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Adenovirus E1A, simian virus 40 tumor antigen, and human papillomavirus E7 protein share the capacity to disrupt the interaction between transcription factor E2F and the retinoblastoma gene product

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ABSTRACT **The adenovirus E1A gene product, the simian virus 40 large tumor antigen, and the human papillomavirus E7 protein share a short amino acid sequence that constitutes a domain required for the transforming activity of these proteins. These sequences are also required for these proteins to bind to the retinoblastoma gene product (pRb). Recent experiments have shown that E1A can dissociate complexes containing the transcription factor E2F bound to pRb, dependent on this conserved sequence element. We now show that the E7 protein and the simian virus 40 large tumor antigen can dissociate the E2F–pRb complex, dependent on this conserved sequence element. We also find that the E2F–pRb complex is absent in various human cervical carcinoma cell lines that either express the E7 protein or harbor an *RB1* mutation, suggesting that the loss of the E2F–pRb interaction may be an important aspect in human cervical carcinogenesis. We suggest that the ability of E1A, the simian virus 40 large tumor antigen, and E7 to dissociate the E2F–pRb complex may be a common activity of these viral proteins that has evolved to stimulate quiescent cells into a proliferating state so that viral replication can proceed efficiently. In circumstances in which a lytic infection does not proceed, the consequence of this action may be to initiate the oncogenic process in a manner analogous to the mutation of the *RB1* gene.**

Considerable attention has been focused on the so-called tumor suppressor genes, such as the retinoblastoma gene, and the roles they play in regulating cellular proliferation and differentiation and in carcinogenic progression. The *RB1* gene product (pRb), a 105-kDa phosphoprotein, appears to play a major role in controlling the growth of normal cells (1, 2), likely as a consequence of the cell-cycle-regulated phosphorylation/dephosphorylation of the protein (3–7). A fruitful approach to the study of *RB1* function has been the analysis of the interaction of certain viral oncoproteins with pRb. Initial studies demonstrated that the adenovirus E1A protein formed a complex with pRb and that this was dependent on E1A sequences that are required for cellular transformation (8). Subsequent studies demonstrated that the simian virus 40 (SV40) large tumor (T) antigen (9) and the human papillomavirus (HPV) E7 protein (10, 11) formed similar complexes with pRb, again dependent on sequences required for oncogenic activity. These observations suggested that the interaction of these viral proteins with pRb inactivated the function of pRb and was thus equivalent to mutation or loss of the *RB1* gene seen in retinoblastomas (12–14) and other human cancers (2, 13, 15–24).

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Recent experiments have provided evidence that the transcription factor E2F is a cellular target for pRb. Extracts of a variety of human cell lines have complexes containing pRb together with transcription factor E2F (25). Using a distinctly different approach, Livingston and colleagues (26) have demonstrated that pRb and cellular proteins that specifically associate with pRb can preferentially bind to DNA sequences that strongly resemble the E2F recognition site. Moreover, a previously identified activity that inhibits E2F DNA binding (27) has been shown to contain pRb (28). Another factor, termed DRTF1, which may be identical to or closely related to the transcription factor E2F, has also been found in association with pRb (29). The significance of the interaction of E2F with pRb, with respect to the role of pRb as a tumor suppressor, was suggested by several observations (25). (*i*) The E2F–pRb complex was not detected in cells expressing a mutant nonfunctional pRb. (*ii*) Analysis of pRb associated with E2F demonstrated that only the underphosphorylated form of pRb was preferentially complexed to E2F. Since underphosphorylated pRb is thought to be functional in growth suppression, this result suggests that the interaction with E2F is functionally important. (*iii*) The adenovirus E1A protein dissociates the E2F–pRb complex, dependent on the E1A sequence that is also important for E1A to function as an oncogene. Thus these results strongly suggest that the interaction of pRb with E2F may be part of the action of pRb to control cell growth.

The sequences in the E1A protein that are essential for dissociation of the E2F–pRb complex and that are also part of the pRb binding site show significant homology to HPV E7 and SV40 T antigen (9, 10, 30, 31) (Fig. 1*B*). Recent experiments have shown that E7 can target E2F, as indicated by the importance of the E2F sites in the E2 promoter for E7-dependent trans-activation (38). Furthermore, the E7 protein can prevent the formation of E2F complexes *in vitro* and can block the activity of a partially purified fraction that inhibits E2F DNA binding (38). We now find that E7 and SV40 T antigen can dissociate the E2F–pRb interaction, suggesting that these distinct viruses have evolved a common activity.

MATERIALS AND METHODS

Cells. Growth of the U937 cell line and the HeLa cell line has been described (25). Details of the human cervical carcinoma cell lines have been described (39). The WI-38 cell line and the SV40-transformed VA13 cell line were obtained

Abbreviations: SV40, simian virus 40; T, tumor; HPV, human papillomavirus; pRb, retinoblastoma gene product; GST, glutathione *S*-transferase.

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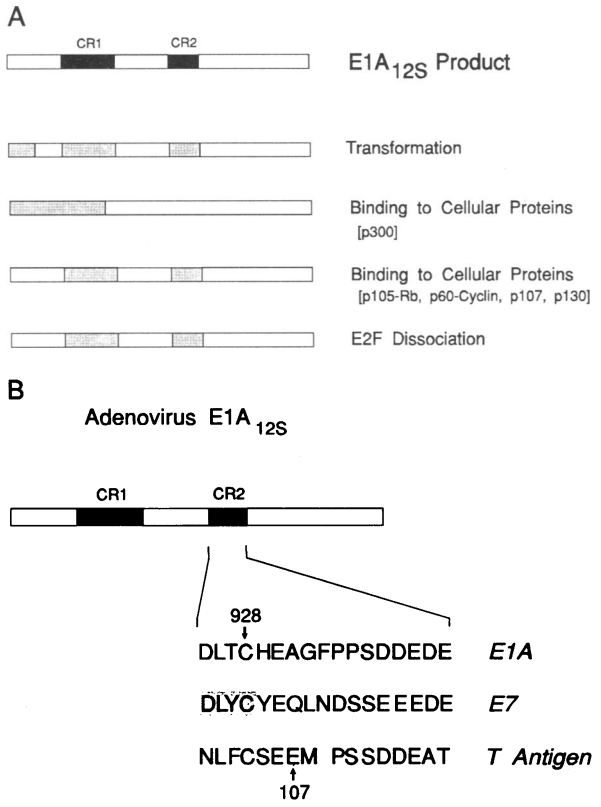

FIG. 1. Relationship between the HPV E7 protein, SV40 T antigen, and adenovirus E1A. (*A*) Functional domains of the adenovirus $E1A_{12S}$ product. The two regions of conserved sequence (CR1 and CR2) (31) are depicted at the top. Shown below are segments (shaded) of the protein that have been shown to be important for transforming activity (32–36, 55, 56), binding to a variety of cellular proteins (34, 36, 57, 58), and dissociation of E2F-containing complexes (27, 37). (*B*) Sequence relationship of E1A, E7, and T antigen. The sequence in the CR2 region of the E1A protein that is involved in pRb binding and that is conserved in the HPV E7 protein and SV40 T antigen is presented. In addition, the position of the E1A 928 mutation in CR2 is indicated and the position of the ΔDLYC mutation of E7 and the T antigen K1 mutation at position 107.

from the American Type Culture Collection. All cells were maintained in Dulbecco's Modified Eagle's medium with 10% (vol/vol) fetal calf serum unless otherwise indicated.

Preparation of Cell Extracts. Preparation of whole cell extracts and assays for E2F, either by gel retardation or by immunoprecipitation, have been described (25).

Isolation of Glutathionine *S*-Transferase (GST) Fusion Proteins. The construction of appropriate expression plasmids and the production and isolation of GST–E7 fusion proteins have been described (38). The SV40 T antigen constructs were derived from the plasmid pSKSVTc, containing the T antigen cDNA (a gift from P. Tegtmeyer, State University of New York, Stony Brook). The T_{k1} point mutant was created by site-directed M13 mutagenesis resulting in a Glu → Lys change at amino acid 107. The GST constructs pGST-T_{wt} and pGST-T_{k1} were constructed by the PCR. Each was cloned into pGEX3 as *Bam*HI–*Eco*RI fragments.

RESULTS

The HPV E7 Product Dissociates the E2F–pRb Complex. Extracts of the human monocytic cell line U937 and several other human cell lines contain an E2F–pRb complex that can be dissociated by the adenovirus E1A protein, dependent on E1A sequence that has been shown to be involved in binding to pRb (25). By using these U937 extracts, the HPV E7 protein was also tested for its ability to dissociate the E2F–pRb complex. The U937 extract contains two specific E2F complexes (Fig. 2). Previous assays have shown that the slow-migrating complex contains the cyclin A protein in association with E2F whereas the fast-migrating complex contains pRb in association with E2F (25). The band migrating between the E2F–cyclin A complex and the E2F–pRb complex appears to result from a nonspecific interaction. Addition of the control GST protein to the extract did not alter either of the E2F complexes. In contrast, addition of the wild-type E7–GST fusion protein eliminated the E2F–pRb complex. Addition of the ΔDLYC mutant of E7, which deletes critical amino acid sequence involved in pRb binding (Fig. 1*B*), did not dissociate the E2F–pRb complex. These data, therefore, demonstrate that the E7 protein can dissociate the E2F–pRb complex, dependent on the E7 domain known to be involved in pRb binding (11). It is also evident from the assay in Fig. 2*A* that the E7 protein had only a modest effect on the E2F–cyclin A complex, in contrast to the efficient dissociation of the E2F–pRb complex. Whether this is an intrinsic property of the E7 protein or reflects a limitation of the GST fusion protein and the *in vitro* assay is not clear at this time.

T Antigen Dissociates the E2F–pRb Complex. The E1A sequences that are important for the dissociation of the E2F–pRb complex are those that are also shared with SV40 T antigen and that are involved in binding to pRb (Fig. 1). Given the similarities between E1A and T antigen, including the fact that the T antigen also trans-activates the adenovirus E2 promoter (40) dependent on the E2F sites (41), we have assayed the ability of T antigen to dissociate the complexes containing the transcription factor E2F.

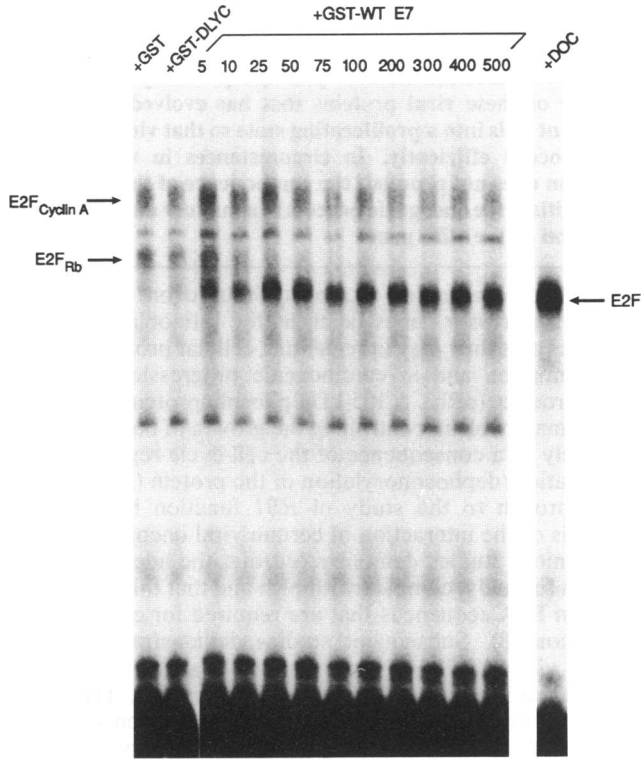

FIG. 2. HPV E7 protein can dissociate the E2F–pRb complex. Extracts of U937 cells were assayed for E2F binding activity as described (25). The binding assays included a control GST protein (500 ng), the ΔDLYC deletion mutant GST–DLYC (500 ng), or increasing amounts (in ng) of the wild-type E7 protein GST–WT E7 (38). In addition, a sample was also incubated with deoxycholate (DOC) prior to the assay. The positions of the E2F–cyclin A complex, the E2F–pRb complex, and free E2F are indicated.

Once again, extracts of human U937 cells served as the source of E2F for these assays. As shown in Fig. 3, addition of the wild-type GST–T antigen fusion protein (T_{wt}) to the U937 extract resulted in the dissociation of the E2F–pRb complex, with a concomitant increase in free E2F. The ability of T antigen to dissociate the E2F–pRb complex was clearly dependent on the T-antigen pRb binding domain, as indicated by the failure of the T_{K1} mutant protein, a mutation that abolishes the ability of T antigen to bind to pRb (9), to dissociate the complex. It was also evident from this experiment that whereas the E2F–pRb complex was effectively eliminated by the addition of the T_{WT} protein, there was little effect on the E2F–cyclin A complex, a result similar to that obtained with the E7 protein.

Absence of the E2F–pRb Complex in Human Cervical Carcinoma Cell Lines. A recent study demonstrated that HPV-positive cervical cancer cells expressing E7 possessed a normal wild-type pRb (42). In contrast, the HPV-negative cervical carcinoma cell lines that did not express E7 contained a mutant pRb, thus providing strong evidence for the role of pRb inactivation, either by mutation or through its association with E7, in the genesis of these tumors (Table 1). If regulation of the transcription factor E2F is a normal function of pRb, then one might expect to find disruptions of E2F–pRb complexes in these carcinoma cell lines.

As shown in Fig. 4*A*, the E2F–cyclin A complex was detectable in each extract with the exception of HeLa cells in which it was greatly reduced. The E2F–pRb complex was absent from each of the samples with the exception of the SiHa extract where there appeared to be a significant amount of the complex, equal to that found in the U937 extract. This may reflect the low level of E7 expression in SiHa cells. It is also evident from these assays that there are significant amounts of free E2F in each of the cervical carcinoma cell extracts. Indeed, it is clear that in most cases the majority of E2F is uncomplexed. In addition to the ability of E7 to dissociate E2F from the E2F–pRb complex as demonstrated in Fig. 2, this appearance of free E2F may also reflect the ability of E7 to block the action of an inhibitor of E2F DNA binding (38), which also appears to involve pRb (28). From these results, we conclude that the E2F–pRb interaction is

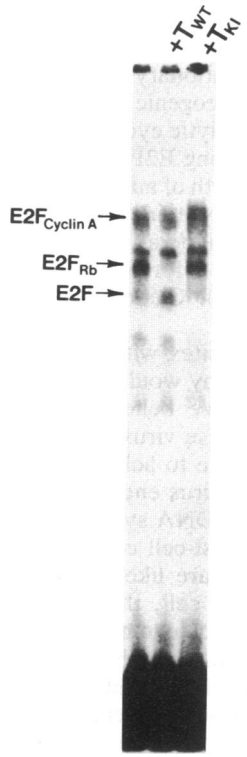

FIG. 3. T antigen dissociates the E2F–pRb complex. A U937 cell extract was assayed for E2F binding activity after incubation with the T_{WT} fusion protein or the T_{K1} fusion protein. An additional sample of the extract was incubated without any addition. E2F binding was measured by gel retardation.

Table 1. Relationship of HPV E7 expression and the state of the *RB1* gene in human cervical carcinoma cell lines

Cell line	HPV DNA/RNA*	*Rb* gene†
HeLa	Yes	Wild type
C4-II	Yes	Wild type
SiHa	Yes	Wild type
CaSki	Yes	Wild type
ME180	Yes	Wild type
C-33A	No	Mutant
HT-3	No	Mutant

*Data are from Yee *et al.* (43) and Reuter *et al.* (44).
†Data are from Scheffner *et al.* (42).

specifically lost in those cells that express an altered form of pRb or in those cells that express high levels of the HPV E7 protein. Coincident with the loss of the E2F–pRb complex is the increase in levels of free E2F.

These results were further verified by E2F coprecipitation experiments using antibodies specific for cyclin A or pRb. Previous experiments have shown that E2F is coimmunoprecipitated with pRb from extracts of cells that express a normal wild-type pRb (25). E2F is also coprecipitated with the cyclin A protein, reflecting the interaction of E2F with cyclin A during S phase of the cell cycle (45). Examples of such assays are shown in Fig. 4*B*. E2F is clearly detected in immunoprecipitates employing either the pRb antibody or the cyclin A antibody and a U937 cell extract. In contrast, there was no evidence of the coimmunoprecipitation of E2F with pRb from extracts of C-33A and HT3 cells, both of which lack a functional pRb. E2F was, however, readily detected in cyclin A immunoprecipitates using these same extracts. Assays for the E2F–pRb complex in the cervical carcinoma cells that possess a normal pRb but express HPV E7 demonstrated a general lack of the E2F–pRb complex. The one exception was the SiHa cell extract in which E2F was recovered in the pRb-specific immunoprecipitate, although the amount was reduced in comparison to that obtained from the U937 extract. Once again, immunoprecipitation of these same extracts with a cyclin A antiserum demonstrated that the E2F–cyclin A interaction persisted in most of the cells, although in some cases the level was reduced (for instance, the HeLa cell sample). Therefore, assays for E2F in the cervical carcinoma extracts, either by direct gel retardation or by coimmunoprecipitation, demonstrate a general loss of the interaction with pRb, variable amounts of the cyclin A complex, and a uniform increase in the level of free E2F.

Reduced Levels of the E2F–pRb and E2F–Cyclin A Complexes in an SV40-Transformed Cell Line. The experiment presented in Fig. 3 demonstrates that T antigen possesses the capacity to dissociate the E2F–pRb complex. To assess the potential *in vivo* relevance of this activity, we have examined the state of E2F in human cells that express T antigen as compared to a control cell line. The VA13 cell line was derived by transformation of the WI-38 cell line with SV40 and expresses T antigen (39). Whole-cell extracts were prepared from each cell line and assayed for E2F binding activity by gel retardation. As shown in Fig. 4*C*, the E2F–cyclin A complex, the E2F–pRb complex, and some free E2F were evident in the WI-38 extract. As in the past, the identity of the complexes was confirmed by the addition of antibodies specific for either cyclin A or pRb (data not shown). In contrast to the pattern observed with the WI-38 extract, an assay of the VA13 cell extract exhibited a much reduced level of the E2F–cyclin A complex and of the E2F–pRb complex but an increased level of the free E2F. In considering this result with the analysis of the cervical carcinoma cell lines, the common finding is the loss of the E2F–pRb interaction and an increase in the amount of free E2F. The absence of the E2F–cyclin A complex is in contrast to the failure of T antigen

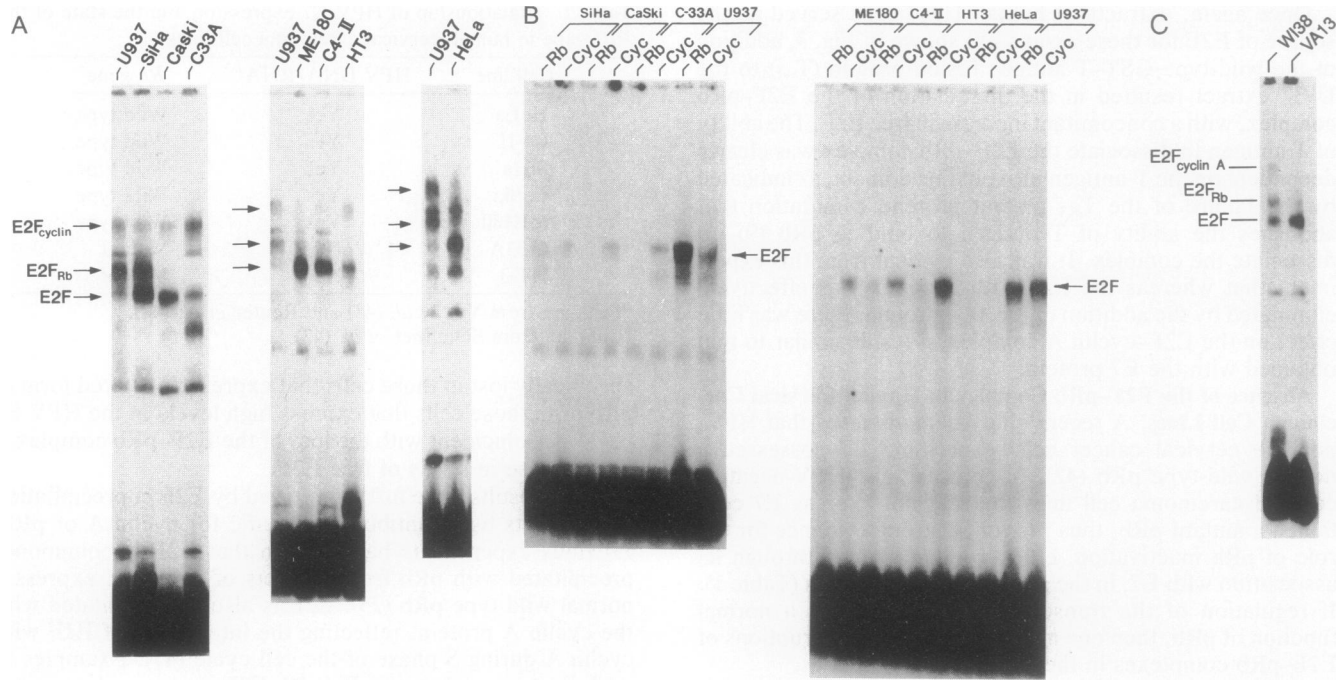

FIG. 4. E2F–pRb complex is reduced or absent from a variety of human cervical carcinoma cell lines and SV40-transformed cells. (*A*) Absence of E2F–pRb complex in human cervical carcinoma cell lines as shown by gel retardation assays. Whole-cell extracts were prepared from each of the indicated cell lines as described (25). The various cell lines were described (42). Extracts were prepared from growing cultures. Cell extracts were assayed for E2F binding activity as described (25). The three panels represent three assays, and in each case, the U937 sample was used as the basis for comparison. The strong band migrating just below the E2F complex in the C-33A sample and in the U937 and HeLa samples to the right appears to be a degradation product of E2F. (*B*) Cell extracts were incubated with either the cyclin A antiserum (Cyc) or the pRb monoclonal antibody (Rb) as described previously (25, 45). Immunoprecipitates were washed and incubated with deoxycholate to release E2F, and then E2F binding activity was measured in the supernatant (25). (*C*) Absence of the E2F–pRb complex in a T-antigen-transformed cell line. Whole-cell extracts were prepared from two human diploid lung fibroblast cell lines: WI-38 and the SV40-transformed WI-38 derivative WI-38 VA13 subline 2RA (39). E2F was assayed in the extracts by gel retardation.

to dissociate the complex *in vitro*. The reason for this difference is not clear although we suspect that it may reflect a reduced ability of T antigen to target this complex, which may then be magnified in the *in vitro* assay. Although this result does not constitute proof that the change in the E2F pattern in the VA13 cell line is a consequence of T antigen expression, this conclusion is, nevertheless, consistent with the ability of T antigen to dissociate the E2F–pRb complex in the *in vitro* assay.

DISCUSSION

Previous experiments have shown that the transcription factor E2F is in a physical complex with pRb (25, 26, 29) and that the E1A protein can disrupt this complex, dependent on E1A domains that are also required for oncogenic activity and trans-activation of the E2 promoter (27). The results presented here demonstrate that the HPV E7 protein and SV40 T antigen can also disrupt the E2F–pRb complex, dependent on the domain in each of these proteins that is homologous to conserved region 2 of adenovirus E1A.

A series of previous experiments have shown that although the adenovirus $E1A_{12S}$ product and SV40 T antigen are distinct proteins, and derive from evolutionarily distinct viruses, the two proteins do share certain similarities that are also shared with the HPV E7 protein. All three proteins possess transforming activity. The ability of E1A, T antigen, or E7 to bind to pRb is dependent on a sequence that is also necessary for oncogenic activity. Each protein functions as a transcriptional trans-activator of the adenovirus E2 promoter, dependent on the E2F sites in the promoter (27, 37, 38, 40, 41). Moreover, previous experiments have shown that trans-activation by E1A (27, 37) or E7 (38) was dependent on the pRb binding domain, and preliminary assays suggest that the same is true for T antigen-mediated activation of the E2 promoter [V.B.K., unpublished results]. We believe that the demonstration that T antigen and E7 can dissociate the E2F–pRb complex, as demonstrated originally for E1A (25, 37), may represent a biochemical basis for these similarities.

In considering that these otherwise unrelated viruses have evolved an apparently identical activity, we are reminded that the lytic function of these viral proteins is their normal function and thus the basis for evolutionary conservation. Although each protein possesses oncogenic activity, it is surely the role they play in the normal lytic cycle that is most relevant. The role of E1A in dissociating E2F from the pRb complex is clearly relevant to the growth of adenovirus. This dissociation allows the adenovirus E4 protein to interact with E2F, forming a complex that binds cooperatively to the E2 promoter (37). A very stable complex is formed resulting in a stimulation of transcription (49–51). Clearly, there is a direct benefit to the virus.

In contrast, we know of no E2F sites within the SV40 genome or the HPV genome. Thus, why would T antigen or E7 target the E2F–pRb complex? We believe the likely answer lies in the observation that these viruses must stimulate cells to enter a proliferative state to achieve efficient viral replication (46). Although each virus encodes proteins that allow for certain aspects of viral DNA synthesis, there remains a requirement for various host-cell components of DNA synthesis. Since these viruses are likely to infect a quiescent cell rather than a dividing cell, the virus must stimulate the cell to enter S phase to achieve maximal DNA replication. In this context, it is possible that E2F may participate in transcription of genes that facilitate S-phase events. A group of cellular genes whose products are important for DNA synthesis, including dihydrofolate reductase,

thymidine kinase, and DNA polymerase α, contain promoters with E2F sites (47, 48, 52, 53). Indeed, at least for the dihydrofolate reductase promoter, the E2F sites are critical for transcriptional activity (48).

Thus, we suggest a model whereby viral oncogenes such as T antigen, E1A, and E7 eliminate control of E2F by liberating E2F from various complexes. Recent studies suggest that the active form of E2F is not complexed to other factors such as pRb or cyclin A (45, 54). The released E2F would then be available to participate in transcriptional activation of cellular genes. Herein may lie an explanation for the sequence and functional similarity of the viral immediate early genes E1A, T antigen, and E7. We propose that this shared ability has evolved to achieve a common goal of these viruses, namely, the stimulation of efficient S phase events. In this context it is clear to see how each of these proteins could function as oncogenes in the absence of a lytic infection. But the role they play in a lytic infection must be the basis for the common evolution.

Finally, perhaps the most significant observation from these studies may be the finding that the E2F–pRb complex is absent from most of the cervical carcinoma cell lines that express E7, thus providing *in vivo* evidence for the action of E7 to disrupt E2F–pRb. Moreover, the analysis of the human cervical carcinoma cell lines described here supports the hypothesis that the disruption of the E2F–pRb complex, either through the action of the HPV E7 protein or through mutation of pRb, is an important event in the genesis of human cervical cancer.

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