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Regions of Human Papillomavirus Type 16 E7 Oncoprotein Required for Immortalization of Human Keratinocytes

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Binding of the retinoblastoma gene product (pRB) by viral oncoproteins, including the E7 of human papillomavirus type 16 (HPV 16), is thought to be important in transformation of cells. One of the steps in transformation is the immortalization process. Here we show that mutations in E7 within the full-length genome which inhibit binding of pRB do not abrogate the ability of the HPV 16 DNA to immortalize primary human epithelial (keratinocyte) cells. A mutation in one of the cysteines of a Cys-X-X-Cys motif which is contained in the carboxy half of the E7 and is part of a zinc finger arrangement completely eliminates the ability of HPV 16 DNA to immortalize cells. The results indicate the importance of E7 in the immortalization of primary keratinocytes but suggest that the binding of pRB is not essential.

Certain human papillomaviruses (HPV) are associated with genital neoplasias, in particular premalignant and malignant disease of the cervix. HPV types 16 and 18 are commonly associated with premalignancies (cervical intraepithelial neoplasia) of all grades and malignancies (5, 10, 19), while HPV 6 and 11 are found in low-grade cervical intraepithelial neoplasia and benign lesions such as genital warts (13, 21).

HPV 16 and 18 have been shown to transform rodent fibroblasts and immortalize primary human epithelial cells in vitro (4, 7, 20, 24, 34). HPV 6 and 11 have been shown to transform rodent cells (21), albeit at a reduced frequency compared with HPV 16 and 18, but do not immortalize primary keratinocytes (14, 36).

The main transforming gene of both HPV 16 and 18 appears to be E7. The gene codes for a protein of approximately 11 kDa which is phosphorylated (29), can bind Zn^{2+} through two Cys-X-X-Cys motifs in the carboxy half of the protein (2, 11), and also binds the cellular retinoblastoma gene product (pRB) (1, 11). E7 of both HPV 16 and 18 can, in cooperation with an activated *ras*, but not v-*myc*, transform rat embryo fibroblasts (REFs) (3, 7) and baby rat kidney cells (31). The protein can also transform mouse NIH 3T3 cells under certain selection conditions (7). However, both E6 and E7 are required for the efficient immortalization of primary human keratinocytes, which are the natural host cell of papillomaviruses infecting mucosal and cutaneous surfaces (15, 22). Like E7, the E6 gene product binds a cellular growth regulatory protein, namely p53, and appears to hasten its breakdown through the ubiquitin pathway (27, 35).

Amino acid changes in E7 produced by site-directed mutagenesis can abrogate its ability to bind pRB (1) and also inhibit the ability to transform NIH 3T3 cells (12) and REFs in cooperation with an activated *ras* (8). For instance, changes in amino acids at positions 24 and 26 which are conserved between other pRB binding regions of the E1a of adenovirus and large T (LT) of simian virus 40 (SV40) inhibits binding of pRB and also transformation of NIH 3T3 cells and REFs. Equivalent mutations in SV40 LT also inhibit binding to pRB and abrogate transformation of primary rodent fibroblasts (9). Recent evidence, however, suggests that SV40 LT with a mutation at amino acid 107 (equivalent to 26 of E7) or with deletions in the pRB region can immortalize primary rodent cells but not transform them, suggesting that pRB is not essential for this function (6, 32). Other results suggest that SV40 LT binds other cellular factors in addition to pRB necessary for immortalization and transformation (25).

Our previous results also showed that changes to the phosphorylated serines at the 3′ end of the pRB region did not affect the ability of E7 to transform primary rodent cells in cooperation with *ras* (8). Similarly, a mutation at amino acid 50 between the pRB binding domain and the Cys-X-X-Cys motifs had no effect on transformation of rodent cells (8). However, mutating a cysteine in either of the motifs reduced transformation significantly, and mutations in both motifs completely inhibited E7 transformation (8).

In this report, we show that mutations in the pRB binding region, in a neutral area in the middle of the protein and in the Zn^{2+} binding region of E7, exhibit differences in their abilities to immortalize human primary keratinocytes. The effects of these mutations on immortalization of human epithelial cells do not concur with their effects on the transformation of rodent fibroblasts.

MATERIALS AND METHODS

Cells and transfections. Primary REFs (Whittaker M. A. Bioproducts, Baltimore, Md.) were grown in Dulbecco's minimal essential medium with 10% fetal bovine serum and then transfected as previously described (8). Briefly, cells were transfected with 25 μg of test DNA and 2 μg of pSV2*neo* as a selectable marker by the calcium phosphate method, after which they were grown for 48 h and split 1:2; one plate was treated with 300 μg of G418 per ml, and the other was left for 2 to 3 weeks for the development of foci. Plates were scored by staining confluent monolayers with 4% Giemsa stain containing formaldehyde for 30 min and counting foci. The G418 plates were similarly treated.

Primary human keratinocytes were cultured from fresh

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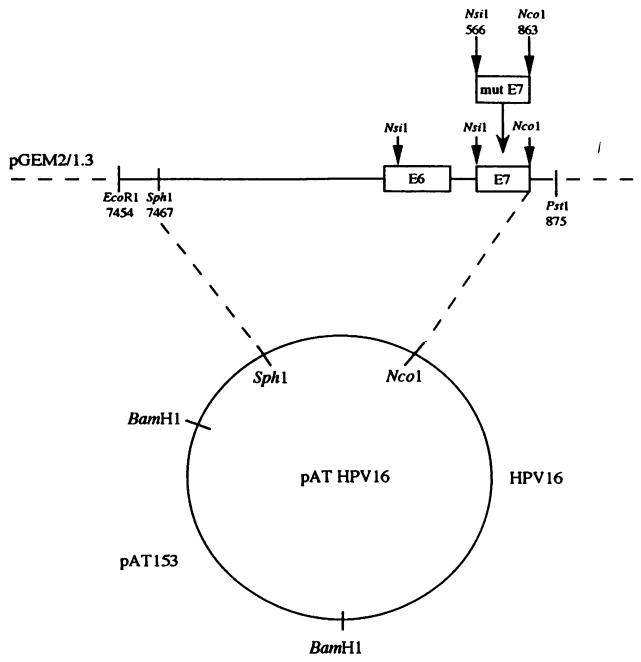

FIG. 1. Cloning of the E7 mutation into the full-length HPV 16 genome to replace the wild-type E7 gene.

foreskin pieces in KGM medium (Clonetics, San Diego, Calif.) and used for experiments at passage 2 or 3. The SCC-13 cell line is from a non-HPV-containing squamous cell carcinoma and was a gift from J. Rheinwald, Harvard University.

Electroporation was used to transfect primary cells. Cells (2×10^6) were electroporated with 10 μg of test plasmid DNA plus 1 μg of pSV2*neo* DNA containing the selectable marker for G418 resistance, using 1,000 V at 25 μF. As a negative control cells were electroporated with 1 μg of pSV2*neo* alone.

Immortalization studies. Following electroporation, the cells were plated into 100-mm petri dishes in E medium (37) with the addition of epidermal growth factor (5 ng/ml) until confluent, when they were split into two groups. One group was selected with G418 at 25 μg/ml for 14 days, and 12 resistant clones were picked and expanded for analysis.

The other group of cells was plated onto 100-mm dishes, allowed to grow to confluence, and then left in E medium for 4 weeks for observation of flat undifferentiated colonies. This is a method described by Schlegel et al. (28) to quantify the number of immortalized cells in a culture, since at confluence the level of calcium plus serum induces differentiation of primary keratinocytes unless they have been stimulated to divide.

Plasmids and E7 mutations. Plasmids (pUC19.MoLTRE7) containing the E7 point mutations at amino acids 24, 26, 50, and 91 were a gift of K. Vousden, Ludwig Institute, St. Mary's Hospital, London, England (12). The mutated E7 genes were cloned into the full-length genome as shown in Fig. 1 and briefly described below. The various E7 mutations were excised from pUC19.MoLTR by using *Nsi*I and *Nco*I and then ligated into a 1.3-kb fragment of HPV 16 (from *Eco*RI at 7454 to *Pst*I at 875) previously cloned into pGEM.2 (Promega, Madison, Wis.), which was digested with *Nsi*I (partial digest) and *Nco*I to accept the E7 insert. This E7 region together with E6 was then excised from pGEM.2/1.3, using *Sph*I and *Nco*I. A full-length copy of HPV 16 was cloned into the *Bam*HI site of plasmid pAT153 from which the *Sph*I site had been deleted and was then digested with *Sph*I and *Nco*I to accept the HPV 16 fragment from pGEM.2/1.3. The E7 genes containing mutations at amino acids 24 and 26 were then sequenced by the double-stranded DNA method, using the Sequenase 2.0 kit and protocol (United States Biochemical, Cleveland, Ohio) to ensure their authenticity. Mutations at amino acids 50 and 91 were tested by restriction analysis as the amino acid changes created *Bst*YI and *Apa*I sites, respectively, in E7.

RNA analysis. RNA was extracted from keratinocytes as previously described (20). The RNA was run on 1% formaldehyde agarose gels, transferred to nitrocellulose filters, and hybridized with ^{32}P-labelled RNA transcribed by T7 RNA polymerase from the E6/E7 region of HPV 16 cloned into pGEM.2.

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1  -  MET HIS GLY ASP THR PRO THR LEU HIS GLU TYR MET LEU ASP LEU GLN PRO GLU

                              24      26
                             GLY     GLY
20 -  THR ASP LEU TYR CYS TYR GLU GLN LEU ASN ASP SER SER GLU GLU GLU ASP GLU ILE

E1a 121 - Asp Leu Thr Cys His Glu Ala Gly Phe Pro Pro Ser Asp Asp Glu Asp Glu -137
LT 102  - Asn Leu Phe Cys Ser Glu Glu Met —  Pro Ser Ser Asp Asp Glu Ala Thr -117

                                                      50
                                                     SER
39 -  ASP GLY PRO ALA GLY GLN ALA GLU PRO ASP ARG ALA HIS TYR ASN ILE VAL THR PHE

58 -  CYS CYS LYS CYS ASP SER THR LEU ARG LEU CYS VAL GLN SER THR HIS VAL ASP ILE

                                                                91
                                                               GLY
77 -  ARG THR LEU GLU ASP LEU LEU MET GLY THR LEU GLY ILE VAL CYS PRO ILE CYS SER

96 -  GLN LYS PRO
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FIG. 2. Amino acid sequence of HPV 16 E7. The site-directed mutations used at amino acids 24, 26, 50, and 91 and the change of amino acid are shown above the normal sequence. Also shown are the pRB binding region of E7 and its homology to the equivalent region of adenovirus E1a and SV40 LT.

Immunoprecipitations. For immunoprecipitations, the cells were labelled with [^{35}S]cysteine (Amersham plc, Amersham, England) for 3 h in cysteine-free E medium. Alternatively, cells were labelled with $^{32}P_i$ in phosphate-free medium. After labelling, each dish was washed three times with ice-cold buffer 1 (40 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.4], 100 mM NaCl), and cells were lysed for 15 min at 4°C in 0.5 of ice-cold buffer 2 (50 mM HEPES [pH 7.0], 250 mM NaCl, 5 mM dithiothreitol, 0.1% Nonidet P-40) containing the protease inhibitors phenylmethylsulfonyl fluoride, aprotinin, and leupeptin at 10 μg/ml each. The lysates were then centrifuged at 4°C for 10 min at 14,000 rpm in a microcentrifuge. For E7 precipitation, aliquots of the clarified supernatants were mixed overnight with a 1:100 dilution of anti-E7 antibodies. Immune complexes were then bound to protein A-Sepharose beads (Sigma, St. Louis, Mo.) for 2 h at 4°C. For pRB precipitation, 2 μg of purified anti-pRB antibodies (RB-PMG-245; PharMingen, San Diego, Calif.) per ml was used. Rabbit anti-mouse immunoglobulin G was then added to this mixture prior to immune complex precipitation with protein A-Sepharose. The beads were then washed five times with buffer 2. Proteins were eluted from the beads by boiling for 2 min in sodium dodecyl sulfate (SDS)-polyacrylamide gel sample buffer (62 mM Tris-HCl [pH 6.8], 3% SDS, 100 mM dithiothreitol, 10% glycerol, 0.01% bromophenol blue) and then loaded onto a 15% SDS-polyacrylamide gel for detection of E7 and onto a 7.5% SDS-polyacrylamide gel for pRB separations. After electrophoresis, the gels were fixed in 7% acetic acid for 30 min, soaked in Amplify (Amersham plc) for 15 min, dried, and exposed to Kodak X-Omat AR film.

RESULTS

We (20) and others (24, 36) have shown that the HPV 16 full-length genome can immortalize and inhibit the differentiation of primary human keratinocytes. Others have shown that E6 and E7 of HPV are sufficient for immortalization (22) and, in the case of HPV 18, also for inhibition of differentiation (15). Now we have cloned some of the site-directed mutants into the full-length HPV 16 genome to replace wild-type E7 (Fig. 2). We show that pRB binding is not essential for immortalization but that the zinc finger region is required.

Transformation of REFs. The E7 gene, when expressed by a strong exogenous promoter, can cooperate with an activated *ras* to transform REFs. We and others had previously shown (8, 12) that mutations in amino acids 24 and 26, among a series of site-directed mutations in E7, were changes which completely abrogated the transformation ability. It was important, therefore, to show whether these same mutations, when in the context of the full-length HPV 16 genome and under the control of the endogenous promoter, had lost transformation ability or whether other HPV 16 genes could compensate for this loss.

To this end, REFs were transfected with wild-type HPV 16 or two full-length constructs with mutations in amino acids 24 and 26 cloned into pAT153 plus pEJ*ras* and the selectable marker pSV2*neo*. After transfection, cells were grown to confluence and then split 1:2. One plate was left for 3 weeks for observation of transformed foci, while the other was treated with G418 (300 μg/ml) to assess efficiency of transfection. As shown in Table 1, wild-type HPV 16 plus pEJ*ras* transformed REFs, as seen by the formation of foci within 3 weeks. The constructs containing E7 mutations at amino acids 24 and 26 (p16.24 and p16.26, respectively) did not transform above the background for pEJ*ras* on its own. However, there was an indication that HPV 16 with a mutation in amino acid 24 when in the context of the full-length genome did have some cooperative effect above background of pEJ*ras* alone.

TABLE 1. Transformation of REFs

Plasmid	No. of foci[a] in expt:			
	1	2	3	4
pEJ*ras* + pLTR*myc*	96	20	58	77
pATHPV16 + pEJ*ras*	21	10	37	53
p16.24 + pEJ*ras*	11	0	0	14
p16.26 + pEJ*ras*	4	ND[b]	0	1
pEJ*ras* alone	3	0	0	4

[a] Corrected for transfection frequency.
[b] ND, not done.

These results demonstrate that the mutations in E7 at amino acids 24 and 26 had a dramatic affect on the ability of the HPV 16 genome to cooperate with *ras* and transform primary rodent cells. We next examined constructs of full-length HPV 16 with E7 mutations at amino acids 24, 26, 50, and 91 for their ability to immortalize primary human keratinocytes.

Immortalization of primary human keratinocytes. Immortalization studies were carried out using human keratinocytes at passage 2 or 3. Cells were electroporated with either pATHPV16 (wild type) or full-length genomes containing an E7 mutation in amino acid 24 (p16.24), 26 (p16.26), 50 (p16.50), or 91 (p16.91). Following electroporation, cells were either passaged as mass cultures or selected with G418 48 h after electroporation, and clones were picked and then passaged individually for immortalization studies. Twelve clones, all of which subsequently proved to be immortalized, were picked for each construct, although data for only three from one of the experiments are presented here.

Table 2 shows that pATHPV16, the mutation in the neutral region of E7 (p16.50), and the two mutations in the pRB binding region of E7 (p16.24 and p16.26) were all able to immortalize keratinocytes in a number of different experiments. However, the HPV 16 DNA containing a mutation in one of the Cys-X-X-Cys motifs did not produce immortalization even though seven separate experiments were carried out. In one experiment, extended life span was observed since the cells survived to the passage 16, while the control cells electroporated with pSV2*neo* alone differentiated and died at passage 9.

To estimate the efficiency of immortalization by constructs p16.24 and p16.26, an assay developed by Schlegel et al. (28) was used. Keratinocytes were electroporated with 10 μg of pATHPV16, p16.24, p16.26 plus 1 μg of pSV2*neo*, and

TABLE 2. Immortalization of human keratinocytes

Plasmid	No. of cultures immortalized[a]/ No. of expts
pATHPV16	7/8
p16.24	3/5
p16.26	2/2
p16.50	3/3
p16.91	0/7

[a] Number of times primary keratinocytes were immortalized with each construct.

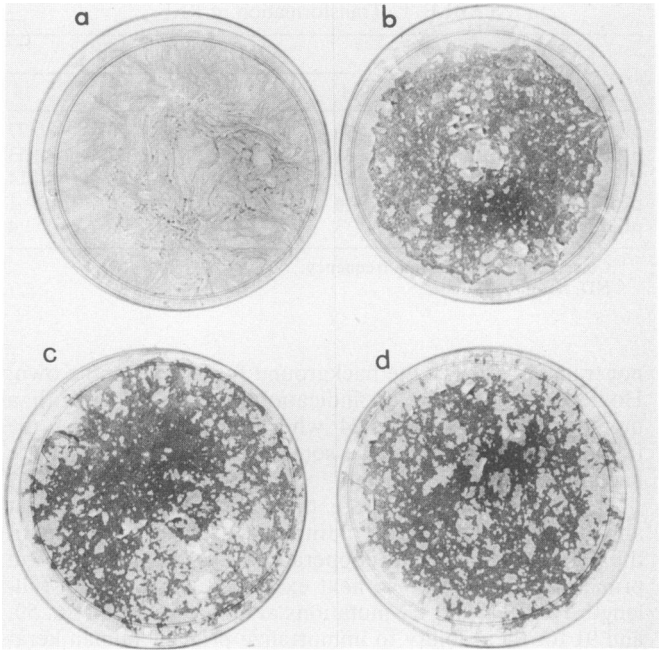

FIG. 3. Foci of dividing cells produced by control pSV2*neo* (a) and by HPV 16 DNA constructs p16.26 (b), p16.24 (c), and pATHPV16 (d).

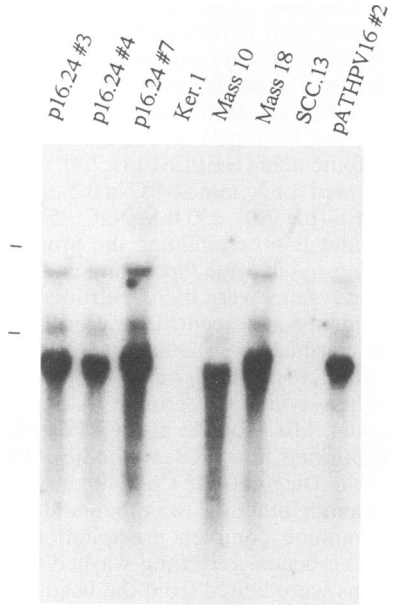

FIG. 4. Northern (RNA) analysis of three clones of cells immortalized with p16.24. Positive control is a clone of cells immortalized with wild-type DNA (pATHPV16). Negative controls are normal primary keratinocytes (Ker.1) and a squamous carcinoma cell line (SCC-13) not containing any HPV DNA. Mass 10 and 18 refer to mass cultures at passages 10 and 18 after transfection with p16.24 and from which clones 3, 4, and 7 were isolated. The filter was hybridized to ^{32}P-labelled RNA transcribed from the E6/E7 region of HPV 16, using the vector pGEM-2. The bars on the left represent the migration distances of 28S and 18S RNAs.

1 μg of pSV2*neo* alone. The cells were plated at 10^6 per 100-mm dish in E medium plus 10% fetal calf serum, allowed to grow to confluence, and then left for 4 weeks, with medium replaced twice weekly. Over this time period, normal keratinocytes differentiate whereas cells containing wild-type HPV 16 DNA continued to divide and push back differentiating cells, which lack HPV DNA. In Fig. 3a, keratinocytes electroporated with pSV2*neo* alone show a uniform pattern of differentiation. Cells transfected with pATHPV16 (Fig. 3d), p16.24 (Fig. 3c), or p16.26 (Fig. 3b) exhibit areas or foci of dividing cells (light areas) against a background of differentiating cells (dark areas). We and others (28) have shown that when the foci are cloned and passaged, immortalized cells result. There are >100 large foci in Fig. 3b to d, indicating that each of the constructs has a similar efficiency of immortalization for primary keratinocytes.

These results, along with those presented above, indicate that pRB binding is not essential for immortalization of primary human keratinocytes but is necessary for the transformation of REFs. However, the Cys-X-X-Cys motifs appear to be required for immortalization, and as determined from previous work (8), when mutated reduce significantly the transformation frequency.

Nucleic acid analysis. Examination of cell lines transfected with pATHPV16, p16.24, p16.26, and p16.50 by Southern blot analysis indicated that the HPV 16 DNA was integrated in all cases (data not shown). RNA analysis was carried out on clones, using ^{32}P-labelled RNA transcribed from an E6/E7 template cloned into pGEM-2 by using the T7 RNA polymerase promoter. Figures 4 and 5B show that three major bands of approximately 1.5, 3.0, and 4.3 kb were seen in pATHPV16, p16.24, and p16.50, while no bands were seen in the controls. The controls included primary human keratinocytes (Ker.1) and an immortalized squamous carcinoma cell line (SCC-13), which does not contain the HPV 16 genome. The number and size of bands are similar to those previously reported by ourselves (19) and others (23, 28). The two mass cultures (Fig. 4, lanes Mass 10 and Mass 18) are cells from one experiment electroporated with p16.24 and from which 12 clones were isolated. The mass cultures were subsequently grown after the cloning, and RNA was extracted at passages 10 and 18. At passage 10, the largest RNA band is either absent or very weak. A similar situation is seen in the clones transfected with p16.26 (Fig. 5A).

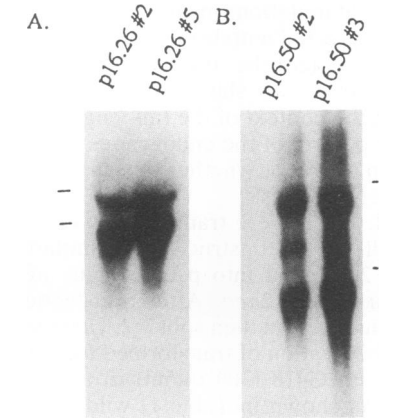

FIG. 5. Northern analysis of clones of cells transfected with p16.26 (A) and p16.50 (B) DNAs. The filters were hybridized as described for Fig. 4; bars represent 28S and 18S RNAs.

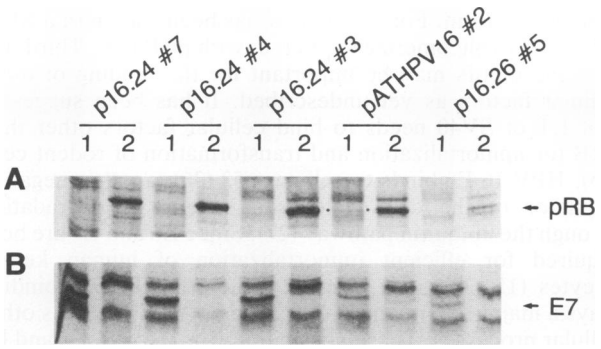

FIG. 6. Immunoprecipitations of [^{35}S]cysteine-labelled immortalized and cloned cells transfected with p16.24, p16.25, or pATHPV16. (A) A 7.5% polyacrylamide gel for separation of pRB (shown by arrow) after immunoprecipitation with anti-E7 (lane 1) or anti-pRB (lane 2) antibodies. (B) A 15% polyacrylamide gel to separate E7 (middle band of three marked by the arrow) after immunoprecipitation with anti-E7 (lane 1) or anti-pRB (lane 2) antibodies. The dots (lane 1, pATHPV16#2) indicate the pRB band immunoprecipitated with the anti-E7 antibodies.

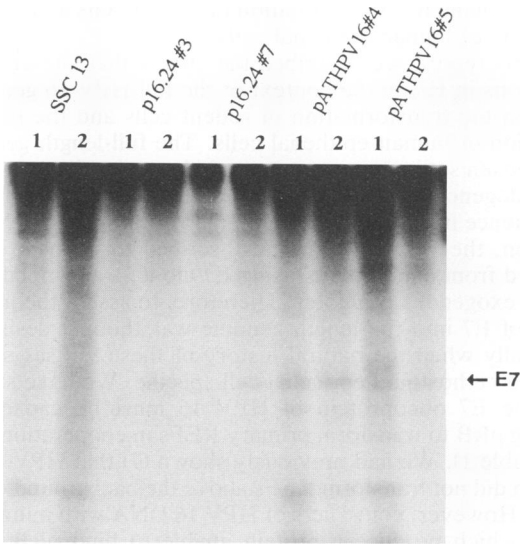

FIG. 7. Immunoprecipitations of ^{32}P-labelled immortalized and cloned cells transfected with either p16.24 or pATHPV16. Control immunoprecipitations were carried out on the SCC-13 cell line, which does not contain HPV DNA. The cell extracts were immunoprecipitated with either anti-pRB (lane 1) or anti-E7 (lane 2) antibodies.

E7 and pRB binding studies. Previous work using in vitro-translated proteins showed that mutations in E7 at amino acids 24 and 26 inhibited or greatly reduced the ability of the protein to bind pRB (1). Alterations in equivalent amino acids of SV40 LT in the homologous pRB binding region were also unable to bind pRB. To confirm that the mutated E7 proteins coded for by the p16.24 and p16.26 constructs did not bind pRB, cells were labeled with [^{35}S]cysteine, proteins were extracted, and pRB and E7 proteins were immunoprecipitated with anti-pRB or anti-E7 antibodies and run on 7.5 and 15% polyacrylamide gels, respectively. Figure 6A (pATHPV16#2, lanes 1 and 2) shows that wild-type E7 contained in pATHPV16 can bind pRB and both proteins are immunoprecipitated together. The two dots on either side of lane 1 indicate the pRB immunoprecipitated in a complex by anti-E7 antibodies. E7 proteins with mutations at amino acids 24 (p16.24) and 26 (p16.26) either were unable to bind detectable amounts or bound no pRB (Fig. 6, p16.24#3 and #4 and p16.26#5, lane 1). It appears that clone p16.24#7 did bind a small amount of pRB, as a faint pRB band is seen with use of anti-E7 antibodies (Fig. 6A, p16.24#7, lane 1), and reciprocally, a faint E7 band is also seen with use of anti-pRB antibodies (Fig. 6B, p16.24#7, lane 2). The result with this clone is different from that reported with use of in vitro-translated mixtures of pRB and E7 and may reflect the obvious differences of the systems used. However, the amount of pRB immunoprecipitated with anti-E7 antibodies is small compared with the levels immunoprecipitated with anti-pRB antisera (Fig. 6, pATHPV16#2) and means that either the small amount of E7 produced in HPV 16-infected cells leaves an excess of unbound pRB or that different optimum immunoprecipitation conditions are needed for each antibody.

In a separate experiment, cells were labelled with $^{32}P_i$ and proteins were immunoprecipitated as described above. It has been shown that HPV 16 E7 (16) and SV40 LT (17) bind to the unphosphorylated form of pRB, which is present in the G_1 phase of the cell cycle. It has also been shown that E7 is phosphorylated at serines 31 and 32 (1) (Fig. 2). The same report showed that mutations in the serines reduced the ability of E7 to transform rodent cells. To ensure that our constructs produced phosphorylated E7, extracts from $^{32}P_i$-labelled keratinocytes containing either wild-type E7 or E7 mutated at amino acid 24 (p16.24 construct) were immunoprecipitated with antibodies against E7 and pRB. Immunoprecipitations show that anti-E7 and anti-pRB antibodies immunoprecipitated phosphorylated E7 (Fig. 7, pATHPV 16#4 and -5, lanes 1 and 2) but that anti-pRB antibodies were unable to precipitate detectable amounts of E7 coded for by p16.24 (Fig. 7, p16.24#3 and -7, lanes 1 and 2). Immunoprecipitation with anti-E7 antibodies did not visualize any pRB, since E7 binds preferentially to unphosphorylated pRB (16; data not shown). Extracts from control cells, SCC-13, when treated with either antibodies, did not immunoprecipitate proteins in the E7 region (Fig. 7, SCC-13, lanes 1 and 2).

The results presented here show that immortalization of primary human keratinocytes by HPV 16 does not depend on the binding of pRB by E7, but the integrity of the Cys-X-X-Cys motifs contained in the carboxy-terminal half of E7 is essential.

DISCUSSION

Certain HPV types, in particular HPV 16 and 18, are associated with malignant disease of the cervix (5, 10, 19). In vitro evidence suggests a causal role for these viruses in the etiology of the cancer, since both can transform rodent cells and immortalize and inhibit differentiation of human epithelial cells (4, 7, 16, 20, 24, 34, 36). The immortalization of keratinocytes on the cervix and the resulting inhibition of differentiation are the first steps in a process which may produce malignant cells. Therefore, these viruses may be involved in the early part of disease, but other factors may dictate progression to malignancy.

HPV 16 E7 has been shown to bind to the unphosphorylated pRB (16), which is a negative regulator of cell division. This finding has lead to the suggestion that this interaction may be important for the in vivo biological properties of

HPV 16, namely, transformation of rodent cells and immortalization of human epithelial cells.

In this report, we described the effects that site-directed mutations in E7, in the context of the full HPV 16 genome, have on the transformation of rodent cells and the immortalization of human epithelial cells. The full-length genome was chosen so that viral genes would be under the control of the endogenous promoter, since this region has been shown to influence immortalization of human keratinocytes (26). In addition, the various properties ascribed to E7 have often resulted from experiments using E7 alone, transcribed by a strong exogenous promoter. Therefore, to assess the importance of E7 in a full-length genome was thought desirable, especially when the natural history of these viruses shows them to be host and epithelial cell specific. We have shown that the E7 oncoprotein of HPV 16 must be capable of binding pRB to transform primary REFs in cooperation with *ras* (Table 1). We had previously shown (7) that HPV 16 on its own did not transform REFs above the background of *ras* alone. However, constructs of HPV 16 DNA with mutations in E7 which produce a protein unable to bind pRB (constructs p16.24 and p16.26), as shown previously by using in vitro-translated proteins (1), can immortalize primary human keratinocytes (Table 2). We repeated the E7/pRB binding experiments, this time immunoprecipitating complexes from in vivo cell extracts, and found that these mutations in E7 bind either undetectable or barely detectable amounts of pRB (Fig. 6A and B; clones p16.24#3, -4, and -7 and p16.26#5 compared with pATHPV16#2; Fig. 7, clones p16.24#3 and -7 compared with pATHPV16#4 and -5). Therefore, the binding of pRB by E7 is required for transformation of rodent cells but is not essential for immortalization of human epithelial cells. Similar results with SV40 LT have been observed: mutations (6) equivalent to mutation in p16.26 in E7 or deletions (30) in the homologous pRB binding region of LT abrogated pRB binding and transformation of rodent cells but did not interfere with the ability of LT to immortalize primary rodent cells. Immortalization is only one of the stages in transformation; therefore, the binding of pRB may be essential, in nature, for the eventual progression to malignancy of HPV 16-infected keratinocytes.

While the binding to pRB was not essential for immortalization, the integrity of the Cys-X-X-Cys motifs are absolutely required for this function. Seven separate experiments were carried out in an attempt to immortalize primary cells with constructs containing a mutation in one of the motifs (p16.91), but all failed to produce immortalized cultures (Table 2). The cells used in these experiments were immortalized by either the wild-type or other mutated constructs, indicating that the source of cells was not the reason for the lack of immortalization. This mutation, at amino acid 91, has previously been shown by us (8) and others (30) to greatly reduce the ability of HPV 16 E7 transcribed by the mouse Moloney leukemia virus long terminal repeat to cooperate with pEJ*ras* and transform primary rodent cells. A number of explanations could account for the biological affect of a mutation in cysteine 91. First, a change in the motif could result in an unstable protein. Our unpublished data show that the E7 protein containing the mutation at amino acid 91 is detectable by immunofluorescence in NIH 3T3 cells transfected by E7 located downstream of the mouse Moloney leukemia virus long terminal repeat, and others have shown that it is capable of binding pRB (33). Second and not mutually exclusive is the possibility that E7 is active as a complex dependent on Cys-X-X-Cys motifs rather than in a monomeric form. For instance, it has been shown that SV40 LT needs to oligomerize to interact with pRB (18). Third, the cysteine motifs may be important for the binding of other cellular factors as yet undescribed. It has been suggested that LT of SV40 needs to bind cellular factors other than pRB for immortalization and transformation of rodent cells (25). HPV 16 E6 binds to cellular p53 (36), another negative regulator of the cell cycle, and speeds its degradation through the ubiquitin pathway (27). Since E6 and E7 are both required for efficient immortalization of human keratinocytes (15, 22), it is possible either that E6/p53 binding plays a major role in immortalization or that E7 affects other cellular processes. These possibilities for HPV 16 E6 and E7 are being investigated.

In summary, mutations in HPV 16 E7 which eliminate binding to pRB do not alter the ability of the constructs to immortalize primary keratinocytes but do eliminate their ability to transform REFs in cooperation with pEJ*ras*. A mutation in one of the Cys-X-X-Cys motifs in the carboxy half of the protein abrogates its immortalization function. The fact that all of these mutations are in the context of the complete genome indicates the importance of E7 in this process but suggests that the interaction with pRB is not essential. As such, dissociation of pRB binding to E7 from its immortalization function presents a unique opportunity to further study how HPV 16 is involved in the initial stages of cervical cancer.

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