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Research Article

Role of the Retinoblastoma Gene in the Initiation and Progression of Human Cancer

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Retinoblastoma, although the most common malignant ocular tumor in childhood, is still a rare human malignancy. Nevertheless, the characterization of the molecular steps involved in the development of this cancer and the isolation of the gene responsible for tumor initiation have provided fundamentally important knowledge regarding a class of human cancer genes which have been defined as tumor "suppressor" or "regulatory" genes by us (1) and "antioncogenes" by others (2). Whatever the terminology one wishes to use, the more important issue is that the retinoblastoma (Rb)[1] gene is representative of a class of cancer genes in which the functional loss of both alleles is critical for tumor formation. In addition, recent evidence suggests that the loss of Rb gene function is not only causally related to the development of retinoblastoma and tumors that occur as second cancers in patients with the hereditary form of retinoblastoma, but also is a key factor in the initiation and/or progression of several of the most common human malignancies including those of lung, breast, and bladder.

As early as 1971 it was postulated that two "mutational" changes were sufficient for the development of retinoblastoma (3). This hypothesis was based primarily upon statistical data indicating that individuals with the hereditary form of retinoblastoma developed tumors earlier than those with nonhereditary disease (3). It was also known that a small portion of individuals with the hereditary form of retinoblastoma had a deletion within chromosome 13 in their constitutional cells, but only in the late 1970s was the common region of deletion in these patients localized to 13q14 by high-resolution chromosome mapping (4). It was not until 1983, however, that data were obtained to enable the conclusion to be reached that the same Rb locus was likely responsible for all forms of retinoblastoma (5).

During the time these studies were ongoing, other findings at both the cytogenetic and molecular biological level strongly suggested that the loss of both Rb alleles was responsible for tumor formation (1, 6–9). This included the fact that there were a significant number of Rb patients who did not have the hereditary deletion form of the disease but in whom a total loss of a 13 chromosome or a deletion including chromosomal region 13q14 was documented in their tumors (6, 7). In addition, highly significant studies using restriction fragment length polymorphisms (RFLPs) located on chromosome 13 indicated that there was a loss of heterozygosity for chromosome 13 in these tumors whether or not there was a loss of a chromosome 13 discernible by cytogenetic analysis (8, 9). Following up on these initial findings, it was possible to show that as many as 75% of the cases in which we were not able to identify chromosome 13 abnormalities in their tumors became homozygous for a portion of chromosome 13 when compared to the heterozygous state of these same chromosome 13 fragments in their constitutional cells (10). Consequently, the second "mutational" event previously postulated as being involved in the development of retinoblastoma (3) appeared to be a gross abnormality of chromosome 13 including loss of the entire 13 chromosome or a mitotic recombination resulting in the loss of the normal Rb allele rather than being a point or frameshift mutation.

Individuals who inherit a mutated Rb allele also develop a significant number of second malignancies of which osteosarcomas and soft tissue sarcomas are the most frequent (11). After the cloning of the putative Rb gene (12–14), the functional loss of the Rb gene was reported in a number of osteosarcomas (12, 14, 15–18) and certain soft tissue sarcomas (16, 19, 20). Moreover, similar changes have been found in tumors such as adenocarcinoma of the breast (21–22), small cell lung carcinoma (SCLC) (23, 24) and bladder carcinoma (25). These later malignancies are not usually associated with a previous history of retinoblastoma, although a higher incidence of lung and bladder cancer has been suggested to occur in relatives of retinoblastoma patients who carried the Rb mutation or in possible Rb carriers (26). Nevertheless, it is unlikely that the loss of the Rb gene is sufficient for tumor development in any of these tumors with the exception perhaps of retinoblastoma. Consequently, one of the critical questions still remaining is whether the changes reported within the Rb gene in different cancers are causally related to the initiation of the tumor or whether they are involved in tumor progression. Therefore, one of the primary goals of this review article will be to discuss not only the abnormalities observed within the Rb gene in retinoblastoma and the tumor types that often occur as second malignancies in patients with hereditary retinoblastoma, but also to comment upon the possible importance of Rb gene loss in other common human cancers. Since we believe it could be of fundamental importance at both the basic and clinical level to know whether the loss of Rb function is associated with the initiation rather than the progression of a specific tumor, the

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1. *Abbreviations used in this paper:* Rb, retinoblastoma; RFLP, restriction fragment length polymorphism; SCLC, small cell lung carcinoma.

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use of immunohistochemical staining to distinguish between these two possibilities will be another major focus.

Rb gene abnormalities in retinoblastoma and second malignancies

The initial report of a total structural loss within a DNA sequence located near the Rb locus used a DNA probe, H3-8, (27) which had been obtained from a chromosome 13 library and mapped to the same region as the Rb gene, namely 13q14. Two retinoblastomas were identified that were missing genetic sequences corresponding to the H3-8 probe (28). These results provided important evidence that not only was the loss of genetic material likely to be involved in retinoblastoma development but also suggested that the H3-8 DNA sequence was near or could even be within the Rb gene itself. In fact, the H3-8 probe has been used as the initial starting point for all three groups who were successful in cloning the putative Rb gene (12–14). Each group used chromosomal walking techniques to identify a cDNA sequence coding for a 4.7-kb mRNA which represented the Rb transcript (12–14).

After the cDNA for the putative Rb gene was obtained, it was divided into a 3.8- and 0.9-kb probe which identified the 3′ and 5′ portions of the Rb gene as well as its transcript (12–14). In the first report on the cloning of the putative Rb gene, retinoblastomas were described which either were missing all the Rb exon fragments or the 3′ portion of the putative Rb gene (12). In addition, a case of a possible hemizygous internal deletion of one Rb allele was presented (12). Another group of researchers noted several retinoblastomas which had no Rb transcript or a truncated transcript, although they found no gross structural abnormalities in the Rb gene using the probes that were available (13). In our initial report of the cloning of the putative Rb gene, we were able to document two cases of retinoblastoma which had homozygous internal deletions within the gene indicating that the gene which had been isolated was indeed the authentic Rb gene (14). In this study 16 of 40 retinoblastomas were found to have structural changes within the Rb gene, and among those tumors without detectable structural changes there was either an absence or truncation of the Rb transcript (14). Subsequently, point mutations of the Rb gene in retinoblastomas have been found using different techniques including amplification of specific portions of the Rb gene followed by DNA sequencing of this amplified region (29, 30) or by the use of the ribonuclease protection assay (31).

The Rb gene also has been transferred into an Rb-negative retinoblastoma cell line (WERI-27) by infection of a defective retrovirus containing the full-length Rb cDNA insert. The recipient retinoblastoma cells were subsequently shown to produce a normal Rb protein with a concomitant decrease in growth rate of the cultured cells to approximately one-third that of WERI-27 cells which were infected with the retrovirus which did not contain the Rb cDNA (32). In addition, 4 wk after infection cells appeared which were enlarged and these cells increased in number over the next 4 wk (32). The enlarged cells disappeared during the subsequent 2 or 4 wk in culture which was correlated with a loss of detectable Rb protein and an increased growth rate in the mass population (32). These results suggested that a restoration of the Rb protein produced morphological changes and significantly inhibited cell growth. We have also recently observed that the transfer of a normal chromosome 13 by microcell fusion into the retinoblastoma cell line, Y-79, resulted in the production of normal Rb protein, the appearance of enlarged cells, and the inhibition of growth rate to a marked extent (Benedict, W. F., A. Banerjee, E. J. Stanbridge, S.-X. Hu, H. J. Xu, manuscript submitted for publication). Finally, Rb-positive WERI-27 cells were nontumorigenic when injected into *nude* mice compared to their Rb-negative parental cells which provided formal proof that the cloned gene was the actual Rb gene (32). We also found that Rb-positive Y-79 were nontumorigenic in *nude* mice (Benedict et al., manuscript submitted for publication).

Since osteosarcomas are the most common second malignancies seen in patients with the hereditary form of retinoblastoma, it was logical to look for chromosome 13 changes in these tumors as well. The first paper to study this possibility used RFLPs located on chromosome 13 to examine whether a reduction to homozygosity occurred for chromosome 13 in osteosarcoma as had been seen in retinoblastoma. It was found that indeed there was a change from heterozygosity in constitutional cells to homozygosity in the osteosarcomas for several chromosome 13 DNA fragments, whether or not the tumors developed in patients who previously had retinoblastoma (33). In addition, in two of the first three reports on the isolation of the Rb gene, osteosarcomas were identified in which homozygous internal deletions of the Rb gene had occurred (12, 14). The first case was an individual who had no previous history of retinoblastoma (12) whereas the second case was a patient who had retinoblastoma (14). These results provided the scientific evidence that loss of Rb function was an important factor in the development of certain osteosarcomas. Subsequently, many additional reports have found abnormalities of the Rb gene in osteosarcomas (15–18).

Other tumor types frequently seen as second malignancies in patients with the hereditary form of retinoblastoma, namely specific soft tissue sarcomas, have been reported to have Rb mutations. This includes a total Rb deletion in a leiomyosarcoma and three malignant fibrous histiocytomas (16, 19) as well as 5′ deletions in an undifferentiated sarcoma, a lymphosarcoma, and an unclassified sarcoma (16, 19). An internal deletion also has been reported in a fibrosarcoma cell line (20). Therefore, similar to osteosarcoma, loss of function of the Rb gene may be critical to the initiation and/or progression of specific types of soft tissue sarcomas or subgroups within these tumor types.

Tumors usually not associated with the predisposition to develop retinoblastoma have Rb gene changes

Although the tumors described above have provided important information concerning the retinoblastoma gene which in turn may be critical to understanding essential mechanisms involved in the development of human malignancies in general, they do not represent particularly common human cancers. More recently, several common malignancies including those of the breast, lung, and bladder have been reported to have Rb mutations. The first study of Rb changes in tumors usually not associated with the development of retinoblastoma was that of SCLC. Structural changes in the Rb gene were seen in a primary SCLC and four SCLC cell lines as well as a pulmonary carcinoid cell line (23). Moreover, the absence of Rb transcripts were noted in 60% of the SCLC lines as well as 75% of the pulmonary carcinoid cell lines (23). Of particular importance was the fact that 5′ and 3′ structural abnormalities

as well as internal deletions within the Rb gene were documented (23). In a separate paper by other investigators, all the SCLC cell lines examined were shown to have an absence of the Rb protein (24). These studies provided the basis for concluding that the loss of Rb function is important in the development of SCLC.

In the reports on SCLC mentioned above, a large cell carcinoma of the lung and an adenocarcinoma of the lung were also found to have no Rb protein (24). In addition, the loss of Rb mRNA was documented in another large cell carcinoma and an adenocarcinoma of the lung (23). Although these data were obtained on cell lines only, it provided the initial data suggesting that the Rb gene could be involved in more common lung cancers than SCLC. This appears to be the case based on a collaborative study we have recently completed with Drs. Peter Reissman and Dennis Slamon and the Lung Cancer Study Group. Out of 160 primary non-SCLCs, 18 were found to have abnormal Rb mRNA expression (Reissman, P. T., R. Takahashi, W. F. Benedict, and D. J. Slamon, manuscript submitted for publication). Further studies are necessary to determine what the actual incidence of Rb loss is in common lung cancers. This analysis will likely be done at the protein level in which single tumor cells in primary tumor will be analyzed as discussed below. However, these results already indicate that the Rb gene is involved in a considerable percentage of lung cancers. It should also be mentioned again that although lung malignancies have not been associated with the development of retinoblastoma, relatives of patients with hereditary retinoblastoma were reported recently to have a statistically significant higher incidence of lung cancer (26).

The breast is also the most frequent sight of malignancy for females in a large number of countries. Therefore, the finding that 25% of the breast tumor cell lines initially examined as well as 7% of primary tumors had Rb abnormalities at the DNA level could be particularly significant (21). Such structural changes included homozygous internal or total deletions resulting in either an absence or truncation of the Rb transcript (21). Breast cancer cell lines examined at the Rb protein level have also been found to be missing Rb protein (22). However, in another collaborative study with Drs. Reissman and Slamon, we could detect structural abnormalities within the Rb gene in only one of over 100 randomly chosen primary breast tumors (Reissman, P. T., R. Takahashi, W. F. Benedict, and D. J. Slamon, manuscript in preparation). The fact that we were unable to document additional tumors with Rb changes could be related to the fact that the normal stromal tissue is frequently found in considerable abundance in such tumor specimens. Another possibility is that the Rb gene is important in the progression of the disease rather than its initiation and therefore a mixed population of tumor cells could be present, some which have normal Rb expression and others which do not. In this latter case, although tumor cells would be present which lost Rb function, they might not be detected by DNA or RNA blot analysis of the tumor tissue.

Bladder cancer is another common malignancy in which Rb gene loss may participate. Although this cancer has not been among those tumors frequently associated with the development of retinoblastoma, it has also been reported that relatives of retinoblastoma patients have a higher incidence of this malignancy than the normal population (26). Therefore, it may not be unexpected that not only have changes within the Rb gene been recorded in bladder cancer cell lines (25), but they may be as frequent in primary bladder cancer (T'Ang, A., et al., manuscript in preparation) as has been reported for breast cancer (21). As additional human malignancies are examined for the status of the Rb gene, the likelihood is high that at least some of these tumors will be found to have lost Rb function. It will then be critical to determine whether this change is etiologically related to the development of this specific cancer, i.e., initiation or whether it is important instead in the progression of the disease. It is our hope that the approach that we shall now discuss will enable us to distinguish between these two possibilities.

Is the loss of Rb function related to initiation or progression of specific cancers?

It is obvious that Rb mutations resulting in the loss of Rb function are important changes in the development of many human malignancies. Although it is likely that these mutations play a role in the initiation of retinoblastoma and other tumors that develop as second malignancies in individuals with the hereditary form of retinoblastoma, the potentially more critical issue is to determine the significance of these mutations in some of the more common human malignancies, including those of the lung and breast. The key issue to us is whether the

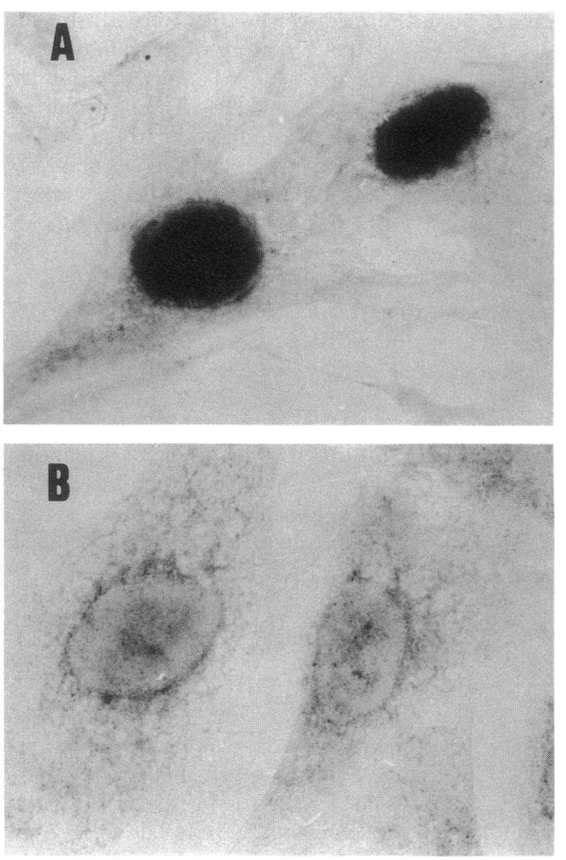

Figure 1. Staining of the Rb protein at the single-cell level. Positive Rb nuclear staining is seen for the normal human fibroblasts, WI-38 (*A*), whereas no nuclear staining is present in the fibrosarcoma cells, Hs 913T (*B*), which has a large homozygous deletion within the Rb gene (20) resulting in the loss of Rb transcripts and Rb gene products. The Rb antibody, WL-1 (18), and a second antibody conjugated with alkaline phosphatase were used for the immunostaining procedures.

loss of Rb gene function is related to its role in the initiation of a tumor vs. the possibility that such loss is associated with tumor progression. We believe that the most reasonable approach to distinguish between these two possibilities may be to examine the status of the Rb protein at the single-cell level in each tumor type. This will require the use of immunohistochemical methods which we believe are now available to perform such studies.

One of the reagents likely to be successfully used for this analysis is the purified high-affinity Rb polyclonal antibody that we have recently described (18). This Rb antibody first allowed us to study the normal Rb protein pattern using both immunoprecipitation and Western immunoblotting (18). Subsequently, we have been able to utilize these antibodies not only to confirm the nuclear staining of the Rb protein (34), but also to extend the findings of previous publications. A typical staining pattern found in Rb-positive cells compared to Rb-negative cells from the same embryonic origin is illustrated in Fig. 1. Clear nuclear staining is seen in the Rb-positive normal WI-38 fibroblasts (Fig. 1*A*), whereas an absence of nuclear staining is apparent in fibrosarcoma cells in which the Rb protein is missing (Fig. 1*B*). More recently, techniques have been developed using these same Rb antibodies to document the presence of the Rb protein at the single cell level in frozen tumor tissue (Xu, H.-J., S.-X. Hu, M. Rieger, and W. F. Benedict, manuscript submitted for publication).

Therefore, it now should be possible to examine various primary human tumors at the single-cell level and to determine whether there are any Rb-positive tumor cells present. If no Rb-positive cells are identified, as is the case in primary retinoblastomas (Xu, H.-J., S.-X. Hu, and W. F. Benedict, manuscript in preparation), this would indicate that the gene was likely causally related to the initiation of the tumor (Fig. 2, *Cancer 1*). In contrast, if the loss of Rb protein can only be primarily observed in focal areas within a tumor or in a metastasis from this primary tumor (Fig. 2, *Cancer 2*), such findings would suggest that the loss of Rb function is involved in tumor progression rather than in its development. This might be the case for breast cancer if an initial study can be confirmed which indicated that certain breast tumors contain a few Rb-positive tumor cells, whereas the majority of tumor cells are Rb negative (35). Should an Rb mutation be associated with tumor progression, it would then be most important to determine if there is a poorer prognosis and/or therapeutic response in patients having a tumor in which a significant portion of their cancer cells are Rb negative compared to those individuals with malignancies of the same tissue type which are Rb positive. If such a correlation could be found, it obviously would have considerable clinical value.

It should be mentioned at this point that, even if all tumor cells in a given cancer are missing the Rb protein, indicating that the Rb gene functional loss is involved in the initiation of this particular tumor, it is likely that there will be several other genes which are also factors in the development of this specific malignancy. The one exception to this generality may be retinoblastoma (Fig. 3). As mentioned above, we have found in our initial study that all tumor cells from primary retinoblastomas examined to date which do not have a truncated Rb protein are Rb negative (Xu, H.-J., S. X. Hu, and W. F. Benedict, manuscript in preparation). In contrast, the development of certain malignancies such as osteosarcoma likely requires the involvement of additional cancer genes. Some of these genes may in fact be similar to the Rb gene in that loss of function could be the basis for their role in tumor formation.

One candidate gene that, in addition to the Rb gene, might be involved in the development of specific tumors is the p53

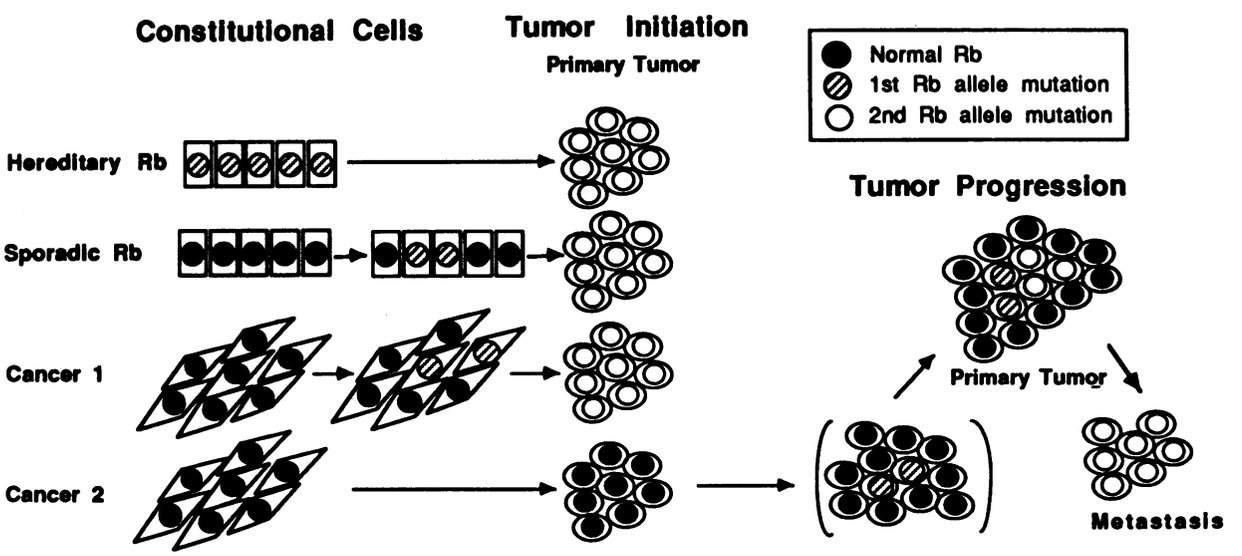

Figure 2. Schematic representation of the expected Rb staining pattern in individual tumor cells depending on whether the loss of Rb function is related to the initiation vs. the progression of a specific malignancy. In primary retinoblastoma all tumor cells should be negatively stained (○) since the loss of Rb function is causally involved in tumor initiation (see text). Rb protein would also be missing in every cell in other malignancies in which Rb functional loss is associated with the tumor development (*Cancer 1*). In contrast, cancers where the loss of Rb function has a role in tumor progression rather than in its initiation, one would expect to find only a portion of the primary tumor in which every cell was missing the Rb protein or malignancies where the total absence of the Rb protein could only be documented in metastasis (*Cancer 2*). The intermediate cell shown in parenthesis would be required in such a scenario, although cells in which functional Rb loss occurs in only one allele (◍) cannot be distinguished from cells containing two normal Rb alleles at the protein level (●).

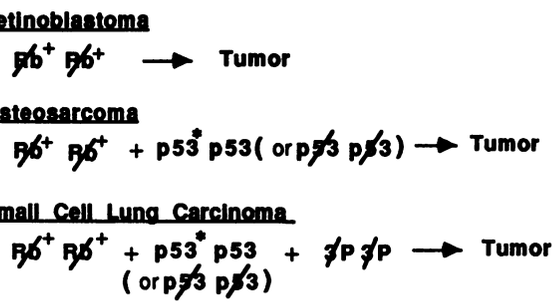

Figure 3. Role of Rb functional loss in the initiation of different tumor types. Retinoblastoma may be unique in that functional loss of both Rb alleles could be sufficient for malignant development. In osteosarcoma a mutation of one p53 allele (*) could indirectly inactivate the wild-type p53 protein encoded for by the remaining normal p53 allele (36) or both p53 alleles could be deleted. In either case a loss of p53 would occur. Therefore, p53 could represent a second "suppressor" gene involved in the development of osteosarcoma (37). Finally, a third recessive "suppressor" gene located on chromosome 3p is also likely to be associated with tumor initiation of SCLC (38).

gene which is located on chromosome 17. Structural changes within p53 have been reported for osteosarcoma resulting in the loss of p53 transcript (37). Moreover, abnormalities in p53 including total homozygous deletions have recently been found in SCLCs (39). It should also be noted that deletions in chromosome 3p have been frequently seen in SCLCs (38), which suggests that a minimum of three "suppressor" genes could be involved in the initiation of this tumor type (Fig. 3). In fact, it is likely that many of the more common human cancers such as those of the lung and breast require additional changes that include not only genetic lesions which result in the loss of gene function, but also mutations which yield an altered gene product.

In conclusion, it should be apparent that the loss of Rb function has a central role in the initiation and/or progression of a significant number of human cancers. The determination of the precise involvement of the Rb gene in these tumors will be a major future task. These studies will include many disciplines as well as approaches. One will need to examine the presence or absence of the Rb protein at the single-cell level in various tumors and correlate these findings with prognosis and/or therapeutic response as mentioned above. In addition, examination of the Rb phosphorylation changes that occur during the cell cycle and the kinases involved in its phosphorylation have already provided interesting leads to the function of the Rb gene (40) and future studies in this area should be equally informative. Moreover, much excitement has recently been generated by the observation that transforming proteins of DNA viruses, in particular those of the adenovirus, polyomavirus, and papillomavirus classes bind to the Rb protein(s) (40). Mutations introduced into these viruses which inhibit the binding of these specific transforming proteins to Rb also reduce or block the transforming capacity of the virus (40). These results imply that DNA viruses transform by blocking the function of the Rb at the protein level which is analogous to the loss of Rb function by genetic deletion. Such binding could aid in the transformation process at several levels in the cell cycle including the movement of cells from the G_0 state into the proliferative pool or the inhibition of differentiation (40). It can be expected that studies that continue to examine the interaction of DNA oncoproteins with the Rb protein(s) will provide important leads to the Rb function and its role in the cell cycle.

A final major goal of future research in the Rb field will be to determine the result of adding a normal chromosome 13 and the Rb gene itself back into the various human tumor types in which loss of Rb function has been shown to occur. If tumor initiation could be inhibited in each case by this manipulation, it would be a major finding and provide the impetus to pursue new diagnostic and therapeutic approaches for many of the most common human malignancies. Whatever the results of these studies, however, one can be assured that life will not be dull or competition lacking for those who are now or later become involved in unraveling the function of the Rb gene and its role in human cancers!

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