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Accelerated Growth of Hepatocytes in Association with Up-Regulation of Cyclin E in Transgenic Mice Expressing the Dominant Negative Form of Retinoic Acid Receptor

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Retinoids play an important role in pathogenesis of liver diseases. To clarify the functional role of retinoic acid (RA) in liver, we developed transgenic mice (Tg) which express the dominant negative form of retinoic acid receptor (RARE) in liver. Here, we report that proliferation of hepatocytes in RARE Tg is greatly enhanced and that cyclin E is up-regulated in RARE Tg. Liver weight, liver/body weight, and proliferating cell nuclear antigen (PCNA) labeling index in RARE Tg were significantly increased, compared to those in wild-type mice ($P < 0.01$, each). Cell cycle analysis showed that 2N DNA content cells and aneuploid area between 2N and 4N DNA, reflecting S phase cells, were significantly increased in RARE Tg, compared to wild-type mice ($P < 0.01$, each). Of G1 phase-related proteins including cyclins, cyclin-dependent protein kinases (CDKs) and cyclin-dependent protein kinase inhibitors (CKIs), cyclin E mRNA and protein was up-regulated in liver from RARE Tg by reverse transcription polymerase chain reaction and Western blot analysis. Furthermore, the immunoprecipitation with anti-cdk2 antibody, followed by Western blot analysis with anti-cyclin E antibody indicated that cyclin E/cdk2 complex is increased in liver of RARE Tg. The results of the present study suggest that cyclin E in association with cdk2 governs cell cycle progression through G1 in hepatocytes where function of RA is inhibited.

Key Words: **retinoic acid receptor; dominant negative; transgenic mouse; cyclin E cdk2; cell cycle.**

Retinoids are important for regulation of normal cellular growth and differentiation (1, 2), and may play an important role in the chemoprevention of certain malignancies (3); retinoids can inhibit the progression of diseases in premalignant lesions of oral cavity, cervix and skin, and are effective in preventing the development of second primary tumors of aerodigestive tract, lung, and breast cancer. In liver, retinoids have been reported to play an important role in regeneration, fibrosis, and cancer (4–7). In addition, Muto *et al.* reported that an acyclic retinoid, polyprenoic acid, prevents second primary hepatocellular carcinoma (HCC) after surgical resection of the original tumor or the percutaneus injection of ethanol (8). Their report is very important, since the prognosis of HCC is still poor and depends on the rates of tumor recurrence and occurrence of second primary tumors (9).

Diversity in the control of gene expression by retinoid signal is generated through complexity at different levels of the signaling pathway (10). A major source of diversity originates from the existence of two families of retinoid acid receptors, the retinoic acid receptor (RAR) isotypes (α, β, and γ) and the retinoid X receptor (RXR) isotypes (α, β, and γ). These RAR/RXR heterodimers bind to the polymorphic *cis*-acting response elements of target genes. To clarify *in vivo* function of retinoids by interfering with retinoid signaling pathway, knockout mice of retinoic acid receptor were generated (11–13); these mice demonstrated growth deficiency, early lethality, and degeneration of testes. Saitou *et al.* found that dominant negative form of RARα (RARE) can suppress the endogenous activities of RARs (α, β, γ) (14). Thereby, circumventing problems with embryonic lethality and increasing the likelihood of observing effects of inhibition of the retinoid signaling pathway in epithelial cells has been enabled by using dominant negative form of RAR (15–17). To investigate retinoid function in the liver, we developed transgenic mice which express RARE under the control of albumin enhancer and promoter. Successful expression of RARE in liver resulted in growth promotion of

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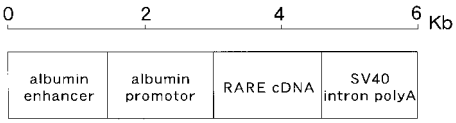

FIG. 1. Scheme of RARE transgene. The transgene contains the albumin enhancer and promoter from pAN/T2-NB', inserted between the indicated *Apa*I and *Hind*III sites (18); the RARE cDNA, included between *Hind*III and *Bam*HI (14); and the SV40 intron and polyadenylation signals from pSV2pgt included between the indicated *Bam*HI and *Mlu*I site (19); the construct is cloned in pGEM-7 (Promega Co., WI), called pAlbRARE. For microinjection, pAlbRARE was cut at the *Apa*I and *Mlu*I sites.

hepatocytes, where cyclin E is up-regulated in association with cdk2.

MATERIALS AND METHODS

Generation of transgenic mice. The transgene, in plasmid pAlbRARE (Fig. 1), contains the albumin enhancer and promoter from pAN/T2-NB', inserted between the indicated *Apa*I and *Hind*III sites (18); the RARE cDNA, included between *Hind*III and *Bam*HI (14); and the SV40 intron and polyadenylation signals from pSV2pgt, included between the indicated *Bam*HI and *Mlu*I sites (19); the construct is cloned in pGEM-7 (Promega Co., WI). For microinjection, pAlbRARE was cut at the *Apa*I and *Mlu*I sites. The microinjection fragment was purified by CsCl gradients purification and dialyzed extensively against injection buffer (5 mM NaCl/0.1 mM EDTA/5 mM Tris–HCl, pH 7.4). The *Apa*I-*Mlu*I fragment of pAlbRARE was microinjected into fertilized oocytes from BDF1 mice (Japan SLC, Shizuoka, Japan) at the single cell stage. Transgenic mice were identified by PCR using the primers, which are specific to RARE transgene but not to endogenous retinoic acid receptor α (shown in Materials and Methods). The size of transcript from transgene is about 3 kb. Five mice each, which were 10 weeks old and male, were used for the experiments. Mice were fed a CE-2 diet (CLEA Japan, Tokyo, Japan) and were allowed to drink water *ad libitum*. Animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985).

Northern blot analysis and reverse transcriptase polymerase chain reaction (RT-PCR). Total cellular RNA was isolated from snap-frozen tissues by using ISOGEN (Nippon Gene Co., Toyama, Japan) according to the manufacturer's instruction. Northern blot analysis was performed using 30 μg total RNA size fractionated on 1% formaldehyde agarose gels. Blots were hybridized to random primed RARE cDNA fragment. As an internal control, the bands of 28S and 18S ribosomal RNA, which were stained with ethidium bromide, were included. For RT-PCR, RNA samples were digested with DNase (Nippon Gene Co.) to remove DNA from samples. One microgram of RNA was converted to complementary DNA and was amplified by using RNA LA PCRTM kit (TaKaRa, Kyoto, Japan) and a thermocycler (Program Temp Control System PC-700, ASTEC, Fukuoka, Japan). The number of cycle in each PCR was 30. The primer sequences and annealing temperatures were the following: RARE primer, forward: TACGACGTGCCCGACTATGCC, reverse: GTGGTAGCCTGAGGACTTGTC, annealing temperature 58°C; albumin primer, forward: TCGGAGACAAGTTATGCGCC, reverse: GCAGCTATTGAGGGCAGATC, annealing temperature 55°C; HGF primer, forward: ATGCTCATGGACCCTGGT, reverse: GCCTGGCAAGCTTCATTA, annealing temperature 55°C; GAPDH primer, forward: CCTTCATTGACCTCAACTAC, reverse: GGAAGGCCATGCCATGCCAGTGAGC, annealing temperature 57°C; cyclin A primer, forward: GAAGCTCAAGACTCGACG, reverse: CCGTGCCTACAAGCTGAA, annealing temperature 55°C; cyclin D1 primer, forward: GTGCAGAGGGAGATTGTGCC, reverse: GCGGCCCAGGTTCCATTTGAG, annealing temperature 59°C, cyclin E primer, forward: AGCGAGGATAGCAGTCAGCC, reverse: TTTCATCCCCGGAGCAAGCG, annealing temperature 59°C; cyclin H primer, forward: CAGAAGCTGGAGCGGTGTCA, reverse: GGTCGTCATCAGTCCATCCC, annealing temperature 59°C; cdk2 primer, forward: CCAGTACTGCCATCCGAGAG, reverse: CGGCGAGTCACCATCTCAGC, annealing temperature 59°C; cdk4 primer, forward: ATCAGCACAGTTCGTGAGGTGGC, reverse: AGCTCGGTACCAGACTGTAACAAC, annealing temperature 59°C; cdk6 primer, forward: TGCACAGTGTCACGAACAGAC, reverse: TGAATGAAAAGCCTGCCTGGG, annealing temperature 57°C; p53 primer, forward: TCTGGGACAGCCAAGTCTGT, reverse: GGAGTCTTCCAGTGTGATGA, annealing temperature 55°C; p21 primer, forward: AGCAAAGTGTGCCGTTGTCT, reverse: AGAAATCTGTCAGGCTGGTC, annealing temperature 55°C; and p27 primer, forward: CAGCTTGCCCGAGTTCTA, reverse: TGGGGAACCGTCTGAAAC, annealing temperature 55°C.

PCNA labeling index. The livers were fixed in 4% paraformaldehyde, and embedded in paraffin. Sections (4 μm thick) were incubated in 0.3% H_2O_2 in methanol for 30 min. They were heated in 10 mmol/l sodium citrate buffer (pH 6.0) at 600 W for 15 min with a microwave oven. Then, they were incubated with normal horse serum. After washing in PBS, they were incubated with a mouse monoclonal antibody against rat PCNA (NCL-PCNA, Novacastra Laboratories, Newcastle, UK) diluted 1:100, at 4°C overnight. Then, they were incubated with biotin-conjugated horse anti-mouse immunoglobulin G (Vector Laboratories, CA) for 60 min. Immunoreacted cells were visualized by a Vectastain ABC kit (Vector Laboratories). Ten high-magnification fields containing a total of at least 1000 hepatocytes nuclei were counted in each group of five mice per group. The labeling index was determined by calculating the mean of the numbers of PCNA-positive hepatocytes divided by the total number of counted hepatocytes in each field.

Isolation of parenchymal and non-parenchymal liver cells. Liver cells were isolated with two-step collagenase perfusion from three transgenic and three control mice as previously described (20). Harvested liver cells from each group were pooled into two fractions. Parenchymal cells were separated from one fraction by isopycnic gradient centrifugation, and non-parenchymal cells were from the other fraction by pronase digestion (20, 21). The yields of parenchymal and non-parenchymal cells were 2 $\times$ 107 and 5 $\times$ 106 cells/g liver, respectively. No preparation was used unless the cell viability exceeded 90% on trypan blue exclusion. These fractions of cells were used for RNA preparation and cell cycle analysis.

Cell cycle analysis. The cells were washed and fixed in 70% ethanol. They were then stained with 5 μg/ml propidium iodine (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) in the presence of RNase A (Nippon Gene) (22). Fluorescence-activated cell sorting was performed on an EPICS-XL flow cytometer (Coulter Electronics Inc., Hialeah, FL).

Immunoprecipitation and Western blot analysis. Liver tissues were homogenized in the TNE buffer (Dojindo Co., Kumamoto, Japan) containing 1% NP-40 (ICN Biomedicals Inc., OH), 10 μg/ml of aprotinin (Sigma Chemical Co., MO) and 1 mM phenylmethylsulfonyl fluoride (Wako Pure Chemical Industries, Ltd.). For immunoprecipitation, 100 μg of protein was incubated with rabbit polyclonal anti-CDK2 IgG (Santa Cruz Biotechnology, Inc., CA) on ice for 1 h, followed by another 1 h-incubation with protein A SepharoseTM CL-4B (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Fifty micrograms of protein and/or immunoprecipitates were boiled for 5 min and electrophoresed on 10% sodium dodecyl sulfate polyacrylamide gels. Then, the proteins were transferred onto Hybond ECL membranes (Amersham Pharmacia Biotech, IL) using a semi-dry blotting apparatus. The membranes were incubated with 10% nonfat milk and 0.2% Tween 20 (Bio-Rad Laboratories, CA) at room temperature for 1 h. Then, they were incubated with a 1:500 dilution of

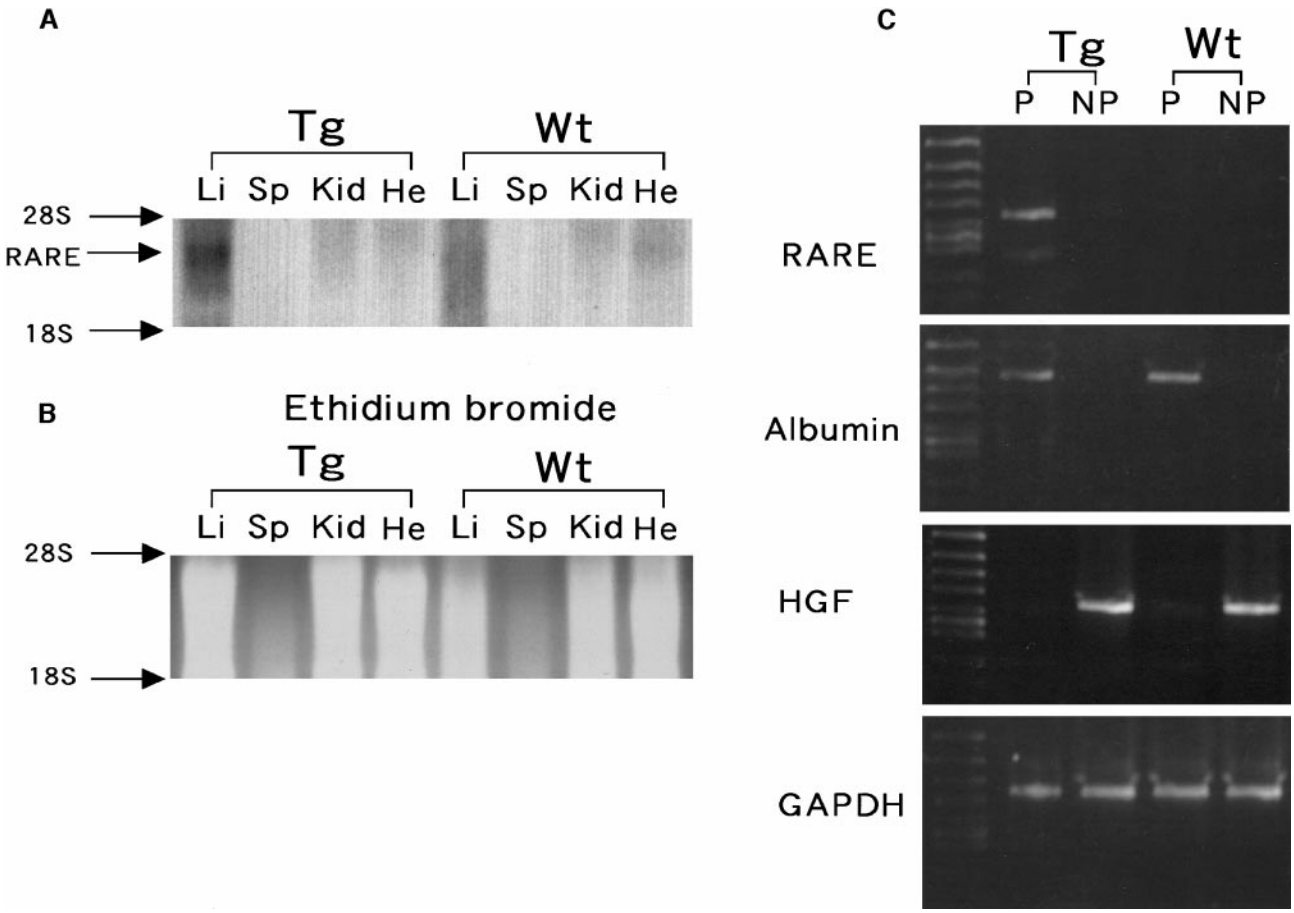

FIG. 2. Expression of RARE transgene in liver tissue and liver parenchymal cells. (A) Northern blot analysis was performed using 30 μg total RNA size fractionated on 1% formaldehyde agarose gels. Blots were hybridized to random primed RARE cDNA fragment. Li, liver; Sp, spleen;, Kid, kidney; He, heart; Tg, transgenic mice; Wt, wild-type mice. (B) As an internal control, the bands of 28S and 18S ribosomal RNA, which were stained with ethidium bromide, were included. (C) Cell type specificity in expression of RARE transgene. P, parenchymal liver cell, NP, non-parenchymal liver cell. RNA extracted from parenchymal and non-parenchymal cells was examined by RT-PCR on expression of each gene. Purity of parenchymal and non-parenchymal liver cells was demonstrated by RT- PCR with albumin as a marker of parenchymal cells and HGF as a marker of non-parenchymal cell.

rabbit polyclonal anti-cyclin E IgG (Santa Cruz Biotechnology, Inc.) at 4°C overnight. After washing, the membranes were incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Pharmacia Biotech) at room temperature for 1 h. Immunoreactive bands were visualized with the ECL Western blotting detection kit (Amersham Pharmacia Biotech) according to the manufacturer's protocol.

Statistics. Data were expressed as mean ± SD. Statistical significances were determined using the unpaired Student's *t* test.

RESULTS

Northern blot analysis showed that RARE mRNA was expressed in liver but not in other tissues including spleen, kidney, and heart from transgenic mice (Fig. 2A). No transcript was seen in wild-type mice. In Fig. 2C, it was shown that expression of RARE transgene was specific to parenchymal liver cells, but not to non-parenchymal liver cells. Purity of parenchymal and non-parenchymal liver cells was demonstrated by RT-PCR with albumin as a marker of parenchymal cells and HGF as a marker of non-parenchymal cell.

Expression of RARE greatly increased hepatocyte proliferation during postnatal liver growth. Parameters of proliferating liver growth were shown in Table 1. The liver weight of transgenic mice was significantly heavier than that of wild-type mice. The liver/body weight of transgenic mice was also increased, compared to that of wild-type mice. PCNA labeling index in transgenic mice was much higher than that in wild-type mice, resulting in an aberrant increase of liver weight.

To confirm DNA synthesizing cells in RARE transgenic mice, the proportions of cells in different phases of cell cycle using DNA content analysis were determined (Fig. 3). Cell cycle analysis in liver is complicated by the presence of resulting polynuclear cells that arise through endomitotic doubling of DNA (23).

TABLE 1

Parameters of Liver Growth in RARE Transgenic and Wild-Type Mice at 10 Weeks of Age

Genotype	Liver weight (g)	Liver/Body weight (%)	PCNA labeling index (%)
Wt[1]	1.36 ± 0.40	5.24 ± 1.22	0.10 ± 0.05
RARE Tg[2]	2.16 ± 0.31[3]	7.76 ± 0.49[3]	3.56 ± 0.80[3]

Note. Data are expressed as mean ± SD.
[1] Wild-type mice.
[2] RARE transgenic mice.
[3] $P < 0.01$, compared to wild-type mice.

Polynuclear cells actually outnumber uninuclear, diploid cells in many mouse strains (24). Accordingly, cells containing a 4N content of DNA were the predominant cell type in the resting liver of control mice (Fig. 3, left), constituting about 50% of the hepatocytes. In contrast, 4N cells were markedly decreased in the transgenic mice (Fig. 3, right). Indeed, 33% of the cells in the RARE transgenic mice contained a 2N content of DNA, whereas 18% of hepatocytes were 2N cells in the wild-type mice. This 2N DNA content is consistent with the G0 and G1 phases of the cell cycle. This change, which is consistent with a reduction in number of nuclei per cell, is known to occur during experimental liver regeneration and in rats treated with chemical carcinogens (25, 26). In addition, the aneuploid area between 2N and 4N DNA contents, reflecting S phase cells, was 3.3-fold increased in RARE transgenic mice, compared to that in wild-type mice. These changes were also observed in transgenic mice expressing HGF (27). The results of present study indicated that blockade of retinoic acid receptor signaling pathway accelerates G1 phase progression of cell cycle.

To clarify the key molecule in the progression of cell cycle in RARE Tg, we firstly performed RT-PCR. In shown in Fig. 4, expression of cyclin A, D1, and H was similar between transgenic and wild-type mice. However, expression of cyclin E is higher in transgenic mice than that in wild-type mice. CDKs including cdk2, cdk4, and cdk6 were not changed (Fig. 5). p53 and CKIs such as p21 and p27 were not also changed. Since expression of cyclin E is increased, we next performed Western blot analysis of this protein. Expression of cyclin E protein is much higher in RARE transgenic mice than that in wild-type mice (Fig. 6A). Furthermore, since cyclin E forms complexes with cdk2 (28), we performed immunoprecipitation with anti-cdk2 antibody, followed by Western blot by using anti-cyclin E antibody (Fig. 6B). Overexpressed cyclin E protein was complexed with cdk2, suggesting that this complex is working in cell cycle progression of hepatocytes expressing RARE.

DISCUSSION

Retinoids play an important role in cell growth and differentiation (1, 2). Recently, receptors for RA, RARs, have been identified and shown to belong to the nuclear receptor superfamily (10). Although levels of gene expression by retinoids are diverse, the RARs and RXRs are the major source of the complexity of pleiotrophic effects of retinoids (10). Saitou *et al.* introduced a mutation, which was originally identified in the thyroid hormone receptor gene, causing dominantly inherited thyroid hormone resistance, to the equivalent position of RAR α (RARE), and showed that RARE, suppressing the endogenous activities of RARs (α, β, γ), demonstrated a dominant negative phenotype (14). By introducing RARE into the transgenic mice using tissue-specific primers, they demonstrated that functional loss of RA dramatically suppresses epidermal maturation and retards skeletal development, and causes cervical abnormalities (15, 17). Thus, this sys-

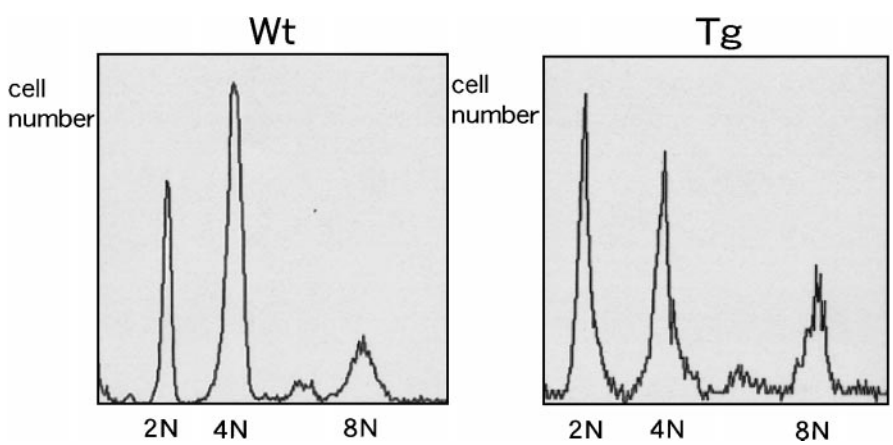

FIG. 3. Cell cycle analysis of hepatocytes. Wt, wild-type mice; Tg, transgenic mice. The cells were washed and fixed in 70% ethanol. They were then stained with 5 μg/ml propidium iodine (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) in the presence of RNase A (Nippon Gene) (7). Fluorescence-activated cell sorting was performed on an EPICS-XL flow cytometer (Coulter Electronics Inc., Hialeah, FL).

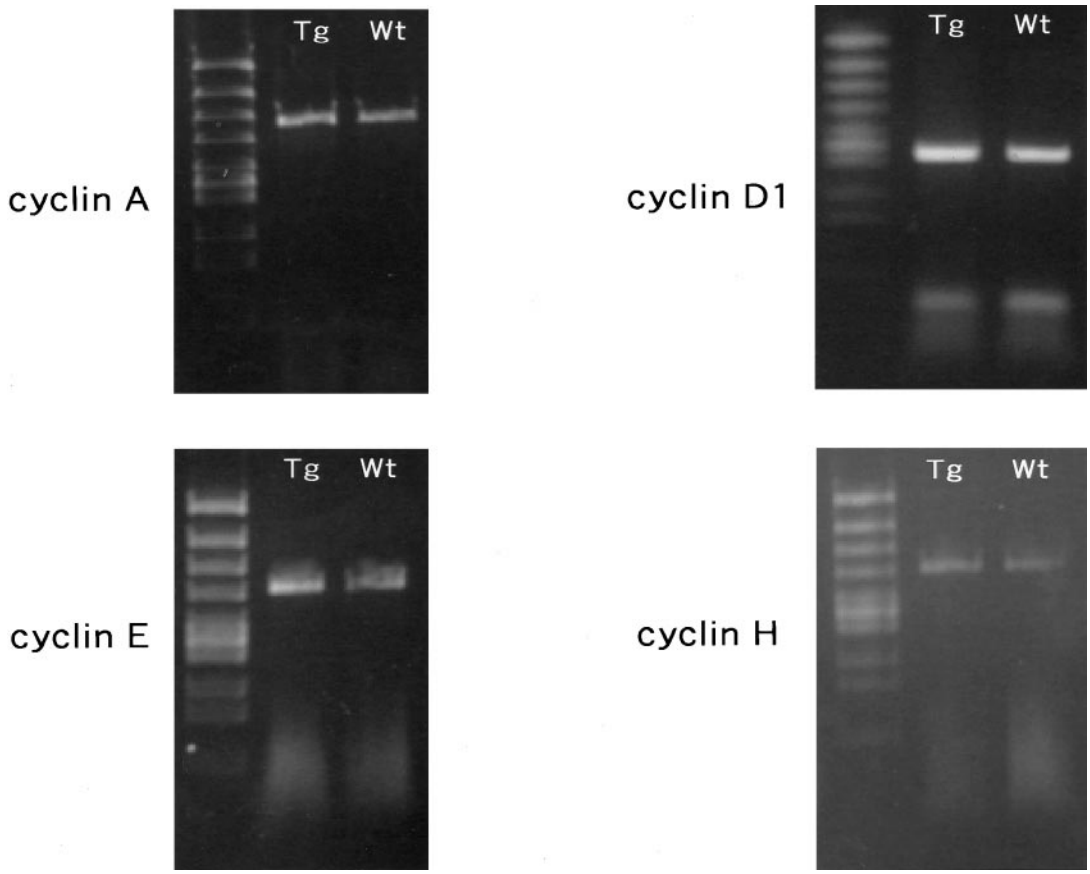

FIG. 4. RT-PCR analysis of cyclin A, D1, E, and H. Tg, transgenic mice; Wt, wild-type mice.

tem is very useful in analysing tissue-specific phenotypes, where function of RA is inhibited. In the present study, we developed transgenic mice, where expression of RARE is targeted to hepatocytes. In this context, these mice are useful in analysing function of RA in liver.

In liver, retinoids play an important role in regeneration, fibrosis, and cancer (4–7). Exogenous administration of RA exerts the antiproliferative activity in the early stage of liver regeneration, accompanied by the repression of *c-fos* and *c-jun* expression (4). Depletion of vitamin A causes cellular necrosis and activation of oval cells after partial hepatectomy, suggesting vitamin A is required for normal liver regeneration (29). Vitamin A deficiency potentiates CCl4-induced liver fibrosis, stimulating the transformation of stellate cells to myofibroblasts and contributing to fibrogenesis of the liver (5). In addition, *in vitro* studies demonstrated that an acyclic retinoid and RARβ gene transfer cause apoptosis and/or antiproliferative effects on HCC cells (6, 7). Muto *et al.* have recently shown that an acyclic retinoid prevents second primary hepatoma after surgical resection of the original tumor or the percutaneus injection of ethanol (8). This observation is very important since tumor recurrence determines the long-term prognosis with primary hepatoma (9). In the present study, transgenic mice expressing RARE exhibited accelerated proliferation of hepatocytes, suggesting that RA functions as an antiproliferative molecule in liver.

Cyclins are the activating partners of a highly conserved family of CDKs (30). A number of cyclins and CDKs play important roles in the regulation of the eukaryotic cell cycle. Cyclin E is expressed in G1 phase of cell cycle, and forms complexes with cdk2, governing the cell cycle progression (31). RA inhibits growth of MCF-7 human breast cancer cells in association with suppression of cdk2 mRNA and protein (32). It has been suggested that growth-inhibitory effects of RA on normal human B lymphocytes are due to the reduced expression of cyclin E and cyclin A in G1 phase, resulting in pRB dephosphorylation (33). RA-mediated G1-S-phase arrest of normal epithelial cells has been reported to be independent of p53 protein expression (34), being identical to no change in p53 mRNA in the present study. The cyclin E/cdk2 complex is the next to appear in the mammalian cell cycle after the D-types in late G1 phase, and is also a potential regulator of Rb (35), and it is reported to be essential for the cells to begin DNA replication (36). Recent data suggest that cyclin E/cdk2 protein kinase activity may be modulated

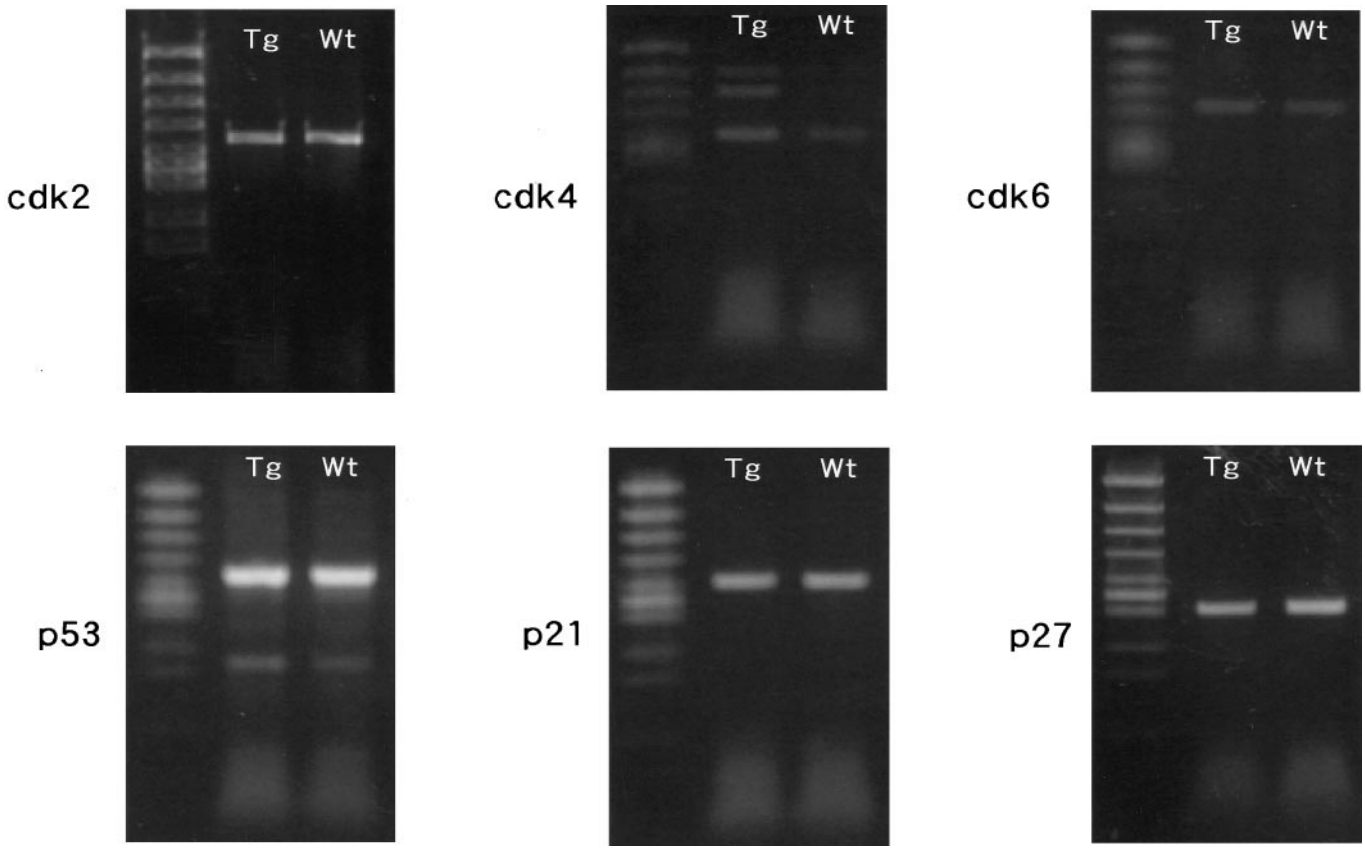

FIG. 5. RT-PCR analysis of cdk2, cdk4, cdk6, p53, p21, and p27. Tg, transgenic mice; Wt, wild-type mice.

by phosphorylation of the cdk2 subunit on Tyr-15, and therefore that the cdc25A phosphatase is required to activate the complex at the end of G1 phase (37). In the present study, we demonstrated that functional loss of RA leads to accelerated growth of hepatocytes in association with up-regulation of cyclin E. Due to the ability to block retinoid action, these transgenic mice provide a means to acquire further insight into molecular mechanisms of gene regulation by RA.

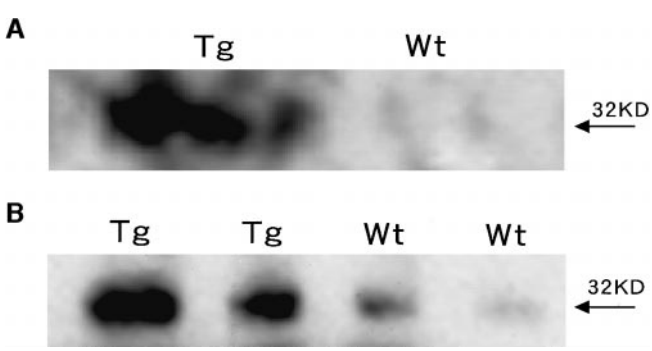

FIG. 6. Expression of cyclin E protein. (A) Western blot analysis of liver protein from transgenic and wild-type mice using anti-cyclin E antibody. Tg, transgenic mice; Wt, wild-type mice. (B) Immunoprecipitation with anti-cdk2, followed by Western blot analysis using anti-cyclin E antibody. Tg, transgenic mice; Wt, wild-type mice.

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