Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 3052–3056, April 1991
Biochemistry

Regulation of transforming growth factor β1 gene expression by the product of the retinoblastoma-susceptibility gene

(promoter/retinoblastoma response element/tumor suppressor/growth factor)

SEONG-JIN KIM*†, HY-DE LEE†, PAUL D. ROBBINS‡, KLAUS BUSAM†, MICHAEL B. SPORN†, AND ANITA B. ROBERTS†

†Laboratory of Chemoprevention, National Cancer Institute, Building 41, Room B1106, Bethesda, MD 20892; and ‡Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261

Communicated by Van Rensselaer Potter, January 8, 1991

ABSTRACT **Transforming growth factor β (TGF-β) isoforms inhibit the growth of many cell types and block progression of the cell cycle by inhibiting events in late G_1 phase. The retinoblastoma gene product, RB, also has properties of a cell-cycle regulatory factor. It remains underphosphorylated in the presence of TGF-β and has been shown to repress the activity of the c-*fos* promoter, resulting in inhibition of transit through the cell cycle. These observations led us to examine effects of human RB on the expression of the human TGF-β1 gene. Using chimeric TGF-β1 promoter–chloramphenicol acetyltransferase gene constructs, we show that RB induces TGF-β1 gene expression in CCL-64 mink lung epithelial cells and A-549 human lung adenocarcinoma cells but represses its expression in NIH 3T3 and AKR-2B mouse cells. Several sequences homologous to the c-*fos* RB control element were identified in the TGF-β1 promoter. These results demonstrate that human RB can regulate TGF-β1 gene expression negatively or positively depending on the cell type.**

Transforming growth factors β (TGF-β1, -β2, and β3) are homodimeric polypeptides belonging to a large family of structurally related multifunctional regulatory peptides (for a recent review, see ref. 1). TGF-βs regulate cell differentiation, cell growth, and cell function and are the most potent growth-inhibitory polypeptides known for a wide variety of cell types including most normal and transformed epithelial, endothelial, fibroblast, lymphoid, and hematopoietic cells (2–6). Although TGF-βs arrest growth in the late G_1 phase of the cell cycle, the mechanism by which they inhibit cell proliferation is virtually unknown (7, 8). In certain cells, such as keratinocytes, TGF-β1 has been shown to reduce the expression of growth-related genes, including c-*myc*, by inhibiting transcriptional initiation (9, 10). This inhibitory activity was abrogated by various viral transforming proteins, including the E7 protein of human papilloma virus type 16, the E1A protein of adenovirus type 5, and the large tumor (T) antigen of simian virus 40, which have been demonstrated to associate with the retinoblastoma gene product RB (10).

Although the RB gene product is synthesized in all phases of the cell cycle, it also has properties of a cell-cycle regulatory protein. Thus whereas underphosphorylated forms of RB are the primary species seen in the G_0 and G_1 phases of the cell cycle, the protein undergoes phosphorylation at multiple sites as cells traverse the G_1/S boundary (11–13). Treatment of cells with TGF-β appears to prevent phosphorylation of RB and retain RB in the underphosphorylated, growth-suppressive state (13). However, there is no direct evidence linking growth arrest by TGF-β1 with underphosphorylation of RB.

Recent results indicate that in mouse NIH 3T3 cells, RB downregulates the activity of AP-1 (14), a heterodimeric transcription factor that is thought to be involved in regulating a set of early response genes required for cell growth (15, 16). The cis-acting element allowing repression by RB was shown to reside in a 31-base-pair sequence, called an RB control element (RCE) lying between nucleotide positions −102 and −71 in the c-*fos* promoter. Further deletion analysis has demonstrated that there are two RCEs in the c-*fos* promoter and that the 5′ RCE (−97 to −86) is sufficient for the regulation of c-*fos* by RB (P.D.R., unpublished data).

Since this same cis-acting element is also found in the human TGF-β1 gene, we examined whether RB might also regulate TGF-β1 promoter activity through this element. The studies presented here demonstrate that transient expression of human RB significantly stimulated the activity of the human TGF-β1 promoter in CCL-64 mink lung epithelial cells and A-549 human lung adenocarcinoma cells but downregulated its activity in several other cell types. Our results suggest that RB can regulate TGF-β1 as well as c-*myc* and c-*fos* gene expression positively or negatively depending on the cell type.

MATERIALS AND METHODS

Cell Culture, Transfections, and Chloramphenicol Acetyltransferase (CAT) Assays. NIH 3T3 mouse fibroblasts, AKR-2B mouse embryo fibroblasts, cells, and CCL-64 mink lung epithelial cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. A-549 human lung adenocarcinoma cells were maintained in Dulbecco's modified Eagle's medium with high glucose, supplemented with 5% fetal bovine serum.

Transfections were performed by the calcium phosphate precipitation method (17). Following incubation with calcium phosphate precipitates and glycerol shock, the cells were incubated for 48 hr in serum-free medium or complete medium. After 48 hr, cells were harvested, and extracts were assayed for CAT activity according to Gorman *et al.* (18). CAT enzyme activity was normalized for transfection efficiency by cotransfection of 1.0 μg of a human growth hormone expression plasmid, pSVGH, and determination of secreted growth hormone in the medium, prior to harvesting for CAT activity (Nichols Institute, San Juan Capistrano, CA).

Plasmids. The construction of a series of deletion mutants of the human TGF-β1 gene promoter linked to the bacterial CAT gene has been described (19). The phTG7-1, phTG7-2, and phTG7-3 plasmids were generated by PCR amplification,

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: TGF, transforming growth factor; RB, retinoblastoma gene product; RCE, RB control element; *tk*, thymidine kinase gene; CAT, chloramphenicol acetyltransferase.

*To whom reprint requests should be addressed.

using oligonucleotides designed to generate ends with *Hind*-III and *Xba* I restriction sites, and cloned into the CAT vector cut with the same enzymes. Each plasmid was purified in two sequential CsCl centrifugation steps for maximal purity. The human RB expression vector phRB and control plasmid (14) and the mutant RB expression plasmid pRBJ82 (20, 21) have been described. The *fos*–CAT gene construct FC4 (22) was kindly provided by M. Fujii (Kanazawa Cancer Center, Japan). The 5′ flanking deletion mutant of the human c-*myc* promoter (pMyc6; ref. 23) was generated by PCR amplification and subcloned as described above.

RESULTS

Effect of Human RB on Human TGF-β1 Gene Expression in Mink Lung Epithelial Cells. To determine whether RB regulates the TGF-β1 promoter, portions of the human TGF-β1 promoter were fused to the bacterial CAT gene. Regulation of these TGF-β1–CAT gene constructs was monitored following transfection into CCL-64 mink lung epithelial cells together with (*i*) vector plasmid DNA (pJ3Ω), (*ii*) this plasmid carrying the human RB cDNA (phRB; ref. 14), or (*iii*) an RB deletion mutant construct (pRBJ82). We used the CCL-64 cells because these cells are sensitive to inhibition of growth by TGF-β. As shown in Fig. 1, cotransfection of cells with two different TGF-β1–CAT reporter constructs and phRB resulted in a 21-fold (phTG5) and 15-fold (phTG7) induction of CAT activity relative to the vector control. To demonstrate that a functional RB protein is required for stimulation, we cotransfected cells with the TGF-β1 promoter–CAT constructs and the plasmid pRBJ82, which produces an aberrant RB protein missing 35 amino acids encoded by exon 21 of the human *RB* gene (20, 21). As expected, the mutant RB failed to induce TGF-β1 promoter activity significantly.

To identify the specific sequences in the TGF-β1 promoter responsible for its stimulation by RB, a series of TGF-β1 deletion constructs was cotransfected into CCL-64 cells with a human RB expression vector or control vector. The activity of phTG2 (−1134 to +11) was induced 22-fold by RB (Fig. 2*A*). The induction of the TGF-β1 promoter activity by RB dropped almost to the basal level when the deletion extended into the region between −100 and −71, suggesting that the RB-responsive elements were located upstream of this point.

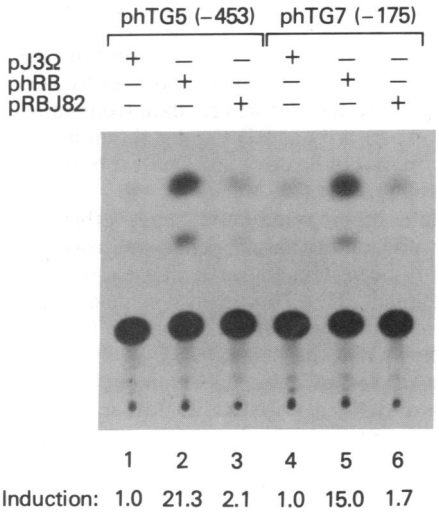

FIG. 1. Effect of human RB on human TGF-β1 gene expression. Reporter construct (phTG5 or phTG7; 10 μg) was cotransfected with 20 μg of RB plasmid (phRB), the control plasmid (pJ3Ω), or the RB mutant construct (pRBJ82) into CCL-64 cells by the calcium phosphate coprecipitation method. Forty-eight hours after transfection, the cells were harvested and CAT activity was determined. The assay was standardized by using equal amounts of protein for each reaction. Transfection efficiency was normalized as described in *Materials and Methods*. Results of a representative experiment are shown.

To examine whether stimulation of the activity of the human TGF-β1 promoter might be mediated through the same or similar sequence elements as the RCEs identified in the c-*fos* promoter (14), we analyzed the TGF-β1 gene for the presence of related sequences of potential regulatory importance. Multiple copies of RCE-like sequences are present in both the direct and inverted forms between −175 and −82 (Fig. 3). To demonstrate that these putative RCEs could mediate the effect of RB on the TGF-β1 promoter, a construct containing sequences of the TGF-β1 gene between −181 and −150 was linked to a *tk* promoter–CAT vector. This construct was inducible by RB (Fig. 2*B*), suggesting that the two copies of the RCE, one direct and one inverted, located between −181 and −150 might be responsible in part for induction of the human TGF-β1 promoter by RB. Further experiments are necessary to determine whether other sequences between −175 and −82 might also be important. A putative RCE was also identified in the c-*myc* promoter (Fig. 3). Moreover, sequence analysis of the 5′ flanking regions of the TGF-β2 (24) and TGF-β3 (25) genes also showed the presence of multiple putative RCEs. Preliminary data demonstrate that, similar to our findings with TGF-β1, appropriate constructs containing these regions of the TGF-β2 and -β3 genes linked to the CAT gene are activated by RB, following cotransfection into CCL-64 cells.

Induction of c-*myc*, c-*fos*, and TGF-β1 Promoter Activity by RB Protein in CCL-64 Cells. To evaluate the generality of the action of the RB protein in regulating transcription, we studied the regulation of the c-*fos* and c-*myc* promoters by RB in CCL-64 cells. Transcription of both a c-*myc* promoter construct containing sequences between −134 and +510 and a c-*fos* promoter construct (−404 to +42) was induced by RB in these cells (Fig. 4, lanes 1–4). A previous report (14) showed that in NIH 3T3 cells, RB represses c-*fos* expression through a cis-acting element between −102 and −71 in the human c-*fos* promoter (14). However, in CCL-64 cells the c-*fos* construct (FC4, −404 to +42) was induced by RB (Fig. 4).

To see whether the effect of RB on TGF-β1 promoter activity was dependent on cell type, we cotransfected the TGF-β1 promoter–CAT construct (phTG5) with phRB or pJ3 into A-549, CCL-64, NIH 3T3, and AKR-2B cells (Fig. 5). RB induced TGF-β1 promoter activity in A-549 and CCL-64 cells but repressed its activity in NIH 3T3 and AKR-2B cells. Averaging the results from four independent experiments indicates that there was a 4.5- and an 18-fold induction of TGF-β1 promoter activity by RB in A-549 and CCL-64 cells, respectively, and a 3- and 1.5-fold reduction of its activity by RB in NIH 3T3 and AKR-2B cells, respectively. We also confirmed that RB repressed c-*fos* promoter activity 1.5-fold in NIH 3T3 cells (data not shown), as previously reported (14).

DISCUSSION

Recent interest in negative regulation of cell growth has intensified investigations into possible mechanistic links between the secreted TGF-β peptides and the nuclear phosphoprotein RB. Previous to this report, attention was focused on possible control by TGF-β of either the function of RB (10) or its state of phosphorylation (13). Here we show the existence of the reciprocal process—namely, regulation of TGF-β1 expression by RB—and demonstrate that sequences homologous to a previously defined RB-responsive element, the RCE (14), mediate the regulation of human TGF-β1 promoter activity by the RB protein.

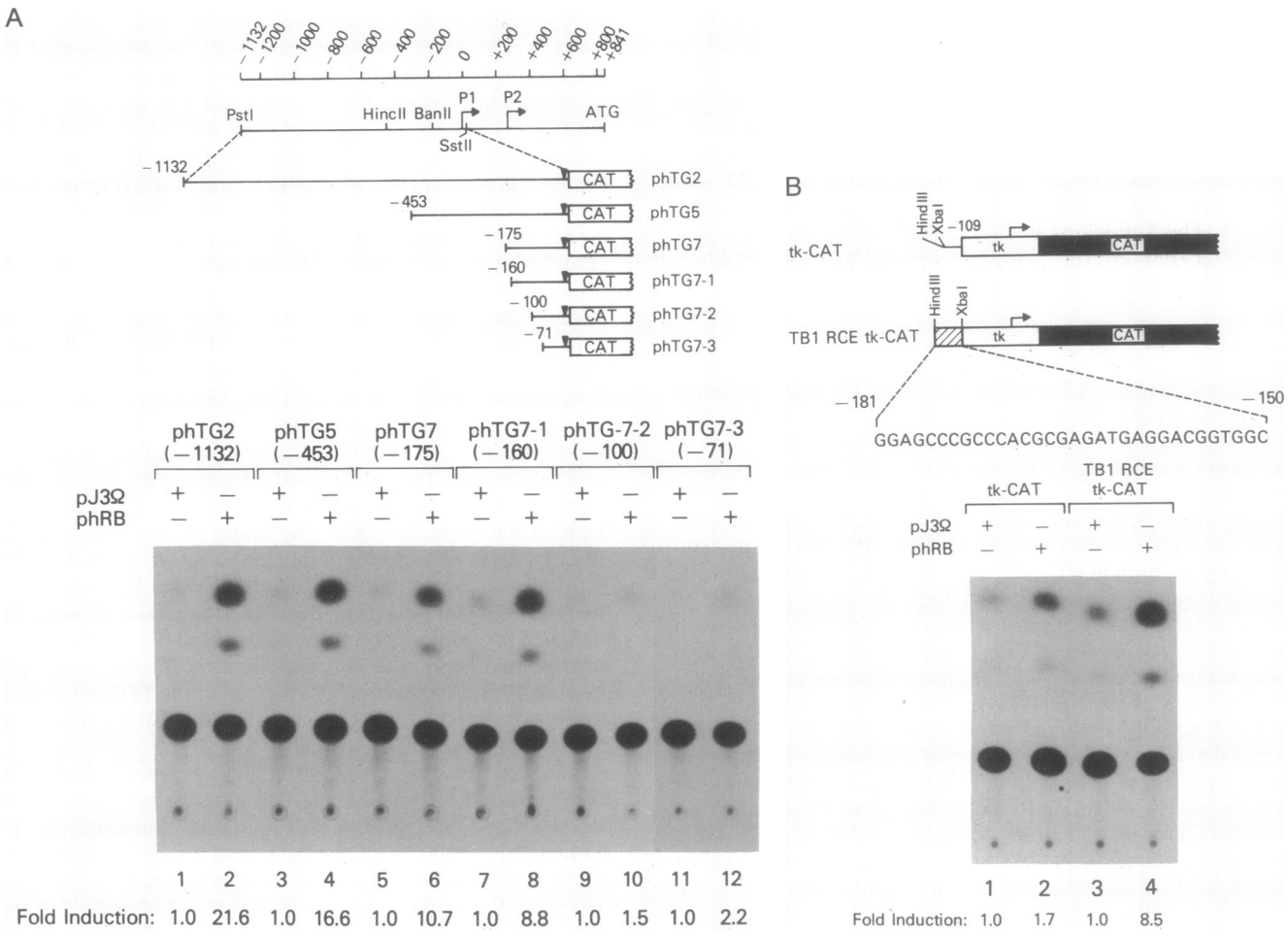

FIG. 2. Mapping of the cis-acting elements mediating induction by RB in the human TGF-β1 promoter. (*A*) At the top, an extended map of the promoter region of the human TGF-β1 gene is shown, indicating the two major transcription start sites (P1 and P2) and several restriction enzyme sites. To analyze the inducibility by RB, 10 μg of each plasmid DNA was cotransfected with 20 μg of the RB expression plasmid (phRB) or the control plasmid (pJ3Ω) into CCL-64 cells; after 48 hr, cells were harvested and CAT enzyme activity was determined. (*B*) TB1 RCE tk-CAT was generated by inserting oligonucleotides containing sequences between positions −181 and −150 of the TGF-β1 promoter into a *Hin*dIII/*Xba* I site 5′ of the *tk* (thymidine kinase gene) promoter of the tk-CAT vector. Activity of the constructs was assayed in CCL-64 cells.

In agreement with the recent report (14) demonstrating that RB downregulates c-*fos* transcription in NIH 3T3 cells, we have shown that RB also downregulates TGF-β1 transcription in both NIH 3T3 and AKR-2B cells. More interesting is our observation that, depending on the particular cells assayed, RB can also upregulate the TGF-β1 gene. Thus RB positively regulates transcription of both the TGF-β1 and c-*fos* genes in CCL-64 and A-549 cells. Induction of TGF-β1 transcription by RB is presumably mediated by underphosphorylated RB, since the basal level of transcription of the TGF-β1 promoter was greater in quiescent cells (unpublished results). Of note, the basal level of TGF-β1 transcription in A-549 and CCL-64 cells is low compared with the level of expression in NIH 3T3 and AKR-2B cells. Moreover, TGF-β1 strongly inhibits the growth of A-549 (3) and CCL-64 (27) cells, whereas it has a weak positive effect on the growth of NIH 3T3 and AKR-2B cells (28). Whether or not any of these parameters are relevant with respect to the effects of RB on TGF-β1 transcription is not understood. In addition, we cannot yet exclude the possibility that the positive and negative effects of RB may be a species-specific effect.

Previous studies have suggested that RB might mediate the effects of TGF-β on growth and that these two peptides might share a common pathway. Thus Laiho *et al.* (13) have proposed that TGF-β might regulate the state of phosphorylation of RB, enhancing the underphosphorylated, growth-inhibitory form of RB. Pietenpol *et al.* (10) have shown that the downregulation of c-*myc* expression associated with inhibition of the growth of keratinocytes by TGF-β might also be mediated by RB. However, data from cells lacking either TGF-β responsivity or RB suggest that links between TGF-β and RB are not obligatory and that each, independently, can inhibit cell growth (29, 30).

Our data, on the other hand, suggest that the TGF-β1 gene may be one of the target genes controlled by RB. It has previously been demonstrated that expression of TGF-β1 is autoregulated (31). This effect is mediated through AP-1 binding sites in the TGF-β1 promoter (26, 32). The present study shows that the human TGF-β1 promoter also contains multiple copies of sequence elements homologous to the previously defined c-*fos* RCE (14), in both the direct and inverted forms (see Fig. 3), and that these elements can also activate TGF-β expression in cells such as CCL-64 and A-549, which are strongly inhibited by TGF-β. Whether other sequences in addition to these putative RCEs are also involved in regulation of the TGF-β1 promoter by RB remains to be determined. However, in contrast to selective activation of the TGF-β1 promoter (as compared with the promoters for TGF-β2 and -β3) through AP-1 sites (24, 25), the presence of multiple RCEs in the 5′ flanking region of the genes for all three mammalian TGF-β isoforms suggests that regulation by RB may be a feature common to all of the TGF-β genes and may have important consequences for control of the cell cycle. Moreover, the ability of a tumor-suppressor gene such

c-fos RCE	$^{-97}$GCGCCACCCCT^{-87}
	$^{-82}$GCGCCACCGTG^{-72}
c-myc RCE	$^{-136}$TCCCCACCCTC^{-126}
	$^{-127}$TCCCCACCCTC^{-117}
	$^{-72}$CCCCCACGCCC^{-82}
TGF-ß1 RCE	$^{-175}$CGCCCACGCGA^{-165}
	$^{-148}$GGGCCACCGTC^{-158}
	$^{-82}$GCCCCACCCAG^{-92}
TGF-ß2 RCE	$^{+255}$TTGCCACCCTA^{+245}
	$^{+324}$CTCCCACCCTT^{+314}
	$^{+365}$TCCCCAGCCAG^{+375}
	$^{+450}$AACCCAGCCAC^{+440}
	$^{+695}$CAGCCACCGCG^{+685}
TGF-ß3 RCE	$^{-688}$CCCCCACCCCG^{-678}
	$^{-674}$CCCCCAGCCCC^{-664}
	$^{-372}$CCCCCAGCCCC^{-382}
	$^{-63}$CCCCCACCCCA^{-52}

FIG. 3. Sequences homologous to the c-*fos* RCE in the c-*myc* promoter and the human TGF-β1, -β2, and -β3 promoters. For the c-*myc* promoter, sequences between -150 and -1 were compared. Sequences between -200 and -71 in the human TGF-β1 promoter, and between $+1$ and $+850$ and between -1 and -700 in the 5′ flanking regions of the TGF-β2 and -β3 genes, respectively, were examined. Note that certain RCEs, such as TGF-β1 -148 to -158, are in the inverse (3′ to 5′) orientation.

as *RB* to induce expression and presumably secretion of a diffusible growth inhibitor such as TGF-β suggests that the RB protein might suppress growth not only of the cells in which RB is expressed but also of adjacent RB-negative cells that might still be sensitive to the inhibitory actions of TGF-β

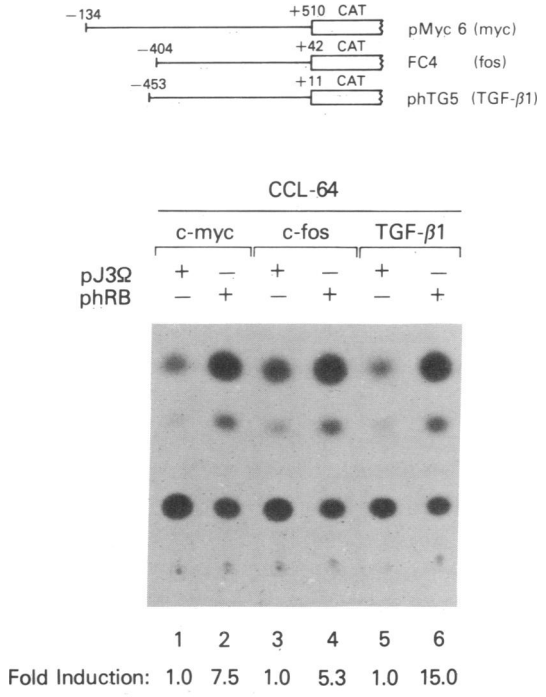

FIG. 4. Effect of RB on the expression of c-*fos*, c-*myc*, and TGF-β1 promoter constructs in CCL-64 cells. Ten micrograms of c-*fos* (FC4), c-*myc* (pMyc6), or TGF-β1 (phTG5) promoter construct was cotransfected with 20 μg of the human RB expression vector (phRB) or the control vector (pJ3Ω). CAT activity was assayed after 48 hr.

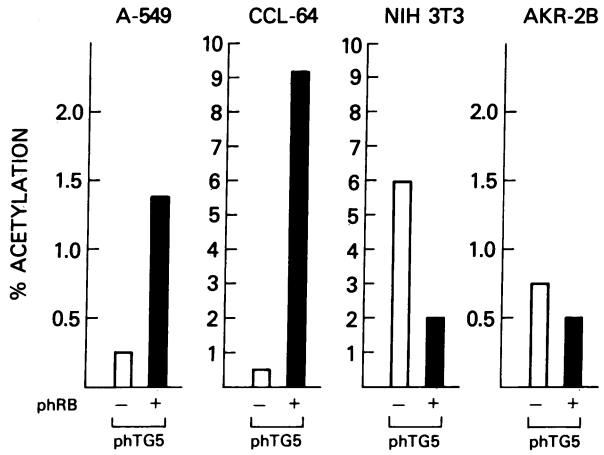

FIG. 5. Effect of human RB on expression of the TGF-β1 promoter in A-549, CCL-64, NIH 3T3, and AKR-2B cells. Cells were transfected with 10 μg of phTG5 and 20 μg of phRB or the control plasmid pJ3Ω. Results (% acetylation of chloramphenicol added to cell lysates) represent the average of three independent experiments.

(29). Suppression of tumorigenesis by RB "in trans" is an exciting possibility.

We are indebted to Robert Weinberg and James Battey for the gifts of the *RB* and c-*myc* constructs, respectively, to Renee Webbink for technical assistance, and to Andrew Geiser, Michael O'Reilly, Lalage Wakefield, and Robert Lafyatis for helpful suggestions.

1. Roberts, A. B. & Sporn, M. B. (1990) *Handb. Exp. Pharmacol.* **95,** 419–472.
2. Massagué, J., Cheifetz, S., Endo, T. & Nadal-Ginard, B. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 8206–8210.
3. Masui, T., Wakefield, L. M., Lechner, J. F., La Veck, M. A., Sporn, M. B. & Harris, C. C. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 2438–2442.
4. Tucker, R. F., Shipley, G. D., Moses, H. L. & Holley, R. W. (1984) *Science* **226,** 705–707.
5. Roberts, A. B., Anzano, M. A., Wakefield, L. M., Roche, N. S., Stern, D. F. & Sporn, M. B. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 119–123.
6. Kehrl, J. H., Wakefield, L. M., Roberts, A. B., Jakowlew, S. B., Alvarez-Mon, M., Derynck, R., Sporn, M. B. & Fauci, A. S. (1986) *J. Exp. Med.* **163,** 1037–1050.
7. Russell, W. E., Coffey, R. J., Ouellette, A. J. & Moses, H. L. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 5126–5130.
8. Silberstein, G. B. & Daniel, C. W. (1987) *Science* **237,** 291–293.
9. Pietenpol, J. A., Holt, J. T., Stein, R. W. & Moses, H. L. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 3758–3762.
10. Pietenpol, J. A., Stein, R. W., Moran, E., Yaciuk, P., Schlegel, R., Lyons, R. M., Pittelkow, M. R., Munger, K., Howley, P. M. & Moses, H. L. (1990) *Cell* **61,** 777–785.
11. Mihara, K., Cao, X.-R., Yen, A., Chandler, S., Driscoll, B., Murphree, A. L., T'Ang, A. & Fung, Y.-K. T. (1989) *Science* **246,** 1300–1303.
12. Ludlow, J. W., Shon, J., Pipas, J. M., Livingston, D. M. & DeCaprio, J. A. (1990) *Cell* **60,** 387–396.
13. Laiho, M., DeCaprio, J. A., Ludlow, J. W., Livingston, D. M. & Massagué, J. (1990) *Cell* **62,** 175–185.
14. Robbins, P. D., Horowitz, J. M. & Mulligan, R. C. (1990) *Nature (London)* **346,** 668–671.
15. Angel, P., Allegretto, E. A., Okino, S. T., Hattori, K., Boyle, W. J., Hunter, T. & Karin, M. (1988) *Nature (London)* **332,** 166–171.
16. Ryseck, R.-P., Hirai, S. I., Yaniv, M. & Bravo, R. (1988) *Nature (London)* **334,** 535–537.
17. Luse, D. S. & Roeder, R. G. (1980) *Cell* **20,** 691–699.
18. Gorman, C. M., Moffat, L. F. & Howard, B. H. (1982) *Mol. Cell. Biol.* **2,** 1044–1051.
19. Kim, S.-J., Glick, A., Sporn, M. B. & Roberts, A. B. (1989) *J. Biol. Chem.* **264,** 402–408.

20. Horowitz, J. M., Yandall, D. W., Park, S.-H., Canning, S., Whyte, P., Buchkovich, K., Harlow, E., Weinberg, R. A. & Dryja, T. P. (1989) *Science* **243,** 937–940.
21. Kaye, F. J., Kratzke, R. A., Gerster, J. L. & Horowitz, J. M. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 6922–6926.
22. Sassone-Corsi, P., Sisson, J. C. & Verma, I. M. (1988) *Nature (London)* **334,** 314–319.
23. Battey, J., Moulding, C., Taub, R., Murphy, W., Stewart, T., Potter, H., Lenoir, G. & Leder, P. (1983) *Cell* **34,** 779–787.
24. Noma, T., Glick, A. B., Geizer, A. G., O'Reilly, M. A., Miller, J., Roberts, A. B. & Sporn, M. B. (1991) *Growth Factors*, in press.
25. Lafyatis, R., Lechleider, R., Kim, S.-J., Jakowlew, S., Roberts, A. B. & Sporn, M. B. (1990) *J. Biol. Chem.* **265,** 19128–19136.
26. Kim, S.-J., Angel, P., Lafyatis, R., Hattori, K., Kim, K. Y., Sporn, M. B., Karin, M. & Roberts, A. B. (1990) *Mol. Cell. Biol.* **10,** 1492–1497.
27. Danielpour, D., Dart, L. L., Flanders, K. C., Roberts, A. B. & Sporn, M. B. (1990) *J. Cell. Physiol.* **138,** 79–86.
28. Shipley, G. D., Childs, C. B., Volkenant, M. E. & Moses, H. L. (1984) *Cancer Res.* **44,** 710–716.
29. Ong, G., Sikora, K. & Gullick, W. J. (1990) *Oncogene,* in press.
30. Lee, E. Y.-H. P., To, H., Shew, J.-Y., Bookstein, R., Scully, P. & Lee, W.-H. (1988) *Science* **241,** 218–221.
31. Van Obberghen-Schilling, E., Roche, N. S., Flanders, K. C., Sporn, M. B. & Roberts, A. B. (1988) *J. Biol. Chem.* **263,** 7741–7746.
32. Kim, S.-J., Jeang, K.-T., Glick, A. B., Sporn, M. B. & Roberts, A. B. (1989) *J. Biol. Chem.* **264,** 7041–7045.