AD-E6AP in cotransfected yeast cells, as measured by

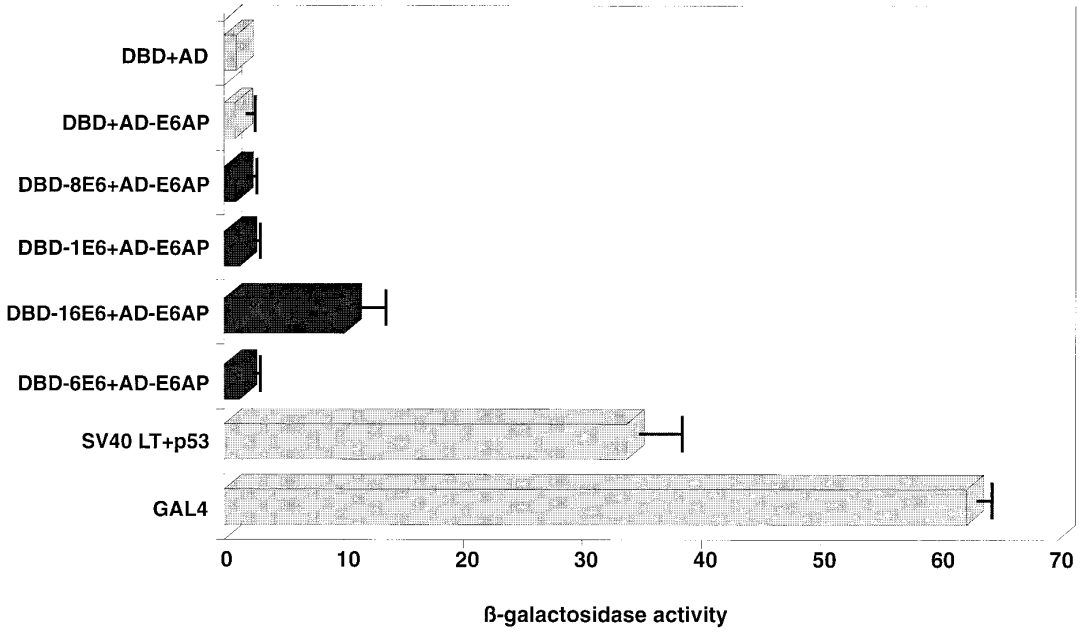


FIG. 4. Interaction between HPV E6 proteins and E6AP in yeast cells. Y190 cells were cotransfected with GAL4-DBD-E6 and GAL4-AD-E6AP (dark bars). Positive and negative controls are represented by grey bars. For other details see legend to Fig. 2A.

the activation of both reporter genes (Fig. 4). Therefore no correlation between E6AP binding and transforming ability could be detected, whereas the property of HPV16 E6 to mediate p53 degradation was clearly a function of E6AP binding.

Influence of E6 on p53-mediated transcriptional activation

Since it is known that the binding of E6 proteins to the tumor suppressor protein mediates the downregulation of p53-dependent transcription of responsive promoters, we tested if the binding behavior of the GAL4-DBD-E6 proteins to GAL4-AD-p53 which we observed in yeast cells is reflected by p53 inactivation in human cells. We transiently cotransfected cells of the human cervical carcinoma cell line C33A with a constant amount of a CAT-reporter construct harboring 13 p53 binding sites upstream of the polyoma virus early promoter (PG13CAT (Kern *et al.,* 1992)) and 10 ng of a p53 expression vector (pC53SN3 (Baker *et al.,* 1990)) together with increasing amounts of the respective unfused E6 protein expression vectors. Although C33A cells contain mutated p53 protein it was shown that this did not interfere with the function of transiently transfected p53 wild-type protein with respect to its transactivating function (Crook *et al.,* 1994; Elbel, 1997). CAT activity was determined and expressed as the relative percentage of conversion rate (from [^{14}C] chloramphenicol to acetylated [^{14}C]chloramphenicol) of the positive control HPV16 E6 in relation to the amounts of cotransfected E6 expression vector (Fig. 5). A clear abrogation of p53-mediated transcriptional activation was observed in a concentration-dependent manner using the expression vector for HPV16 E6 (Fig. 5, rhombs). HPV6 E6 was only able to repress p53 activity at high intracellular concentrations (Fig. 5, squares), which could be a reflection of the lower binding affinity to p53 as we observed in the yeast two-hybrid system. No effect on p53-mediated transactivation could be detected even at high amounts of cotransfected expression vectors encoding HPV1 E6 or 8 E6 (Fig. 5, triangles and circles, respectively). These results are colinear with our findings from the yeast two-hybrid system where no interaction between the E6 proteins of the cutaneous HPV types 1 and 8 and the tumor suppressor protein p53 could be detected.

Investigation of in frame deletion mutants of HPV16 E6

The transforming E6 proteins of HPV1 and 8 neither interact with p53 or E6AP nor do they have an influence on the stability or sequence-specific DNA binding of the p53 tumor suppressor protein. As a further step to elucidate if the oncogenic properties of E6 in rodent fibroblasts involve binding to p53, in frame deletions covering the entire length of HPV16 E6 were generated. Because it has been observed that mutations of cysteines in Cys-X-X-Cys-motifs resulted in protein instability, our strategy was to delete short amino acid stretches within E6, but to leave the conserved Cys-motifs intact. A map of five independent in frame deletion mutants of HPV16 E6 is depicted in Fig. 6, upper panel, with the positions of the deleted amino acids indicated. All mutants and wild-type

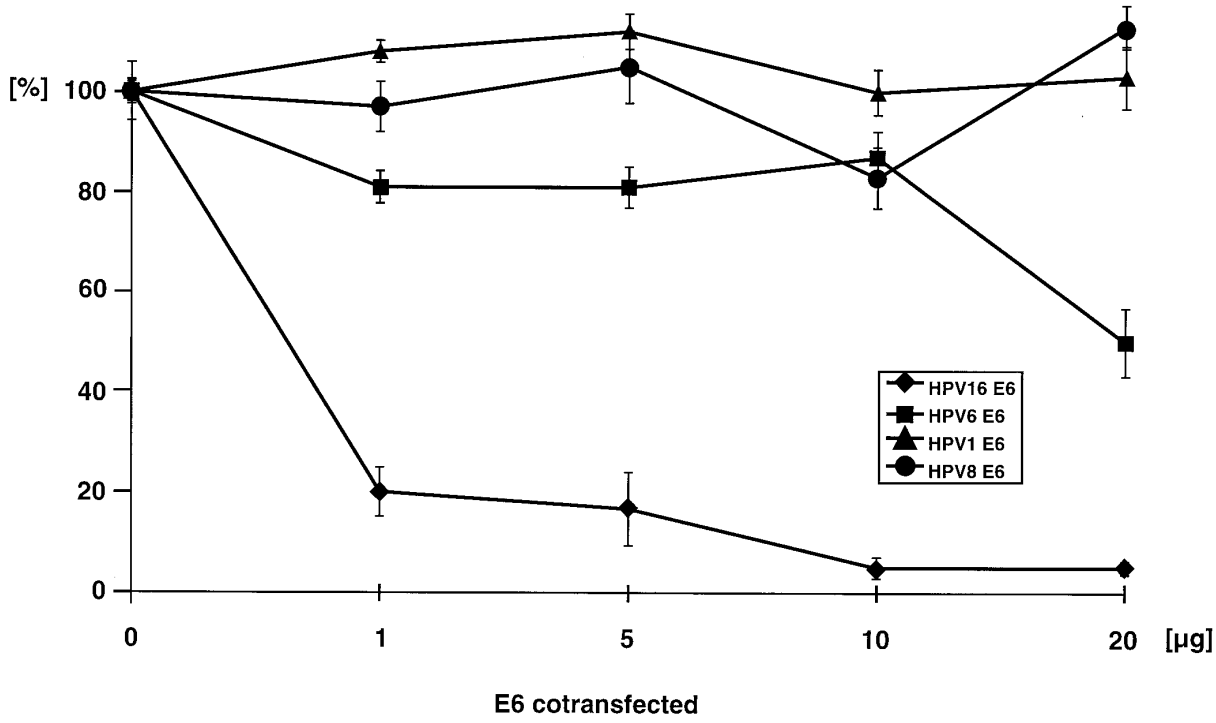


FIG. 5. Dose-dependent inhibition of p53-mediated transactivation by different E6 proteins. Cells were cotransfected with 1 μg reporter plasmid (pG13CAT), 10 ng p53 expression vector (pC53SN3), and increasing amounts of expression vectors for HPV1 E6 (triangles), HPV8 E6 (circles), HPV6 E6 (squares), or HPV16 E6 (rhombs). CAT activity was determined with the help of a phosphoimager (Fuji) and expressed as the conversion rate from nonacetylated to acetylated [^{14}C]chloramphenicol. The amount of acetylated [^{14}C]chloramphenicol produced by pC53SN3 without E6 was set to 100%. Values represent the average of four independent experiments, with minimal duplicate transfections. Standard deviations are depicted as black lines.

HPV16 E6 were cloned as fusion proteins with the hemagglutinin (HA) epitope at the C-terminus in the eukaryotic expression vector pLXSN. First we transfected NIH 3T3 cells with the individual mutant constructs and established pools of G418-resistant cells which were tested for anchorage-independent growth in softagar (Fig. 6, lower panel). Although all five in frame deletions affected the transformation efficiency in comparison to wild-type 16 E6, the mutants 35/56 and 71/90 still led to a fair number of colonies in the softagar assay. The N-terminal deletion (18/25) and the deletion in the second zinc finger (108/126) and in the C-terminal domain (142/149) resulted in a complete abolishment of the transforming potential of HPV16 E6. The number of colonies formed with these mutants was not significantly different from the background observed with pools established from HPV6 E6-transfected cells. These data indicate that integrity of the aminoterminal as well as the carboxyterminal domain, including the second Cys-X-X-Cys-motif, is important to maintain the transforming function.

To establish a correlation between the transforming properties of the mutant HPV16 E6 proteins and their p53 or E6AP binding abilities we expressed all mutants as fusion proteins with the DBD of GAL4 in yeast Y190 cells cotransfected with human GAL4-AD-p53 or GAL4-AD-E6AP. The amount of β-galactosidase produced due to the interaction of p53 or E6AP with the respective mutant E6 proteins is shown in Fig. 7, upper panel. Only the deletion mutant 142/149 was still able to interact with the p53 tumor suppressor protein, which indicates that the extreme C-terminal domain of HPV16 E6 is dispensable for an association with p53. When we tested the ability of the GAL4-DBD-16 E6 mutants to interact with GAL4-AD-E6AP we received similar results. Besides the wild type only mutant 142/149 was still able to interact with GAL4-AD-E6AP; in general, however, the affinity of the GAL4-DBD-16 E6 mutant to E6AP seems to be lower than to p53.

To ensure that the lack of interaction between the four deletion mutants and p53 is not simply due to protein instability, we performed Western blotting analysis which revealed that all proteins are stably expressed in yeast cells (data not shown).

To next address the question whether the in frame deletion mutants of HPV16 E6 can inhibit p53-mediated transcriptional activation, we transfected C33A cells with 10 μg of each of the E6 mutant pLXSN-constructs, together with 1 μg of CAT reporter and 10 ng of p53 expression vector as described before (Fig. 7, lower panel). In agreement with our data from the yeast two-hybrid sys-

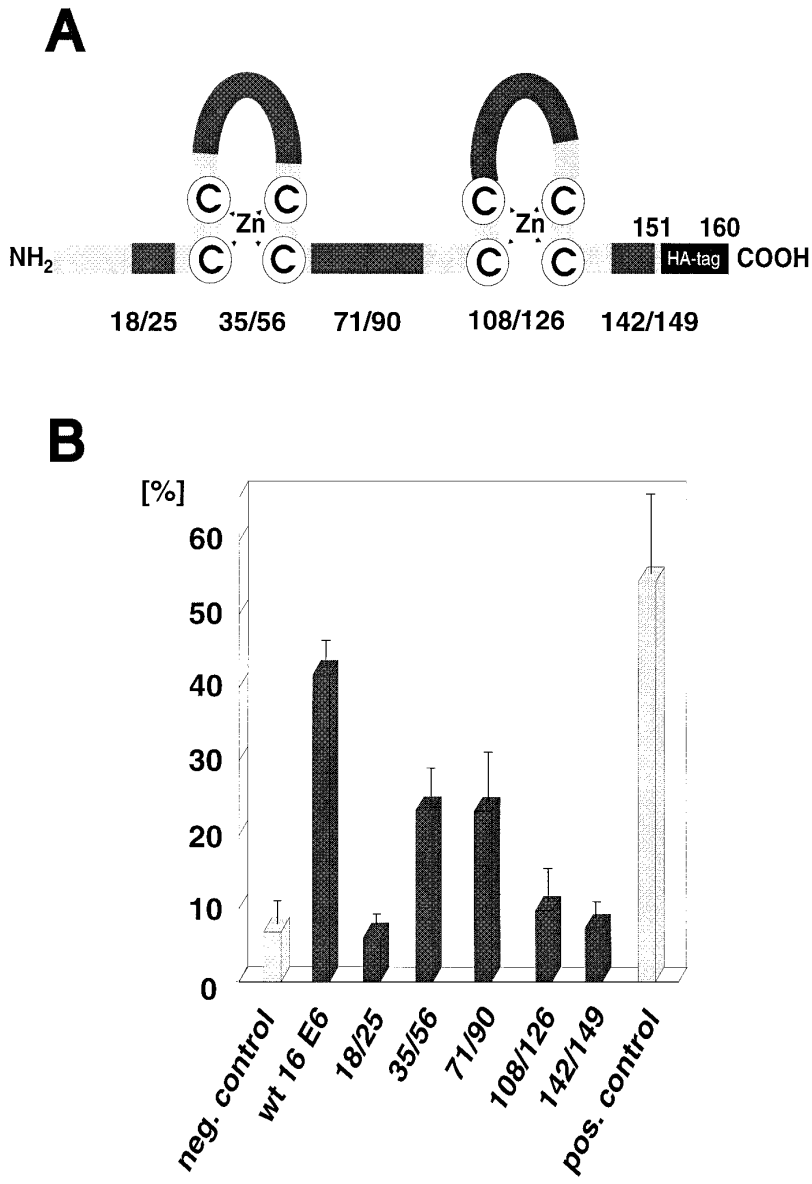


FIG. 6. In frame deletion mutants of HPV16 E6 and their interaction with p53. (A) Dark colored parts represent deleted portions of the HPV16 E6 protein. The first and the last deleted amino acid in each mutant construct is indicated. Encircled "C" represents conserved cysteine residues, which form two zinc binding fingers (Zn). A hemagglutinin epitop (HA-tag) is fused to the last amino acid of all mutated and the wild-type HPV16 E6 protein. (B) Anchorage-independent growth of NIH 3T3 cells transfected with wild-type HPV16 E6-HA (wt 16 E6) or one of five individual in frame deletion mutants of HPV16 E6. Softagar assays were carried out as described in the legend to Fig. 1. Negative control, NIH 3T3 cells were transfected with HPV6 E6. Positive control, established cell line harboring HPV16 E6 (Schmitt *et al.,* 1994).

tem only the in frame deletion mutant 142/149 inhibited p53 transactivation to the same extent as wildtype 16 E6. The other mutant E6 proteins 71/90 and 108/126 showed no effect on p53, whereas 18/25 and 35/56 roughly repressed p53-mediated transactivation by ~40%. To verify the expression of the important mutant 142/149 in mammalian cells, the E6 mutant 142/149 and wild-type 16 E6 were subcloned in an eukaryotic expression vector allowing high levels of expression in cells containing the large T-antigen of SV40 (Mizushima and Nagata, 1990) and transiently transfected into COS7. The cells were labeled with [^{35}S]cysteine and cellular extracts were directly immunoprecipitated with a HA-epitope-specific antibody in the presence of 2 μg anti-IgG antibody (Fig. 8A, lane 4, wild-type 16 E6, and lane 6, 142/149). No precipitation of E6 without additional anti-IgG antibody was observed (Fig. 8A, lanes 3 and 5). As controls, mock-transfected cell extracts were incubated with anti-HA- and anti-IgG-antibody (Fig. 8A, lane 2). Figure 8A, lane 1, shows an incubation of 16 E6-transfected cells with an unrelated antibody. This analysis clearly showed the presence of detectable amounts of both E6 proteins in

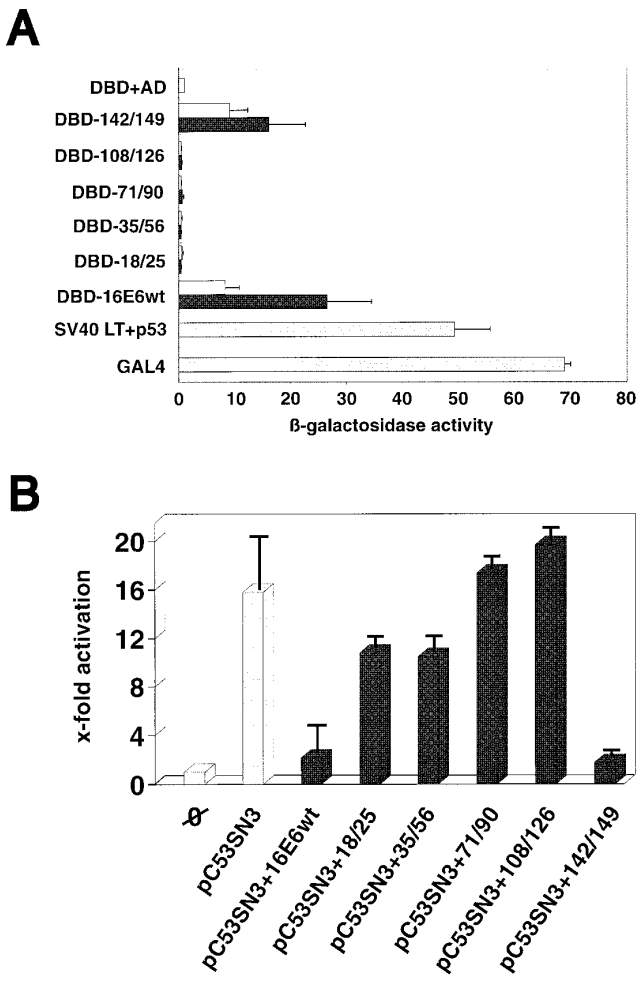


FIG. 7. Interaction between wild-type p53 or E6AP and in frame deletion mutants of HPV16 E6. (A) Y190 yeast cells were cotransfected with GAL4-DBD-in frame deletion mutants of 16 E6 and GAL4-AD-p53 (dark bars) or GAL4-AD-E6AP (white bars). Controls, grey bars. For other details see the legend to Fig. 2A. (B) Cells were transfected with 1 μg pG13CAT, 10 ng pC53SN3, and 10 μg of expression vectors for wild-type HPV16 E6 or each in frame deletion mutant. The basal CAT activity of pG13CAT ($\varnothing$) was set to "1." Activities of the other constructs are given as *x*-fold values relative to pG13CAT. Details on determination of CAT activity are described in the legend to Fig. 5.

the transfected cells, although it cannot be ruled out that the mutant E6 protein may be functionally inactive due to defective subcellular localization.

Since the transforming potential of HPV16 E6 mutants in rodent fibroblasts does not correlate with the capacity to directly bind to p53, we wanted to investigate whether the interaction between mutant 142/149 and p53 induces the ubiquitin-dependent degradation of the tumor suppressor protein *in vitro.* Thus p53 was translated in reticulocyte lysate in the presence of [^{35}S]methionine/cysteine and incubated with wild-type 16 E6 or 142/149 at 25°C (Fig. 8B). Increasing amounts of nonhydrolyzeable ATPγS were added and the reactions were stopped after 60 min. The total reaction mixtures were analyzed on SDS–PAGE and the p53 proteins were visualized by fluorography. With increasing amounts of ATPγS the ubiquitination of p53 becomes visible in the presence of wild-type 16 E6 and 142/149. In a time course experiment we observed a slower kinetic of mutant 142/149 in degrading p53 as with wild-type 16 E6 (Fig. 8C). After 60 min 96% of the input amount of p53 are degradated when wild-type 16 E6 was present, whereas only 83% are degra-

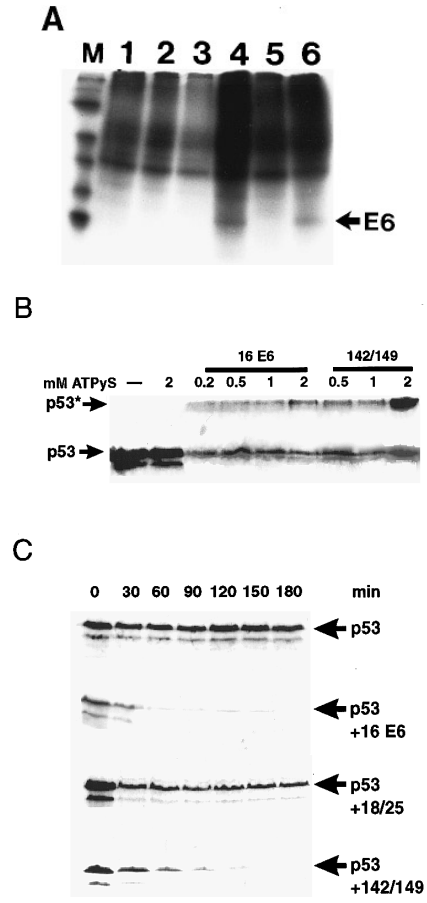


FIG. 8. Expression of wild-type HPV16 E6 and 142/149 and degradation of p53. (A) Immunoprecipitation of wild-type 16 E6 and 142/149 in COS7 cells. The E6 proteins were immunoprecipitated with a HA-specific antibody. The immunoprecipitation in lane 4 and 6 contained in addition 2 μg anti-IgG antibody allowing an efficient precipitation of wt 16 E6 (lane 4) and 142/149 (lane 6). The E6 proteins are indicated by an arrow. No precipitation occurred without anti-IgG antibody (lane 3 and 5). A reaction with an unrelated antibody (lane 1) and mock-transfected COS7 cells (lane 2) served as negative controls. M, molecular weight markers from top to bottom in kDa: 220, 97, 66, 46, 30, 19. (B) Ubiquitination of p53 in the presence of wild-type 16 E6 and 142/149. Ubiquitination reactions were performed in the presence of increasing amounts of ATPγS as indicated. Details as described in the legend to Fig. 1B. (C) Time course experiment of 142/149-induced degradation of p53. p53 was incubated in the absence or in the presence of equal amounts of HPV16 E6, 18/25, or 142/149 at 25°C as described under Material and Methods. 18/25 is depicted as a representative for all other in frame deletion mutants, which led to neither ubiquitination nor degradation of p53 *in vitro* (data not shown). Details on degradation assay as described in the legend to Fig. 1C.

dated in the case of 142/149. All other mutants, which revealed no binding to p53 in the yeast two-hybrid system nor influenced the p53-mediated transcriptional activity were also tested in the ubiquitination assay. Even in the presence of high amounts of ATPγS no ubiquitinated forms of p53 appeared when 18/25, 35/56, 71/90, or 108/126 were present in the reaction (data not shown). These results reveal that binding of E6 to p53 and E6AP clearly correlates with the ubiquitination and degradation of the tumor suppressor protein but not with the transforming potential of E6 in NIH 3T3 rodent fibroblasts.

DISCUSSION

A common necessity for all small DNA tumor viruses is to induce DNA synthesis in quiescent cells to provide cellular enzymes for the replication of the viral DNA. All papillomaviruses investigated so far encode an E7 gene whose protein product interferes with the function of members of the retinoblastoma family controlling the transition from the G1-phase into the S-phase of the cell cycle. Uncontrolled entry into S-phase, however, activates negative growth control signals which have to be bypassed by the virus to achieve production of progeny viruses. Interestingly, while high risk genital HPV16 E7 is able to overcome different negative growth signals, low risk HPV6 E7 does not exhibit such properties (Demers *et al.,* 1996). In addition to these remarkable properties of the E7 protein, high risk genital types encode an E6 protein that overcomes p53-mediated G1-arrest and apoptosis in concert with the cellular E6AP factor by targeting p53 for the enhanced ubiquitin-dependent degradation (Huibregtse and Scheffner, 1994). Although many properties of the E6 and E7 proteins of high risk genital types have been discovered, the key question, which of these functions is responsible for the carcinogenic phenotype, is still discussed. One important difference between low and high risk genital types is the ability of the latter to immortalize primary human keratinocytes (Halbert *et al.,* 1992, 1991; Sedman *et al.,* 1992; Hawley-Nelson *et al.,* 1989; Watanabe *et al.,* 1989; Munger *et al.,* 1989). Second, the continuous E6-mediated degradation of p53 may lead to an accumulation of genetic mutations in the infected cell, which clearly has to be regarded as an important contribution to the carcinogenic action of high risk genital HPV types (zur Hausen, 1994). In addition other cellular ligands, like E6BP and paxillin, recently have been described that bind specifically to the transforming E6 protein of BPV1 and to the E6 protein of high risk genital types but not to low risk types (Chen *et al.,* 1995; Tong and Howley, 1997). These newly identified interactions may also contribute to the high risk phenotype either by altering the differentiation behavior of infected keratinocytes or by disrupting the actin cytoskeleton.

Comparatively little is known about the functions of the E6 and E7 proteins of cutaneous papillomaviruses that primarily infect the keratinizing skin outside of the genital tract. In contrast to high risk genital types no immortalizing or transforming activities have been found for the E7 proteins of the high risk types 8 and 47 (Iftner *et al.,* 1988; Schmitt *et al.,* 1994; Hiraiwa *et al.,* 1993). On the other hand the ability of the E6 protein to transform established rodent fibroblasts seems to be a property shared by genital and cutaneous types (Schmitt *et al.,* 1994; Iftner *et al.,* 1988; Kiyono *et al.,* 1992). The mechanism of rodent cell transformation by the E6 protein, however, remains to be elucidated.

In the case of the cutaneous HPVs almost nothing is known about the interactions of the E6 proteins with cellular ligands. One study only investigated the interaction of *in vitro*-translated HPV8 E6 with murine p53 (Steger and Pfister, 1992), but did not check for degradation of p53 or interaction with E6AP. We therefore compared the properties of the cutaneous E6 proteins with already known functions of E6 proteins of genital viruses and found no correlation between p53-inactivation and transforming potential neither for cutaneous nor for genital E6 proteins (summarized in Table 1).

When we tested the E6 protein of HPV1, 6, and 8 for interaction with p53 in reticulocyte lysates we observed neither ubiquitination nor degradation of the p53 molecule, whereas HPV16 E6 efficiently degradated 96% of the input amount of p53 protein within 1 h. This lack of function of the E6 proteins of HPV1, 6, and 8 to degradate p53 correlated well with the inability of these E6 proteins to interact with E6AP, as measured in the yeast two-hybrid system.

It has been reported, however, that the nontransforming HPV6 E6 in spite of having no influence on the stability of p53 nevertheless can bind to the tumor suppressor protein (Li and Coffino, 1996; Crook *et al.,* 1991). This complex formation was shown to affect the sequence-specific DNA binding of p53, which is essential for its transactivating function (Lechner and Laimins, 1994). Therefore the ability to form a complex with p53 seems to be a property shared by all genital types. To investigate cutaneous E6 proteins for direct p53 binding we used the yeast two-hybrid system, which allows measurement of E6AP-independent complex formation between E6 and p53. In agreement with other studies, we observed a strong binding of HPV16 E6 and a weaker binding of HPV6 E6 to human p53 expressed in yeast cells. None of the cutaneous E6 proteins revealed a detectable interaction with p53. Transient transactivation assays with a p53 reporter construct in a human epithelial cell line were in line with these findings from the yeast two-hybrid system. Whereas HPV16 E6 had a strong suppressive effect on p53-mediated transactivation, HPV6 E6 exhibited a minor influence only at high

TABLE 1

p53-Inactivation Does Not Correlate with Transforming Potential

	p53-binding	p53-ubiquitination/ p53-degradation	Inhibition of p53-transactivation	Transformation of NIH3T3 fibroblasts
HPV1 E6	−	−	−	+
HPV8 E6	−	−	−	+
HPV6 E6	+	−	±	−
HPV16 E6	+	+	+	+
mt 18/25[a]	−	−	±	−
mt 35/56[a]	−	−	±	+
mt 71/90[a]	−	−	−	+
mt 108/126[a]	−	−	−	−
mt 142/149[a]	+	+	+	−

[a] In frame deletion mutants of HPV16 E6.

concentrations and the cutaneous E6 proteins were completely inactive in this assay (Table 1).

It has been reported (Li and Coffino, 1996) that the p53 molecule contains two binding sites for the E6 proteins, one in the core region and another one at the carboxyterminus, but only E6AP-mediated binding to the core region resulted in degradation of p53. To investigate if the complex between E6 and p53 found under physiological conditions in yeast cells depends on identical regions of the p53 molecule we analyzed different mutants of p53. In contrast to Li and Coffino (1996) we found no interaction between E6 and the exactly same p53 core mutant (Cys-135-Tyr). This may be explained by the different experimental conditions used; however, earlier reports using the same *in vitro* techniques as Li and Coffino (1996) were also not able to demonstrate E6 binding to the p53 (Cys-135-Tyr) core mutant (Scheffner *et al.,* 1992; Huibregtse *et al.,* 1993a, 1991; Slebos *et al.,* 1995). Taken together with other existing data (Mansur *et al.,* 1995) the core region seems to be essential to mediate interaction between E6 and p53 at least in the yeast two-hybrid system. Interestingly, by using other p53 mutants with deletions either at the amino- or carboxyterminal end we confirmed in part the finding of Li and Coffino (1996) that the carboxyterminus of p53 has an influence on the stability of the p53–E6 complex. A deletion of the first 100 amino acids of p53 abolished the complex formation, whereas deletion of the last 67 carboxyterminal amino acids only reduced the binding affinity of HPV16 E6 to p53. In the case of HPV6 E6 the same carboxyterminal deletion resulted in a complete loss of detectable interaction with p53. Because binding of HPV6 E6 to p53 could not be observed with the N-terminal and the core mutant of p53, one might conclude that these regions of the p53 protein are essential to mediate the weak interaction with HPV6 E6. The core binding also correlates with the observed effect of HPV6 E6 to inhibit at high intracellular levels the transactivation function of p53, which requires sequence-specific binding through an unoccupied core domain. Removal of the carboxyterminal domain of p53, which had a modulatory effect on the stability of the complex between 16 E6 and p53, may in the case of the weaker binding of HPV6 E6 simply cause reduction of binding affinity below the level of detection.

Because we could not establish a correlation between the transforming functions of the cutaneous E6 proteins with any of the activities known from genital E6 proteins, we next tested deletion mutants of the high risk HPV16 E6 protein for their respective activities. For this, we constructed a number of in frame deletions of the HPV16 E6 gene and tested them for the ability to transform NIH 3T3 cells, bind p53 or E6AP, degradate p53, or inhibit p53-mediated transactivation. A number of *in vitro* studies have already been performed to localize the domains within the E6 molecule that are responsible for p53 binding or degradation; however, the results obtained were somewhat controversial (Slebos *et al.,* 1995; Foster *et al.,* 1994; Crook *et al.,* 1991; Dalal *et al.,* 1996; Nakagawa *et al.,* 1995). Using the yeast two-hybrid system and other techniques we now were clearly able to separate p53 and E6AP binding from transforming activity. The HPV16 E6 mutant 142/149 with a deletion of eight amino acids at the very end of the molecule was still able to bind to p53 and E6AP, to degradate p53, and to inhibit its transactivating function, but could no longer transform NIH 3T3 cells. In contrast, two other deletion mutants (35/56 and 71/90) efficiently induced softagar growth of transfected NIH 3T3 cells, but were no longer able to bind p53 or E6AP or degradate p53. These results are partially in agreement with earlier experiments that showed that the carboxyterminus of HPV16/18 E6 is not necessary for the interaction with p53 (Foster *et al.,* 1994; Pim *et al.,* 1994). Interestingly the binding profile of the E6 mutants to p53 and E6AP was the same and did not support a possible role of E6AP interaction for the transformation of rodent fibroblasts by E6. This indepen-

dence of E6-mediated rodent cell transformation from p53 or E6AP binding is, however, contrasted by the necessity of p53 degradation in the immortalization of human embryonic kidney cells (Nakagawa *et al.,* 1995) or mammary epithelial cells (Dalal *et al.,* 1996). Finally, we could demonstrate that the carboxyterminus of HPV16 E6 is important for the inherent transforming function of the protein, which is in agreement with a study from Sherman and Schlegel (1996) showing that the carboxyterminus of HPV16 E6 is required for the induction of calcium and serum resistance of human keratinocytes and for the immortalization in conjunction with E7.

In summary binding to p53 seems to be a prerequisite for inhibition of p53-mediated transactivation but not to be sufficient for degradation of p53, which is clearly a function of E6AP. Both activities, however, are dispensable for the transformation of rodent fibroblasts. Studies are in progress to narrow down the domains in the E6 protein which are responsible for rodent cell transformation and to search for new cellular ligands of the E6 proteins with the aim to further elucidate the pathway of E6-mediated transformation.

ACKNOWLEDGMENTS

We thank M. Scheffner for kindly providing plasmids encoding p53(Cys-135-Tyr), human E6AP, and helpful technical advice and comments, B. Vogelstein and L. Laimins for the plasmids pG13CAT and pC53SN3. We thank A. Schmitt and T. A. D. Siepen for excellent technical assistance, B. Rapp, A. Keller, and K. Lorz for critical reading of the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft DFG If1/3-1 to T.I.

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