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Genetics

The retinoblastoma gene functions as a growth and tumor suppressor in human bladder carcinoma cells

(antioncogene/gene transfection/tumorigenicity/growth regulation)

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ABSTRACT **The product of the human retinoblastoma gene (*RB*) is a nuclear phosphoprotein that is thought to function as a tumor suppressor. Mutations of *RB* frequently occur in human bladder carcinoma. To investigate the significance of the functional loss of this gene in bladder cancer, an *RB* expression plasmid (pBARB) under control of the human β-actin promoter was transfected into the bladder carcinoma cell line HTB9, which lacks *RB* expression. Marker-selected transfectants that expressed RB protein were identified by immunoblotting and immunohistochemical staining. In selected clones, stable RB expression has persisted over 1 yr under standard culture conditions with 10% serum. However, RB expression caused major alterations of HTB9 growth properties both *in vitro* and *in vivo*. RB^+ transfectants lacked the ability to form colonies in semi-solid medium, and their growth rate was significantly decreased in 3% serum. In addition, the tumorigenicity of these transfectants was markedly decreased. Tumors that formed in nude mice were much smaller and had a longer latency period but were indistinguishable microscopically from those produced by parental cells. Slower growing tumors were RB^+, as measured by nuclear staining of their RB protein and by a normal RB protein pattern on immunoblots. These findings support the concept that the *RB* gene acts as both a growth and tumor suppressor in bladder cancer cells.**

The retinoblastoma gene (*RB*) was the first human tumor suppressor gene isolated (1–3). Several tumor types, including those of the eye, lung, breast, prostate, bone, and bladder, have been shown to frequently lose *RB* function. In bladder cancer, mutation of *RB* was reported in the J82 bladder carcinoma cell line, which contained a point mutation resulting in production of a truncated retinoblastoma (RB) protein (4). RB inactivation was later found in 5 of 16 independent human bladder carcinoma cell lines (5). Moreover, RB inactivation in primary bladder tumors has recently been documented (J. Ishikawa, H.-J.X., D. W. Yandell, S.-X.H., S. Maeda, S. Kamidono, W.F.B., and R.T., unpublished data). Based on these findings, the loss of RB function presumably plays a role in bladder cancer, either at its initiation or in its progression.

The RB protein has been shown to be phosphorylated in a cell cycle-specific manner (6–10), to have DNA-binding properties (11), and to be localized primarily within the nucleus (11). The function of the *RB* gene is presumably linked to these properties of the RB protein, although there is no direct evidence for its specific role(s). Although a functional test is critical for defining its role in tumorigenesis, establishing such a functional assay of the *RB* gene has not been straightforward. This lack is due partly to the delay in successful development of a stable RB expression system in human cancer cells.

Thus far, only a few studies have shown phenotypic alterations in recipient cells after *RB* was introduced into tumor cells in which loss of *RB* function had occurred (12, 13). These studies used a defective retroviral vector infected into target cells. In the first study, *RB* was reexpressed in both the retinoblastoma line WERI-Rb27 and the osteosarcoma line Saos2. Introduction of *RB* into RB-deficient (RB^-) cells produced phenotypic changes, including complete suppression of tumorigenicity in nude mice (12) for WERI-Rb27. In the second study, which involved prostate carcinoma cell line DU145, all tumors that developed in nude mice were RB^- (13). More recently, plasmid transfection of *RB* was attempted in a small-cell lung carcinoma cell line, but phenotypic alterations were not examined (14). Although the results of Huang *et al.* (12) were reproduced partly by another group (15) with the same retroviral vector, we felt it was important not only to carry out a similar study in an independent system but also to analyze another tumor type. Furthermore, several questions, especially those concerning stability of the RB^+ cells previously obtained and interpretation of their tumorigenicity data, remained unanswered from the initial studies.

In this paper, we report the stable transfection of *RB* in the bladder carcinoma cell line HTB9 as well as the phenotypic changes that occur after reexpression of RB protein. Introduction of *RB* markedly decreased the ability of these cells to grow in low concentrations of serum and completely inhibited colony formation in soft agar. In addition, when injected s.c. into nude mice, tumor formation was significantly inhibited when compared with that of parental cells. The small tumors that did develop were still RB^+, and their malignant nature could be documented histologically. Our results support the proposed function of *RB* as a tumor and growth suppressor but suggest that replacement of *RB* function in RB^- tumor cells may not be sufficient to revert completely their malignant phenotype.

MATERIALS AND METHODS

Construction of an RB Expression Vector. A cDNA library derived from mRNAs of human embryo fibroblasts (16) was screened by using the 3.8 kilobase (kb) RB-cDNA probe (3). Isolated RB-cDNAs were sequenced by the Sanger method (17). The 5′ end of the RB-cDNA insert was located 57 base pairs (bp) upstream from the first start codon (ATG) of translation. The 3′ end of the insert was located 3684 bp downstream from the first ATG. The RB expression vector,

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Abbreviation: RB, retinoblastoma.

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pBARB, was constructed by ligating this RB-cDNA fragment into a mammalian expression vector, pHβAPr-1-neo, which contained the human β-actin promoter, and neomycin- and ampicillin-resistant sequences (18).

Stable Transfection in HTB9 Cells. The control plasmid pHβAPr-1-neo and the *RB* gene-containing plasmid pBARB were transfected by the $Ca^{2+}PO_4$ method (19) using a mammalian transfection kit (Stratagene). Exponentially growing HTB-9 (5637) cells, obtained from the American Type Culture Collection, that were incubated with 1.37 pmol (10 μg or 13.6 μg) of pHβAPr-1-neo or pBARB, respectively, for 12 hr in a 3% CO_2 atmosphere, were treated with 20% dimethyl sulfoxide for 5 min, washed twice with medium, and cultured in 5% CO_2/atmosphere for 48 hr. The cells were subcultured into five 100-mm dishes. After 24 hr, the medium was changed to G418-containing Dulbecco's minimal essential medium at an active concentration of 200 μg/ml. After 2 weeks, several colonies were seen in each dish, and each of the G418-resistant colonies was transferred separately to an individual well of 24-well plates. A mass cell culture from each dish was also maintained. Cells were subsequently maintained in G418 medium and expanded for further analyses.

RNA Blotting. Total cytoplasmic RNA was isolated by the guanidium thiocyanate method (20). RNA blotting was done as described (21). Membranes were then hybridized with a ^{32}P-labeled, 3.8-kb RB probe and exposed to Kodak XAR-5 film at −80°C.

Immunoblotting. Fifty micrograms of cellular protein was electrophoresed in an 8% SDS/polyacrylamide gel and electroblotted to Immobilon polyvinylidene difluoride membranes (Millipore). After being blocked with 4% bovine serum albumin/1% normal goat serum in Tris-buffered saline, membranes were incubated overnight with the RB-WL-1 antibody (6) at a final concentration of 0.4 μg/ml. The blot was then probed by the ProtoBlot Western blot alkaline phosphatase system (Promega).

Immunohistochemistry. Cells grown on coverslips were fixed in 45% (vol/vol) acetone/10% (wt/vol) formaldehyde/0.1 M phosphate buffer for 5 min. After being washed six times with phosphate-buffered saline, cells were preincubated in 5% bovine serum albumin/4% normal goat serum in phosphate buffer for 4 hr at room temperature. The MAb-1 anti-Rb monoclonal antibody (Triton Biosciences) was diluted 1:25 in 3% bovine serum albumin/1.5% normal horse serum/0.02% Triton X-100/phosphate buffer and was incubated with the cells overnight. After being washed, the coverslips were processed for immunostaining with the avidin biotinylated peroxidase complex (ABC) method according to the technical manual (Vector Laboratories).

Growth Curve and Growth in Soft Agar. Cells were seeded at 1×10^5 cells per 60-mm dish in either 3% or 10% fetal bovine serum/medium. Cells from replicate dishes were counted each day for 6 days with a hemocytometer. For the studies in soft agar, cells were seeded at 1×10^5 cells per dish into 35-mm dishes containing 0.33% agarose with complete medium/20% fetal bovine serum. The medium was replenished every 7 days, and colonies (>50 cells) were counted after 3 weeks. Results were calculated as the average of two dishes per cell strain.

Tumorigenicity Test in Nude Mice. A variable number of RB^+ cells (1×10^6, 2×10^6, or 1×10^7) were injected s.c. in 0.2 ml phosphate buffered saline into the right flank of nude mice. Control RB^- HTB9, HTB9-PL-M, or HTB9-PL-M-CL1 cells were injected at an identical concentration into the left flank of the same mice. Tumors were measured every 1–2 weeks and excised at various times for further analysis. To reestablish a culture line from the tumors, they were minced under sterile conditions and transferred to dishes in the presence of G418-containing complete medium. All cell strains, both before injection and after reestablishment from tumors, were found to be free from mycoplasma contamination by using a mycoplasma-specific DNA fragment as a probe (22).

RESULTS

Plasmid Construction and Transfection. A 4.74-kb cDNA containing the complete RB coding sequence and both the 5′ and 3′ untranslated regions (Fig. 1*A*) was isolated from a normal human embryo fibroblast cDNA library. *In vitro* expression of a full-length RB protein (110 kDa) was achieved with this RB-cDNA by using a rabbit reticulocyte system (data not shown). To assay its function, we constructed an RB-expression plasmid, pBARB (Fig. 1*B*), by inserting the isolated RB-cDNA under control of the human β-actin promoter into expression vector pHβAPr-1-neo (18). This vector also included a dominant selection marker gene *neo* under control of the simian virus 40 early promoter. pBARB and the control vector were each introduced by the $Ca^{2+}PO_4$ method (19) into human bladder carcinoma cell line HTB9. This cell line lacks detectable RB expression, as reported by Horowitz *et al.* (5) and confirmed by us both at the protein (Fig. 2*A*, lane 2) and RNA (Fig. 2*B*, lane 1) levels. Stable transfection efficiencies with HTB9 cells were extremely low but not appreciably different for RB and control vectors. The mean number of colonies per dish was 8.4 and 7.4 in the five dishes plated each with pHβAPr-1-neo- and pBARB-transfected cells, respectively, using 1.37 pmol of plasmid DNA. Colony sizes were also similar.

A mass culture, HTB9-PL-M, and five separate clones, HTB9-PL-M-CL1, -CL2, -CL3, -CL4, and -CL5, that were

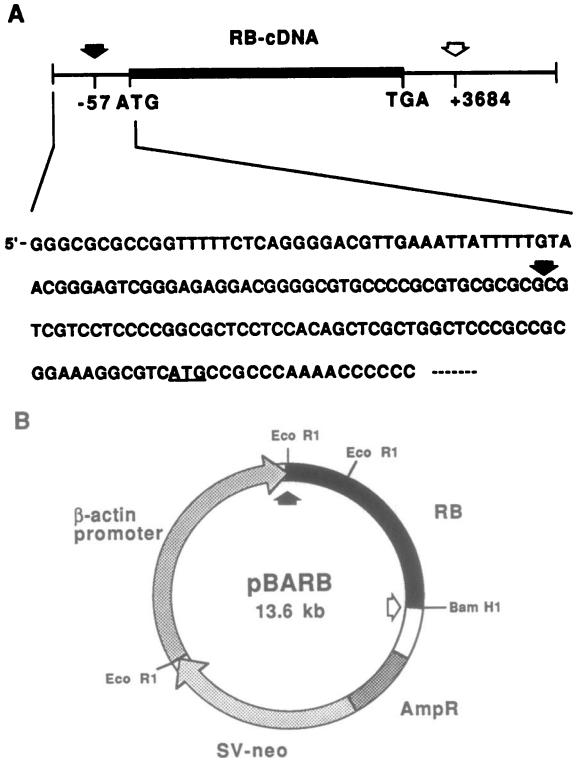

FIG. 1. (*A*) RB-cDNA used for construction showing the 5′ integration site (closed arrow) into RB expression plasmid. The 3′ integration site used is also shown (open arrow). (*B*) Schematic representation of RB expression plasmid. RB, RB-cDNA insert defined in *A*; AmpR, ampicillin-resistance gene; SV-neo, simian virus Tn5 neomycin-resistant gene. The specific 5′ and 3′ integration sites shown in *A* are depicted similarly as closed and open arrows, respectively.

derived from it, were obtained by transfection with pHβAPr-1-neo and were used as controls. Attempts to isolate and propagate individual RB^+ clones directly from the original pBARB-transfected mass culture in five dishes were unsuccessful. Thus, we used a mass culture of G418-resistant cells from one of the dishes, H/RB-M, that grew up after transfection with pBARB. The cells were then diluted and seeded at a concentration that would yield an average of less than a single cell per well in microtiter plates. Five clones were selected randomly and analyzed along with the mass culture H/RB-M by immunoblotting, as described below. Four of these clones were positive for RB protein expression. The mass culture, H/RB-M, and one of the isolated RB^+ clones, H/RB-M-CL5, were used for further studies. We also isolated a second RB^+ clone, H/RB-CL2, from an independent G418-resistant mass culture.

Expression by HTB9 Transfectants of Apparently Normal RB Protein. As shown in Fig. 2*A* (lanes 3–5), both H/RB-CL2 and H/RB-CL5 as well as the mass culture, H/RB-M, produced readily detectable RB proteins that apparently were identical in size and in phosphorylation pattern to control WI-38 cells (Fig. 2*A*, lane 1). This similarity in phosphorylation pattern included both the underphosphorylated and phosphorylated forms of the RB protein, recognized as a 110-kDa band and a smeary region between 110 and 116 kDa, respectively (6). In addition, there was little variation in the level of RB expression (Fig. 2*A*, lanes 3–5). The RB^+ transfectant clones were also immunohistochemically stained by anti-RB antibody to determine the subcellular localization of RB protein. Each cell line that was RB^+ by immunoblotting showed positive nuclear RB protein staining, whereas the parental HTB9 cells exhibited no nuclear staining under identical conditions (Fig. 3). This pattern of RB protein expression was similar to that of normal fibroblasts and consistent with previous studies (6–11).

RNA blotting showed that both the parental HTB9 and control plasmid-transfected HTB9-PL-M lacked any RB transcript (Fig. 2*B*, lanes 1 and 2). The RB-cDNA used for the pBARB construction predicted a transcript size of 3.8 kb, in contrast to the 4.7-kb endogenous RB transcript. This exogenous truncated RB transcript was detected in each RB^+ transfectant (Fig. 2*B*, lanes 3 and 4), all of which also lacked the normal-sized RB transcript. In addition, the RNA expression level of RB^+ transfectants was equivalent to that of normal fibroblast WI-38 cells (Fig. 2*B*, lane 6), whereas HeLa cells showed higher RB expression (Fig. 2*B*, lane 5).

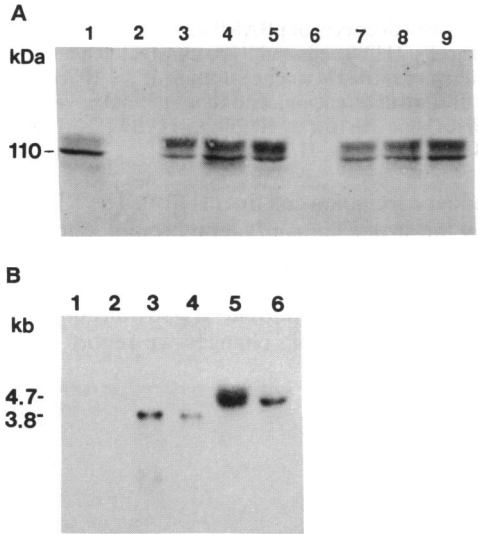

FIG. 2. (*A*) Expression of RB protein in RB-transfected bladder carcinoma cells by immunoblotting. Lanes: 1, WI-38 RB^+ control (human fibroblast); 2, parental RB^- HTB9. RB^+ isolates, lanes: 3, H/RB-M; 4, H/RB-M-CL5; and 5, H/RB-CL2. Tumor derived from injection of H/RB-M, lanes: 6, T2; 7, T5; 8, T7; and 9, T8. (*B*) Demonstration of endogenous and exogenous RB transcripts by RNA blotting. Lanes: 1, parental HTB9; 2, plasmid-transfected HTB9-PL-M; 3, H/RB-M; 4, H/RB-CL2; 5, HeLa; 6, WI-38. Twenty micrograms of total RNA was loaded into each lane. Equal loading was confirmed by staining the gel and measuring the band intensities of ribosomal RNA (data not shown).

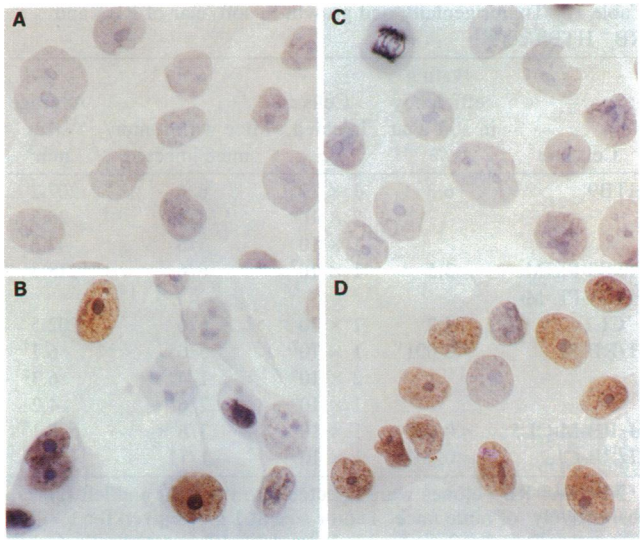

FIG. 3. Immunohistochemical detection of nuclear RB protein in HTB9 cells and RB^+-transfected HTB9 cells. (*A*) Parental HTB9 (RB^-). (*B*) H/RB-M (RB^+). (*C*) T2 (RB^-). (*D*) T5 (RB^+).

Each RB^+ transfectant was independently maintained in G418-containing medium, and RB expression was monitored sequentially by RNA blotting, immunoblotting, and immunohistochemical staining for over a year. For >1 yr, these RB^+ transfectants have been maintained as the dominant cells under standard culture conditions. Viable freezing also did not affect the stability of RB expression.

Phenotypic Properties of RB^+ Cells in Culture. *In vitro* properties, such as anchorage-independent growth and continued proliferation despite growth-limiting serum concentration, have been correlated with the malignant phenotype *in vivo*. Table 1 shows that the ability of RB^+ transfectants to form colonies in soft agar was completely suppressed, whereas parental HTB9 cells, plasmid-control HTB9-PL-M and HTB9-PL-M-CL1, produced large colonies with high plating efficiency under the same conditions. The other four plasmid-control clones (CL-2 through CL-5) also grew well in soft agar (data not shown).

The effect of RB expression on cell proliferation was measured at serum concentrations of 10% and 3%. As shown in Fig. 4*A*, there was no significant difference in growth rates of parental HTB9 cells and RB^+ clones when cultured in 10% fetal bovine serum. However, the proliferation of RB^+ lines H/RB-M, H/RB-M-CL5, and H/RB-CL2 was, in each case, more significantly reduced than that of parental RB^- HTB9 cells when cultured in 3% fetal bovine serum (Fig. 4*B*). Five plasmid control clones, HTB9-PL-M-CL1, -CL2, -CL3, -CL4, and -CL5, showed no significant differences in growth properties in 10% or 3% serum when compared with parental HTB9 and HTB9-PL-M cells (data not shown). Thus, although cell proliferation under standard culture conditions was not affected, it was markedly impaired by RB expression under restrictive conditions.

Tumorigenicity in Nude Mice. We next investigated the effect of stable RB expression on tumorigenicity of HTB9 cells. RB^- parental HTB9 cells and RB^- control plasmid-transfected HTB9-PL-M and one of the clone HTB9-PL-M-

Table 1. Tumorigenicity and growth in soft agar of RB^+ and RB^- HTB9 cells

Cell line	Plating efficiency in soft agar, %	Cells injected, no.	Mice with tumor/ mice injected	Average size, mm^3
HTB9	6.3	1×10^7	6/6	207.2
		2×10^6	4/4	28.3
		1×10^6	5/6	47.6
HTB9-PL-M	3.5	1×10^7	6/6	218.3
HTB9-PL-M-CL1	4.2	1×10^7	6/6	202.5
H/RB-M	<0.001	1×10^7	5/6*	6.1†
		2×10^6	1/4	6.3‡
		1×10^6	2/6§	4.2
H/RB-M-CL5	<0.001	1×10^7	3/6	5.1¶
H/RB-CL2	<0.001	1×10^7	1/11	5.3‖

RB^+ and RB^- clones were injected into the right and left flanks, respectively, of nude mice. Tumor size at 1 mo is given for 1×10^7 injected cells and at 2 mo for 1×10^6 and 2×10^6 injected cells. Cubic volumes (mm^3) are given based on a three-dimensional measurement.

*Tumor T1 (130 mm^3) was removed at 1 mo (not included in size calculation).

†All remaining tumors (T3–T6) were removed at 2 mo.

‡Tumor T8 was removed at 2 mo.

§Tumor T2 (57 mm^3) was removed at 2 mo (not included in size calculation).

¶Tumor T7 was removed at 3 mo.

‖Tumor appeared at 5 mo but did not progress.

CL1 cells consistently formed large, progressively growing tumors after s.c. inoculation of nude mice. At 1 mo, tumor volume averaged 207.2 mm^3, 218.3 mm^3, and 202.5 mm^3, respectively, when 1×10^7 cells were injected (Table 1). RB^+ cells produced much smaller and nonprogressive tumors under the same conditions (Table 1). For example, H/RB-M and H/RB-M-CL5 formed tumors with an average size of 6.1 mm^3 and 5.1 mm^3, respectively, during the same period. With the exception of two progressively growing tumors (T1 and T2), which arose rapidly in animals inoculated with H/RB-M, all other tumors (T3–T8) from H/RB-M cells remained small over an extended period (Table 1). H/RB-CL2 cells formed only one small tumor after 5 mo, and this tumor did not progress. By histopathologic analysis, all small tumors developing after injection retained the malignant characteristics of the parental cells. For example, T5 tumor was composed of moderately differentiated bladder carcinoma cells with focal necrosis and invasion into adjacent connective tissue (Fig. 5).

To further analyze tumors that formed after injection of RB^+ cells, cell lines were established from rapidly growing T1 and T2, as well as six small, nonprogressing tumors (T3–T8). The cell lines obtained from tumors T1 and T2 were found to be RB^- as determined by immunoblotting (Fig. 2, lane 6). In contrast, cell lines derived from the small, nonprogressive tumors were RB^+ and had normal RB patterns by immunoblotting (Fig. 2, lanes 7–9). These results were confirmed by immunohistochemical staining. For example, the RB^- cell line obtained from T2 is shown in Fig. 3*C*, and the RB^+ cell line established from T7 is shown in Fig. 3*D*. Similarly, cell lines established from small H/RB-M-CL5- and H/RB-CL2-derived tumors were analyzed by immunoblotting and immunohistochemical staining. All of these cell lines were found to be RB^+ (data not shown).

DISCUSSION

In this study, we investigated the functional effects of a human RB-cDNA expression construct transfected into an RB^- bladder carcinoma cell line, HTB9. The RB-cDNA was driven by the strong human β-actin promoter and linked to a dominant selection marker. Both RB RNA and protein expression levels in the RB^+ cell lines were similar to that found, for example, in normal WI-38 fibroblasts (Fig. 2). Therefore, the phenotypic changes we report are probably

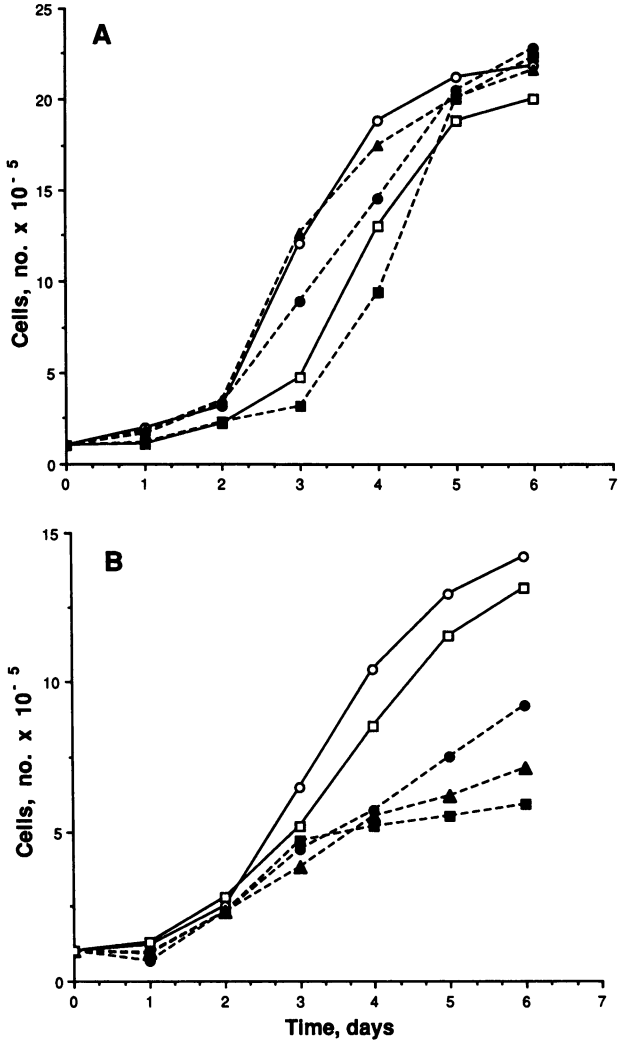

FIG. 4. Growth effects of pBARB and pHβAPr-1-neo on bladder carcinoma cells. HTB9 cells and RB^+-transfected lines derived from HTB9 cells were seeded at a concentration of 1×10^5 cells per 60-mm dish (day 0). Two different concentrations of fetal bovine serum were used, 10% (*A*) and 3% (*B*). ○, HTB9; □, HTB9-PL-M; ●, H/RB-M; ■, H/RB-M-CL5; and ▲, H/RB-CL2.

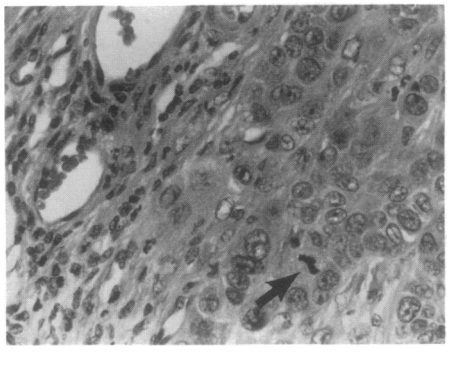

FIG. 5. Histopathology of RB^+ tumor (T7) growing in nude mice. Infiltrating tumor cells exhibited marked nuclear atypism, and mitotic figures (arrow) were frequent. (Hematoxylin/eosin; ×100.)

not the result of aberrantly high levels of RB expression in these transfectants. RNA blotting also established the specific expression of the exogenous RB-cDNA. These findings excluded the possibility that occult normal RB-expressing cells were selected from the parental HTB9 cell population or that an intact endogenous *RB* gene in these cells (5) was switched on by some unknown mechanism due to transfection.

The data obtained from this study are based mainly on cells that were stably isolated from parental HTB9 cells. If heterogeneity of parental HTB9 cells exists, our results might need to be interpreted differently. However, five separate plasmid-control clones and a plasmid-control mass culture gave similar growth rates to that of parental HTB9 cells in both 10% and 3% serum. In addition, because the tumorigenicity of two plasmid-control strains was similar to HTB-9 parental cells, we believe that control plasmids containing the β-actin promoter do not have adverse effects on this study.

We demonstrated that the propagation of RB^+ transfectant clones under standard culture conditions yields stable RB^+ cells for >1 yr. These findings differ from previous reports in which RB^- revertants occurred rapidly both *in vitro* and *in vivo* (12, 13, 15). Whether the differences reflect the constructs used, the methods of introducing the *RB* gene, the levels of RB expression, or the target cells used must still be resolved. However, developing a stable RB transfection system makes analyses of the effects of RB expression on phenotypic properties of previously RB^- tumor cells more readily interpretable.

Several important effects of RB expression on HTB9 growth properties were observed. RB^+ transfectants lost the ability to form colonies in semi-solid medium and showed partial inhibition of their growth when cultured at a low serum concentration. The inability of RB^+ HTB9 cells to grow in soft agar may provide a particularly sensitive assay to screen various RB constructs for biological activity. The difference in phenotype found between RB^+ HTB9 cells and their RB^- parental counterpart could also provide rapid, functional tests to examine the effect of introducing the *RB* gene into a variety of other RB^- tumor types.

We further demonstrated that introducing a functional *RB* gene caused the cells to form substantially smaller and nonprogressive tumors with a longer latency period. Moreover, tumors developed less frequently from RB^+ cells than their RB^- parental counterparts. These results strongly argue for an important role of RB functional loss in bladder cancer.

We observed two rapidly growing neoplasms among those induced by a mass population of marker-selected cells (H/RB-M) in which a minority of RB^- clones had been initially identified. Because both tumors were found to be RB^-, it is likely that they arose by selection of RB^- cells in the original population. In contrast, cells from the small, nonprogressively growing tumors in each case were found to express a normal RB protein. Such RB^+ tumors were histologically malignant and invasive. Thus, it is difficult to distinguish RB^+ tumors microscopically from tumors induced by RB^- parental cells. These findings are more consistent with a model by which *RB* acts as a growth suppressor rather than by induction of terminal differentiation. In any case, at least in this tumor cell type, introduction of a stable *RB* gene and its subsequent normal expression is not sufficient to completely suppress the malignant phenotype. Because multiple events are usually involved in the initiation and progression of most human cancers in which *RB* plays a role, inability of *RB* to suppress tumorigenicity completely is not unexpected.

Significant evidence has accumulated that the *RB* gene is closely related to cell growth regulation and suppression of tumorigenicity. We believe our RB^+ and RB^- cells provide an important system with which to analyze directly the effects of various growth factors and cytokines on signaling pathways in which RB plays an important role. These cell lines should also be valuable for testing the differential sensitivity of RB^+ and RB^- tumor cells to anticancer agents. Because patients with RB^- tumors may have a poorer prognosis than patients with RB^+ tumors of the same tumor type (23, 24), the results of such testing could become important in determining the optimal clinical therapy for a given tumor.

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