Cell-Restricted Immortalization by Human Papillomavirus Correlates with Telomerase Activation and Engagement of the hTERT Promoter by Myc

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The high-risk human papillomaviruses (HPVs; e.g., HPV type 16 [HPV-16] and HPV-18) are associated with anogenital carcinomas (65, 66), laryngeal carcinomas, and head and neck carcinomas (15). These HPVs carry two oncogenes, E6 and E7, which are retained and expressed in HPV-positive cervical cancers (1, 2, 50) and are required for maintenance of the tumorigenic phenotype (35, 36). The E6 and E7 proteins were first identified as targeting the p53 and Rb tumor suppressor pathways in host cells (8, 9, 35, 36, 47, 48), thereby disrupting cell cycle controls. Telomerase is a specialized reverse transcriptase that synthesizes repeat DNA sequences at the ends of chromosomes termed telomeres (17). The absence of telomerase activity in most normal human cells results in the progressive shortening of telomeres with each cell division (19, 56, 60), eventually leading to growth arrest or replicative senescence (6, 19). In contrast to most human somatic cells, immortalized and cancer cells contain detectable telomerase activity and consequently maintain their telomere length and proliferative potential (18, 25, 52, 63).

The high-risk papillomaviruses (HPVs; e.g., HPV type 16 [HPV-16] and HPV-18) are etiologically linked to additional human cancers, including those of anal, oral, and laryngeal origin. The main transforming genes of the high-risk HPVs are E6 and E7. E6, in addition to its role in p53 degradation, induces hTERT mRNA transcription in genital keratinocytes via interactions with Myc protein, thereby increasing cellular telomerase activity. While the HPV type 16 E6 and E7 genes efficiently immortalize human keratinocytes, they appear to only prolong the life span of human fibroblasts. To examine the molecular basis for this cell-type dependency, we examined the correlation between the ability of E6 to transactivate endogenous and exogenous hTERT promoters and to immortalize genital keratinocytes and fibroblasts. Confirming earlier studies, the E6 and E7 genes were incapable of immortalizing human fibroblasts but did delay senescence. Despite the lack of immortalization, E6 was functional in the fibroblasts, mediating p53 degradation and strongly transactivating an exogenous hTERT promoter. However, E6 failed to transactivate the endogenous hTERT promoter. Coordinately with this failure, we observed that Myc protein was not associated with the endogenous hTERT promoter, most likely due to the extremely low level of Myc expression in these cells and/or to differences in chromatin structure, in contrast with hTERT promoters that we found to be activated by E6 (i.e., the endogenous hTERT promoter in primary keratinocytes and the exogenous hTERT core promoter in fibroblasts), where Myc is associated with the promoter in either a quiescent or an E6-induced state. These findings are consistent with those of our previous studies on mutagenesis and the knockdown of small interfering RNA, which demonstrated a requirement for Myc in the induction of the hTERT promoter by E6 and suggested that occupancy of the promoter by Myc determines the responsiveness of E6 and the downstream induction of telomerase and cell immortalization.

Our previous studies and those of other laboratories have shown that E6-mediated hTERT transactivation is independent of p53 degradation and interactions with PDZ proteins (12, 14, 22, 26, 30). However, as demonstrated in studies of small interfering RNA (siRNA) knockdown, hTERT transactivation by E6 requires the cellular ubiquitin ligase E6AP as well as Myc (12, 14, 30, 58). We have also shown that E6 and Myc associate in vivo and bind coordinately with promoter activation to the hTERT promoter in primary human foreskin keratinocytes (HFKs) (58).

The HPV-16 E6 oncoprotein increases cellular telomerase activity (27, 54), predominantly by inducing transcription of the hTERT gene (13, 32, 42, 57). The hTERT protein is the catalytic, rate-limiting subunit of the telomerase enzyme complex and is selectively expressed in a small subset of normal cells (stem cells), tumor tissues, and tumor-derived cell lines (33, 40, 45, 55). Interestingly, overexpression of hTERT protein or an hTERT promoter transactivator (Myc) can substitute for E6 in the immortalization of primary HFKs (26, 28), indicating that telomerase activation constitutes a major immortalizing activity of E6.

To further explore the relationship between E6, Myc, telomerase, and cell immortalization, we transduced primary HFKs and human foreskin fibroblasts (HFFs) with E6, E7, or both E6 and E7. Although the E6 and E7 genes can immortalize human foreskin keratinocytes (20, 37), they (HPV-16...
DNA fail to immortalize HFFs (43). The intent of this study was to determine whether genital keratinocytes and fibroblasts differ in their regulation of telomerase and their response to E6 expression. We found that E6 and E7 were expressed in both keratinocytes and fibroblasts and induced the degradation of p53 and pRb, respectively. In addition, the E6 protein effectively induced an exogenous hTERT promoter in fibroblasts, and both E6 and Myc associated with this promoter. However, E6 could not induce the endogenous hTERT promoter or increase cellular telomerase activity in fibroblasts. This failure to induce the hTERT promoter correlated with a lack of promoter-broad Myc protein, an observation that is compatible with recent findings showing that overexpression of Myc can induce the fibroblast hTERT promoter, activate telomerase, and facilitate immortalization (4). Overall, our data demonstrate that E6 induces the hTERT promoter and activates telomerase, but only in cells in which Myc resides on the hTERT promoter.

MATERIALS AND METHODS

Plasmids and retroviruses. We used the following previously described (57, 58) vectors and plasmids: the pBS55 vector, pBS55-16E6, pBS55-16E7-AU1, pBS55-16E7, the pLXSN vector and pLXSN-16E6, pLXSN-16E7, pLXS58, pLXS58-16E6E7, pGL3-basic (pGL3B), and the pGL3B-hTERT core promoter (previously defined as pGL3B-255). Retrovirus-packaging cells (SD3443 cells) were transfected with a pLXS5 vector containing either E6 or E7 or both E6 and E7, as described above, using Lipofectamine 2000 (Invitrogen) as instructed by the manufacturer (54). Culture supernatants containing retroviruses were collected 24 h after transfection.

Cell cultures and generation of stable cell lines. Primary HFKs and HFFs were cultured from neonatal foreskins as described previously (49) and were maintained in keratinocyte growth media (Invitrogen) supplemented with gentamicin (50 μg/ml) and complete Dulbecco’s modified Eagle’s medium, respectively. Primary HFKs (passage 2) and HFFs (passage 5) were transduced with amphotropic LXSN retroviruses expressing HPV-16 E6, E7, or both E6 and E7 (see above). Retrovirus-transduced cells were selected in G418 (100 μg/ml) for 5 days. Resistant colonies were pooled and passaged every 3 to 4 days (at a 1:4 ratio for HFKs and a 1:8 ratio for HFFs). HeLa cells were maintained in keratinocyte growth media (Invitrogen) supplemented with gentamicin (50 μg/ml) and complete Dulbecco’s modified Eagle’s medium, respectively. HeLa cells were grown to 80 to 90% confluence in 100-mm dishes. Chromatin immunoprecipitation (ChIP) assays were performed as described previously (56). Normal rabbit IgG (Santa Cruz Biotechnology), rabbit anti-Myc polyclonal antibody (N-262; Santa Cruz Biotechnology), and monoclonal anti-AU1 (Covance) were used for immunoprecipitation assays for HFKs, HFFs, and HeLa cells, respectively. PCR products were separated on a 1.8% agarose gel and visualized by ethidium bromide staining.

RESULTS

E6 and E7 disrupt the p53 and pRb pathways in both HFK and HFF cells. The HPV-16 E6 and E7 genes are necessary and sufficient to immortalize primary HFKs and ectocervical keratinocytes (20, 38). While E6 directs the ubiquitin-dependent degradation of p53, it also has functions that are p53 independent, including telomerase activation and cell transformation (12, 35, 36). To determine whether the immortalizing activity of E6 and E7 correlated with tumor suppressor inactivation or telomerase induction, we used retroviruses to generate cell strains expressing an empty retroviral vector (pLXSN), E6 (pLXSN-16E6), E7 (pLXSN-16E7), or E6 and E7 (pLXSN-16E6E7), as described in Materials and Methods. The above cell lines were then passed serially in vitro to assay for immortalization. When cells reached 80% confluence, they were split at a 1:4 ratio for HKFs and a 1:8 ratio for HFFs. Therefore, one split would correspond to two or three cell population doublings (PDs) for HKFs and HFFs, respectively.

As shown in Fig. 1, HKFs and HFFs transduced with LXSN ceased proliferating at 22 to 24 PDs and 61 to 64 PDs, respectively. E6 alone extended the life span of HKFs to 32 to 34 PDs and HFFs to 82 to 85 PDs, and E7 alone extended cell divisions to 36 to 38 PDs and 67 to 70 PDs, respectively. Neither E6 nor E7 could independently immortalize HKFs or HFFs. As anticipated, the combined activity of E6 and E7 allowed HKFs, but not HFFs, to bypass senescence and become immortalized. We explored the possibility that the E6 and E7 proteins might be differentially targeting the p53 and pRb pathways in these two different human cell types or differentially inducing the hTERT promoter and thereby activating telomerase.

First, we confirmed that E6 and E7 were expressed in both HKF and HFF cells. RT-PCR was performed with E6- or
E7-specific primers. As anticipated, E6 mRNA was expressed in HFKs and HFFs transduced with the E6 and E6E7 retroviruses, while E7 mRNA was expressed in the E7- and E6E7-transduced cells. HFKs and HFFs transduced with the pLXSN vector served as the control, and GAPDH was used to normalize gene expression (Fig. 2A). We also performed reactions without reverse transcriptase to confirm that PCR products came from mRNA, not DNA (data not shown).

To determine whether the E6 and E7 proteins were functional in both cell types, we assayed the levels of tumor suppressors p53 and pRb by Western blotting, using β-actin as a loading control (Fig. 2B). Regardless of cell type, the p53 levels were decreased in E6-transduced cells, and the pRb protein levels were reduced in E7-expressing cells, indicating that the HPV oncoproteins were functional in both cell types and also that inactivation of p53 and pRb is insufficient for immortalization of HFFs.

**E6 is sufficient to induce the exogenous hTERT promoter in both HFKs and HFFs.** To determine whether the failure of E6E7 to immortalize HFF cells might be due to the inability of E6 to activate the hTERT promoter, we first analyzed the effect of E6 and E7 on an exogenous hTERT promoter luciferase construct. HFKs and HFFs were transduced with the pLXSN vector serving as the control, and GAPDH was used to normalize gene expression (Fig. 2A). We also performed reactions without reverse transcriptase to confirm that PCR products came from mRNA, not DNA (data not shown).

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**E6 cannot induce endogenous hTERT transcription or telomerase activity in HFFs.** To validate the studies with the exogenous promoter, we performed qRT-PCR with endogenous hTERT mRNA in HFF and HFK cells. Unexpectedly, we found that neither the E6 nor E6E7 genes could induce the hTERT promoter (Fig. 4A). This finding contrasted with the ability of these genes to effectively induce both the endogenous (Fig. 4A) and exogenous hTERT promoters in HFKs (Fig. 3). To further confirm that this mRNA analysis reflected the downstream activation of telomerase, we prepared lysates from the cells and quantified the telomerase activity by using a q-TRAP assay (see Materials and Methods). As anticipated, E6 increased telomerase activity in HFKs (Fig. 4A). However,
Myc resides on the endogenous HFK hTERT promoter but not the HFF promoter. Myc is known to be a direct activator of telomerase in both human keratinocytes and fibroblasts (4, 5, 28, 61), and our previous studies have shown that E6 and Myc physically interact and bind to the hTERT promoter (30, 51, 58). Indeed, the presence of Myc is required for E6-mediated induction of the hTERT promoter. Thus, it was possible that E6 was unable to activate the hTERT promoter because Myc was not present on the hTERT promoter in HFF cells, as it is in noninduced HFF cells.

To test this hypothesis, we performed ChIP assays on both cell types with a Myc antibody. In primary HFKs expressing either the vector or E6, Myc was bound to the endogenous hTERT promoter (Fig. 5A), as reported previously (30, 58). However, we did not observe a signal for Myc binding to the endogenous hTERT promoter in HFFs, even when they expressed E6 (Fig. 5A). Myc was bound to the endogenous hTERT promoter in telomerase-positive HeLa cells (used as a positive control). Therefore, these experiments indicate that Myc is present on the hTERT promoter of telomerase-quiescent HFK cells but not on the promoter of telomerase-quiescent HFF cells. The inaccessibility of Myc to the hTERT promoter in HFF cells could derive from either differences in cellular Myc expression or differences in chromatin structure, which prevent Myc from accessing the promoter. We evaluated Myc expression levels, as discussed in the next section.

*Myc protein is expressed at very low levels in HFF cells.* Our initial experiments employed RT-PCR to detect Myc mRNA expression in the two cell types, using both qualitative and qRT-PCR (Fig. 5B and C). In addition, we assayed the abundance of mRNA for two other transcription factors that modulate Myc activity, Max and Mad (11, 16, 21, 31, 39, 44, 46). In Fig. 5B and C, it is apparent that Myc is expressed at the mRNA level in both HFK and HFF cells. It is also apparent that there are similar levels of Mad and Max mRNA in these cells. It is also important to note that E6, E7, and E6E7 do not alter the expression of Myc or the Myc-related proteins.

However, the critical question is whether the Myc protein levels were different between HFK and HFF cells, which we explored by using Western blotting with a Myc antibody. As shown in Fig. 5C, Myc protein was detectable in HFKs expressing vector, E6, E7, or E6E7, and the viral oncoproteins did not alter the expression of Myc or its partners, Max and Mad. Unlike the Myc mRNA studies, we found gross differences in the levels of Myc protein between HFK and HFF cells. That is, there were very low levels of Myc protein in HFF cells compared to that in HFK cells, and it was extremely difficult to detect Myc protein in fibroblasts without the use of MG-132 proteasome inhibitor to prevent Myc degradation. p53 protein was blotted as a control, since it is degraded by E6, and as expected, MG-132 led to an increased level of p53 protein in E6-expressing cells. Thus, our data suggest that the low levels of Myc protein in HFFs might be due to rapid protein turnover. Interestingly, there is a coordinately low level of the Myc-associated Max protein in HFFs, and we could visualize Max reproducibly only when using a proteasome inhibitor. Unlike Myc and Max, however, the Mad protein is expressed at similar levels in both HFK and HFF cells, and there is little or
no change in the level of this protein with either E6, E7, or E6E7 or in the presence of a proteasome inhibitor. In summary, the low levels of Myc protein detected in HFF cells might be the etiologic basis for its absence on the endogenous hTERT promoter and the nonresponsiveness of this promoter to E6.

**Myc protein expression increases the ability of E6 to engage the endogenous hTERT promoter.** One possibility for the lack of E6’s ability to induce the HFF hTERT promoter is that it cannot associate stably with the promoter without Myc binding, which is clearly lacking in HFF cells. To determine if Myc protein might modulate the association of E6 with the hTERT promoter, we transfected both HFF and HFK cells with epitope-tagged E6 (E6-AU1), which is known to retain its ability to induce the hTERT promoter. ChIP experiments with monoclonal anti-AU1 antibody or an IgG control demon-
strated that E6-AU1 bound to the hTERT promoter in both types of cells (Fig. 6A), suggesting that E6 might associate with other promoter-associated proteins (e.g., NFX1-91), without the activation of telomerase. However, when the PCR signal was normalized to input, it appeared that E6 binding to the HFK promoter was stronger than that to the HFF promoter (Fig. 6A). This moderate, quantitative difference in E6 binding to the HFK and HFF promoters, however, does not seem to explain the complete absence of hTERT induction by E6 in HFF cells. More likely, it is the absence of Myc on the HFF promoter that is responsible for its lack of responsiveness to E6. Most important, while Myc was sufficient to induce telomerase in both HFKs and HFFs by itself (Fig. 6B), the forced expression of Myc in HFFs significantly increased E6 association with the endogenous hTERT promoter (Fig. 6A, right). The E6-activated, exogenous hTERT promoter in HFF cells is associated with Myc. The above data strongly suggest that Myc occupancy on the hTERT promoter correlates with the ability of E6 to induce hTERT transcription. However, as shown in Fig. 3, E6 is sufficient to induce an exogenous hTERT core promoter in HFFs, despite their very low level of Myc protein. This observation provides a unique system to define whether Myc protein levels or the chromatin structure might be the principal determinants of Myc promoter binding. If Myc is associated with the exogenous hTERT promoter in HFF cells, the “open” chromatin conformation of transfected plasmid DNA may be more accessible to low levels of Myc than the endogenous promoter. If Myc is not associated with the exogenous promoter, it indicates not only that Myc levels regulate hTERT promoter association but also that E6 can induce the hTERT promoter without Myc. To test these possibilities, we transfected the hTERT core promoter and E6 into HFFs and performed a ChIP assay with a Myc antibody. Our data showed that Myc could be detected on the exogenous hTERT core promoter (Fig. 6C), although it was not detectable on the endogenous hTERT promoter (Fig. 5A). Thus, it appears that only hTERT promoters associated with Myc protein are inducible by E6. This conclusion, however, is qualified by the possibility that our ChIP assay conditions may not have been sufficiently sensitive to detect very low levels of Myc on the endogenous promoter.
DISCUSSION

The activation of telomerase in epithelial cells by HPV E6 is believed to be critical for cell immortalization (26). Although this function of E6 is independent of p53 degradation and PDZ binding ability, it appears to require E6AP (3, 12, 22, 24, 30). In contrast to the above studies, a single recent report suggests that E6AP is not required for hTERT promoter induction (51). The etiology of these experimental differences is currently unclear. However, independent studies have shown that the specific knockdown of E6AP interferes with hTERT promoter induction by E6 (3, 12, 22, 24, 30).

We, as well as other researchers, have shown that E6 activates hTERT transcription through Myc binding sites in the hTERT promoter (13, 32, 42, 57). However, E6 neither induces Myc expression (Fig. 5C) (12, 13, 42, 51, 57, 58) nor changes Myc binding to the hTERT promoter (12, 51, 58). Myc protein binds to the promoter in the presence or absence of E6 protein in keratinocytes (12, 51, 58). Interestingly, E6 associates with Myc in vivo and in vitro, and both bind to the core hTERT promoter (58), suggesting that there is a functional, cooperative interaction between these proteins on the hTERT promoter. The importance of Myc/hTERT promoter binding has been verified not only by promoter mutagenesis (13, 32, 42, 57, 59, 61) but also by siRNA knockdown of Myc expression and the expression of Mad and Mnt (Myc antagonists), all of which significantly inhibit E6-induced telomerase activity (29, 58; our unpublished data). E6 also increases the acetylation of histones resident on the promoter (12, 22; our unpublished data), suggesting that it might alter local chromatin structure and enhance promoter activity.

In this study, we examined the correlation between the ability of E6 to induce the hTERT promoter and to immortalize two very different cell types, HFKs and HFFs. It is well documented that the efficient immortalization of HFK cells requires the E6 and E7 genes and that these same two genes are incapable of immortalizing HFF cells (7, 26). Our current study provides a potential model to explain this differential cellular immortalization (Fig. 7).

First, our data indicate that the E6 and E7 genes are expressed in both HFK and HFF cells and that two tumor-suppressor proteins, p53 and pRb, are similarly inactivated. When considered in the context of previous publications, our study suggests that p53 inactivation is neither necessary nor sufficient for cell immortalization. In this study, p53 inactivation (along with E7 expression) was not sufficient to induce the immortalization of HFF cells, and in previous studies of HFK cells, E6-mediated degradation of p53 was not required for cell immortalization (26, 28). The critical conclusion, however, is that the differential abilities of E6 and E7 to immortalize HFK and HFF cells do not reside in the differential expression levels/activities of these genes in the corresponding cells.

Second, the difference between HFK and HFF cells actually appears to derive from variations in the association of Myc protein with the hTERT promoter. HFK cells have Myc associated with the endogenous hTERT promoter, whereas HFF cells do not. The etiology of this difference might derive from the low level of Myc protein that we observed in the HFF cells.
(Fig. 5C) and that others have observed in normal human WI-38 fibroblasts (34). Compatible with the proposal that Myc protein levels might regulate hTERT responsiveness is the observation by several laboratories that Myc overexpression is sufficient to induce telomerase in these cells (Fig. 6B) (4, 5). More importantly, transduction of Myc into such fibroblasts is sufficient for cell immortalization (4, 23, 34, 41).

Interestingly, our study indicates that the low level of Myc protein in foreskin fibroblasts derives from posttranscriptional controls. The Myc gene is expressed similarly at the mRNA level in HFF and HFK cells, but the level of Myc protein is dramatically different. The ability to restore higher Myc protein levels in HFF cells with proteasome inhibitors suggests that proteolysis might contribute to the very low levels of Myc protein in HFF cells.

However, the level of Myc protein cannot be the complete explanation for the difference between HFF and HFK responses to E6. While HFF cells have nearly undetectable Myc protein levels, they provide a very suitable environment for the transactivation of an exogenous hTERT promoter by E6. Indeed, the exogenous hTERT promoter is activated to an even greater extent in HFF cells than in HFK cells, which suggests that there might be another mechanism regulating Myc access to the endogenous hTERT promoter, such as chromatin structure. In general, transfected plasmids are not highly decorated with chromatin proteins and exhibit a more “open” conformation than endogenous genes (53). It is therefore possible that the “open” nature of the exogenous hTERT promoter permits access to the lower levels of Myc protein in HFFs and thereby allows the transfected promoter to respond to E6 activation.

It is interesting that we found E6 protein associated with the endogenous hTERT promoter in both HFK and HFF cells, although there may be some quantitative differences (Fig. 6A). If Myc were the sole binding site on the hTERT promoter for E6, we should have found little E6 bound to the endogenous hTERT promoter. While forced expression of Myc does significantly enhance E6 binding, we speculate that E6 might modulate hTERT promoter acetylation is E6AP dependent, increased in later passage of immortal cells (33). It is therefore possible that the “open” nature of the exogenous hTERT promoter permits access to the endogenous hTERT promoter, such as chromatin structure. In general, transfected plasmids are not highly decorated with chromatin proteins and exhibit a more “open” conformation than endogenous genes (53). It is therefore possible that the “open” nature of the exogenous hTERT promoter permits access to the lower levels of Myc protein in HFFs and thereby allows the transfected promoter to respond to E6 activation.

In summary, the differential abilities of the HPV E6 and E7 genes to immortalize HFK and HFF cells correlates with Myc binding to the hTERT promoter, which provides the appropriate promoter environment for E6 responsiveness.

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