Transcriptional Regulation of *neu* by RB and E1A in Rat-1 Cells

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Abstract

Functional inactivation of the tumor-suppressing retinoblastoma gene (*RB*) is involved in the etiology of many types of human cancers, including hereditary retinoblastomas. The *neu* gene is a dominant transforming oncogene, and we previously found that the *RB*-encoded protein (RB) suppresses *neu*-induced transformation in NIH3T3 cells by repressing transcription of the *neu* oncogene. We report here that RB was unable to repress *neu* oncogene transcription in Rat-1 cells but could functionally antagonize transcriptional repression of *neu* by the adenovirus E1A. Mutant forms of RB that have mutations in either the E1A-binding or carboxy-terminal regions had less or no antagonizing effects on E1A-mediated repression of *neu* in Rat-1 cells. Results of focus-formation assays showed that the transformation activity of the *neu* oncogene in Rat-1 cells could be regulated by E1A and RB in accordance with their transcriptional regulation activities. The data demonstrate that RB can regulate transcription of *neu* in a negative or positive manner depending on the cell type. Carboxy terminus of RB as well as the E1A-binding region can mediate transcriptional regulation. Based on these results, we propose a model for the complex transcriptional regulation of *neu* by RB and E1A.

Introduction

The *RB* gene is a prototypic tumor suppressor gene that encodes a 105 kD nuclear phosphoprotein, RB (1–5). Inactivation of *RB* plays an important role in the evolution of several types of human cancers (3, 6–17), and reintroduction of a wild-type *RB* allele into tumor cells in which *RB* has been inactivated inhibits tumor formation by the recipient cells in nude mice (7, 18). Therefore, *RB* inactivation plays an essential role in oncogenesis. The *RB* gene can also function as a cell cycle control element, and RB protein shows cyclical changes in phosphorylation (19–24). Microinjection of RB can inhibit cell cycle progression during a specific time window in G1 (25). It has been proposed that the underphosphorylated RB is the functional form that limits the progression of cells through the cell cycle by sequestering a variety of nuclear proteins involved in transcription of growth regulatory genes (26–32). Transforming proteins of several DNA tumor viruses such as the adenovirus E1A protein, the SV40 large T antigen, other papovaviral T-antigens, and the human papillomavirus E7 protein can bind to RB at the region extending from residue 379 to 792 (33–39). It is believed that these viral proteins induce transformation at least in part by binding to the RB protein and inactivating the tumor-suppressing function of RB. In addition, several cellular proteins can also interact with the E1A-binding domain of RB to control cell cycle progression (40–43).

The *neu* oncogene encodes a 185 kD transmembrane protein homologous to the epidermal growth factor receptor. Amplification and overexpression of the human *neu* gene (also named as NGL, HER-2, and c-erbB-2) are frequent events in many types of human cancers (44–51). We previously found that RB can suppress *neu*-induced transformation in NIH3T3 cells by repressing *neu* oncogene transcription (52). We demonstrate here that neu gene transcription and neu-induced transformation can be modulated by RB and E1A in Rat-1 cells in a cell type-specific manner, i.e., RB cannot repress *neu* oncogene transcription in Rat-1 cells but can antagonize transcriptional repression of *neu* by the adenovirus E1A.

Results

Regulation of *neu* Oncogene Expression in Rat-1 Cells Is Different from That in NIH3T3 Cells. We previously found that the mouse RB protein suppresses *neu*-induced transformation in NIH3T3 cells by repressing transcription of the *neu* oncogene (52). To further study the transcription repression of *neu* by RB, increasing amounts (2, 5, 10, and 15 μg) of hRB were cotransfected with the pNeuEcoRI-CAT reporter plasmid into NIH3T3 and Rat-1 cells (Fig. 1). Like mouse RB protein, hRB also repressed *neu* promoter activity in a concentration-dependent manner in NIH3T3 cells (Fig. 1A). However, both hRB (Fig. 1B) and mRB4 had no effect on CAT activity in Rat-1 cells. The results indicate that the RB repressed *neu* oncogene transcription in NIH3T3 cells but not in Rat-1 cells. Therefore, the regulation of the *neu* gene expression by RB is a cell-type-specific phenomenon.

RB Antagonized E1A-Mediated Repression of *neu* Expression in Rat-1 Cells. We previously found that the adenovirus E1A gene product can repress *neu* gene expression in many cell types, including Rat-1 and NIH3T3 cells (53–55). It is

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3 The abbreviations used are: RB, retinoblastoma susceptibility gene; RB, retinoblastoma susceptibility gene product p105 protein; E1A, adenovirus early region 1A; CAT, chloramphenicol acetyltransferase; hRB, human retinoblastoma susceptibility gene.

4 D. Yu and M.-C. Hung, unpublished data.
well known that E1A can form protein complexes with RB and that this binding activates a transcription factor, DRTE/E2F, which binds to RB in the absence of E1A and is released from RB when E1A binds to RB (36, 56). Therefore, we asked whether the E1A/RB interaction was involved in the transcriptional regulation of neu expression in Rat-1 cells. To study the effects of the E1A/RB complex on the transcriptional repression of neu, we performed transient transfection CAT assays in which 3 μg of pNetEcoR-CAT reporter plasmid was cotransfected either with E1A-expressing plasmids or hRB-expressing plasmids or a combination of both. Cotransfection of 3 μg of E1A-expressing plasmid, as expected, led to a significant decrease in relative CAT activity (Fig. 2a, no. 2) compared with basal CAT activity from transfection of pNetEcoR-CAT reporter plasmids (Fig. 2a, no. 1). Cotransfection with RB-expressing plasmid alone had no significant effect on relative CAT activity, which is consistent with the results shown in Fig. 1 (Fig. 2a, no. 3). However, CAT activity decreased less when 3 μg of RB-expressing plasmid was cotransfected with the same amount of E1A-expressing plasmid (Fig. 2a, no. 4). This derepression was more pronounced when more RB-expressing plasmid was used (Fig. 2a, no. 5). Although increasing amounts (6, 9, and 12 μg) of E1A-expressing plasmid cotransfected with 3 μg of RB-expressing plasmid were able to decrease relative CAT activities, the CAT activities were higher than that of transfecting with 6, 9, and 12 μg of E1A-expressing plasmid alone without RB-expressing plasmid. Fig. 2b shows the results of a representative CAT assay experiment, which indicates a clear derepression of neu when RB-expressing plasmids were cotransfected with pE1A. These data suggest that the RB gene product antagonized the repressing effect of E1A on neu gene transcription.

It is possible that the derepression of neu gene transcription is due to repression of E1A by RB which might occur
when E1A-expressing and RB-expressing plasmids were cotransfected. To examine this possibility, cell lysates were made from the E1A transfecteds and the E1A plus RB cotransfectants. The E1A protein levels in each transfecant were examined by immunoblotting analysis with anti-E1A antibody M73. The E1A protein levels in the E1A plus RB cotransfectants were similar to that in the E1A transfecant (Fig. 2c). These results indicate that the derepression of neu gene transcription in the E1A plus RB cotransfection was not due to decreased expression of E1A protein but was probably the result of inhibition of E1A by RB.

**Requirement of E1A-binding Region and Carboxy Terminus of RB for Derepression.** The wild-type RB protein contains 928 amino acids and consists of an amino-terminal region, an E1A-binding region (amino acids 393 to 792), and a carboxy-terminal region (Fig. 3A; Ref. 57). To determine which region of the RB protein was required for the neu derepression, we examined derepression effect of a panel of RB mutants that express RB proteins that have mutations in either the amino-terminal, the E1A-binding, or the carboxy-terminal regions. The RB insertional mutants HX108, MX496, and MX620 were constructed by in-frame insertion of an XhoI linker (6 base pairs; two amino acid insertions) at positions corresponding to amino acids 108, 496, and 620, respectively (Fig. 3A). The MX496 and MX620 mutants had insertions inside the E1A-binding region, and their E1A binding activity varied as indicated in Table 1. To first examine whether the E1A-binding region of RB was required for derepression, the MX496 and MX620 mutants were cotransfected with the reporter plasmid pNeuEcoRICAT and with E1A-expressing plasmids (+E1A) or with control plasmids (-E1A). The relative CAT activity is the average from three sets of transient transfections and is shown as the percentage of CAT activity from transfection of pNeuEcoRICAT and 10T, which is defined as 100%

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<th>Relative CAT activity (%)&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>HX108</td>
<td>95 ± 9</td>
<td>84 ± 16</td>
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<td>E1A-binding region</td>
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<td>MX496</td>
<td>110 ± 21</td>
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<td>MX620</td>
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<td>Δ787–833</td>
<td>97 ± 18</td>
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<td>90 ± 17</td>
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<sup>a</sup> Each control or mutant was cotransfected with the reporter plasmids pNeuEcoRICAT and with E1A expressing plasmids (+E1A) or with control plasmids (-E1A). The relative CAT activity is the average from three sets of transient transfections and is shown as the percentage of CAT activity from transfection of pNeuEcoRICAT and 10T, which is defined as 100%.

<sup>b</sup> CAT activity fell within the range of CAT activity of wild-type RB (wt RB); - , CAT activity fell within the range of CAT activity of 10T.

<sup>c</sup> The relative E1A-binding activities of these RB mutants were determined by commounoprecipitation of RB and E1A +, binds to E1A efficiently; ±, binds to E1A but not as efficiently; -, does not bind to E1A.

![Diagram](image)

**Fig. 3.** A, schematic diagrams of the RB mutants used in this study. The wild-type RB protein has 928 amino acids, and its E1A-binding pocket extends from amino acids 393 to 792 (black region). The RB insertional mutants were constructed by inserting a 6-base pair XhoI linker at positions corresponding to amino acids 108, 496, and 620. All mutants have been tested to express mutated forms of the RB protein. B, RB termination mutants that expressed RB proteins that are truncated at amino acids 10 and 743 and were, therefore, named 10T and 743T, respectively. The RB deletion mutants Δ76–302, Δ787–833, and Δ830–882 have deletions at amino acids 76 to 302, 787 to 831, and 830 to 882, respectively. The RB deletion mutants Δ22 has deletion of exon 22, which approximately corresponds to amino acid 624 to 803.

<p>| Table 1 Effects of mutations on the ability of RB to antagonize E1A-mediated repression of neu |
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showed); and (b) the deletional mutants Δ76–302 that had deletion from amino acid 76 to 302 and could bind E1A (Ref. 58; Fig. 3B). Both the insertional mutant HX108 and deletional mutant Δ76–302 reduced the effect of E1A-mediated repression of neu, as did the wild-type RB, when they were cotransfected with the reporter plasmid pNeuEcorICAT and with E1A-expressing plasmid (+E1A) (Table 1). These data suggest that the amino-terminal region of RB is not critical for derepression.

The RB carboxy-terminal region outside of the minimal E1A-binding pocket has been shown to contribute to the growth suppression function of RB (57). To examine whether this region is required for neu derepression, we performed transfection experiments with RB carboxy-terminal mutants. The deletional mutants Δ787–833 and Δ830–882 had deletions from amino acids 787 to 833 and 830 to 882, respectively (Fig. 3B). Both the Δ787–833 and Δ830–882 mutants could bind to E1A (Table 1 and data not shown). The RB terminational mutant 743T expressed RB proteins that were truncated at amino acid 743 (Fig. 3B). Interestingly, none of these carboxy-terminal mutants had the derepression effect of wild-type RB (Table 1). Therefore, the carboxy-terminal portion of the RB protein is also required for derepression in addition to the E1A-binding region. Moreover, this is consistent with the observation that the E1A binding function of RB was not a determinant for derepression.

Modulation of neu-induced Transformation by RB and E1A in Rat-1 Cells. The above-mentioned results from transient transfection assays, although reproducible, were surprising in that the tumor suppressor RB did not repress neu oncogene transcription but blocked the repressing effect of E1A in Rat-1 cells. To determine whether derepression by RB is a physiologically relevant phenomenon that also occurs in stable transfection experiments, we examined the ability of a mutation-activated genomic rat neu oncogene to induce foci in Rat-1 cells after cotransfection with human RB, E1A, or both. Transfection of 0.5 μg of genomic neu (cNeu104) with the 10T plasmid plasmid induced about 50 to 80 foci (defined as 100%) in different transfection experiments in Rat-1 cells (Table 2). When cNeu104 was cotransfected with 1, 3, or 9 μg of wild-type hRB-expressing plasmid (RB), there was no significant change in the number of foci (Table 2). Cotransfection of 3 μg of E1A-expressing plasmid resulted in a decrease in the number of foci to 36–59% (Table 2, E1A, 3). However, when 1, 3, or 9 μg of hRB-expressing plasmid were cotransfected with 3 μg of E1A-expressing plasmid, the number of foci was greater than when only E1A-expressing plasmid was transfected (Table 2), and the increase in the number of foci was dependent on the amount of cotransfection plasmid hRB. The results suggest that RB and E1A modulate neu-induced transformation in Rat-1 cells in a direction consistent with the transient transfections.

The different RB mutants that were used in the transient transfections (Table 1) were also tested for their effects on focus formation by cNeu104 when they were cotransfected with or without E1A. The RB mutants also modulated cNeu104-induced foci number in a direction consistent with the transient transfections (Table 2). Therefore, derepression by RB requires the E1A-binding and the carboxy-terminal regions in both transient and stable transfection assays.

Discussion

In further studying the regulation of neu gene expression by RB, we found that RB was unable to repress neu oncogene transcription in Rat-1 cells as it did in NIH3T3 cells but that RB could antagonize E1A-mediated repression of neu in Rat-1 cells. This observation is in accord with the previous observation that RB protein can modulate transcription of growth-regulatory genes such as c-fos, c-myc, and transforming growth factor β1 in either a positive or negative manner, depending on the cell type (30–32). Taken together, these results suggest that RB is a cell-type-specific transcription factor that controls expression of growth-regulatory genes.

The derepression effect of RB was abolished or reduced by different mutations in the E1A-binding pocket or the carboxy-terminal region of RB. These results indicate that derepression of neu transcription requires these two regions of RB and that both RB domains may mediate transcription regulation by interacting with different proteins. In support of this notion, it was recently reported that in addition to the E1A-binding region, the 792–928 carboxy-terminal RB segment is also required for efficient association with the transcription factor E2F and for the ability to suppress cell growth (57). Recently, it was also shown that the biological functions of RB are dependent on both its E2F-binding domains and amino-terminal region, which is required for phosphorylation (59). In the study reported here, we found that derepression by RB was dependent upon two of the biochemically defined RB domains, including the E1A-binding region and the carboxy-terminal segment. Our results demonstrate that in addition to the well-known E1A-binding region, the carboxy-terminal region outside the E1A-binding region is also involved in transcriptional and growth regulation.

Table 2 Modulation of neu-induced focus formation by E1A and hRB in Rat-1 cells

| Cotransfected genes, μg | Relative focus formation (% of 10T) | Derepression*
|------------------------|------------------------------------|-----------------
|                        | Exp. 1 | Exp. 2 | Exp. 3 |                |                |
| 1OT, 9                 | 100    | 100    | 100    | NA             |                |
| E1A, 3                 | 42     | 59     | 36     | NA             |                |
| E1A + RB, 1            | 49     | 78     | 85     | +              |                |
| E1A + RB, 3            | 51     | 116    | 84     | +              |                |
| E1A + RB, 9            | 83     | 94     | 91     | +              |                |
| E1A + HX108, 9         | ND     | 86     | 87     | +              |                |
| E1A + Δ76–302, 9       | 91     | 95     | ND     | +              |                |
| E1A + MX496, 9         | 49     | 47     | ND     | –              |                |
| E1A + Δ22, 9           | 47     | 34     | 59     | –              |                |
| E1A + Δ787–833, 9      | 55     | 59     | ND     | –              |                |
| E1A + Δ830–882, 9      | 51     | 54     | 49     | –              |                |
| RB (wt), 1             | 100    | 138    | 108    | NA             |                |
| RB (wt), 3             | 81     | 134    | ND     | NA             |                |
| RB (wt), 9             | 96     | 138    | 112    | NA             |                |
| HX108, 9               | ND     | 89     | 86     | –              |                |
| Δ76–302, 9             | 99     | 101    | ND     | NA             |                |
| MX496, 9               | 81     | 84     | ND     | NA             |                |
| Δ22, 9                 | 75     | 116    | 111    | NA             |                |
| Δ787–833, 9            | 92     | 109    | ND     | NA             |                |
| Δ830–882, 9            | 141    | 122    | 133    | NA             |                |

+ foci formation fell within the range of foci formation with cotransfection of 10T; –, foci formation fell within the range of foci formation with cotransfection of E1A. ND, not determined; NA, not applicable (controls).
with RB to modulate transcription of growth-related genes (26–29, 57). In addition, RB has been shown to interact with nuclear proteins or general transcription factors such as Myc, ATF-2, Sp1, and MyoD (43, 61–63). We did not find consensus binding sites for E2F or ATF-2 in the neu promoter and upstream regulatory region (64). However, a consensus E12-MyoD-binding site (CCNNNTG at position +248 to +253) and a Sp1-binding site (GGGCGG at position +199 to +204) are present in the neu promoter (64). Since a large number of cellular proteins can bind to E1A or RB and many of them have not been well characterized, it is not yet clear which specific protein is involved in transcriptional regulation of neu by E1A and RB. However, based on the data presented in this report, we propose a model of how RB and E1A may regulate neu transcription in Rat-1 cells (Fig. 4). In this model, we hypothesize that in Rat-1 cells, some cellular proteins (X proteins in Fig. 4) are involved in regulation of neu gene expression. We propose that RB interacts with the X proteins to maintain basal level transcription of neu in Rat-1 cells, and the equilibrium between RB/X complexes and free X proteins may control the transcription of neu (Fig. 4A). E1A can repress neu gene transcription by complexing with endogenous RB to liberate X proteins from the RB/X complex; the free X proteins cannot maintain basal level neu transcription (Fig. 4B). Adding an excess amount of wild-type RB will relieve the transcriptional repression by E1A, since there will be excess RB to interact with X proteins to form RB/X complex and maintain basal level neu transcription in addition to binding to E1A (Fig. 4C). However, mutant RB in the absence of E1A, regardless of their E1A-binding capability, cannot interact with the X proteins. Therefore, transfecting mutant RB will not interfere endogenous wild-type RB interaction with the X proteins to maintain basal level transcription of neu in Rat-1 cells. Adding an excess amount of non-E1A-binding mutant RB in the presence of E1A will not relieve the transcriptional repression by E1A, because E1A will bind to the endogenous wild-type RB and the mutant RB cannot bind to X proteins. This will produce uncomplexed free X proteins which cannot maintain basal level neu transcription. Transfecting an excess amount of E1A-binding RB mutants cannot derepress E1A-mediated transcriptional repression because these types of RB mutants either may bind to E1A and undergo conformational changes to form nonfunctional E1A mutant-RB/X complexes and, therefore, could not maintain basal level neu transcription; or may bind to E1A with lower affinity than wild-type RB, and hence, the majority of the endogenous wild-type RB will bind to E1A and result in uncomplexed free X proteins. In accordance with our experimental results, we propose that the E1A-binding and the carboxy-terminal regions of RB are required for interaction with the X proteins to form functional RB/X complex. However, the amino-terminal mutants (HX108 and Δ76–302) can still form functional RB/X complexes and behave like the wild-type RB, i.e., have derepression function (Fig. 4C). Based on this model, a possible explanation for the cell type specificity of RB effect on neu transcription in Rat-1 cells and NIH3T3 cells may be that these cells can have different cellular proteins (X proteins in Fig. 4) that are involved in regulation of neu gene expression. There may be other alternative modes of regulation of neu gene by RB and E1A. However, the above model fits our experimental data most properly. This model suggests that the neu gene may be a target gene for the RB protein and for RB-binding proteins. Since the neu gene is a member of the growth factor receptor tyrosine kinase gene family (65) that plays important roles in cell growth control, regulation of neu gene expression may be one of the important pathways by which RB and RB-binding proteins exert their growth regulatory effects.

Materials and Methods

Plasmids. pNeuEcoRI-CAT was used as reporter gene in our early studies and it contains the 2.2-kilobase rat neu promoter and upstream sequences linked to the CAT reporter gene (66). pNeuEcoRV-CAT was used in later studies and is constructed by deletion of 1.7-kilobase upstream sequences from pNeuEcoRI-CAT, and the remaining 0.5-kilobase rat neu promoter and upstream sequences contain E1A an RB responding elements (66). The wild-type hRb expression construct phRbc-SVE has been described previously (67) and was used in this study to express wild-type hRb. The insertion mutant forms of RB complementary DNA were constructed in hRb vector by inserting an Xhol linker in frame, and the termination mutants were made by inserting an Xbal linker with stop codons at a given restriction site (58). The mutants are named for the restriction site disrupted and the position of amino acid disrupted. The deletional mutant Δ76–302 has been described previously (58). The deletional
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mutant Δ787–833 and Δ830–882 were generated by polymerase chain reaction mutagenesis and confirmed by DNA sequencing; then the deletional mutants were subcloned into pRbc-SVE vector to replace the wild-type RB. The deletional mutants Δ22 were derived from the NCI-H69C small cell lung carcinoma (3) and were subcloned into pRbc-SVE vector. pE1A is a plasmid that expresses the adenovirus type-5 E1A gene (68). pSV2E contains only the SV40 promoter, which is the promoter used to drive RB expression in the RB expressing plasmid; this plasmid was used as a control to assure that the differences in CAT activity with addition of hRb plasmid were due to RB expression but not the existence of the SV40 promoter. cNeu-104 (genomic neu) is a cosmid clone containing the 30-kilobase activated genomic rat neu gene, including the 2.2-kilobase rat neu promoter and upstream regulatory sequences (66, 69).

Cell Cultures and DNA Transfection. Cells were cultured as described previously (70). Transfections were carried out by the calcium-phosphate precipitation technique (71) with minor modifications as described previously (53). pRSVLacZ contains the bacterial β-galactosidase gene driven by the Rous sarcoma virus long terminal repeat (72) and was used to monitor transfection efficiency. pGEM4 plasmid vector (Promega) was used as an internal quantity control in transfection experiments to bring the total amount of transfected DNA to the same quantity. Each experiment was repeated at least four times.

CAT Assays. The CAT assays were carried out as described previously (53). After transfection, cell lysates were assayed for β-galactosidase activity from the cotransfected pRSVLacZ plasmid to monitor the transfection efficiency (73). Aliquots of cell extracts with equal β-galactosidase activity were then used for CAT assays. CAT activity was quantitated by analyzing the spots on thin-layer chromatography plates corresponding to the positions of 14C-chloromphenicol and its acetylated products on a Betascope 603 blot analyzer (Betagen). Each experiment was reproducibly repeated at least three times.

Immunoblotting. Immunoblot analyses were performed by published techniques as described previously (55). Each sample (100 μg) was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The nitrocellulose filter was incubated with primary monoclonal antibody M73 against the E1A proteins (a generous gift from Dr. L.-S. Chang, Ohio State University) and was then incubated with a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Bio-Rad Laboratories); then it was subjected to a color-developing reaction with horseradish peroxidase (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD).

Focus Formation Assay. Focus formation assays were performed as described previously (52). Rat-1 cells (5 × 104) were cotransfected with cNeu-104, a cosmid clone containing 30-kilobase activated genomic rat neu gene, including 2.2-kilobase rat neu promoter and upstream regulatory sequences (69) and wild-type or mutated hRB or E1A-expressing plasmids. pSV2-neo plasmid DNA (1 μg) carrying the neomycin-resistant marker gene was cotransfected in all of the transfections. Total transfected DNAs in each transfection were kept constant to 11 μg with pGEM DNA. Forty-eight h after transfection, the cells were split 1 to 10 and were fed every 3 days with fresh culture medium. One-half of the cultures were grown in media with G418 (800 μg/ml) for selection of G418-resistant colonies to measure transfection efficiency, and the other one-half was cultured without G418 to allow foci formation. G418 colonies and foci were observed 14–16 days after transfection and were stained with 1% crystal violet, fixed with 20% ethanol, and counted. The number of foci in each transfection was corrected by transfection efficiency as indicated by the number of G418 colonies. Results are the average of three experiments and are shown as percentages of the number of foci in each transfection versus the number of foci in the control, which is the number of foci induced by activated genomic neu without addition of wild-type or mutated hRB or E1A-expressing plasmids.

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References


