BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **252,** 269–273 (1998)
ARTICLE NO. RC989623

Stable Transfection of Retinoblastoma Gene Promotes Contact Inhibition of Cell Growth and Hepatocyte Nuclear Factor-1-Mediated Transcription in Human Hepatoma Cells

Satoshi Awazu,* Keisuke Nakata,† Daisaku Hida,† Tomoko Sakamoto,‡ Kimiko Nagata,‡ Nobuko Ishii,‡ and Takashi Kanematsu*,[1]

**Department of Surgery II, Nagasaki University School of Medicine, Nagasaki 852-8501, Japan; †The First Department of Internal Medicine, Nagasaki University School of Medicine, Nagasaki 852-8501, Japan; and ‡Health Research Center, Nagasaki University, Nagasaki 852-8501, Japan*

Received September 23, 1998

The retinoblastoma (Rb) gene is a key factor involved in cell cycle regulation. In the present study, alterations in cell growth and expression of the α-fetoprotein and albumin genes by the stable transfection of the Rb gene were analyzed in HuH-7 human hepatoma cells. Cell growth was almost similar in the Rb gene-transfected and nontransfected cells at the sparse cell density. However, under a confluent condition, contact inhibition of cell growth was stimulated by the Rb gene transfection. By Northern blotting, the levels of the α-fetoprotein and albumin mRNA as well as the transcript of hepatocyte nuclear factor-1 (HNF-1), a liver-specific transcriptional factor regulating both genes, were apparently elevated in the Rb gene-transfected cells, compared with the nontransfected cells. These results suggest that the Rb gene transduction promotes contact growth inhibition in conjunction with the enhanced HNF-1-mediated liver-specific gene transcription in human hepatoma cells. © 1998 Academic Press

The retinoblastoma (Rb) gene originally identified and eventually cloned by virtue of its absence in a number of retinoblastoma cell lines is characterized as a tumor suppressor gene (1). The Rb protein is known to play a crucial role in the regulation of cell cycle, particularly, at the G_1-S phase transition. It is now well established that activity of the Rb protein during cell cycle is regulated by its level of phosphorylation; it is underphosphorylated in G_1 phase and hyperphosphorylated at the G_1-S phase transition (2,3). In addition, subsequent studies have shown that the Rb gene expression is relevant to induction of cell differentiation. For example, the Rb protein, in association with myogenic factor such as MyoD, is required to induce terminal differentiation of muscle cells (4).

Previous studies showed that inactivation of the Rb gene was a frequent event in tumorigenesis and found in a variety of tumor cell types including human hepatoma cells (5,6). In fact, Kaino et al. (7) reported the disrupted expression of the Rb gene in HuH-7 human hepatoma cells as well as other human hepatoma cell lines. However, little is known about the Rb gene-mediated alterations in cell growth or expression of liver-specific genes such as α-fetoprotein (AFP) and albumin in these cells.

In the present study, the Rb gene was stably transfected into HuH-7 human hepatoma cells, and cell growth and expression of the AFP and albumin genes were compared in the Rb gene-transfected and nontransfected cells.

MATERIALS AND METHODS

Chemicals. The Rb gene expression plasmid, pBARB, in which the β-actin promoter and the simian virus promoter regulate the Rb gene expression and the neomycin-resistant gene expression, respectively (8), was a generous gift from Dr R.Takahashi (Kobe University School of Medicine, Kobe, Japan). [α-^{32}P]-deoxycytidine triphosphate and D-threo-[dichloro-acetyl-1-^{14}C]-chloramphenicol were purchased from Amersham Japan (Tokyo, Japan). Lipofectin reagents were purchased from GIBCO BRL (Geithersburg, MD).

Cell culture and stable transfection in HuH-7 cells. The HuH-7 cells were maintained in a chemically defined medium, IS-RPMI (9). The Rb gene-containing plasmid, pBARB, or the control plasmid, pSV-neo, in which the simian virus promoter regulates the neomycin-resistant gene expression were transfected into HuH-7 cells by the lipofection method. Two days later, the cells were sub-

[1] Address correspondence to Takashi Kanematsu, Department of Surgery II, Nagasaki University School of Medicine, Nagasaki 852-8501, Japan. Fax: +81-95-849-7319. E-mail: http://WWW.med.nagasaki-u.ac.jp/surgery 2.

0006-291X/98 $25.00

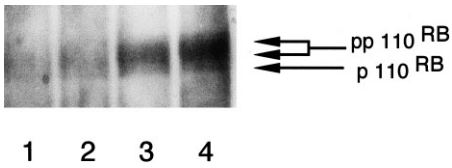

FIG. 1. Immunoblotting of RB protein. $pp110^{RB}$ and $p110^{RB}$ indicate hyperphosphorylated and hypophosphorylated forms of the RB protein, respectively. Lane 1, HuH-7 wild; lane 2, HuH-7 neo; lane 3, HuH-7 RB-1; and lane 4, HuH-7 RB-2.

cultured into 100mm-dishes. After 24 hours, the medium was replaced with the fresh medium containing 400 μg/ml G418 (GIBCO BRL), and the cells were subsequently maintained in G418-containing medium. After 4 weeks, the G418-resistant colonies were selected and expanded for further analyses.

Immunoblotting. Fifty μg of cellular protein was electrophoresed in an 7.5% SDS/polyacrylamide gel and electroblotted to a nitrocellulose membrane (Amersham Pharmacia Biotech Ltd, England). Nonspecific binding of the membrane was blocked with 5% skim milk for 1 hour at room temperature. The membrane was then probed with a mouse monoclonal anti-Rb protein (10) at 4°C overnight. The signals were then visualized by the enhanced chemiluminescence system (Amersham Pharmacia Biotech Ltd, England). As shown in Figure 1, the Rb protein was overexpressed in the pBARB-transfected cells, HuH-7 RB-1 and RB-2, compared with the non-transfected cells, HuH-7 wild, or the pSV-neo-transfected cells, HuH-7 neo.

Cell growth. Cell growth was analyzed using 24-well multiplates, and 2×10^4 cells were placed into each well and incubated at 37°C in 5% CO_2. One day later, the medium was replaced with fresh medium or fresh medium supplemented with 5% fetal bovine serum. The medium was changed at 2 days interval, and numbers of viable cells were counted at 1,2,4 and 6 days after incubation, respectively using the trypan blue dye exclusion method. In addition, the cells were incubated under a sparse condition (5×10^5 cells/100-mm dish) or a confluent condition (2×10^6 cells/100-mm dish). Four days later, cell cycle was analyzed by the flow cytometric method. Briefly, the cells were trypsized, pelleted and washed with phosphate buffered saline (PBS). The resuspended cells were centrifugated at 500g for 2 minutes at 4°C and fixed with cold ethanol (70%, final concentration). The fixed cells were centrifugated, washed with PBS, pretreated with RNase (100 μg/ml) at 37°C for 30 minutes, and then stained with propidium iodide (100 μg/ml). Samples were stored on ice prior to analysis by the flow cytometry with an EPICS PROFILE scan (Coulter Co., Hileah, FL).

Northern blotting. Total RNA was isolated from the cultured cells by the guanidium isothiocyanate method. Total RNA (10 μg) was fractionated on a 1% formaldehyde agarose gel, transferred to a nylon membrane, and hybridized with a [^{32}P]-labeled cDNA probe. AFP cDNA (pHAF-2) (11), albumin cDNA (palb-7) (12), HNF-1 cDNA (a generous gift from prof. T. Tamaoki, University of Calgary, Calgary, Canada), and human β-actin cDNA (Wako Chem, Ltd., Osaka, Japan) were used as probes.

Chloramphenicol acetyltransferase plasmids and cell transfection. The chloramphenicol acetyltransferase (CAT) plasmids used in this study were described previously (13,14). pBR-CAT plasmid contains the CAT coding sequence and the simian virus 40 polyadenylation signal but no upstream regulatory sequences. pAF0.2-CAT contains 169bp of the AFP promoter sequence linked to the CAT gene in pBR-CAT. pAL0.3-CAT contains 288 bp fragment of the human albumin 5′-flanking sequence inserted at the 5′end of the CAT gene in pBR-CAT. pSV2-CAT was also used as a control plasmid in this study (15).

The HuH-7 wild cells were co-transfected with 5 μg CAT plasmid and 10 μg pBARB plasmid or 10 μg pSV-neo plasmid as a control by the lipofection method. Two days later, the cells were harvested and lysed by five cycles of freezing and thawing. The lysate was heated at 63°C for 10 minutes, and the supernatant was used for determination of the CAT activity as described previously (13,14). The amounts of protein and incubation times were 20 μg and 20 minutes (pSV2-CAT), 30 μg and 120 minutes (pAF0.2-CAT and pAL0.3-CAT).

RESULTS

Comparison of Cell Growth in the Rb Gene-Transfected and Nontransfected Cells

Cell growth was analyzed in the Rb gene-transfected cells (HuH-7 RB-1 and RB-2) and the nontransfected cells (HuH-7 wild and HuH-7 neo) in the absence or presence of 5% FBS (Figure 2A). Cell growth was almost similar in both cell lines until 4days after incubation. However, the growth curves in HuH-7 RB-1 and RB-2 cells reached a plateau 4 days after incubation, whereas HuH-7 wild cells as well as HuH-7 neo cells could proliferate over 4days after incubation. The same results were observed irrespective of the absence or presence of 5% FBS. By the flow cytometric analysis, the patterns of cell cycle in HuH-7 wild and HuH-7 neo cells were not affected by cell densities. In contrast, the patterns in HuH-7 RB-1 and RB-2 cells were shifted to G_1 phase at the confluent cell density, compared with those at the sparse cell density (Figure 2B).

Increase in the Levels of AFP, Albumin, and HNF-1 mRNA in the Rb Gene-Transfected Cells

Total cellular RNA was extracted from the cells cultured at the confluent cell density. The levels of both AFP and albumin mRNA were apparently elevated in HuH-7 RB-1 and RB-2 cells, compared with HuH-7 wild or HuH-7 neo cells (Figure 3). Since HNF-1 is a liver-specific transcriptional factor regulating both the AFP and albumin genes, the levels of HNF-1 mRNA were compared in the Rb gene-transfected and nontransfected cells. The levels of HNF-1 mRNA in HuH-7 RB-1 and RB-2 cells were higher than those in HuH-7 wild or HuH-7 neo cells. The levels of β-actin mRNA used as a control did not differ in the cell lines.

Effects of Rb Gene-Transfection on AFP- and albumin-CAT Expression

The AFP and albumin promoter sequences have the HNF-1 binding motifs. pAF0.2-CAT and pAL0.3-CAT contain the AFP promoter and the albumin promoter sequence, respectively. When HuH-7 wild cells were co-transfected with the CAT plasmid and the Rb gene expression plasmid, the co-transfection resulted in the increase in CAT expression from the AFP- and albumin-CAT plasmids (Figure 4). CAT

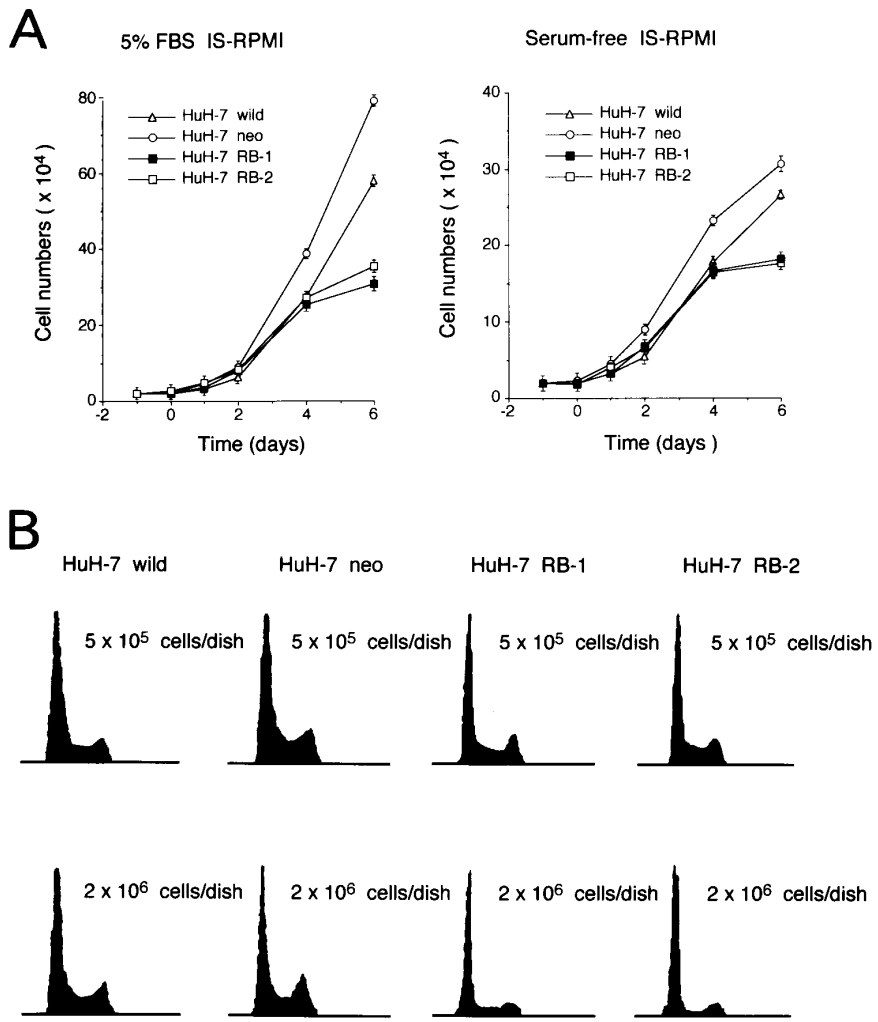

FIG. 2. Effects of Rb gene transfection on cell growth. (A) The cells were incubated in IS-RPMI with 5% FBS (left column) or serum free IS-RPMI (right column). Numbers of viable cells were counted at indicated times using the trypan blue dye exclusion method. Each value is expressed as mean±SD (n=4). HuH-7 wild (△); HuH-7 neo (○); HuH-7 RB-1 (■); and HuH-7 RB-2 (□). (B) The cells were incubated at the sparse condition (5×10^5 cells/100-mm dish) or the confluent condition (2×10^6 cells/100-mm dish). Four days later, cell cycle was analyzed by flow cytometry as described in Materials and Methods.

expression from pSV2-CAT used as a control plasmid was not affected by this treatment.

DISCUSSION

In this study, the effects of the Rb gene transduction on cell growth and expression of the AFP and albumin genes were analyzed in HuH-7 human hepatoma cells. Cell growth was almost similar in the Rb gene-transfected and nontransfected cells at the sparse cell density. However, when cell culture became near confluent, cell growth was suppressed in the Rb gene-transfected cells, but cell proliferation continued in the nontransfected cells. Similar results were reported previously (8), where the Rb gene functioned as a growth suppressor in human bladder carcinoma cells, resulting in growth inhibition of the Rb gene-transfected cells over 3 days after incubation with the medium containing a relatively lower concentration of FBS. To evaluate whether the growth inhibition was associated with the concomitant activation of contact growth inhibition in the Rb gene-transfected cells, subsequent studies were performed using the flow cytometric analysis as described previously (16). The patterns of cell cycle did not differ at the sparse or confluent cell densities in the nontransfected cells. In contrast, the patterns were shifted to G_1 phase at the confluent cell density in the Rb gene-transfected cells. These results suggest that the Rb gene transduction enhances the ability of contact growth inhibition and suppresses cell growth under a confluent condition in HuH-7 cells.

Expression of many liver-specific genes is regulated by HNF-1, a liver-specific transcriptional factor. In the AFP and albumin gene expression, HNF-1 up-

regulates both gene expression through the activation of the AFP promoter and enhancer and the albumin promoter, each of which contains the HNF-1 binding motif (13). By Northern blot analysis, the levels of both the AFP and albumin transcripts as well as the levels of HNF-1 transcripts were elevated in the Rb gene-transfected cells, compared with the nontransfected cells. In addition, co-transfection experiments using CAT plasmid and the Rb gene expression plasmid revealed the increased CAT expression from the CAT plasmids containing the HNF-1 binding motif in the 5′-flanking sequences. Thus, our results indicate that the Rb gene transduction stimulates the HNF-1-mediated transcription including the AFP and albumin genes. Recent studies have demonstrated that the Rb gene product can either positively or negatively regulate expression of several genes through *cis*-acting elements in a cell-type-dependent manner (17–21). The nucleotide sequence of the retinoblastoma control element is considered to be GCCACC or CCACCC (22). By the analysis of the 5′-flanking sequence of the HNF-1 gene, it contains the CCACCC motif between-187 and -182bp in addition to HNF-4 and HNF-3 binding sites (23). This would account for the Rb gene-mediated stimulation of HNF-1 expression at the transcriptional level.

It was reported that the albumin synthesis was cell density-dependent in primary culture of rat hepatocytes. When rat hepatocytes were cultured at the confluent cell density, the tight cell-to-cell contact resulted in the decreased DNA synthesis but the increased albumin synthesis (24). This coincides with our results that the Rb gene functions as both a mediator of contact growth inhibition and an inducer of the liver-specific genes, although human hepatoma cells were used in this study.

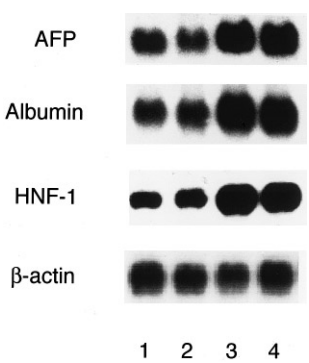

FIG. 3. Comparison of the levels of AFP, albumin and HNF-1 mRNA in Rb gene-transfected and nontransfected HuH-7 cells. Total cellular RNA was extracted from each cell line, and the levels of AFP, albumin and HNF-1 mRNA as well as the levels of β-actin mRNA as a control were analyzed by Northern blotting as described in Materials and Methods. Lane 1, HuH-7 wild; lane 2, HuH-7 neo; lane 3, HuH-7 RB-1; and lane 4, HuH-7 RB-2.

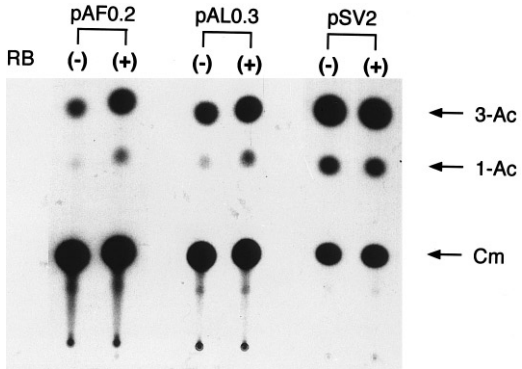

FIG. 4. Effects of Rb gene expression vector transfection on AFP- and albumin-CAT expression. The HuH-7 cells were co-transfected with each indicated CAT plasmid and control vector or with each indicated CAT plasmid and Rb gene expression vector. Two days later, CAT activities were determined as described in Materials and Methods. 3-Ac, 3-acetylchloramphenicol; 1-Ac,1-acetylchloramphenicol; Cm, chloramphenicol.

The AFP and albumin genes are believed to be derived from a common ancestral gene. However, the AFP expression in normal hepatocytes is repressed by the AFP silencer existing between its enhancer and promoter regions (25). The AFP silencer interacts with its enhancer to inactivate the enhancer-mediated activation of the promoter. In the absence of the AFP silencer activity as is seen in HuH-7 cells (25), the degree of AFP expression largely depends on its enhancer activity, because the enhancer is much stronger than the promoter. Based on these facts, it is likely that the Rb gene-mediated stimulation of the AFP gene expression is less efficient in normal hepatocytes than that in hepatoma cells.

In summary, the current study shows that the Rb gene transduction leads to contact inhibition of cell growth together with the enhanced expression of the HNF-1-mediated liver-specific genes in HuH-7 human hepatoma cells. In mammalian cells, cell cycle is tightly controlled by a variety of factors. Among the factors, the Rb gene seems to participate in a regulator of not only cell growth but also liver-specific gene expression in hepatocytes.

REFERENCES

1. Weinberg, R. A. (1991) *Science* **254,** 1138–1146.
2. DeCaprio, J. A., Ludlow, J. W., Lynch, D., Furukawa, Y., Griffin, J., Piwnica-Worms, H., Huang, C.-M., and Livingston, D. M. (1989) *Cell* **58,** 1085–1095.
3. Chen, P.-L., Scully, P., Shew, J.-Y., Wang, J. Y. J., and Lee, W. H. (1989) *Cell* **58,** 1193–1198.
4. Gu, W., Schneider, J. W., Condorelli, G., Kaushal, S., Mahdavi, V., and Nadal-Ginard, B. (1993) *Cell* **72,** 309–324.
5. Yokota, J., Akiyama, T., Fung, Y. K., Benedict, W. F., Namba, Y., Hanaoka, M., Wada, M., Terasaki, T., Shimosato, Y., Sugimura, T., and Terada, M. (1988) *Oncogene* **3,** 471–475.

6. Murakami, Y., Hayashi, K., Hirohashi, S., and Sekiya, T. (1991) *Cancer Res.* **51,** 5520–5525.
7. Kaino, M. (1997) *J. Gastroenterol.* **32,** 40–46.
8. Takahashi, R., Hashimoto, T., Xu, H.-J., Hu, S.-X., Matsui, T., Miki, T., Bigo-Marshall, H., Aaronson, S. A., and Benedict, W. F. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 5257–5261.
9. Nakabayashi, H., Taketa, K., Yamane, T., Miyazaki, M., Miyano, K., and Sato, J. (1984) *Gann* **75,** 151–158.
10. Xu, H.-J., Hu, S.-X., Hashimoto, T., Takahashi, R., and Benedict, W. F. (1989) *Oncogene* **4,** 807–812.
11. Morinaga, T., Sakai, M., Wegmann, T. G., and Tamaoki, T. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 4604–4608.
12. Urano, Y., Sakai, M., Watanabe, K., and Tamaoki, T. (1984) *Gene* **32,** 255–261.
13. Sawadaishi, K., Morinaga, T., and Tamaoki, T. (1988) *Mol. Cell. Biol.* **12,** 5179–5187.
14. Watanabe, K., Saito, A., and Tamaoki, T. (1987) *J. Biol. Chem.* **262,** 4812–4818.
15. Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982) *Mol. Cell. Biol.* **2,** 1044–1051.
16. Deffie, A., HAO, M., deoca Luna, R. M., Hulboy, D., Lozano, G. (1995) *Mol. Cell. Biol.* **15,** 3926–3933.
17. Pietenpol, J. A., Stein, R. W., Moran, E., Yaciuk, P., Schlegel, R., Lyons, R. M., Pittelkow, M. R., Münger, K., Howley, P. M., and Moses, H. L. (1990) *Cell* **61,** 777–785.
18. Robbins, P. D., Horowitz, J. M., and Mulligan, R. C. (1990) *Nature* **346,** 668–671.
19. Pietenpol, J. A., Münger, K., Howley, P. M., Stein, R. W., and Moses, H. L. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 10227–10231.
20. Kim, S. J., Onwuta, U. S., Lee, Y. I., Li, R., Botchan, M. R., and Robbins, P. D. (1992) *Mol. Cell. Biol.* **12,** 2455–2463.
21. Udvadia, A. J., Rogers, K. T., Higgins, P. D., Murata, Y., Martin, K. H., Humphrey, P. A., and Horowitz, J. M. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 3265–3269.
22. Chen, L. I., Nishinaka, T., Kwan, K., Kitabayashi, I., Yokoyama, K., Fu, Y. H., Grünwald, S., and Chiu, R. (1994) *Mol. Cell. Biol.* **14,** 4380–4389.
23. Kuo, C. J., Conley, P. B., Chen, L., Sladek, F. M., Darnell, J. E. Jr., and Crabtree, G. R. (1992) *Nature* **355,** 457–461.
24. Takehara, T., Matsumoto, K., and Nakamura, T. (1992) *J. Biochem.* **112,** 330–334.
25. Nakabayashi, H., Hashimoto, T., Miyao, T., Tjong, K. K., Chan, J., Tamaoki, T. (1991) *Mol. Cell. Biol.* **11,** 5885–5893.