Positive and Negative Regulation of Cell Proliferation by E2F-1: Influence of Protein Level and Human Papillomavirus Oncoproteins

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E2F-1 is a member of a family of transcription factors implicated in the activation of genes required for the progression through the S phase of the cell cycle. We have examined the biological activities of E2F-1 with short-term colony-forming assays and long-term immortalization assays. High levels of E2F-1, produced by transfection of the *E2F-1* cDNA under the control of a strong promoter, reduced colony formation in normal human foreskin keratinocytes (NHFKs). This inhibition could not be overcome by wild-type human papillomavirus type 16 (HPV16) E6 and E7, two proteins which cooperate to immortalize NHFKs, or by a transdominant p53 mutant. High levels of E2F-1 also inhibited growth of primary and established fibroblasts. The growth-inhibitory activity required the DNA binding function of E2F-1 but not its transactivation or pRB binding activities. A positive role for lower levels of E2F-1 in NHFK immortalization was established by examining the ability of E2F-1 to complement HPV16 E7 mutants that were unable to cooperate with HPV16 E6 to immortalize NHFKs. Although E2F-1 was unable by itself to cooperate with E6, it did, in conjunction with E6, complement a p24GLY mutant of E7 that is defective for immortalization and binding of pRB and pRB-related proteins. By contrast, E2F-1 was unable to complement two other E7 mutants, p2PRO and p31/32ARG/PRO, which are also defective in the immortalization assay, although their proteins display wild-type binding of pRB in vitro. Since the binding of E7 to pRB results in disruption of pRB-E2F interaction and release of transcriptionally active E2F, the data support the hypothesis that binding of pRB by E7 and the consequent increase in E2F activity are important but not sufficient for E7-induced keratinocyte immortalization.

The influence of viral oncoproteins on cellular proteins has elucidated important features of cell function, as well as contributing to our understanding of the mechanisms underlying cellular transformation by viruses. E2F represents a widely expressed transcription factor that was initially identified in adenovirus-infected cells because the adenoviral E1A proteins stimulated its DNA binding activity (33). E2F DNA binding sites have been found in the promoters of several cellular genes implicated in growth, including *c-myc*, *cdc2*, *RB-1*, and the DNA polymerase α gene, and E2F activity appears to participate in the transcriptional regulation of these and other genes (23, 35).

The transcriptional activity of E2F appears to be negatively regulated by the formation of complexes with cellular proteins that participate in growth regulation (3, 5, 11, 13). The interaction between E2F and pRB, the product of the *RB-1* retinoblastoma susceptibility gene, has been examined in greatest detail. E2F binds preferentially to the underphosphorylated form of pRB, which is believed to inhibit transit through the cell cycle (11, 26). Hyperphosphorylation of pRB as cells progress through G₁ leads to its dissociation from E2F and a concomitant increase in E2F transcriptional activity (38, 52). E2F also forms complexes with the pRB-related proteins p107 and p130 (10, 14). The stimulation of E2F activity during adenovirus infection results from the ability of the adenoviral

E1A proteins to bind pRB, p107, and p130, which leads to the release of E2F (2).

Recent molecular analysis has identified several related genes with E2F activity (35). *E2F-1* (26, 32, 51), whose protein product possesses many of the features established for E2F, is the most intensively characterized member of this multigene family. The *E2F-1* product can stimulate transcription that depends upon the presence of E2F DNA binding sites and bind pRB (26, 51). Microinjection of *E2F-1* cDNA into serum-starved REF-52 rodent fibroblasts induces S-phase entry (31), while SAOS-2 cells arrested in G₁ by overexpression of pRB can be rescued by cotransfection of *E2F-1* (58).

As with adenovirus E1A, the E7 proteins of the genital human papillomaviruses (HPVs) implicated in cervical cancer also bind pRB, p107, and p130 (16, 18, 40), and E7 disrupts the E2F-pRB complex. Aside from their role in neoplastic disease (59), genital HPVs have been used to study the biology of normal human foreskin keratinocytes (NHFKs), which have a limited life span in culture. Transfection of DNA from the HPVs associated with cervical cancer (high-risk HPV types, especially HPV type 16 [HPV16] and HPV18) can induce immortalization of NHFKs, while DNA from genital HPVs that are not associated with cervical cancer (low-risk types, especially HPV6 and HPV11) fail to immortalize under similar conditions. Efficient NHFK immortalization can be achieved by cooperation between the two major oncogenes, *E6* and *E7* (22, 29, 39), which are the two viral genes that are preferentially expressed in HPV-positive tumors and in tumor-derived cell lines (48).

In addition to participating in NHFK immortalization, *E7* can induce anchorage-independent growth of NIH 3T3 cells

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and transform primary rodent cells in cooperation with a *ras* oncogene. *E7* genes from high-risk HPVs have much stronger transforming and immortalizing activities than do the *E7* genes of low-risk HPV types, which correlates with a greater efficiency of binding of high-risk *E7* protein with pRB. Analysis of HPV16 *E7* mutants in rodent fibroblasts has suggested that although *E7*-mediated transformation requires binding between pRB and *E7* protein, other *E7* activities may also be required, since some *E7* mutants that bind pRB in vitro are greatly reduced in their ability to induce fibroblast transformation (55).

Given that both *E2F-1* and *E7* can induce DNA synthesis in fibroblasts (7, 31), we have sought in the current study to determine whether *E2F-1* can substitute for HPV16 *E7* in NHFK immortalization. Unexpectedly, we find that high-level expression of *E2F-1* induces growth inhibition in NHFK and other cell types. However, lower levels of *E2F-1* can complement a specific *E7* mutant in the NHFK immortalization assay but cannot substitute completely for *E7*.

MATERIALS AND METHODS

Plasmids. The Moloney murine leukemia virus long terminal repeat-activated HPV16 *E6* and *E7* gene expression vectors pSDE6 (49) and pE7Mo and the *E7* mutants p21SER, p24GLY, p26GLY, p58/91GLY/GLY (19), p31/32ARG/PRO (8), and p2PRO (7) have been described elsewhere. Plasmids pCMVneoBam (4), which contains the cytomegalovirus (CMV) immediate-early gene enhancer-promoter and the neomycin resistance gene, and pCMVE2F-1, a derivative of pCMVneoBam that contains the human *E2F-1* cDNA coding sequence, have also been reported previously (25). To generate the plasmid pE2F-1Mo, which places *E2F-1* under control of the Moloney murine leukemia virus long terminal repeat, the *E2F-1* cDNA was removed from pCMVE2F-1 by digestion with *Bam*HI and cloned into the *Bam*HI site of the plasmid pMo (19) in the proper orientation.

The *E2F-1* mutant E132 has been previously described (15). It was cloned into the pCMVneoBam vector to create pCMVE2F-1(E132) (24). The other *E2F-1* mutants, plasmids pCMVE2F-1(Y411C), pCMVE2F-1(1-417), pCMVE2F-1(1-374), and pCMVE2F-1(1-284), have been described elsewhere (25, 27). They are also isogenic to pCMVE2F-1.

The human wild-type and mutant p53 clones pC53-SN3 and pC53-SX3 were also previously described (4). Clone pC53-SCX3 has an alteration in codon 143 which results in a valine-to-alanine change in the mutant p53 protein.

Cells and transfections. CaSki cells, an HPV16-positive human cervical carcinoma cell line, and NIH 3T3 cells were obtained from the American Type Culture Collection and maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. Primary human keratinocytes were cultured from fresh foreskins in keratinocyte serum-free medium (GIBCO Keratinocyte-SFM) and used for transfection at the third passage.

For stable transfections of human keratinocytes, cells were seeded in 60-mm-diameter dishes and transfected when 80% confluent. Briefly, plasmid DNA (15 μ g total) was diluted in 1.5 ml of keratinocyte basal medium (KBM; Clonetics) together with 50 μ l of lipofectin reagent (GIBCO). After a 10-min incubation, the solution was added to the cells, and the plates were incubated at 37°C for 5 h in a 5% CO₂ incubator. Four milliliters of complete medium (Keratinocyte-SFM) was then added, and the cells were incubated overnight. After 24 h, the lipofectin-containing medium was replaced with fresh medium.

Transient transfections were performed as described elsewhere (26), with minor modifications. Briefly, keratinocytes grown in 60-mm-diameter dishes were transfected with 10 μ g of expression plasmid as described above. Thirty-six hours after transfection, the cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris hydrochloride [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], and protease inhibitors). Western blot (immunoblot) analysis was performed as described below.

For growth inhibition assays, NIH 3T3 cells were transfected by calcium phosphate precipitation as described previously (49). Transfected cells were selected with G418. Drug-resistant colonies were stained and counted as described below.

Immortalization assay. The immortalization assay was performed essentially as described previously (50). Following transfection, confluent cells from each plate were dispersed with trypsin-EDTA and seeded in two 100-mm-diameter plates. Selection with G418 (100 μ g/ml) was applied when the cells were approximately 50 to 70% confluent. Colony counting was performed by fixing and staining one G418-treated plate in a solution containing 5% methylene blue and 2.5% carbol fuchsin in methanol. The colonies from the second plate were pooled, fed twice weekly, and split approximately every 7 to 10 days. Immortalization was defined as the ability of cells to be propagated for at least 16 passages (4 months) beyond control cells.

Protein analysis. To detect the *E7* protein, subconfluent pooled cultures of G418-selected keratinocytes were lysed in RIPA buffer. The total protein concentration was determined spectrophotometrically with bicinchoninic acid (Pierce). Equal aliquots (100 μ g) of each lysate were incubated for 1 h with a rabbit polyclonal anti-HPV16 *E7* serum raised against a trp-*E7* fusion protein (53). Antigen-antibody complexes were recovered with protein A-Sepharose beads, and the precipitated proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (15% polyacrylamide) and transferred to an Immobilon P membrane (Millipore Corp.). The blot was incubated with a monoclonal antibody against the HPV16 *E7* protein (Triton Biosciences) and then with an anti-mouse biotinylated antibody (Pierce). The antibody-protein complexes were detected by the ABC (avidin-biotinylated alkaline phosphatase complex) staining method (Pierce).

For *E2F-1*, protein immunoblots were performed. Established cell lines were lysed in RIPA buffer, and the protein concentration was determined as described above. Proteins (100 μ g) were separated by SDS-10% PAGE and electrotransferred to a nitrocellulose membrane (Amersham). *E2F-1* protein was identified by using mouse monoclonal antibody KH95 (27) followed by an anti-mouse horseradish peroxidase-conjugated whole antibody (Amersham) and detected by chemiluminescence. Proteins were quantified with an Ultra-scan-XL enhanced laser densitometer (LKB Bromma).

RESULTS

Immortalizing activity of *E7* mutants. The HPV16 *E7* protein is structurally and functionally related to the adenovirus E1A and the simian virus 40 large T proteins (44). It is a nuclear protein (98 amino acids [aa]) consisting of three regions (Fig. 1): region 1 (CRI, aa 1 to 20), which shows similarity to part of E1A conserved region I; region 2 (CRII, aa 21 to 40), which shows homology with E1A CRII (44); and region 3 (aa 41 to 98), which contains a Cys-X-X-Cys zinc-binding motif (9). The CRII domain mediates the association of *E7* with pRb, p107, and p130 (16, 18, 40). The region also

FIG. 1. Schematic representation of HPV16 E7 protein showing the conserved regions CRI and CRII, homologous to E1A CRI and CRII, respectively, and the carboxy-terminal Cys-X-X-Cys zinc-binding domain. Amino acid numbers and changes for the mutants analyzed in the study are indicated. Wild-type and mutant amino acids are indicated in single-letter code above and below the arrows, respectively.

contains two serines, adjacent to the pRB binding domain, which are substrates for casein kinase II (8, 20). The carboxy-terminal region is required for protein stability (45) and has been implicated in other functions of the protein, including dimerization, binding of pRB, and disruption of the pRB-E2F complex (36, 43). No biochemical activities have been identified for CRI.

In fibroblasts, abrogation of the pRB-E7 interaction abolishes the transforming activity of E7 (7, 8, 45). However, mutations in aa 2 of CRI or the sites of casein kinase II phosphorylation (aa 31 and 32) also eliminate or decrease the transforming potential of E7 without affecting its in vitro pRB binding activity (8).

Since no comparable analysis of E7 mutants has been carried out with NHFKs, we determined which HPV16 E7 domains are required for immortalization. NHFKs were co-transfected with an expression plasmid containing the HPV16 wild-type E6 gene and an expression plasmid containing a mutant or wild-type E7 gene. After G418 selection, colonies were stained and counted; comparable numbers of colonies were obtained for each of the mutants tested (Fig. 2 and data not shown). As previously demonstrated, transfection of E6 alone or E7 alone was unable to induce immortalization of the NHFKs, while at least some cells from every dish cotransfected with E6 and wild-type E7 gave rise to an immortal line (Table 1).

Cotransfection of E6 with a variety of E7 mutants resulted in two distinct phenotypes, depending on the E7 mutant: immortalization similar to that of wild-type E7 or the failure to immortalize any cultures (Table 1). Codon 21 encodes an aspartate that is conserved in all high-risk HPVs. However, the p21SER mutant, which has been reported to have wild-type activity with respect to rodent fibroblast transformation and pRB binding of its encoded protein, was indistinguishable from the wild-type gene in the immortalization assay. By contrast, cotransfection of E6 and E7 mutant p24GLY or p26GLY

FIG. 2. G418-selected normal human keratinocyte colonies. After cotransfection, cells were split and selected in G418 as described in Materials and Methods. The cultures were grown for approximately 2 weeks and then stained. Plates transfected with E2F-1 alone or in conjunction with other genes developed fewer colonies than did the other transfectants.

TABLE 1. NHFK immortalization assay

Constructs transfected	No. of immortalized lines/no. of independent transfections
pMo + <i>neo</i>	0/8
E6.....	0/8
E7wt.....	0/8
E6 + E7wt.....	8/8
E6 + E7p2.....	0/5
E6 + E7p21.....	4/5
E6 + E7p24.....	0/6
E6 + E7p26.....	0/6
E6 + E7p31/32.....	0/6
E6 + E7p58/91.....	0/6

failed to immortalize in six independent experiments. These two mutants affect the region of the protein that is homologous to the CRII domain of adenovirus E1A (Fig. 1); they are deficient for E7-induced transformation of NIH 3T3 cells, for binding to pRB, and for association with histone H1 kinase activity (16, 18, 40). E7 mutants p2PRO and p31/32ARG/PRO were also unable to cooperate with E6 to induce immortalization, although their mutant proteins are capable of wild-type binding to pRB in vitro (8). The E7 zinc finger mutant p58/91GLY/GLY was also defective in the immortalization assay.

To determine the levels of wild-type and mutant E7 protein in the transfectants, extracts from G418-selected cultures were analyzed by immunoprecipitation from metabolically labeled keratinocytes (data not shown) and by sequential immunoprecipitation and immunoblotting (Fig. 3). The amounts of the p2PRO, p21SER, p24GLY, and p31/32ARG/PRO mutant proteins detected were similar to those of the wild type (Fig. 3).

FIG. 3. Detection of E7 proteins by immunoprecipitation followed by immunoblotting. The levels of wild-type and mutant E7 proteins in the NHFKs transfected with E6 plus E7 were compared with those of the controls transfected with E6 plus *neo*. Extracts of subconfluent cultures of G418-selected keratinocytes were immunoprecipitated with a rabbit polyclonal anti-HPV16 E7-specific serum, separated by SDS-15% PAGE, and transferred to an Immobilon P filter. The filter was probed with a mouse monoclonal antibody raised against an HPV16 E7 bacterial fusion protein. Molecular size markers are indicated on the left.

Therefore, the loss of biological activity for the p2PRO, p24GLY, and p31/32ARG/PRO mutants did not result from protein instability. However, much lower levels of E7 protein were detected in cells transfected with the mutants p26GLY and p58/91GLY/GLY, which suggests that these two mutant proteins may be unstable in vivo, so no specific conclusions can be drawn from the lack of biological activity of the mutants.

E2F-1 inhibits growth of keratinocytes and other cell types.

Since E7 disrupts the E2F-pRB complex (12, 42) and E2F-1 can stimulate DNA synthesis in fibroblasts by increasing E2F transcriptional activity (37), we sought to examine a possible role for E2F in immortalization of NHFKs by transfecting them with the cloned *E2F-1* gene. When NHFKs were cotransfected with pCMVE2F-1, which contains the *E2F-1* cDNA, under control of a strong CMV promoter, linked to the *neo-R* gene, and selected with G418, we reproducibly obtained the unexpected result that fewer G418-resistant colonies developed than from cells cotransfected with E7 plus *neo-R* or those that received *neo-R* alone (Fig. 2). The observed decrease in the number of G418 colonies generated probably resulted from the high levels of E2F-1 protein expressed from the CMV promoter-activated construct (Fig. 5). More G418-resistant colonies were obtained when less pCMVE2F-1 plasmid was used for the transfection or when the *E2F-1* gene was expressed from the less active retroviral long terminal repeat promoter (pE2F-1Mo; data not shown). For each set of transfections, parallel experiments in which equal amounts of pSV2neo were cotransfected with pCMVE2F-1 were done. No differences in the number of colonies after G418 selection were observed, whether pCMVE2F-1 was transfected alone or cotransfected with a *neo-R* gene contained in the pSV2neo plasmid. These experiments indicate that the reduced number of G418-resistant colonies is not simply due to *trans* repression of the *neo-R* transcription by E2F-1.

These results suggested that high-level expression of E2F-1 induced growth inhibition. It has been reported that E2F-1 induces apoptosis in murine fibroblasts in cooperation with wild-type p53 (57). Therefore, one possibility is that E2F-1 can also induce apoptosis in NHFKs. It is also possible that the growth inhibition is due to competition between E2F-1 and other, related transcription factors. Since the p53 gene has been implicated in apoptosis in several systems, and since inactivation of the p53 gene may overcome the apoptotic block (56), we cotransfected an HPV16 E6 gene or a dominant mutant p53 gene-expressing plasmid and pCMVE2F-1. HPV16 E6 protein induces rapid degradation of p53 in NHFKs (28). However, it was unable to overcome the inhibitory effects of the pCMVE2F-1 construct (Fig. 2). Similarly, the transdominant mutant p53 gene in clone pC53-SCX3, which is able to cooperate with E7 to immortalize NHFK, was unable to overcome the E2F-1 inhibition (data not shown). We also cotransfected E2F-1 and the neomycin resistance gene into C33A, an HPV-negative human cervical carcinoma-derived cell line which contains endogenous mutant p53. Colony formation was inhibited approximately 10-fold by E2F-1 (data not shown). Therefore, inactivation of p53 may not relieve the block induced by high-level expression of E2F-1.

As described above, the inability of the E7 mutants p2PRO and p31/32ARG/PRO to cooperate with E6 to immortalize the NHFKs, although they encode stable mutant proteins that bind pRB, suggested that the wild-type E7 protein provides a function(s) in addition to pRB binding when it participates in NHFK immortalization. To determine if this putative function(s) might be required to overcome the inhibitory effects of pCMVE2F-1, pCMVE2F-1 was cotransfected with wild-type HPV16 E7 plus HPV16 E6. Even this combination failed to

FIG. 4. Growth-inhibitory activity of E2F-1 mutants on NIH 3T3 cells and structure of the human E2F-1 protein. The functional domains of the human E2F-1 protein and the structures of the E2F-1 mutants are shown. aa 120 to 191 contain the DNA binding domain. The dimerization domain encompasses aa 191 to 284. The carboxy-terminal region contains the transactivation domain and the pRB binding domain. The locations of the point mutations in Y411C and E132 (arrows) are indicated. The properties of the E2F-1 mutants are summarized below. Their growth-inhibitory activity was determined in this study. A positive result (+) for growth inhibition means that there was at least a fivefold reduction in the number of G418-resistant colonies compared with the control pCMVneoBam plasmid, and a negative result (-) means less than a twofold reduction. The other properties of the mutants have been reported previously (24, 26, 27, 32, 51). n.d., not determined.

overcome the inhibitory effects of pCMVE2F-1 (Fig. 2). A reduction in the ability to form colonies after coselection, which is a phenomenon that also occurs after overexpression of other cell cycle regulatory genes (46), was also seen when several established lines and nonestablished strains, including REF, WI38, CV-1, BRK, 1634, and NIH 3T3, were tested by transfection of pCMVE2F-1 and selection in G418 (data not shown).

Genetic localization of E2F-1-mediated growth inhibition. Several functional domains have been identified within the wild-type, 437-aa E2F-1 protein (Fig. 4). These include a DNA binding domain, which has a helix-loop-helix structure (aa 120 to 191), a dimerization domain (aa 191 to 284), a transactivation domain (aa 284 to 417), and a pRB binding domain (aa 409 to 426). To localize the E2F-1 sequences that were responsible for growth inhibition, NIH 3T3 cells were transfected with a series of previously described E2F-1 mutants in plasmids that were isogenic with pCMVE2F-1 (Fig. 4). Selection for G418-resistant colonies indicated that E2F-1 mutant E132, which contains a point mutation in the DNA binding domain and fails to bind E2F binding sites, did not inhibit cell growth. The E2F-1 protein levels in cells transiently transfected with the E132 mutant were similar to those in cells transfected with the wild-type gene, as measured by sequential immunoprecipitation and immunoblotting (data not shown).

The other E2F-1 mutants tested retained the inhibitory activity. These mutants included a point mutant with a Y-to-C change at position 411 (Y411C), whose lesion abolishes pRB binding while leaving other functions intact, and three premature termination mutants, 1-417, 1-374, and 1-284. The latter two mutants lack pRB binding and transactivating activity, but all three retain DNA binding activity. The results suggest that sequence-specific DNA binding activity is necessary, and perhaps sufficient, for growth inhibition.

Complementation of E7 mutants by E2F-1. Although E2F-1 reduced the number of G418-resistant colonies in the various cell types tested, it remained formally possible that the protein might still contribute to immortalization of NHFKs. Indeed, the G418-resistant colonies that did develop appeared normal in size and in the morphology of the individual cells. However, the cells transfected with E2F-1 alone or with E2F-1 plus E6 senesced upon further passage (Table 2), suggesting that E2F-1 alone is unable to immortalize NHFKs and that it is unable to substitute for E7 in the immortalization assay.

Given these negative results, we sought to determine if E2F-1 might complement any of the defective E7 mutants in the immortalization assay. The most likely candidate was the E7 mutant p24GLY, which encodes a stable mutant protein but does not bind pRB. When E2F-1 was cotransfected with the p24GLY mutant and E6, low numbers of colonies were

TABLE 2. NHFK immortalization assay with E2F-1

Constructs transfected	No. of immortalized lines/no. of independent transfections
<i>neo</i>	0/4
E6 + E7wt.....	8/8
E2F-1.....	0/2
E6 + E2F-1	0/4
E7 + E2F-1	0/2
E6 + E7 + E2F-1	2/2
E6 + E7p2 + E2F-1	0/4
E6 + E7p24 + E2F-1	3/6
E6 + E7p26 + E2F-1	0/6
E6 + E7p31/32 + E2F-1	0/4

obtained, as expected (Fig. 2). Nevertheless, immortal cell lines did arise from these colonies (Table 2). To generate a rapidly growing culture, the transfectants required several more passages than the positive controls in which *E6* was cotransfected with wild-type *E7*. In a limited number of experiments, *E2F-1* was added together with *E6* plus wild-type *E7*, resulting in immortalized cells each time. This latter cotransfection was not studied further because immortalization of the transfectants could arise from cells that contained only the wild-type HPV genes, in contrast to the NHFKs cotransfected with the p24GLY mutant.

If the observed ability of *E2F-1* to complement the defect in the p24GLY mutant resulted from a specific replacement of an activity related to the pRB binding function of *E7* that is defective in this mutant protein, then *E2F-1* should fail to complement the p2PRO and p31/32ARG/PRO mutants, which bind wild-type levels of pRB in vitro, or the p26GLY mutant, which encodes an unstable *E7* protein. Results consistent with this hypothesis were obtained, since *E2F-1* was unable to immortalize cells cotransfected with any of these mutants plus *E6* (Table 2).

To verify that the *E2F-1* protein product was expressed in the transfected keratinocytes, pooled G418-resistant colonies were passaged until the control nonimmortal cells senesced, and immunoblot analysis of protein extracts from the immortalized cells was performed. In contrast to the large amount of *E2F-1* detected in transiently transfected cultures, despite the fact that only a minority of cells expressed the *E2F-1* gene (Fig. 5), the level of *E2F-1* in the immortalized lines was found to be only twofold higher than those in the *neo*-R-transfected pre-immortal cells or a line immortalized by *E6* plus *E7* (Fig. 6). These results support the notion that there was selection against high levels of *E2F-1* during establishment of the immortal lines. The level of the *E7* proteins in the cells expressing *E6* plus *E7* plus *E2F-1* was comparable to that of the control cells expressing *E6* plus *E7* (data not shown).

DISCUSSION

In this study, we have found that high levels of *E2F-1* protein inhibit cell growth, obtained evidence that DNA binding is required for this activity, and, through the use of HPV16 *E7* mutants, identified a role for low levels of *E2F-1* in keratinocyte immortalization.

Although most previous studies have emphasized the growth-stimulatory activities of *E2F*, we observed that transfection of *E2F-1* under control of the strong CMV promoter had an inhibitory effect on the efficiency of forming G418-resistant colonies in several nonestablished and established cell lines, including NHFKs. One possible explanation for the

FIG. 5. Detection of *E2F-1* protein in transiently transfected keratinocytes. *E2F-1*-transfected cells were compared with cells transfected with *neo*-R alone or with *neo*-R plus HPV16 *E7*. Keratinocytes were transfected and harvested 36 h later. Total proteins (100 μ g) were separated by SDS-10% PAGE and transferred to a nitrocellulose membrane. The proteins were visualized by chemiluminescence with a mouse monoclonal antibody specific for *E2F-1* (27). The CaSki cell line was used as a positive control for *E2F-1* expression. Molecular size markers are indicated on the left.

inhibition could be that *E2F-1*, when overexpressed, competes with the other endogenous *E2Fs* by titrating endogenous DP-1, a recently identified transcriptional partner for *E2F* (21). The *E2F-1* and DP-1 proteins form heterodimers that lead to cooperative DNA binding and transactivation of *E2F*-dependent promoters (6, 27).

However, genetic localization of the *E2F-1* sequences that mediate growth inhibition of NIH 3T3 cells did not support this hypothesis, since an *E2F-1* point mutant that retains its DP-1 binding activity but lacks DNA binding activity was unable to inhibit cell growth. If the *E2F-1*-mediated growth inhibition results from limiting amounts of DP-1, overexpression of DP-1 should be able to rescue the toxicity observed after *E2F-1* overexpression. However, in preliminary experiments in which *E2F-1* was cotransfected with DP-1 in NHFKs, no significant difference in the inhibitory activity of *E2F-1* (or in immortalization) has been noted (unpublished data).

A second possible explanation for the observed inhibition could be that high levels of the *E2F-1* protein titrate out an associated factor involved in other essential functions. Our finding that premature termination mutants of *E2F-1* that lack transactivation and pRB binding domains but retain the *E2F-1* DNA binding function were still competent for growth inhibition argues against a squelching-type mechanism of inhibition. Instead, the results of the mutational analysis indicate that the sequence-specific DNA binding activity of *E2F-1* is essential for this phenotype and suggest that the growth inhibition results from *E2F-1* competing with similar DNA binding by

FIG. 6. Detection of E2F-1 proteins in immortalized keratinocytes. Extracts from CaSki cells and E6 plus *neo*, E6 plus E7 wild type, and E6 plus E7p24 plus E2F-1 transfectants were prepared, and the E2F-1 proteins were detected by immunoblot as described in Materials and Methods. Molecular size markers are indicated on the left.

endogenous transcription factors, which may include other E2Fs.

Another hypothesis is that the E2F-1-mediated toxicity may result from the induction of apoptosis. This hypothesis is supported by recent reports showing that E2F-1 cooperates with p53 to induce apoptosis in murine fibroblasts and would be analogous to observations made with the *c-myc* proto-oncogene, whose overexpression is known to induce programmed cell death in certain cell types deprived of specific growth factors or of serum (56). HPV16 E6 or the mutant p53 gene was unable to reverse the growth-inhibitory effects of E2F-1, and E2F-1 also inhibited growth of C33A, a p53 mutant-containing cell line. Therefore, E2F-1-induced growth inhibition in our assays appears to be a p53-independent phenomenon, since the E6 protein inactivates endogenous p53 in NHFKs by targeting it for ubiquitin-mediated degradation (47), and the mutant p53 gene tested acts as a transdominant gene in cooperation with E7 to immortalize NHFKs (28, 50).

The requirement for HPV16 E6 and E7 for NHFK immortalization (22, 29, 39) suggests that these cells may be more resistant to immortalization than cell types that can be immortalized simply by inactivation of p53, which is a function supplied by E6. The results obtained with the HPV16 E7 mutants imply that E7 contributes at least two functions to NHFK immortalization. One is related to pRB binding of E7 (and/or the binding of some other cellular protein, such as p107 or p130, to the pRB binding site), while the other is independent of this activity.

Previous studies with fibroblasts have suggested that the ability of E7 to bind pRB is required for anchorage-independent growth of NIH 3T3 cells, transformation of REF or BRK cells in cooperation with an activated *ras* oncogene, and transactivation of the adenovirus E2 promoter (55). The data

also imply that regions of E7, in addition to the pRB binding domain, are required for immortalization. The mutants p2PRO and p31/32ARG/PRO were defective in our assay, despite having normal pRB binding activity in vitro and normal stability in vivo. Because of the low level of expression of the E7 protein in the human keratinocytes, we were unable to investigate the in vivo interaction of wild-type and mutant E7 proteins with pRB and p107. Therefore, we cannot formally rule out the possibility that reduced pRB binding by these two mutant proteins in vivo may be responsible for the loss of the immortalizing activity.

Although high levels of E2F-1 were growth inhibitory, we found that lower levels of E2F-1 can replace the pRB-associated function of E7. However, E2F-1, when transfected alone or in cooperation with E6, is not able to fully replace E7, although E2F-1 and E7 can each stimulate DNA synthesis in fibroblasts. The other function(s) of E7 may be required for the progression through the later stages of the cell cycle. The pRB-E2F complex is absent from most cervical carcinoma cell lines, supporting the idea that disruption of the pRB-E2F interaction, either through E7 or mutations in pRB, is an important step in cervical carcinogenesis (12, 42). E7 can also disrupt, in vitro and in vivo, complexes containing p107-E2F, although less efficiently than pRB-E2F (1, 12).

The ability of E2F-1 to complement the immortalization defect of the E7 mutant p24GLY, which does not bind pRB and has a reduced but detectable ability to bind p107 in vitro (7, 8, 16, 45), indicates that deregulated expression of E2F-1 may substitute for the pRB, and perhaps p107, binding activity of E7 and that activation of E2F-1 is one of the critical mechanisms through which E7 induces immortalization.

The failure of cotransfected E2F-1 to complement E7 mutants p2PRO and p31/32ARG/PRO argues for the specificity of its activity with the p24GLY mutant. The results also make it unlikely that changes in pRB binding in vivo for p2PRO and p31/32ARG/PRO account for their loss of biological activity or that these mutants are defective because they are unable to induce the release of E2F when they bind pRB.

In a previous report, it was shown that E7 p24GLY was able to immortalize human primary keratinocytes, with reduced efficiency, when it was transfected in the context of the full-length HPV16 genome (30). Together with the results reported here, this observation raises the possibility that the activity of another HPV gene may inefficiently substitute for the pRB binding function of E7. One possible candidate could be E5, which, for HPV16, has been shown to potentiate the activity of growth factor receptors (54). One possible outcome of increased downstream signaling from the activated growth factor receptors is activation of E2F.

The E7 p24 mutant retains a small amount of p107 binding activity in vitro (16), and this could be sufficient to activate the p107-specific E2F activity that is probably necessary for progression through later stages of the cell cycle. Alternatively, E2F-1 could substitute also for the p107 binding activity of E7, although, unlike pRB, p107 does not appear to interact with the E2F-1 protein but with a distinct E2F-like protein (17). Indeed, there are indications that pRB-E2F and p107-E2F complexes might regulate different target promoters. In NIH 3T3 cells, disruption of p107-E2F, but apparently not pRB-E2F, complexes by E7 appears to activate the transcription of the *B-myb* gene, which is necessary for cell growth and is potentially important in the regulation of the cell cycle (34). In contrast, it has been reported that activation of thymidine kinase gene expression requires disruption of the pRB-E2F complex but not the p107-E2F complex (41). It will be interesting to determine whether other recently identified E2F

genes (35) have the same activity as E2F-1 in the immortalization assay, which would suggest functional redundancy, or whether these clones do not complement the p24GLY mutant, which would suggest that they have distinct functions in promoting progression through the cell cycle.

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