Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 2290–2295, March 1998
Cell Biology

Human papillomavirus type 16 *E6* and *E7* oncogenes abrogate radiation-induced DNA damage responses *in vivo* through p53-dependent and p53-independent pathways

SHIYU SONG, GENE A. GULLIVER, AND PAUL F. LAMBERT*

McArdle Laboratory for Cancer Research, University of Wisconsin Medical School, 1400 University Avenue, Madison, WI 53706

Edited by Paul Ahlquist, University of Wisconsin, Madison, WI, and approved December 17, 1997 (received for review August 28, 1997)

ABSTRACT **E6 and E7 oncoproteins from high risk human papillomaviruses (HPVs) transform cells in tissue culture and induce tumors *in vivo*. Both E6, which inhibits p53 functions, and E7, which inhibits pRb, can also abrogate growth arrest induced by DNA-damaging agents in cultured cells. In this study, we have used transgenic mice that express HPV-16 E6 or E7 in the epidermis to determine how these two proteins modulate DNA damage responses *in vivo*. Our results demonstrate that both E6 and E7 abrogate the inhibition of DNA synthesis in the epidermis after treatment with ionizing radiation. Increases in the levels of p53 and p21 proteins after irradiation were suppressed by E6 but not by E7. Through the study of p53-null mice, we found that radiation-induced growth arrest in the epidermis is mediated through both p53-dependent and p53-independent pathways. The abrogation of radiation responses in both E6 and E7 transgenic mice was more complete than was seen in the p53-null epidermis. We conclude that E6 and E7 each have the capacity to modulate p53-dependent as well as p53-independent cellular responses to radiation. Additionally, we found that the conserved region (CR) 1 and CR2 domains in E7 protein, which are involved in the inactivation of pRb function and required for E7's transforming function, were also required for E7 to modulate DNA damage responses *in vivo*. Thus pRb and/or pRb-like proteins likely mediate both p53-dependent and p53-independent responses to radiation.**

Human papillomaviruses (HPVs) are the causative agents of warts. A subset of HPVs, referred to as high risk HPVs, are associated with human anogenital cancers such as cervical cancers in women and penile cancers in men (1). Two genes of these high risk HPVs, *E6* and *E7*, are expressed in the cells derived from HPV-associated cancers (2, 3). *E6* and *E7* cooperate with each other (4, 5) and with other oncogenes (6–9) in the immortalization or transformation of cells. The transforming activities of E6 and E7 correlate, at least in part, with their inactivation of two cellular tumor suppressor gene products, p53 and pRb, which regulate the processes of division, differentiation, and/or death in cells (10–12). E6 binds to p53 (13) and mediates degradation of p53 through the ubiquitin–proteasomal degradation pathway (14). E7 binds to pRb (15), inhibits pRb's function (16), and promotes degradation of pRb (17, 18). *In vivo* experiments using mice transgenic for E6 and E7 have shown that E6 and E7 together can induce tumors in the targeted tissues (19–21). Our laboratory has generated transgenic mice in which the expression of HPV-16 E6 (S.S. and P.F.L. unpublished work) or E7 (22) singly was directed to mouse squamous epithelial cells by the human keratin 14 (K14) promoter. These mice develop papillomas and carcinomas in the late stage of their life, indicating that E6 and E7 each is sufficient to induce tumors. Because of the long latent period of tumor induction, it is proposed that other genetic changes must contribute to the carcinogenesis induced by E6 or E7.

One property of E6 and E7 that may contribute to genetic changes is their modulation of cellular responses to DNA damage. Cells often respond to DNA damage by undergoing growth arrest in either G_1 or G_2; this response is thought to give cells time to repair their damaged DNA. Cell cycle arrest in G_1 can be mediated by p53, which is increased after DNA damage (23). In cell culture experiments, high risk E6, which inactivates p53, abrogates DNA damage-induced cell cycle arrest (24). Transition from G_1 to S phase is regulated by pRb, which is a distal target of p53 through its induction of p21, an inhibitor of cyclin-dependent kinases (CDKs) that phosphorylate and thereby inactivate pRb. It is not surprising, therefore, that high risk E7 protein which binds to and inactivates pRb, has been shown to abrogate DNA damage responses in tissue culture (25, 26).

To determine whether E6 and E7 can affect DNA damage responses *in vivo*, we tested the cellular responses to ionizing radiation in the epidermis of K14HPV16E6 and K14HPV16E7 transgenic mice, in which HPV-16 E6 or E7 is expressed, respectively, in epidermis. We found that both E6 and E7 can modulate responses to DNA damage *in vivo*. Of particular note, we found that E6 and E7 each abrogated DNA synthesis inhibition in the epidermis more completely than does the loss of p53 function. This result indicates radiation-induced growth arrest in the epidermis is mediated through both p53-dependent and p53-independent pathways, and E6 and E7 must inactivate both pathways. Furthermore, we found that both the conserved region CR1 and CR2 domains of E7 protein, which are each required for cellular transformation, were also required for E7 to modulate DNA damage responses.

MATERIALS AND METHODS

Mice. K14HPV16E7 mice transgenic for the wild-type HPV-16 E7 gene or mutant HPV-16 E7 genes $E7^{\Delta PTHLE}$ and $E7^{\Delta DLYC}$ were described previously (22, 27). K14HPV16E6 mice transgenic for wild-type HPV16 E6 were generated similarly (S.S. and P.F.L., unpublished work). In all cases, the viral genes were placed under the control of the human keratin 14 (K14) promoter, which directs transgene expression to the undifferentiated compartment of stratified squamous epithelia such as the epidermis. Expression of E6 (S.S. and P.F.L., unpublished results) or E7 (22, 27) in the epidermis was confirmed by *in situ* hybridization. All transgenes were main-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

This paper was submitted directly (Track II) to the *Proceedings* office.
Abbreviations: HPV, human papillomavirus; CR, conserved region; K14, keratin 14.
*To whom reprint requests should be addressed. e-mail: Lambert@oncology.wisc.edu.

tained in the inbred FVB/N mouse strain in the American Association for the Accreditation of Laboratory Animal Care (AAALAC)-approved McArdle Laboratory Animal Care Facility. FVB/N mice carrying the p53-knock-out allele generated by Tyler Jacks (28) were obtained from Anne Griep (University of Wisconsin). These mice were backcrossed to FVB/N more than 10 generations, making their genetic background congenic to the FVB/N HPV transgenic mice. P53-null ($p53^{-/-}$) mice were produced by interbreeding $p53^{+/-}$ mice, and genotyped by PCR.

Treatment of Mice with Ionizing Radiation. Eight-day-old mice were irradiated with γ rays from a ^{137}cesium (^{137}Cs) source at a dose rate of 3.1 gray (Gy)/min. A total dose of 5 Gy was delivered to the whole body of each mouse individually. The mice were sacrificed at 4, 24, or 48 hr after irradiation. Groups of unirradiated mice were used as controls. One hour prior to sacrifice, mice were injected with 5-bromo-2′-deoxyuridine (BrdUrd; Sigma, catalog no. B-5002) at a dose of 100 μg/g of body weight, to measure DNA synthesis in tissues. Skin samples were obtained from the dorsal area and fixed in 10% buffered formalin. Skin sections 5 μm in thickness were cut from paraffin-embedded samples.

Immunohistochemistry for BrdUrd. Skin sections were deparaffinized in xylenes and rehydrated in graded alcohol and PBS. Endogenous peroxidase was quenched by treatment of skin sections with 3% hydrogen peroxide for 15 min. BrdUrd was detected by using the protocol provided with the BrdUrd staining kit (Calbiochem catalog no. HCS24). Briefly, the tissue sections were digested with trypsin and treated with a denaturing solution. After incubation with biotinylated mouse anti-BrdUrd antibody (3 hr) and streptavidin-peroxidase, the slides were exposed to the peroxidase substrate (diaminobenzidine) mixture for 5 min and counterstained with hematoxylin. To arrive at the percentage of BrdUrd-positive cells, the total numbers of cells and the number of BrdUrd-positive cells in the epidermis were counted in 30 randomly selected microscopic fields (×400 magnification) of skin sections from three to six mice for each time point. The statistical significance of the reduction in the percentage of BrdUrd-positive cells was calculated by using the normal approximation for the binomial models (29).

Immunohistochemistry for p53 and p21. The tissue slides were deparaffinized, rehydrated, and quenched the same way as for BrdUrd staining. The slides were incubated in a boiling 0.01 M citrate buffer (pH 6.0) in a microwave oven for 20 min to unmask antigens. Tissue sections were blocked with 5% nonfat dry milk/PBS and 5% normal goat serum for 30 min. After blocking, rabbit anti-mouse p53 antibody (CM5, catalog no. NCL-p53-CM5p, Novocastra Laboratories, Newcastle upon Tyne, U.K.), diluted 1:500, or anti-mouse p21 antibody (M-19, catalog no. sc-471, Santa Cruz Biotechnology), diluted 1:100 (1 μg/ml), was added, and the slides were incubated for 3 hr at room temperature in a humid chamber. After incubation with secondary antibody (30 min), then with Vectastain ABC reagents (30 min), the slides were exposed to diaminobenzidine substrate. p53- and p21-positive cells were examined, photographed, and counted.

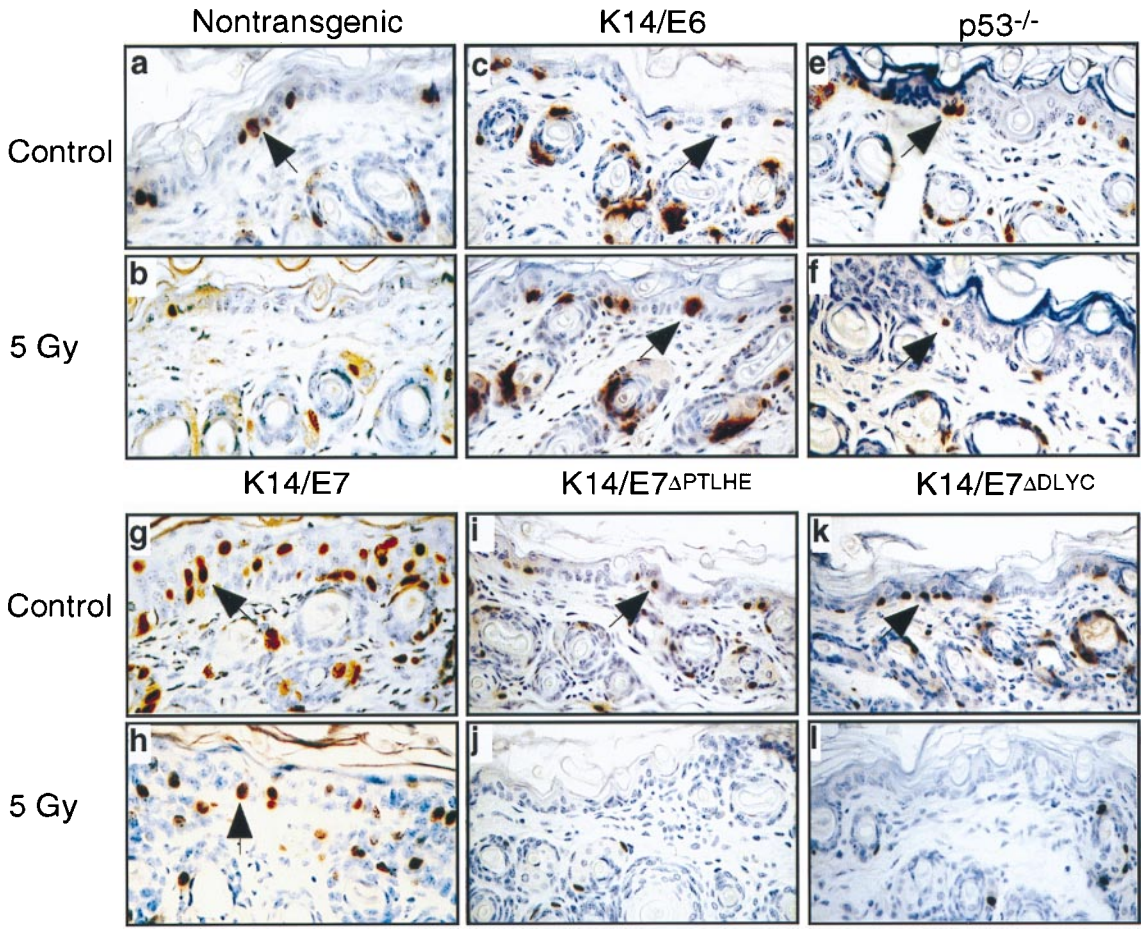

FIG. 1. Comparison of levels of DNA synthesis in nontransgenic, E6-transgenic, p53-null, and E7 transgenic epidermis after treatment with radiation. Shown are high-power magnification (×400) images of cross sections of skin from nontransgenic, K14HPV16E6 transgenic, $p53^{-/-}$, K14HPV16E7, K14HPVE7$^{\Delta PTHLE}$, and K14HPV16E7$^{\Delta DLYC}$ transgenic mice stained immunohistochemically for BrdUrd. Mice were either not treated (*a*, *c*, *e*, *g*, *i*, and *k*) or treated with 5 Gy of ionizing radiation 24 hr prior to sacrifice (*b*, *d*, *f*, *h*, *j*, and *l*). Arrows indicate examples of BrdUrd-positive (brown-stained nuclei) cells in the epidermis.

Table 1. Decreases in the levels of DNA synthesis after ionizing radiation in mouse epidermis

Mouse strain	BrdUrd-positive cells,* %		*P* value
	Unirradiated	Irradiated	
Nontransgenic	4.52 ± 1.69	0.39 ± 0.59	<0.001
E6-transgenic	5.64 ± 1.84	5.05 ± 1.83	>0.05
p53-null	4.37 ± 2.05	2.86 ± 2.21	<0.001
E7-transgenic	9.42 ± 3.21	8.40 ± 2.63	>0.05
$E7^{\Delta PTLHE}$-transgenic	4.25 ± 2.29	0.40 ± 0.75	<0.001
$E7^{\Delta DLYC}$-transgenic	5.50 ± 2.57	0.45 ± 0.66	<0.001

*Shown are the average percentage of BrdUrd-positive epidermal cells in unirradiated and irradiated mice (24 hr after irradiation). Data were obtained from analysis of paraffin-embedded skin sections stained for BrdUrd.

RESULTS

HPV16 E6 Abrogates Radiation-Induced Inhibition of DNA Synthesis *in Vivo*. We compared levels of DNA synthesis in nontransgenic and E6-transgenic (K14HPV16E6) mouse epidermis after treatment with 5 Gy of ionizing radiation. In the nontransgenic mice, 4.5% of the epidermal cells in control (unirradiated) mice were labeled with BrdUrd (Table 1); all of these cells were in the stratum basale (Fig. 1). These cells represent the fraction of cells that were going through S phase during the 1-hr BrdUrd labeling period just prior to sacrifice of the mice. After irradiation, the number of BrdUrd-positive cells was greatly reduced, with maximal reduction at 24 hr after radiation (Fig. 2, Table 1). This reduction indicates that there was an arrest in the cell cycle within this normally proliferative tissue. By 48 hr, a partial recovery of DNA synthesis was observed (Fig. 2), indicating that cells were being released from this growth arrest. In contrast to nontransgenic mice, there was no significant change ($P = 0.26$) in the number of BrdUrd-positive cells in the epidermis of E6 transgenic mice after irradiation (Figs. 1 and 2; Table 1), at any time points up to 48 hr. These results demonstrate that E6 efficiently abrogates the inhibition of DNA synthesis induced by ionizing radiation *in vivo*, correlative with findings made in tissue culture (25).

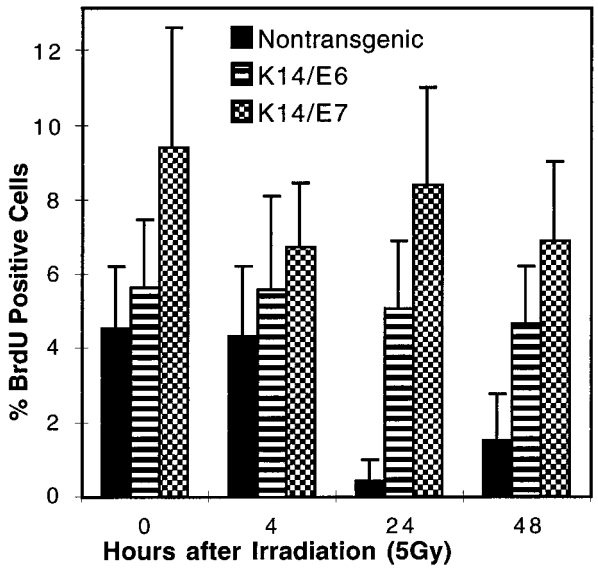

FIG. 2. Time course of changes of BrdUrd-positive cells in the epidermis of nontransgenic and E6- and E7-transgenic epidermis treatment with radiation (5 Gy). Graphed are the average percentage of epidermal cells positively stained for BrdUrd at different times after treatment. Error bars indicate standard deviation (SD) among samples from three different mice.

HPV-16 E6 Abrogates Radiation-Induced Inhibition of DNA Synthesis More Efficiently Than Does Loss of p53. Because E6's effects are predicted to be through its inactivation of p53, we compared the radiation responses of epidermis of E6-transgenic mice and p53-null mice. There was still a significant reduction in number of BrdUrd-positive cells in the epidermis of p53-null mice after irradiation ($P < 0.001$, Fig. 1 and Table 1), but less severe than in p53-sufficient mice. The percentage of BrdUrd-positive cells in p53-null epidermis at 24 hr after irradiation was reduced less than 2-fold, whereas the reduction was 10-fold in p53-sufficient mice (Table 1, Fig. 3). The partial inhibition of DNA synthesis in p53-null mice indicates that ionizing radiation affects DNA synthesis through p53-independent as well as p53-dependent pathways. In comparison with p53-null epidermis, however, there was a complete abrogation in the DNA damage-induced inhibition of DNA synthesis in E6-transgenic epidermis (Fig. 3). Thus, E6 must possess p53-independent activities that modulate DNA damage responses.

HPV-16 E7 Abrogates DNA Synthesis Inhibition After Radiation *in Vivo*. To understand the *in vivo* effects of E7 on DNA damage responses, we carried out experiments on our E7-transgenic (K14HPV16E7) mice with the same protocol described above for the E6-transgenic mice. E7 abrogated radiation-induced cell growth arrest (Fig. 1). At 24 hr after radiation, there was no significant decrease in the number of BrdUrd-positive cells ($P = 0.91$, Table 1; Fig. 2). In comparison to p53-null epidermis, there was a more complete abrogation of radiation-induced growth arrest in E7 transgenic epidermis, suggesting that, like E6, E7 must affect both p53-dependent as well as p53-independent pathways.

Both CR1 and CR2 Domains Are Required for E7 to Modulate DNA Damage Responses. The CR1 and CR2 domains in E7 protein are important for transformation of cells in culture. We have shown in transgenic mice that mutations in either the CR1 ($E7^{\Delta PTHLE}$) or the CR2 ($E7^{\Delta DLYC}$) domain abolish E7's ability to induce epidermal hyperplasia and skin tumors (27). To test whether these mutations in the CR1 and CR2 domains also affect E7's ability to modulate DNA damage responses *in vivo*, K14HPV16$E7^{\Delta PTHLE}$ and K14HPV16$E7^{\Delta DLYC}$ transgenic mice, respectively, were irradiated. The percentage of BrdUrd-positive cells in the unirradiated epidermis of the mutant E7 mice was not statistically

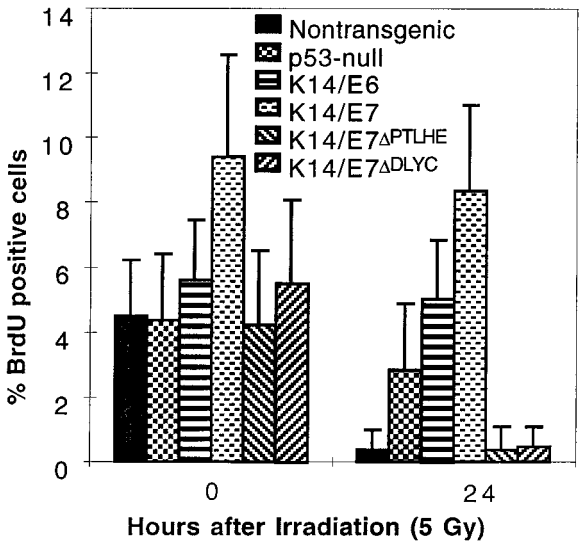

FIG. 3. Comparison of decreases in the number of BrdUrd-positive cells in the epidermis of nontransgenic, E6-transgenic, p53-null, E7- and mutant E7-transgenic ($E7^{\Delta PTLHE}$ and $E7^{\Delta DLYC}$) epidermis at 24 hr after irradiation. Graphed are the mean percentage of epidermal cells positively stained for BrdUrd.

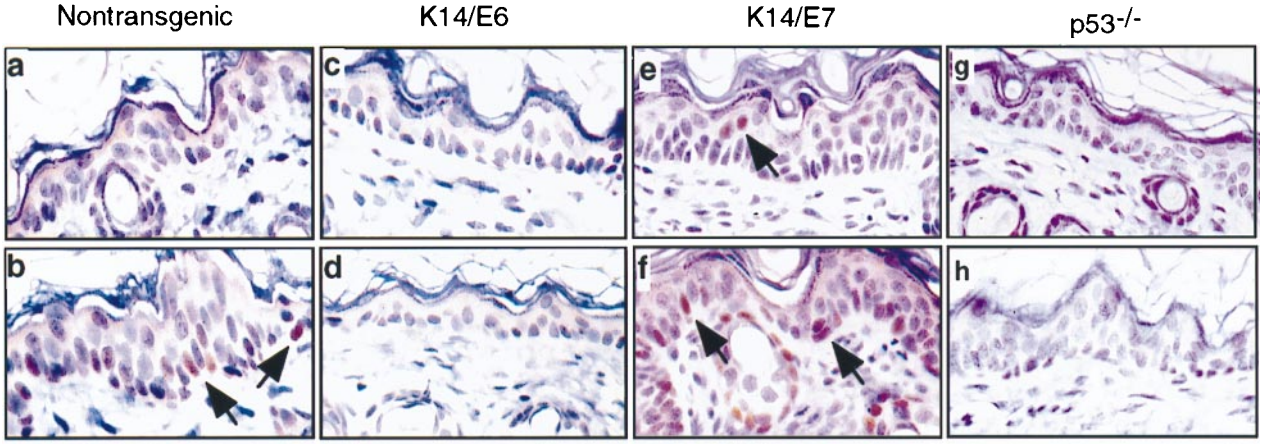

FIG. 4. Levels of p53 in the skin of nontransgenic, E6- and E7-transgenic, and p53-null mice after treatment with radiation. Shown are high power magnifications (×400) of cross sections of skin from nontransgenic, K14HPV16E6 transgenic, and K14HPV16E7 transgenic mice stained immunohistochemically for p53. Mice were either not treated (*a*, *c*, *e*, and *g*) or treated with 5 Gy of ionizing radiation 24 hr prior to sacrifice (*b*, *d*, *f*, and *h*). Examples of p53-positive cells are indicated by arrows.

different than that seen in the unirradiated nontransgenic mice (Figs. 1 and 3), consistent with the prior observation (27) that these E7 mutants are defective in inducing epithelial hyperplasia. After irradiation, the number of BrdUrd-positive cells in the epidermis of the CR1 and CR2 E7 mutant transgenic mice was significantly ($P < 0.001$) reduced (Table 1). This reduction was comparable to that seen in the epidermis of irradiated nontransgenic mice (Fig. 3). This result demonstrates that both the CR1 and CR2 domains are required for E7 to modulate DNA damage responses *in vivo*.

Radiation Induces an Increase of p53 and p21 Levels in E7 but Not in E6 Transgenic Epidermis. To understand how p53 responds to ionizing radiation in E6- and E7-transgenic mice, we monitored the level of p53 after irradiation by immunohistochemical staining. A significant increase in the number of p53-positive cells was seen 4 hr after irradiation in nontransgenic mice and E7-transgenic mice (Figs. 4 and 5), indicating that E7 does not affect p53 accumulation. In the nontransgenic epidermis, levels of p53 decreased by 48 hr (Fig. 5), when recovery from growth arrest was observed (Fig. 2). In the E7-transgenic epidermis, high levels of p53 protein persisted until 48 hr after irradiation (Fig. 5), indicating that mechanisms involved in reestablishing normal steady state of p53 in E7-expressing cells are affected. In E6-transgenic epidermis, no p53-positive cells were observed before or up to 48 hr after radiation (Figs. 4 and 5), presumably because E6 had led to efficient degradation of p53 protein in this tissue.

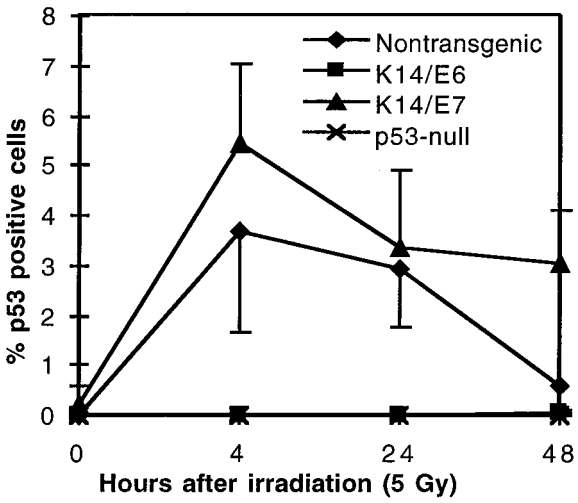

FIG. 5. Quantification of p53-positive cells in the epidermis of nontransgenic (◆), E6-transgenic (■), E7-transgenic (▲), and p53-null (×) epidermis after treatment with radiation. Graphed are the mean percentage of epidermal cells positively stained for p53 at different times after treatment of mice with 5 Gy of radiation. Error bars indicate SD among samples from different mice.

p21 protein levels fluctuate with cellular differentiation as well as with cellular responses to DNA damage. P53 contributes to an increase of p21 following some forms of DNA damage (30). We monitored p21 protein levels by immunohistochemical staining (Fig. 6). In nontransgenic epidermis, radiation-induced increases in the number of p21-positive cells were seen by 4 hr, reached a maximum by 24 hr, and were sustained at that level up to 48 hr after irradiation (Fig. 7). The cells that accumulated p21 after irradiation were in suprabasal layers as well as in the stratum basale. Increases in p21-positive cells after irradiation were also seen in E7-transgenic mice but to lesser of a degree in p53-null or E6-transgenic mice (Fig. 7). Thus p21 induction after irradiation is at least partially dependent on p53, and this induction is strongly inhibited by E6 but not by E7.

In our experiments, we also found that the p21 levels in unirradiated epidermis of E7-transgenic mice (average 4.82% positive) were significantly higher than in the epidermis of unirradiated nontransgenic (average 0.88% positive) and E6-transgenic mice (average 0.60% positive) (Figs. 6 and 7). The mechanisms for the increased level of p21 in E7 transgenic epidermis is not known. One mechanism may be the increased expression of p21 induced by p53. Prior to radiation, we observed some, though not many, p53-positive cells in E7-transgenic epidermis (0.41% of cells in epidermis were p53-positive; see Fig. 6), but not in nontransgenic or E6-transgenic epidermis.

DISCUSSION

In this study, we monitored the responses to radiation in the mouse epidermis and the influence on that response by p53 status and the HPV-16 oncogenes *E6* and *E7*. Exposure of nontransgenic FVB/N mice to ionizing radiation led to a nearly complete inhibition of DNA synthesis in the epidermis 24 hr after treatment, with a partial recovery of DNA synthesis by 48 hr. Inhibition of DNA synthesis after irradiation was only partially abrogated in the epidermis of p53-null mice. Thus the inhibition of DNA synthesis in the epidermis induced by radiation occurs through both p53-dependent and p53-independent pathways. In contrast, both HPV-16 oncogenes,

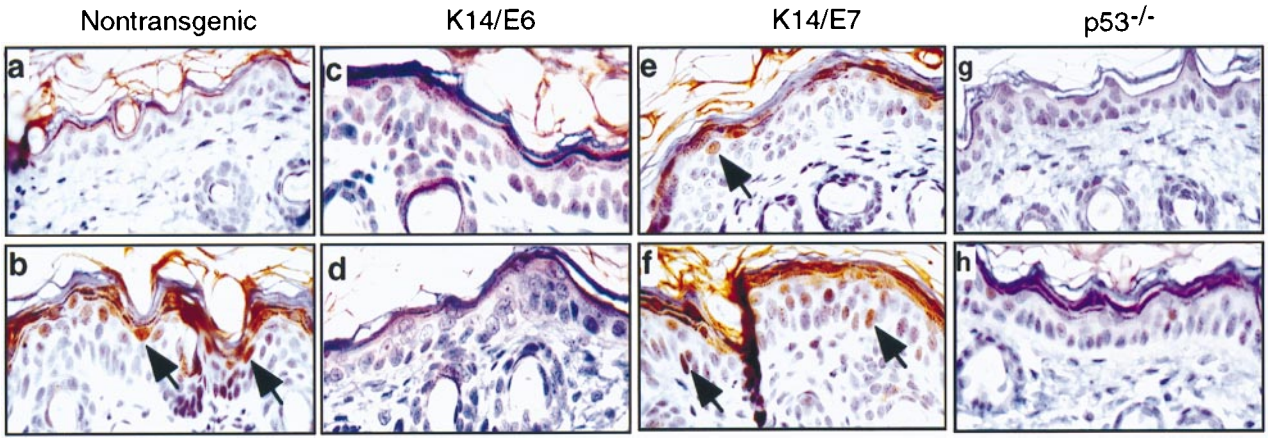

FIG. 6. Levels of p21 protein in the epidermal cells of nontransgenic, E6- and E7-transgenic, and p53-null mice after radiation. Shown are high power magnifications (×400) of cross sections of skin from nontransgenic, K14HPV16E6, and K14HPV16E7 transgenic mice that were stained immunohistochemically for p21. Mice were either not treated (*a*, *b*, *c*, and *g*) or were treated with 5 Gy of ionizing radiation 24 hr prior to sacrifice (*d*, *e*, *f*, and *h*). Examples of p21-positive cells are indicated by arrows.

E6 and *E7*, were found to abrogate completely the inhibition of DNA synthesis after irradiation. We interpret these findings to indicate that E6 and E7 each can modulate DNA damage responses mediated through both p53-dependent and p53-independent pathways.

Role of p53 in Mediating Radiation-Induced Responses in the Epidermis. After irradiation there was an induction in p53 protein levels, maximal at 4 hr (Fig. 5), followed by an increase in levels of p21, a p53-responsive protein (Fig. 7). High levels of p21 were reached at 24 hr after irradiation, and this correlated with the timing of maximal inhibition of DNA synthesis (Figs. 2 and 7). The temporal order of the induction of p53, and the subsequent induction of p21 and coincident reduction in DNA synthesis, suggests a cause and effect relationship between p53 protein and radiation-induced inhibition of DNA synthesis. This relationship was substantiated by the finding that p53-null mice were impaired in their ability to respond to radiation (Fig. 3; Table 1). p53, however, can mediate only part of the cellular response, because the epidermis of p53-null mice still display a partial inhibition of DNA synthesis after irradiation. Therefore, there must be a p53-independent pathways that mediate in part the growth arrest in response to this form of DNA damage in this cell type.

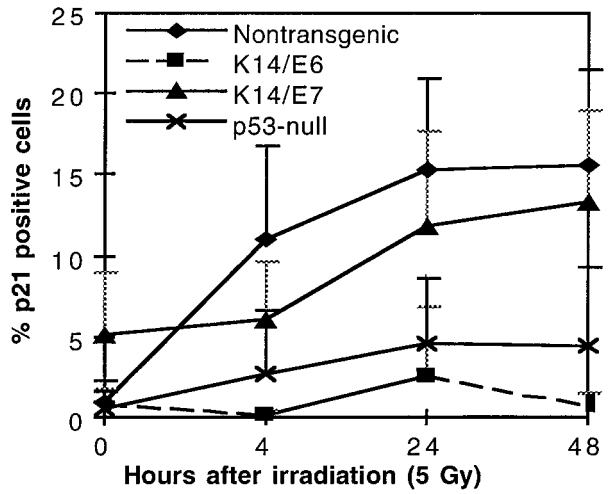

FIG. 7. Quantification of p21-positive cells in the epidermis of nontransgenic (◆), E6-transgenic (■), E7-transgenic (▲), and p53-null (×) epidermis after treatment with radiation. Graphed are the percentage of epidermal cells positively stained for p21 at different times after treatment of mice with 5 Gy of radiation.

Mechanism of Action of E6 and E7 in Modulating Radiation Responses in the Epidermis. E6 and E7 both could abrogate radiation-induced inhibition of DNA synthesis more completely than was seen in p53-null mice (Table 1; Fig. 3). These findings indicate that both oncogenes modulate p53-independent as well as p53-dependent response pathways. That E6 inhibits p53-dependent processes is predictable, given its capacity to bind and target for degradation p53. Not surprisingly, we observed that the induction in p53 protein levels and the subsequent induction of p21 after irradiation are suppressed in the K14HPV16E6 mice (Figs. 4–7). Consistent with our results, E6 was recently found to abrogate radiation-induced G_2 arrest in cultured cells more efficiently than p53-dominant negative mutants (31). Thus, like the inhibition of apoptosis (32), the inhibition of growth arrest induced by radiation represents another biological activity of E6 mediated through p53-dependent as well as p53-independent response pathways. E6's transforming or transactivating activities may also require p53-independent activities (5, 33). The fact the E6 affects multiple pathways, not exclusively the p53-dependent pathway, should be taken into consideration when E6 is employed to inhibit p53 function.

E7 is likely to modulate p53-dependent responses to DNA damage through its direct interaction with pRb and pRb-like proteins, and/or its modulation of p21 activity. pRb, a critical regulator of the G_1-to-S transition, is phosphorylated and inactivated by cyclin-dependent kinases, which in turn are inhibited by p21, a p53-responsive protein. Therefore, p53, p21, and pRb are proposed to be interconnected in DNA damage response pathways (25, 26), with pRb acting downstream of p53 and p21. Any factors that can inhibit the function of pRb may override the inhibitory signals from upstream p53 and p21. We found E7's ability to modulate DNA damage responses depended on the integrity of its CR1 and CR2 domains (Figs. 1 and 3). Cell culture experiments have demonstrated that E7's abrogation of cell cycle arrest after treatment with actinomycin D also requires the CR1 and CR2 domains (34). Both the CR1 and CR2 domains of E7 have been implicated in its modulation of pRb protein; the CR2 domain is required for E7's binding to pRb (35), whereas the CR1 and CR2 domains are both required for E7 to induce the degradation of pRb protein (17). Therefore, our data are consistent with the hypothesis (25, 34) that E7 abrogates p53-dependent responses to radiation through its binding and inactivation of pRb. E7 is also known to bind and inactivate p21 (36, 37). However, p21 appears to be functional in the cells expressing E7, because kinase activity associated with cyclins is reduced after DNA damage in E7-expressing cells (38, 39). Also, E7's

capacity to bind p21 maps to the CR2 but not the CR1 domain (36). Thus, the lack of DNA synthesis inhibition in E7 transgenic epidermis after irradiation correlates with E7's interaction with pRb, not p21.

Given that E7 can abrogate nearly completely the inhibition of DNA synthesis induced by radiation, we deduce that E7 suppresses cellular responses to radiation that are mediated through both p53-dependent and p53-independent pathways. While current knowledge of p53-induced growth arrest points to pRb and p21 as likely downstream targets, little is known about p53-independent pathways. As indicated above, our genetic analysis of E7 is consistent with E7 abrogating cellular responses to radiation through its inactivation of pRb. Therefore we propose that the p53-independent response pathway may converge on the same downstream targets of the p53-dependent pathway targeted by E7, e.g., pRb.

E7 Alters p53 and p21 Levels in the Epidermis. Levels of p53 and p21 in the epidermis of the K14HPV16E7-transgenic mice were aberrant (Figs. 4 and 6). p53-positive cells were observed in the unirradiated epidermis of E7-transgenic mice, whereas they were not seen in the epidermis of unirradiated nontransgenic mice. Consistent with our observations, others have found recently that E7 mediates the destabilization of pRb and this is coupled with stabilization of p53 (42). The increase in p53-positive cells in the K14HPV16E7-transgenic epidermis may explain why we observed increased numbers of p21-positive cells in the same tissue. This increased p21 appears not to inhibit the proliferative status of the E7 epidermis, which is hyperplastic. This disparity may be explained by reports that E7 disrupts the formation of complexes of p21 and cyclin/cyclin-dependent kinases (CDKs) and/or abrogates p21-mediated inhibition of CDKs (36, 37), or, as mentioned above, by E7's inhibition of pRb. The majority of p21-positive cells in the unirradiated E7-transgenic epidermis were suprabasal (Fig. 6). This finding suggests that signals leading to the increase in p21 protein are related to those that normally drive cell differentiation. After treatment of the K14HPV16E7-transgenic mice, we noted an induction in the levels of p53 in the epidermis that was commensurate, in timing and levels, with its induction seen in nontransgenic epidermis (Fig. 5). Also there was an induction of p21 in the irradiated E7-transgenic epidermis (Fig. 7). These results are consistent with ones in tissue culture studies (38, 39). However, whereas the number of p53-positive cells in the nontransgenic epidermis returned to pretreatment levels by 48 hr after treatment, the number of p53-positive cells remained high in the K14HPV16E7-transgenic epidermis (Fig. 5). Thus E7 appears to block or retard normal cellular processes that lead to reestablishing normal p53 steady-state levels after radiation. Recently, the mdm2 protein has been identified to modulate the stability of p53 protein (40, 41). It is interesting to speculate that E7 may alter the function or levels of mdm2 and thereby cause this dysregulation of p53 protein levels.

We thank Dr. Karl Münger for communication of unpublished results, Dr. Yunlai Zhang for statistic analysis, Drs. Janet Mertz and Bill Sugden for helpful discussions, and Drs. Norman Drinkwater and Mary Ellen Perry for critical review of this manuscript. This study was supported by a grant from the American Cancer Society (VM164) and by grants from the National Institutes of Health (CA22443 and CA07175).

1. zur Hausen, H. (1996) *Biochim. Biophys. Acta* **1288,** F55–F78.
2. Woodworth, C. D., Doniger, J. & DiPaolo, J. A. (1989) *J. Virol.* **63,** 159–164.
3. van den Brule, A. J., Cromme, F. V., Snijders, P. J., Smit, L., Oudejans, C. B., Baak, J. P., Meijer, C. J. & Walboomers, J. M. (1991) *Am. J. Path.* **139,** 1037–1045.
4. Münger, K., Phelps, W. C., Bubb, V., Howley, P. M. & Schlegel, R. (1989) *J. Virol.* **63,** 4417–4421.
5. Sedman, S. A., Hubbert, N. L., Vass, W. C., Lowy, D. R. & Schiller, J. T. (1992) *J. Virol.* **66,** 4201–4208.
6. Bedell, M. A., Jones, K. H., Grossman, S. R. & Laimins, L. A. (1989) *J. Virol.* **63,** 1247–1255.
7. Phelps, W. C., Yee, C. L., Münger, K. & Howley, P. M. (1988) *Cell* **53,** 539–547.
8. Storey, A., Pim, D., Murray, A., Osborn, K., Banks, L. & Crawford, L. (1988) *EMBO J.* **7,** 1815–1820.
9. Storey, A. & Banks, L. (1993) *Oncogene* **8,** 919–924.
10. Heck, D. V., Yee, C. L., Howley, P. M. & Münger, K. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 4442–4446.
11. Nakagawa, S., Watanabe, S., Yoshikawa, H., Taketani, Y., Yoshiike, K. & Kanda, T. (1995) *Virology* **212,** 535–542.
12. Mansur, C. P. & Androphy, E. J. (1993) *Biochim. Biophys. Acta* **1155,** 323–345.
13. Werness, B. A., Levine, A. J. & Howley, P. M. (1990) *Science* **248,** 76–79.
14. Scheffner, M., Huibregtse, J. M., Vierstra, R. D. & Howley, P. M. (1993) *Cell* **75,** 495–505.
15. Dyson, N., Howley, P. M., Münger, K. & Harlow, E. (1989) *Science* **243,** 934–937.
16. Chellappan, S., Kraus, V. B., Kroger, B., Münger, K., Howley, P. M., Phelps, W. C. & Nevins, J. R. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 4549–4553.
17. Jones, D. L. & Münger, K. (1997) *J. Virol.* **71,** 2905–2912.
18. Boyer, S. N., Wazer, D. E. & Band, V. (1996) *Cancer Res.* **56,** 4620–4624.
19. Arbeit, J. M., Münger, K., Howley, P. M. & Hanahan, D. (1993) *Am. J. Path.* **142,** 1187–1197.
20. Griep, A. E., Herber, R., Jeon, S., Lohse, J. K., Dubielzig, R. R. & Lambert, P. F. (1993) *J. Virol.* **67,** 1373–1384.
21. Comerford, S. A., Maika, S. D., Laimins, L. A., Messing, A., Elsasser, H. P. & Hammer, R. E. (1995) *Oncogene* **10,** 587–597.
22. Herber, R., Liem, A., Pitot, H. & Lambert, P. F. (1996) *J. Virol.* **70,** 1873–1881.
23. Gottlieb, T. M. & Oren, M. (1996) *Biochim. Biophys. Acta* **1287,** 77–102.
24. Kessis, T. D., Slebos, R. J., Nelson, W. G., Kastan, M. B., Plunkett, B. S., Han, S. M., Lorincz, A. T., Hedrick, L. & Cho, K. R. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 3988–3992.
25. Slebos, R. J., Lee, M. H., Plunkett, B. S., Kessis, T. D., Williams, B. O., Jacks, T., Hedrick, L., Kastan, M. B. & Cho, K. R. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 5320–5324.
26. Demers, G. W., Foster, S. A., Halbert, C. L. & Galloway, D. A. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 4382–4386.
27. Gulliver, G., Herber, R., Liem, A. & Lambert, P. F. (1997) *J. Virol.* **71,** 5905–5914.
28. Jacks, T., Remington, L., Williams, B. O., Schmitt, E. M., Halachmi, S., Bronson, R. T. & Weinberg, R. A. (1994) *Curr. Biol.* **4,** 1–7.
29. Lehmann, E. L. (1986) *Testing Statistical Hypotheses* (Wiley, New York).
30. Macleod, K. F., Sherry, N., Hannon, G., Beach, D., Tokino, T., Kinzler, K., Vogelstein, B. & Jacks, T. (1995) *Genes Dev.* **9,** 935–944.
31. Thompson, D. A., Belinsky, G., Chang, T. H.-T., Jones, D. L., Schlegel, R. & Münger, K. (1997) *Oncogene* **15,** 3025–3036.
32. Pan, H. & Griep, A. E. (1995) *Genes Dev.* **9,** 2157–2169.
33. Ishiwatari, H., Hayasaka, N., Inoue, H., Yutsudo, M. & Hakura, A. (1994) *J. Med. Virol.* **44,** 243–249.
34. Demers, G. W., Espling, E., Harry, J. B., Etscheid, B. G. & Galloway, D. A. (1996) *J. Virol.* **70,** 6862–6869.
35. Münger, K., Werness, B. A., Dyson, N., Phelps, W. C., Harlow, E. & Howley, P. M. (1989) *EMBO J.* **8,** 4099–4105.
36. Jones, D. L., Alani, R. M. & Münger, K. (1997) *Genes Dev.* **11,** 2101–2111.
37. Funk, J. O., Waga, S., Harry, J. B., Espling, E., Stillman, B & Galloway, D. A. (1997) *Genes Dev.* **11,** 2090–2100.
38. Hickman, E. S., Bates, S. & Vousden, K. H. (1997) *J. Virol.* **71,** 3710–3718.
39. Ruesch, M. N. & Laimins, L. A. (1997) *J. Virol.* **71,** 5570–5578.
40. Kubbutat, M. H., Jones, S. N. & Vousden, K. H. (1997) *Nature (London)* **387,** 299–303.
41. Haupt, Y., Maya, R., Kazaz, A. & Oren, M. (1997) *Nature (London)* **387,** 296–299.
42. Jones, D. L., Thompson, D. A. & Münger, K. (1997) *Virology* **239,** 97–107.