JOURNAL OF VIROLOGY, Nov. 1996, p. 7535–7539 Vol. 70, No. 11
0022-538X/96/$04.00+0

Human Papillomavirus Type 11 and 16 E5 Represses $p21^{WafI/SdiI/CipI}$ Gene Expression in Fibroblasts and Keratinocytes

YEOU-PING TSAO, LONG-YUAN LI, TZUNG-CHIEH TSAI, AND SHOW-LI CHEN*

Department of Microbiology and Immunology, National Defense Medical Center, Taipei, Taiwan, Republic of China

Received 12 April 1996/Accepted 29 July 1996

Here we report that the E5 proteins of human papillomavirus type 11 (HPV-11) and HPV-16 suppressed the expression of the $p21^{WafI/SdiI/CipI}$ tumor suppressor gene in NIH 3T3 cells and immortalized human keratinocytes. The promoter activity of p21 was repressed by E5 of HPV-11 and -16, suggesting that p21 gene suppression by E5 was at the transcriptional level. Using an inducible system, we demonstrated that increased induction of HPV-11 E5 in NIH 3T3 cells and keratinocytes led to increased repression of p21 promoter activity. The repression of p21 promoter activity by a series of E5 mutants was somewhat correlated with their respective transforming activities. Previously, we and other investigators showed that the E5 proteins of HPV-11 and -16 can activate the expression of c-*jun*. The repression of p21 gene expression might be a mechanism of oncogene-mediated growth promotion, since the expression of c-*jun* also led to a reduction of the levels of p21 RNA and protein in keratinocytes. This is the first demonstration that E5 proteins of HPV-11 and -16 repress p21 gene expression, and this might be one of the mechanisms by which E5 stimulates cell proliferation. In addition, this is also the first report of c-*jun* repression of p21.

In mammalian cells, progression through the G_1 phase of the cell cycle is dependent on the sequential activation and inactivation of a series of protein kinase complexes (for a review, see reference 24). These complexes contain a catalytic unit, a cyclin-dependent kinase (CDK), and a regulatory subunit known as a cyclin; these include CDC2, CDK2, CDK4, and CDK5 and cyclins A, B, D, E (13). Association of CDKs with cyclins, as typified by cyclin D-CDK4 and cyclin E-CDK2 complexes, is required for the activation of CDKs. In addition to positive regulation by cyclins, CDK activity is also downregulated by association with inhibitory proteins, such as $p21^{WafI/SdiI/CipI}$, p53, p16, p15, and p27 (for a review, see reference 24). The protein p21 is encoded by a recently cloned gene (Sdi) which is overexpressed in senescent fibroblast (21, 28). Another group also showed that the same p21 protein encoded by the CipI gene can associate with different CDK-cyclin complexes and inhibit the kinase activity that is required for cell cycle progression (13, 30, 31). Independent research has shown that p53 causes induction of the WafI gene, a gene later found to be identical to the p21 gene (12). p21 can mediate the cell proliferation arrest induced by p53 (11). The p21 protein binds to and inhibits the activity of CDKs, preventing phosphorylation of critical CDK substrates and blocking cell cycle progression (13, 30, 31). A recent report has shown that p21 is preferentially expressed in senescent cells and can block growing cells from entering the S phase when transiently overexpressed (21).

The E5 genes of human papillomavirus type 11 (HPV-11) and HPV-16 have been shown to be transforming oncogenes (3, 4, 19, 20, 22, 26, 29). The protein E5 is a highly hydrophobic protein and is found in the membrane compartment of cells (9). Recent reports have shown that the E5 protein of HPV-6 can bind to epidermal growth factor and erbB2 receptors (9) and that the E5 protein of HPV-16 can increase the half-life of the epidermal growth factor receptor (25). Epidermal growth factor receptors, along with other tyrosine kinase growth factor receptors, initiate diverse biochemical events that ultimately result in the transcription of a variety of proto-oncogenes, including c-*jun* and c-*fos*. Recent reports have shown that the expression of c-*jun*, *junB*, and c-*fos* is induced by the E5 proteins of HPV-11 and -16 (1, 3, 7), supporting E5 potentiation of the signaling pathways downstream from growth factor receptors. However, since c-*jun* antisense DNA treatment can only partially suppress the transformation function of HPV-11 E5 (6), we suspect the existence of other mechanisms for E5 transformation of cells.

We previously showed that HPV-11 E5 can partially complement the function of a temperature-sensitive T antigen at the nonpermissive temperature in primary human keratinocytes containing a temperature-sensitive simian virus 40 (SV40) T antigen (29). Repression of p21 gene expression occurs in HPV-11 E5-transformed cells (29). There does not appear to be any information regarding possible interactions between E5 and tumor suppressor genes. It is important to determine whether the p21 concentration can be affected by E5 in other cell types. An even more interesting question is whether the effect of E5 on p21 protein concentration is one of the mechanisms by which E5 promotes cell transformation. In this work, we addressed these questions by measuring the expression of p21 in response to E5 of HPV-11 and -16. We also studied the correlation between the transformation activity and p21 suppression effect of HPV-11 E5 by using a series of E5 mutants (5).

MATERIALS AND METHODS

DNAs and plasmids. The pC11E5 and pC16E5 plasmids are pCEP4 (Invitrogen, San Diego, Calif.)-based plasmids which contain an HPV-11 E5 and an HPV-16 E5 open reading frame (ORF), respectively, as well as the nucleotide sequence encoding the T7 epitope, which is cloned in frame to the 5′ end of each E5 ORF (5, 29). The T7 epitope tag is an 11-amino-acid sequence from the natural amino-terminal end of the T7 major capsid protein, against which a highly reactive monoclonal antibody (Novagen, Madison, Wis.) has been raised.

* Corresponding author. Mailing address: Department of Microbiology and Immunology, National Defense Medical Center, Taipei, Taiwan, Republic of China. Phone: 886-2-3683465. Fax: 886-2-3686028.

The plasmid pC-jun was constructed by transferring the 2,604-bp *Eco*RI fragment derived from JAC-1 (23) into the *Eco*RI site of pCEP4 (Invitrogen). The orientation of this insert was determined by dideoxynucleotide sequencing. The plasmids pC11E5, pC16E5, and pC-jun all carry a hygromycin resistance marker and utilize the human cytomegalovirus immediate-early gene promoter to direct HPV-11 E5, HPV-16 E5, or c-*jun* gene expression.

HPV-11 E5 site-specific mutants were constructed by using PCR and single-mutant primers (2). The plasmid pC11E5aQ was made by changing cysteine at position 73 to serine, pC11E5aC was made by changing cysteine at position 75 to serine, and pC11E5aY was made by changing cysteines at both positions 73 and 75 to serines (5). A deletion mutant, pC11E5aT, containing only the first 34 amino acids was also constructed. All of the mutant fragments of the HPV-11 E5 gene were cloned into the eucaryotic expression vector pCEP4 (Invitrogen).

Cell culture and transfection. NIH 3T3 cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% bovine calf serum and antibiotics. Z134 cells, which are SV40-immortalized human epidermal keratinocytes (kindly provided by V. Defendi), were maintained in Dulbecco modified Eagle medium supplemented with 10% NuIV serum (Collaborative Research) and antibiotics.

Immunoblots. Cellular proteins were extracted in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. After being boiled for 10 min, about 100 μg of each crude protein lysate was separated by SDS-PAGE, transferred to a nitrocellulose filter, reacted with mouse monoclonal antibodies, and visualized with the enhanced chemiluminescence system (Amersham) by procedures recommended by the manufacturer. An antibody recognizing p21 was purchased from Pharmingen (San Diego, Calif.). A T7 antibody recognizing T7-tagged E5 fusing protein was purchased from Novagen.

Analysis of E5 protein expression. Cell extracts were prepared by rinsing cells twice with phosphate-buffered saline, lysing in 1 ml of radioimmunoprecipitation assay buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS) for 20 min on ice, and spinning out cell debris. An antibody to T7 peptide (Novagen) was added to the extracts, which were then incubated for 2 h at 4°C. After rotation at 4°C for 2 h, 50 μl of a 1:1 suspension of protein A-Sepharose beads (Pharmacia) in 10 mM Tris-HCl (pH 7.4)–165 mM NaCl–10% (wt/vol) bovine serum albumin was added, and the mixture was rotated for 45 min at 4°C. The beads were pelleted and washed five times with cold radioimmunoprecipitation assay buffer plus protease inhibitors and then resuspended in 75 μl of sample buffer with β-mercaptoethanol. Samples were heated to 100°C for 4 min and analyzed by SDS–15% PAGE. Gels were subjected to immunoblot analysis with T7 mouse monoclonal antibodies (1:500 dilution) and visualized with the enhanced chemiluminescence system (Amersham) by procedures recommended by the manufacturer.

p21 promoter activity assay. WWP-Luc is a p21 promoter luciferase reporter construct that was kindly provided by Bert Vogelstein, Johns Hopkins School of Medicine (12). NIH 3T3 cells or Z134 keratinocytes (10^6) were transfected with 3 μg of WWP-Luc by using the calcium phosphate and Lipofectin (GIBCO) methods, respectively. Transfected cells were cultured for 48 h and then lysed with 100 μl of 1× CCLR (Promega) for 10 min at room temperature. After this mixture was spun for 5 s to pellet large debris, 20 μl of supernatant was added to 100 μl of luciferase assay reagent (Promega). As an internal control for the variation in transfection efficiency, 2 μg of pCH110, a plasmid containing the *Escherichia coli lacZ* gene that was used to monitor β-galactosidase activity, was cotransfected with the test plasmids to normalize the luciferase assay. Light emission was detected in a luminometer (BioOrbit, Turku, Finland).

RESULTS

p21 is repressed by both HPV-11 and HPV-16 E5 genes. We previously found that the concentration of the p21 protein was lower in HPV-11 E5-transfected DA518/KT3 cells than that in the parental human keratinocytes immortalized with a temperature-sensitive SV40 T antigen (29). In the present study, we investigated the effect of E5 on the p21 concentration in human keratinocytes. First, we generated E5-containing keratinocytes. The plasmids pC11E5 (containing the HPV-11 E5 ORF with the T7 epitope at its amino terminus) and pC16E5 (containing the HPV-16 E5 ORF with the T7 epitope at its amino terminus) and the pCEP4 vector were separately transfected into keratinocytes (Z134, passage 67) by Lipofectin transfection with selection for resistance to hygromycin (200 μg/ml). After 3 weeks, at least 80 hygromycin-resistant colonies were pooled, and stable cell lines were generated; in general these were analyzed within a few passages after their derivation. Cells expressing epitope-tagged HPV-11 E5 and HPV-16 E5 fusion proteins were named Z11E5 and Z16E5, respectively. Control cells containing the vector only were named ZC. Transforming activity was characterized by anchorage-independent growth. Both Z11E5 and Z16E5 cells, but not ZC cells, exhibited anchorage-independent growth (data not shown). We had previously shown that the T7-tagged HPV-11 E5 fusion protein retains 100% of transforming activity in NIH 3T3 cells (5). Likewise, in this study, both the T7-tagged HPV-11 E5 fusion protein and the HPV-16 E5 fusion protein also retained their transforming activity in immortalized human epidermal keratinocytes. Figure 1 reveals that the concentrations of p21 RNA (Fig. 1B) and protein (Fig. 1A) were decreased in Z11E5 and Z16E5 cells, which could express HPV-11 and -16 T7-tagged E5 fusion proteins, respectively (Fig. 1A). The decreased levels of p21 RNA and protein were also observed in the cells containing wild-type HPV-11 E5 or HPV-16 E5 (data not shown).

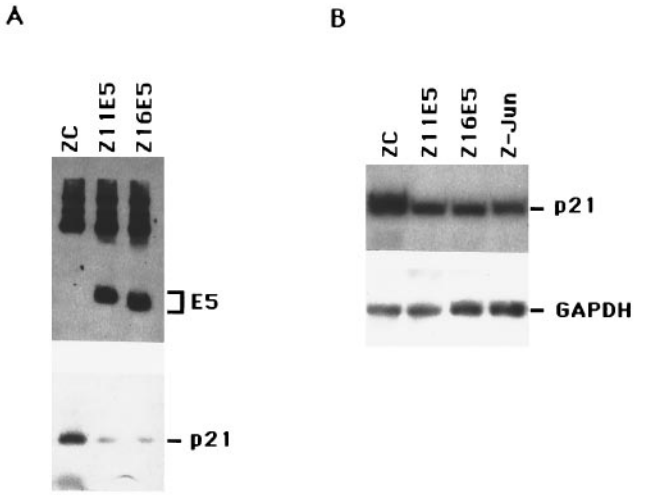

FIG. 1. Concentrations of p21 RNA and protein in HPV-11 and HPV-16 E5-transformed human keratinocytes. (A) E5 proteins of HPV-11 and HPV-16 in immunoprecipitated cell extracts were measured with T7 antiserum (Novagen) and then electrophoresed and immunoblotted with T7 antibody. p21 proteins were assessed by immunoblotting with p21 antiserum (Pharmingen). (B) Analysis of p21 RNA by Northern (RNA) blotting. RNA (20 μg) was fractionated by agarose gel electrophoresis, blotted, and hybridized with ^{32}P-labeled p21 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs separately. A GAPDH probe was used to ensure equal RNA loading. Z11E5, keratinocytes containing the HPV-11 E5 gene; Z16E5, keratinocytes containing the HPV-16 E5 gene; Z-Jun, kerationocytes containing the c-*jun* gene; ZC, keratinocytes containing the vector alone (pCEP4).

The decreased concentrations of p21 RNA and protein in cells containing the E5 gene of HPV-11 or -16 could be due to repression of p21 promoter activity. The promoter activity of p21 gene was therefore investigated by transient-transfection assays. We cotransfected the p21 promoter luciferase reporter plasmid (WWP-Luc) (3 μg) and plasmid pC11E5 (containing HPV-11 E5) (5 μg) or pC16E5 (containing HPV-16 E5) (5 μg) into NIH 3T3 cells and Z134 cells, respectively. Luciferase reporter analysis shows that both HPV-11 E5 and HPV-16 E5 repressed p21 promoter activity in NIH 3T3 cells and keratinocytes (Fig. 2).

p21 can be suppressed by HPV-11 E5 in an inducible system. We previously demonstrated that NE5a1 cells, which are NIH 3T3 fibroblasts containing the HPV-11 E5 gene, have a transformed morphology, exhibit anchorage-independent growth, and are tumorigenic in nude mice. In addition, KE5a cells, which are Z134 keratinocytes that contain the HPV-11 E5 gene, exhibit anchorage-independent growth but are not tumorigenic in nude mice (26). NE5a1 and KE5a cells contain the HPV-11 E5 gene under the control of the mouse metallothionein promoter, with which expression of the E5 gene can

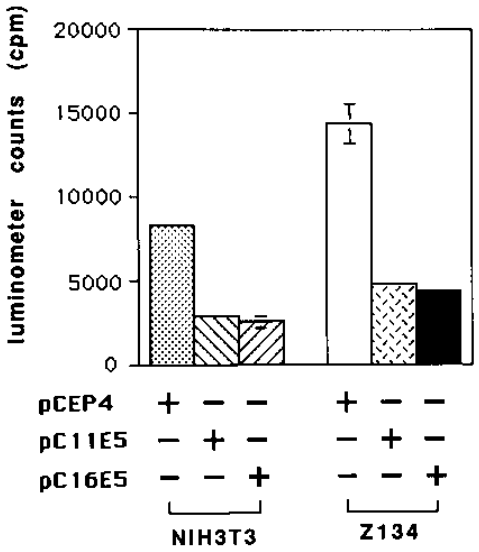

FIG. 2. p21 promoter activity is repressed by HPV-11 and -16 E5 transfection in NIH 3T3 cells and keratinocytes. A luciferase reporter plasmid (3 μg) under the control of the p21 promoter was transiently cotransfected with an HPV-11 E5 (pC11E5) (5 μg) or HPV-16 E5 (pC16E5) (5 μg) expression vector and an internal control plasmid (pCH110) (2 μg) in NIH 3T3 cells or Z134 keratinocytes, respectively. Cell extracts were then prepared, and the luciferase activity assay was performed as described in Materials and Methods. Cell extracts derived from three repeats of experiments were assayed for luciferase activity.

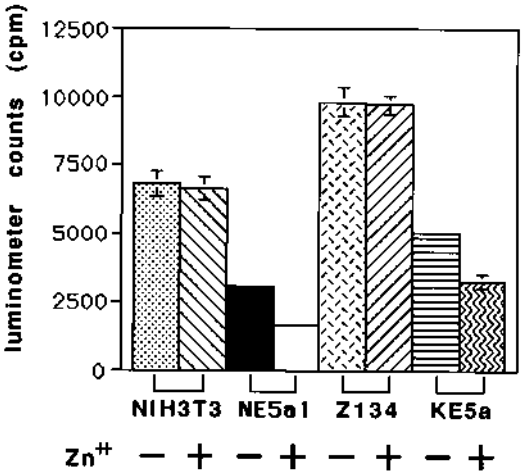

FIG. 3. Repression of p21 promoter activity by HPV-11 E5 in an inducible system. NE5a1 cells and KE5a keratinocytes are NIH 3T3 cells and Z134 keratinocytes, respectively, containing an HPV-11 E5 gene which is regulated by the mouse metallothionein promoter. A luciferase reporter plasmid (3 μg) and internal control plasmid pCH110 (2 μg) were transfected into NIH 3T3 cells, NE5a1 cells, Z134 keratinocytes, or KE5a keratinocytes with or without treatment with 100 μM zinc chloride. Luciferase activities from three independent transfection experiments were measured as described for Fig. 2.

be induced by zinc treatment (4, 7). In this system, although a substantial level of E5 gene expression was driven by the metallothionein promoter in NE5a1 and KE5a cells, exposure of these cells to zinc led to further augmentation of E5 expression (7). Using this zinc-inducible system, we have shown that the induction of c-*jun* gene expression, which is one of the biological effects of E5, is proportional to the concentration of E5 (7). To establish the relationship between E5 and p21 gene expression, we also employed this inducible system and assayed the activity of the p21 promoter. WWP-Luc DNA (3 μg) was transfected into NE5a1 and KE5a cells; 40 h later, the cells were exposed to 100 μM zinc chloride for 3 h, and p21 promoter activity was then determined by the luciferase assay. Although baseline expression of HPV-11 E5, driven by basal metallothionein promoter activity, somewhat suppressed p21 promoter activity in NE5a1 and KE5a cells, zinc induction of E5 gene expression led to further suppression of p21 promoter activity (Fig. 3). In addition, p21 promoter activity was not affected by zinc treatment in the control cells. These observations provide convincing evidence that the expression of E5 correlates with the repression of p21 promoter activity.

E5 transforming activity is related to the repression of p21 gene expression. To further establish the repression of p21 gene expression by E5, we investigated the effects of several HPV-11 E5 mutants on p21 promoter activity by cotransfection of the HPV-11 E5 mutant (5 μg) and p21 promoter constructs (WWP-Luc) (3 μg). As shown in Fig. 4, mutant T, an HPV-11 E5 deletion mutant, completely lacks transformation activity (5) and could not repress p21 promoter activity. Point mutants of HPV-11 E5 (such as mutants Q, Y, and C) preserve their transformation activities to different degrees (5). Cotransfection with p21 promoter constructs reveals that the repression of p21 expression by these three E5 mutants appeared to be somewhat correlated to their respective transformation activities. This result implies that the repression of p21 promoter activity may be involved in the transformation mechanism of E5.

c-*jun* gene expression reduces the intracellular concentration of p21. The E5 genes of HPV-11 and -16 have been shown to activate c-*jun* gene expression (1, 3, 7). The activation of c-*jun* expression has been proposed to be involved in the cell transformation induced by E5 (7). Since E5 was found to repress p21 gene expression, we investigated the relationship between c-*jun* and p21 gene expression. We generated cell lines overexpressing c-*jun* (named Z-Jun) by transfecting Z134

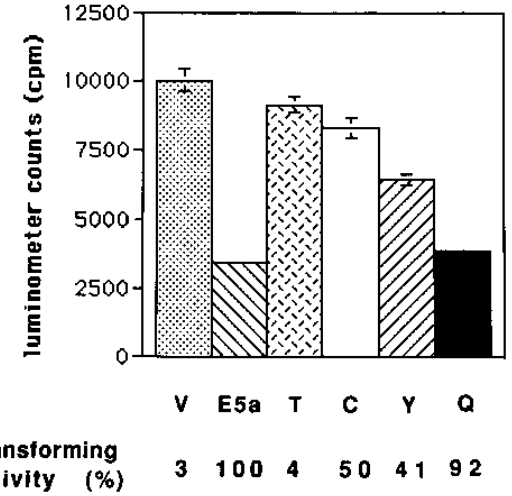

FIG. 4. Repression of p21 promoter activity by HPV-11 E5 mutants. A luciferase reporter plasmid (3 μg) was transiently cotransfected with wild-type HPV-11 E5 or E5 mutants and internal control plasmid pCH110 (2 μg) into NIH 3T3 cells. E5a, wild-type HPV-11 E5; V, vector; T, deletion mutant containing the first 34 amino acids of E5; Q, point mutant with a substitution of Ser for Cys-73; C, point mutant with a substitution of Ser for Cys-75; Y, mutant with substitutions of Ser for both Cys-73 and -75. Transforming activities of E5 and the E5 mutants are shown at the bottom. Luciferase activities were measured as described for Fig. 2.

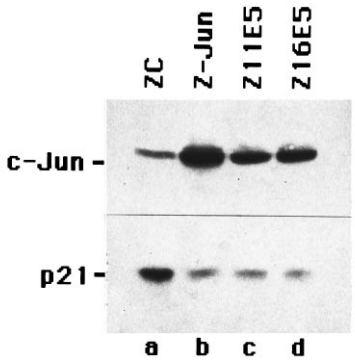

FIG. 5. c-*jun* gene expression reduces the intracellular p21 protein concentration as determined by immunoblot analysis. Cellular proteins were extracted and subjected to SDS-PAGE and Western blotting with either monoclonal anti-p21 serum (bottom) or polyclonal anti c-Jun serum (Oncogene Science) (top). ZC, keratinocytes containing vector only; Z-Jun, keratinocytes containing the c-*jun* gene; Z11E5, keratinocytes containing the HPV-11 E5 gene; Z16E5, keratinocytes containing the HPV-16 E5 gene.

cells with pC-jun plasmid DNA. After 3 weeks, at least 80 hygromycin-resistant clones were pooled and assayed for p21 protein concentration. As shown in Fig. 5, Z-Jun cells (lane b), like Z11E5 (lane c) and Z16E5 (lane d) cells, were found to possess significantly lower levels of p21 protein and higher levels of c-Jun protein than the control ZC cells (lane a). Similarly, the concentration of p21 RNA was also decreased in Z-Jun cells (Fig. 1B). To further establish the relationship between c-*jun* and p21 expression, the effect of c-*jun* on p21 promoter activity was assayed by cotransfecting Z134 cells with 5 μg of pC-jun plasmid DNA and 3 μg of WWP-Luc plasmid DNA. The results indicate that c-*jun* expression led to a significant reduction in p21 promoter activity (Fig. 6). These results show that c-*jun* gene expression can lead to repression of p21 gene expression, and they suggest that HPV-11 and -16 E5 may repress the expression of p21 by inducing c-*jun* gene expression.

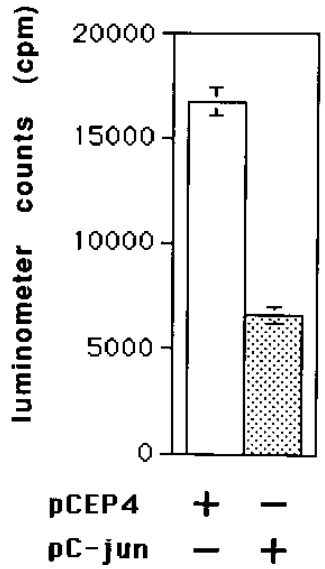

FIG. 6. Repression of p21 promoter activity by c-Jun. A luciferase reporter plasmid (3 μg) was transiently cotransfected with a c-*jun* expression vector (pC-jun) (5 μg) or a vector without an insert (pCEP4) (5 μg) into keratinocytes.

DISCUSSION

In this work, we show that the E5 proteins of HPV-11 and -16 repressed the expression of the p21 tumor suppressor gene. p21 gene repression by E5 appeared to be at the transcriptional level, since the promoter activity of p21 was repressed by E5 (Fig. 2). Using an inducible expression system, we observed that induction of the E5 gene of HPV-11 in both NIH 3T3 cells and immortalized keratinocytes led to further repression of p21 promoter activity (Fig. 3). This demonstrates that the expression of E5 correlates with the repression of the p21 promoter activity. In addition, the repression of p21 promoter activity by HPV-11 E5 was somewhat correlated with the transforming effects of E5; HPV-11 E5 mutant T, which lost transforming activity, was unable to repress p21 promoter activity. However, there is no significant correlation between the transforming activity of E5 mutant C and its repression of p21 promoter activity. This can be explained by the existence of a transformation mechanism other than repression of the p21 gene. For example, we and other investigators previously showed that E5 of HPV-11 and HPV-16 can constitutively activate c-*jun* and c-*fos* gene expression (1, 3, 7), which may be involved in transforming activity in cells expressing E5.

The biological significance of p21 gene repression by HPV-11 and -16 E5 is unclear. There are three oncogenes, E5, E6, and E7, encoded by HPV (14, 15, 32). HPV-16 E6 and E7 can immortalize and transform primary cells, whereas E5 can transform established cells (28). The mechanisms of immortalization by HPV-16 E6 and E7 involve E6-mediated degradation of p53 and E7-mediated inactivation of the pRb protein (15, 32). Since p21 has been identified as an effector of the p53-mediated suppression of cell proliferation, E6, by degrading p53, may also prevent the activation and function of p21. The Z134 cells used in this work are SV40-immortalized human epidermal keratinocytes (26), and the p53 protein should be inactivated by the association of p53 with SV40 T antigen. It is unlikely that the HPV E5 protein regulates the expression of p21 through the function of p53. Moreover, there is evidence that p21 may be activated by a p53-independent pathway (18). This indicates that E6 may not be able to completely abolish p21 synthesis through the inactivation of p53 under certain conditions. The repression of p21 gene expression by HPV-11 and -16 E5 that we observed suggests that E5 may augment the effect of E6 by further reducing p21 expression.

The observation of E5 repressing the p21 gene also suggests a role of E5 in supplementing the function of E7 in the immortalization of human genital keratinocytes. HPV-16 E7 has been shown to bind and inactivate pRb (15), which is a negative regulator of cell division. This finding has led to the suggestion that E7-pRb interaction may be important for the immortalization of human keratinocytes. However, 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 keratinocytes (17). Moreover, intradermal injection of the DNA of cottontail rabbit papillomavirus, which has a natural history of infection very similar to that of HPV-16, with mutations in E7 gene sequences critical for the binding of pRb still induces papillomas in rabbits (10). Taken together, the repression of the p21 gene by E5 that we observed suggests a potential role of E5 in complementing the mutation of the pRb binding activity of E7 in the above-described situations. The repression of the p21 gene by E5 may result in more active CDK4-cyclin D complexes, which are known to phosphorylate pRb and thereby inactivate it. Hence, whether E5 might supplement the immortalization efficiency of E6 and E7 remains to be determined.

Studies of clinical specimens have indicated that HPV-11 causes benign tumors, whereas HPV-16 is an etiological agent of malignant tumors (32). We and other investigators have shown that the E5 proteins of HPV-11 and -16 act similarly to induce the expression of the cellular oncogenes c-*jun*, *junB*, and c-*fos* (1, 3, 7). In the current study, we also show that the E5 proteins of both HPV-11 and -16 repressed the expression of the p21 tumor suppressor gene. These results provide mechanistic explanations for the similar in vitro transforming capabilities of HPV-11 and HPV-16 E5. The difference in the pathogeneses of HPV-16 and HPV-11 seems to depend on factors other than HPV-11 and HPV-16 E5. For instance, it was reported that the E6 and E7 proteins of HPV-11 have lower binding affinities to p53 and pRb than those of HPV-16 (15).

As addressed in the introduction, E5 of HPV-11 and -16 can promote cell growth by activating growth factor receptor signaling pathways (9, 16). These pathways eventually lead to the activation of cellular transcription factors such as c-*jun* and c-*fos*, which may in turn activate genes that are essential for cell growth. Interestingly, the cell proliferation and cell cycle progression of normal cells are also tightly regulated through the activity of tumor suppressor genes. However, the interaction between growth factor receptor signaling and tumor suppressors during the course of transformation is unclear. We previously showed that the E5 proteins of HPV-11 and -16 can activate the expression of c-*jun* through growth factor receptor signaling pathways (27). The repression of p21 by c-*jun* observed in the present work indicates that cellular oncogenes can also affect the expression of tumor suppressor genes. This provides an example of the interaction between an oncogene and a tumor suppressor gene and provides clues on how growth factor receptors and oncogene products counteract the function of tumor suppressor genes to control cell cycle progression.

In summary, this is the first demonstration of the repression of the tumor suppressor gene p21 by the E5 proteins of HPV-11 and -16. This might be a mechanism by which E5 stimulates cell proliferation during the transformation of established cells. In addition, this is also the first report of c-*jun* repression of p21.

ACKNOWLEDGMENTS

We thank Bert Vogelstein for providing WWP-Luc DNA and V. Defendi for providing Z134 keratinocytes. We are also grateful to Steve Roffler for editing the English in the manuscript.

This work was supported by National Science Council grants NSC 85-2331-B-016-024 MH and NSC 85-2331-B-016-090 M22.

REFERENCES

1. **Bouvard, V., G. Matlashewski, Z. M. Gu, A. Storey, and L. Banks.** 1994. The human papillomavirus type 16 E5 gene cooperates with the E7 gene to stimulate proliferation of primary cells and increases viral gene expression. Virology **203:**73–80.
2. **Chen, S. L., C. P. Han, and Y. P. Tsao.** 1993. Construction of site-specific mutants of E5a protein of HPV-11 by polymerase chain reaction and a single mutant primer. J. Virol. Methods **42:**89–94.
3. **Chen, S. L., C. H. Huang, T. C. Tsai, K. Y. Lu, and Y. P. Tsao.** 1996. The regulation mechanism of c-jun and junB by human papillomavirus type 16 E5 oncoprotein. Arch. Virol. **141:**791–800.
4. **Chen, S. L., and P. Mounts.** 1990. Transforming activity of E5a protein of human papillomavirus type 6 in NIH 3T3 cells and C127 cells. J. Virol. **64:**3226–3233.
5. **Chen, S. L., T. C. Tsai, C. P. Han, and Y. P. Tsao.** 1996. Mutational analysis of human papillomavirus type 11 E5a oncoprotein. J. Virol. **70:**3502–3508.
6. **Chen, S. L., L. T. Tsao, and Y. P. Tsao.** 1994. Antisense oligodeoxynucleotides to c-jun inhibits proliferation of transformed NIH 3T3 cells induced by E5a of HPV-11. Cancer Lett. **85:**119–123.
7. **Chen, S. L., Y. P. Tsao, C. M. Yang, Y. K. Lin, C. H. Huang, and S. W. Kuo.** 1995. Differential induction and regulation of c-jun, junB, junD and c-fos by human papillomavirus type 11 E5a oncoprotein. J. Gen. Virol. **76:**2653–2659.
8. **Conrad, M., V. J. Bubb, and R. Schlegel.** 1993. The human papillomavirus type 6 and 16 E5 proteins are membrane-associated proteins which associate with the 16-kilodalton pore-forming protein. J. Virol. **67:**6170–6178.
9. **Conrad, M., D. Goldstein, T. Andresson, and R. Schlegel.** 1994. The E5 protein of HPV-6, but not HPV-16, associates efficiently with cellular growth factor receptors. Virology **200:**796–800.
10. **Defeo-Jones, D., G. A. Vuocolo, K. M. Haskell, M. G. Hanobik, D. M. Kiefer, E. M. McAvoy, M. Ivey-Hoyle, J. L. Brandsma, A. Oliff, and R. E. Jones.** 1993. Papillomavirus E7 protein binding to the retinoblastoma protein is not required for viral induction of warts. J. Virol. **67:**716–725.
11. **El-Deiry, W. S., J. W. Harper, P. M. O'Connor, V. E. Velculescu, C. E. Canman, J. Jackman, J. A. Pietenpol, M. Burrell, D. E. Hill, and Y. Wang.** 1994. WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis Cancer Res. **54:**1169–1174.
12. **El-Deiry, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler, and B. Vogelstein.** 1993. WAF1, a potential mediator of p53 tumor suppression. Cell **75:**817–825.
13. **Harper, J. W., G. R. Adami, N. Wei, K. Keyomarsi, and S. J. Elledge.** 1993. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell **75:**805–816.
14. **Hawley-Nelson, P., K. H. Vousden, N. L. Hubbert, D. R. Lowy, and J. Schiller.** 1989. HPV 16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. EMBO J. **8:**3905–3910.
15. **Howley, P. M.** 1991. Role of the human papillomaviruses in human cancer. Cancer Res. **51**(Suppl)**:**5019s–5022s.
16. **Hwang, E. S., T. Nottoli, and D. DiMaio.** 1995. The HPV-16 E5 protein: expression, detection, and stable complex formation with transmembrane proteins in COS cells. Virology **211:**227–233.
17. **Jewers, R. J., P. Hildebrandt, J. W. Ludlow, B. Kell, and D. J. McCance.** 1992. Regions of human papillomavirus type 16 E7 oncoprotein required for immortalization of human keratinocytes. J. Virol. **66:**1329–1335.
18. **Jiang, H., J. Lin, S. Zao-Zhong, F. R. Collart, E. Huberman, and P. B. Fisher.** 1994. Induction of differentiation in human promyelocytic HL60 leukemia cells activates p21, WAF1/CIP1, expression in the abscence of p53. Oncogene **9:**3397–3406.
19. **Leechanachai, P., L. Banks, F. Moreau, and G. Matlashewski.** 1992. The E5 gene from human papillomavirus type 16 is an oncogene which enhances growth factor-mediated signal transduction to the nucleus. Oncogene **7:**19–25.
20. **Leptak, C., Y. Ramon, S. Cajal, R. Kulke, B. H. Horwitz, D. J. Riese II, G. P. Dotto, and D. DiMaio.** 1991. Tumorigenic transformation of murine keratinocytes by the E5 genes of bovine papillomavirus type 1 and human papillomavirus type 16. J. Virol. **65:**7078–7083.
21. **Noda, A., Y. Ning, S. F. Venable, S. O. Pereira, and J. R. Smith.** 1994. Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. Exp. Cell Res. **211:**90–98.
22. **Pim, D., M. Collins, and L. Banks.** 1992. Human papillomavirus type 16 E5 gene stimulates the transforming activity of the epidermal growth factor receptor. Oncogene **7:**27–32.
23. **Ryder, K., and D. Nathans.** 1988. Induction of protooncogene c-jun by serum growth-factors. Proc. Natl. Acad. Sci. USA **85:**8464–8467.
24. **Sherr, C.** 1994. GI phase progression: cycling on cell. Cell **79:**551–555.
25. **Straight, S. M., P. M. Hinkle, R. J. Jewers, and D. J. McCance.** 1993. The E5 oncoprotein of human papillomavirus type 16 transforms fibroblasts and effects the downregulation of the epidermal growth factor receptor in keratinocytes. J. Virol. **67:**4521–4532.
26. **Tsao, Y. P., T. Y. Chu, T. M. Chen, Y. F. Yang, and S. L. Chen.** 1994. Effects of E5a and E7 of human papillomavirus type 11 on immortalized human epidermal keratinocytes and NIH 3T3 cells. Arch. Virol. **138:**177–185.
27. **Tsao, Y. P., C. H. Huang, Y. K. Lin, and S. L. Chen.** 1995. Protein kinase C- and ras-dependent activation of c-jun gene by human papillomavirus type 11 E5a oncoprotein. Cancer Lett. **95:**201–205.
28. **Tsao, Y. P., S. W. Kuo, S. F. Li, J. C. Liu, S. Z. Lin, K. Y. Chen, and S. L. Chen.** 1995. Differential regulation of cyclin A, cyclin B, and p21 concentrations in a growth-restricted human fibroblast cell line. Biochem. J. **312:**693–698.
29. **Tsao, Y. P., S. W. Kuo, S. F. Li, T. C. Tsai, L. Y. Li, and S. L. Chen.** 1995. Human papillomavirus 11 E5a delays the growth restriction induced by temperature shift in temperature sensitive simian virus 40 T antigen immortalized keratinocytes. Biochem. Biophys. Res. Commun. **217:**712–720.
30. **Xiong, Y., G. J. Hannon, H. Zhang, D. Casso, R. Kobayashi, and D. Beach.** 1993. p21 is a universal inhibitor of cyclin kinases. Nature (London) **366:**701–704.
31. **Xiong, Y., H. Zhang, and D. Beach.** 1993. Subunit rearrangement of the cyclin-dependent kinases is associated with cellular transformation. Genes Dev. **7:**1572–1583.
32. **zur Hausen, H.** 1991. Human papillomaviruses in the pathogenesis of anogenital cancer. Virology **184:**9–13.