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Overexpression of Regucalcin Modulates Tumor-Related Gene Expression in Cloned Rat Hepatoma H4-II-E Cells

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Abstract Regucalcin is a regulatory protein in intracellular signaling pathway which is related to various protein kinases and protein phosphatases in many cells. The effect of regucalcin on the expression of tumor-related genes was investigated in the cloned rat hepatoma H4-II-E cells and the hepatoma cells (transfectants) overexpressing regucalcin. Hepatoma cells were cultured for 24–72 h in the presence of fetal bovine serum (FBS; 10%). The proliferation of hepatoma cells was significantly suppressed at 24–72 h of culture in regucalcin transfectants as compared with that of wild-type or mock-type cells. Western blot analysis showed that regucalcin was markedly expressed in transfectants. The expression of c-*myc*, c-*fos*, c-*jun*, Ha-*ras*, and *p53* mRNAs was determined using reverse transcription-polymerase chain reaction (RT-PCR). Of these genes, the expression of c-*myc* or Ha-*ras* mRNAs was significantly suppressed in regucalcin transfectants. The suppression of c-*myc* mRNA expression in transfectants was confirmed by using Northern blot analysis; significant suppression was seen at 24, 48, or 72 h of culture in the presence of 10% FBS. Culture with 10% FBS significantly enhanced c-*myc* mRNA expression in the hepatoma cells (wild-type) as compared with that of 1% FBS. The enhancement was significantly abolished in the transfectants. Meanwhile, the expression of *p53* mRNA in the hepatoma cells was significantly enhanced in regucalcin-overexpressing hepatoma cells. This study demonstrates that the expression of oncogene c-*myc* and Ha-*ras* mRNA in hepatoma cells overexpressing regucalcin is suppressed, and that the tumor suppression gene *p53* is enhanced in the transfectants. J. Cell. Biochem. 90: 619–626, 2003.

Key words: regucalcin; oncogene; c-*myc*; c-*fos*; c-*jun*; Ha-*ras*; *p53*; hepatoma cells; transfectant

Calcium ion (Ca^{2+}) plays a pivotal role in the mechanism of intracellular signaling induced by hormone and cytokine stimulation in many cells [Cheung, 1980; Heizman and Hunziker, 1991; Kraus-Friedman and Feng, 1996]. Regucalcin, which was found as a novel Ca^{2+}-binding protein [Yamaguchi and Yamamoto, 1978; Yamaguchi, 1988], has been shown to play a multifunctional role as a regulatory protein for Ca^{2+}/calmodulin-dependent protein kinase, protein kinase C, MAP kinase, protein tyrosine phosphatase, calcineurine, and function of nucleus in many cells [Yamaguchi, 2000a,b; in review]. The role of regucalcin is also demonstrated in regucalcin transgenic rats overexpressing endogenous regucalcin in vivo [Yamaguchi et al., 2002a,b].

Regucalcin, which is largely expressed in liver [Shimokawa and Yamaguchi, 1992; Yamaguchi and Isogai, 1993], has been demonstrated to translocate to the nucleus of rat liver [Tsurusaki et al., 2000]. Regucalcin has been shown to have an inhibitory effect on protein kinase and protein phosphatase activities in the nucleus of normal and regenerating rat liver which is modulated through signal transduction for hormonal stimulation [Katsumata and Yamaguchi, 1998; Omura and Yamaguchi, 1999]. Endogenous regucalcin, moreover, has been shown to have an inhibitory effect on DNA and RNA synthesis in the nucleus of normal and regenerating rat liver [Tsurusaki and Yamaguchi, 2002a,b]. Thus, regucalcin may play a regulatory role in proliferative liver cells.

Recent studies have demonstrated that regucalcin has a suppressive effect on the function of nucleus in the cloned rat hepatoma H4-II-E

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cells with proliferation. Endogenous regucalcin has an inhibitory effect on the enhancement of protein phosphatase activity [Inagaki and Yamaguchi, 2000], protein kinase activity [Inagaki and Yamaguchi, 2001b], and inhibits the proliferation of cloned rat hepatoma H4-II-E cells. Furthermore, endogenous regucalcin has been shown to have a suppressive effect on cell proliferation and DNA synthesis in the cloned rat hepatoma H4-II-E cells overexpressing regucalcin [Misawa et al., 2002].

This study demonstrates the role of endogenous regucalcin in the regulation of the expression of tumor-related genes in the cloned rat hepatoma H4-II-E cells overexpressing regucalcin stably. We found the suppression of tumor stimulator gene c-*myc* and Ha-*ras* mRNAs expression and the enhancement of tumor suppressor gene *p53* mRNA expression in the regucalcin-overexpressing hepatoma cells.

MATERIALS AND METHODS

Chemicals

α-Minimum essential medium (α-MEM) and penicillin–streptomycin solution (5,000 U/ml penicillin; 5,000 μg/ml streptomycin) were obtained from Gibco Laboratories (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Sigma Chemical Co. (St. Louis, MO). Calcium chloride and other chemicals were purchased from Wako Pure Chemical Co. Ltd. (Osaka, Japan).

Animals

Male Wistar rats, weighing 90–100 g, obtained commercially from Japan SLC (Hamamatsu, Japan). Rats were given a single intraperitoneal administration of calcium chloride (5 mg Ca/0.5 ml/100 g body weight), and 60 min later they were killed by bleeding. Livers were frozen immediately and total liver cell RNA was extracted.

Cell Culture

The cloned rat hepatoma H4-II-E cells and the regucalcin-overexpressing H4-II-E cells (3.0×10^5) were maintained for 24–72 h in α-MEM supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin in humidified 5% CO_2/95% air at 37°C to obtain confluent monolayers [Yamaguchi and Nakajima, 1999; Misawa et al., 2002]. After culture, cells were washed three times with phosphate-buffered saline (PBS), scraped into 0.5 ml of ice-cold 0.25 M sucrose solution, and disrupted for 30 s with an ultrasonic device. Scrapped cells were also homogenized in Potter–Elvehjem homogenizer with a Teflon pestle. The homogenates were spun at 5,500*g* in a refrigerated centrifuge for 5 min to remove nuclei and mitochondria. The 5,500*g* supernatant was pooled to analyze regucalcin protein by Western blot. Protein concentration in the 5,500*g* supernatant of cell homogenate was determined by the method of Lowry et al. [1951].

Regucalcin Transfectants

The cDNA encoding rat regucalcin was isolated and cloned into the pBluescript vector [Misawa and Yamaguchi, 2000]. The regucalcin cDNA contains *Pst* I site downstream of the translational stop codon, and a *Pst* I site, and an Eco RI upstream of the regucalcin cDNA. The Eco RI fragment (containing the complete coding cDNA) was cloned into the Eco RI site of the pEXN2 expression vector [Niwa et al., 1991]. The resultant plasmid was designated as regucalcin (RC)/pCXN2 [Misawa et al., 2002].

For transient transfection assay, the H4-II-E cells were grown on 35-mm dishes to approximately 70% confluence. Each of RC/pCXN2 and pCXN2 vector alone was transfected into H4-II-E cells using the synthetic cationic lipid components, a Tfx-20 reagent, according to the manufacturer's instructions (Promega, Madison, WI) cells were harvested and used for subsequent experiments. H4-II-E cells were transfected with RC/pCXN2 vector alone using a Tfx-20 reagent. After 24 h, neomycin (1.0 mg/ml Geneticin G418, Sigma) was added to cultures for selection and cells were plated at limiting dilution. Multiple surviving clones were isolated, transferred to 35-mm dishes, and grown in the medium without neomycin. Regucalcin was stably expressed in the transfectants. In experiments, transfectants were cultured for 24–72 h in -MEM containing 10% FBS.

Determination of Cell Numbers

After trypsinization using 0.2% trypsin plus 0.02% ethylenediamine–tetraacetic acid in Ca^{2+}/Mg^{2+}-free PBS, cell numbers were determined by electronic particle counter.

Western Blot Analysis

The homogenate from the cloned rat hepatoma cells and transfectants cultured with 10% FBS was centrifuged for 10 min at 5,500*g* at 4°C,

and the supernatant was used for Western blot analysis [Wessendorf et al., 1993]. Aliquots of protein (20 μg) were mixed with 5× Laemmli sample buffer, boiled for 5 min, and SDS–PAGE was performed by the method of Laemmli [1970] using 12% polyacrylamide resolving gel. After SDS–PAGE, the proteins were then transferred onto a polyvinylidene difluoride membrane at 100 mA for 4 h. The membranes were incubated with a polyclonal rabbit anti-regucalcin antibody [Yamaguchi and Isogai, 1993], which was diluted 1:2,000 in 10 mM Tris-HCl, pH 8, containing 150 mM NaCl, 0.1% (w/v) Tween 20 (washing buffer), and 5% (w/v) skim milk for 1 h. The membranes incubated with antibody were washed four times with washing buffer. Then membranes were incubated for 1 h with horseradish peroxidase linked anti-rabbit IgG which was diluted 1:5,000 with washing buffer containing 5% (w/v) skim milk, and again they were washed. Detection of the protein bands was performed using an enhanced chemiluminescent kit following the manufacture's instruction. The molecular size of the detecting protein was determined by running the standard protein molecules of known sizes in parallel.

Quantification of Specific mRNA by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed with a Titan™ One Tube RT-PCR Kit (Roche Molecular Biochemicals, Indianapolis, IN) as recommended by the supplier to investigate the gene expression of c-*myc*, c-*fos*, c-*jun*, Ha-*ras*, or *p53*. The primers generated based on the published rat sequences. Primers for amplication c-*myc* c-DNA were 5′-(1036)-CCTGTACCTCGTCCGATTC-CAC-(1057)-3′ and 5′-(1509)-GCTACGCTTCA-GCTCGTTTCTCC-(1531)-3′ of cDNA sequense [Hayashi et al., 1987]. Primers for c-*fos* c-DNA were 5′-(142)-TCTCGGGTTTCAACGCGGAC-TAC-(165)-3′ and 5′-(540)-CCTTCGGATTCT-CCGTTTCTCTTCCTC-(566)-3′ [Curran et al., 1987]. Primers for c-*jun* cDNA were 5′-(2521)-CACTACACCGACCCCCACTCAGTTCTTG-(2548)-3′ and 5′-(2936)-GTACCGTCTGCGGC-TCTTCCTTCAG-(2960)-3′ [Kitabayashi et al., 1992]. Primers for Ha-*ras* cDNA were 5′-(43)-TCCTAGCAGCGGCTTCTGAC-(62)-3′ and 5′-(337)-TCTACAGGGGCTGCCAAACC-(356)-3′ [Damante et al., 1987]. Primers for *p53* cDNA were 5′-(1202)-AAGGCCTCATTCAGCTCTCG-(1221)-3′ and 5′-(1202)-AAGGCCTCATTCAG-CTCTCG-(1221)-3 [Hulla and Schneider, 1993]. Primers for regucalcin cDNA were 5′-(618)-AGATGAACAAATCCCAGAT-(636)-3′ and 5′-(924)-TCACCCTGCATAGGAATAT-(906)-3′ [Misawa and Yamaguchi, 2000]. Primers for G3PDH cDNA were 5′-TGAAGGTCGGTGT-GAACGGATTTGGC-3′ (sense strand) and 5′-CATGTAGGCCATGAGGTCCACCAC-3′ (antisense strand) from the G3PDH Amplimer Set (Clontech, Palo Alto, CA). RT-PCR was performed by using reaction mixture (20 μl) containing 1 μg of total RNAs, supplied RT-PCR buffer, Titan™ enzyme mix (AMV and Expand™ High Fidelity), 0.2 mM deoxynucleotide triphosphate, 5 mM dithiothreitol, 5 U RNase inhibitor, 2.5 U Taq DNA polymerase, and 0.3 μM primers. Samples were incubated at 50°C for 30 min and at 94°C for 2 min, and then amplified for 35 cycles under the following conditions; denaturation for 30 s at 94°C, annealing for 30 s at 60°C, and extension for 60 s at 68°C. The amplified PCR products were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining and quantitated using a densitometer.

Northern Blot Analysis

Thirty micrograms of total RNAs extracted from the cloned rat hepatoma H4-II-E cells or RC/pCXN2 transfectants were electrophoresed in 1.2% agarose denaturing gels containing 2.2 M formaldehyde in MOPS buffer (pH 7, containing 20 mM 3-morpholinopropanesulfonic acid, 5 mM sodium acetate, and 1 mM EDTA) with 3 V/cm^3 for 3 h [Sambook et al., 1989]. The electrophoresed gels were transferred to nylon membranes by blotting. Northern blots were probed with DIG-labeled c-*myc* and G3PDH cDNA, respectively [Misawa and Yamaguchi, 2001]. The c-*myc* cDNA probe was corresponding to the position of 1,036–1,531 in rat c-*myc* cDNA antisense sequence. The blots were detected with alkaline phosphatase-conjugated anti-DIG antibody and a chemiluminescent substrate according to the manufacturer's instructions (Roche). The X-ray films were exposed for 60 min. The size of mRNA was determined using an RNA ladder (Promega). Data for blots were quantitated by using a densitometer.

Statistical Analysis

Data were expressed as the mean ± SEM. The significance of difference between the values

was estimated by Student's *t*-test or by analysis of variance (ANOVA) for comparing multiple groups. *P*-values of <0.01 were considered to indicate statistically significant difference.

RESULTS

Modulation of Proliferation of Hepatoma Cells Overexpressing Regucalcin

The change in the proliferation of the cloned rat hepatoma H4-II-E cells overexpressing regucalcin was examined. The hepatoma cells (wild-type), pCXN2 transfected cells (mock-type), or stable regucalcin/pCXN2 transfectants were cultured for 24–72 h in medium containing 10% FBS. The proliferation of the cells was significantly suppressed in transfectants cultured for 24, 48, or 72 h (Fig. 1). Cell proliferation was not significantly altered in mock-type cells (Fig. 1). The expression of regucalcin in the cells was remarkable in transfectants cultured for 24, 48, or 72 h (data not shown), as shown previously [Izumi et al., 2003].

Change in the Expression of c-*myc*, c-*jun*, or c-*fos* mRNAs in Hepatoma Cells Overexpression Regucalcin

The expression of *regucalcin* gene in rat liver has been shown to be modulated by calcium administration [Shimokawa and Yamaguchi, 1992]. We first examined the effect of calcium chloride treatment on the expression of c-*myc*, c-*jun*, or c-*fos* mRNAs in the liver of rats (Fig. 2). Based on the RT-PCR analysis, c-*myc*, c-*jun*, or c-*fos* mRNA was not detected in normal rat liver. A single intraperitoneal administration of calcium chloride (5 mg Ca/100 g body weight) caused a significant increase in the expression of c-*myc*, c-*fos*, or c-*jun* mRNAs in rat liver. The expression of regucalcin or G3PDH mRNAs was seen in normal rat liver. Regucalcin mRNA expression was not significantly enhanced by the dose of 5 mg calcium per 100 g body weight.

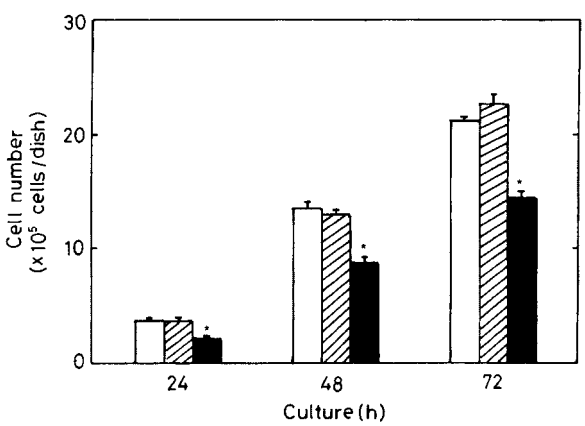

Fig. 1. Change in cell number of the cloned rat hepatoma H4-II-E cells (wild-type), pCXN2-transfected cells (mock type), or stable RC/pCXN2- transfected cells (3.0×10^5) were cultured for 24, 48, and 72 h in the presence of 10% FBS. Each value is the mean ± SEM of five experiments. $^*P < 0.01$, compared with the value obtained by culture of wild type or mock-type cells. Open bars, wild; hatched bars, mock; black bars, transfectant.

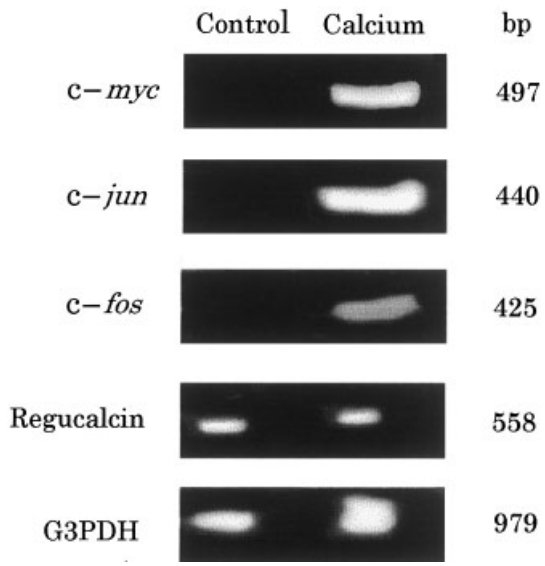

Fig. 2. Expression of c-*myc*, c-*jun*, or c-*fos* mRNAs in rat liver. Rats were intraperitoneally administered calcium chloride (5 mg Ca/100 g body weight), and 60 min later the animals were killed by bleeding. Total RNAs (1 μg) extracted were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) using specific primers. The housekeeping gene *G3PDH* or *regucalcin* was used as an internal control. The figure shows one of four experiments with separate rat liver.

The expression of c-*myc*, c-*jun*, or c-*fos* mRNA was detected by using RT-PCR in the cloned rat hepatoma H4-II-E cells (wild-type) cultured for 72 h in the presence of 10% FBS (Fig. 3). In regucalcin transfectants, c-*myc* mRNA expression was significantly suppressed, while the expression of c-*fos*, c-*jun*, and G3PDH mRNAs expression were not changed in transfectants.

The suppression of c-*myc* mRNA expression in regucalcin transfectants was further supported by using Northern blot analysis (Fig. 4). When the hepatoma cells (wild-type), pCXN2 transfected-cells (mock-type), or stable regucalcin/pCXN2 transfectants were cultured for 24, 48, or 72 h in medium containing 10% FBS, c-*myc* mRNA was expressed in the wild or

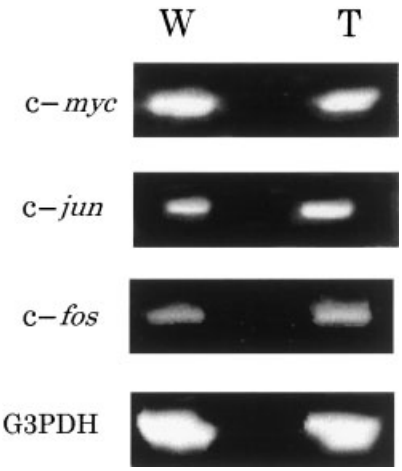

Fig. 3. Expression of c-*myc*, c-*jun*, or c-*fos* mRNAs in the cloned rat hepatoma H4-II-E cells (wild-type; W) or stable RC/pCXN2 transfectants (T). Cells were cultured for 72 h in the presence of 10% FBS. Total RNAs (1 μg) extracted from the cells were analyzed by RT-PCR using specfic primers. The figure shows one of four experiments with separate samples. The densitometric data for c-*myc*, c-*jun*, or c-*fos* mRNAs expression in transfectants (T) was 75 ± 2.5, 128 ± 7.7, or 120 ± 15.6 (% of wild-type cells; mean ± SEM for four experiments), respectively. The suppression of c-*myc* mRNA levels was significant ($P < 0.01$) as compared with that of wild-type cells.

mock-type cells. The expression in transfectants was significantly suppressed at 24, 48, or 72 h of culture.

The expression of c-*myc* mRNA in the hepatoma cells (wild-type) cultured for 72 h in the presence of 1% FBS was clearly decreased as compared with that of 10% FBS; the densitometric data showed a significant ($P < 0.01$) reduction $55 \pm 3.8\%$ (mean ± SEM of four experiments) in comparison with that of 10% FBS (Fig. 5).

Change in the Expression of Ha-*ras* or *p53* mRNAs in Hepatoma Cells Overexpression Regucalcin

The expression of Ha-*ras* or *p53* mRNAs in normal rat liver and the cloned rat hepatoma H4-II-E cells was examined by using RT-PCR analysis (Fig. 6). The hepatoma cells were cultured for 72 h in the presence of 10% FBS. The expression of *p53* mRNA was detected in normal rat liver (A) and hepatoma cells (B). Ha-*ras* mRNA expression in normal rat liver was negligible, and was only slightly expressed in H4-II-E hepatoma cells.

The cloned rat hepatoma H4-II-E cells (wild-type) or transfectants were cultured for 72 h in the presence of 10% FBS (Fig. 7). The expression of *p53* mRNA was significantly increased in transfectants as compared with that of wild-type cells. Ha-*ras* mRNA expression was completely abolished in transfectants. The expression of G3PDH mRNA was not changed in transfectants.

DISCUSSION

The role of regucalcin in proliferative cells has been shown in the cloned rat hepatoma H4-II-E cells. The expression of regucalcin in

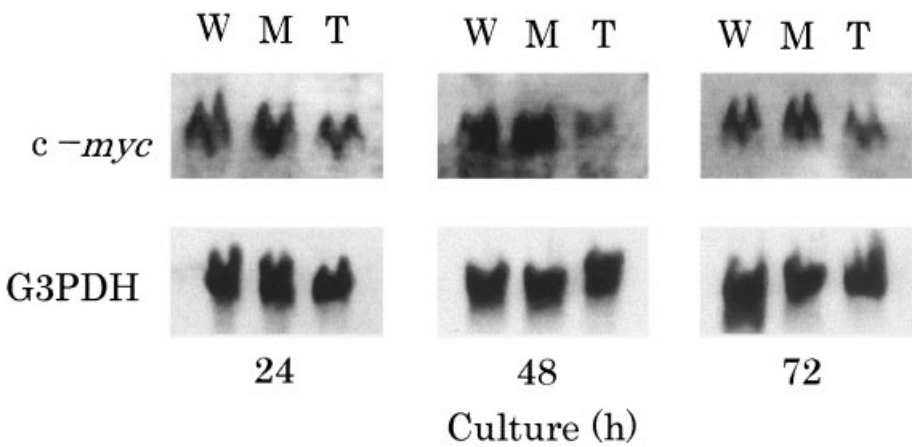

Fig. 4. Change in c-*myc* mRNA expression in the cloned rat hepatoma H4-II-E cells (wild-type; W), pCXN2 transfected cells (mock-type; M), or stable RC/pCXN2 transfectants (T). Cells were cultured for 24, 48, or 72 h in the presence of 10% FBS. Total RNAs (30 μg) extracted from the cells were analyzed by Northern blot. The figure shows one of four experiments with separate samples. The densitometric data for c-*myc* mRNA band in mock-type cells or transfectants cultured with 24, 48, or 72 h were 92 ± 6.8 (M) or 62 ± 3.5 (T), 114 ± 7.9 (M) or 73 ± 4.3 (T), or 88 ± 9.1 (M) or 65 ± 3.9(T) (% of wild-type (W); mean ± SEM for four experiments), respectively. The decrease in c-*myc* mRNA levels seen at 24, 48 or 72 h of culture was significant ($P < 0.01$) as compared with that of wild-type cells.

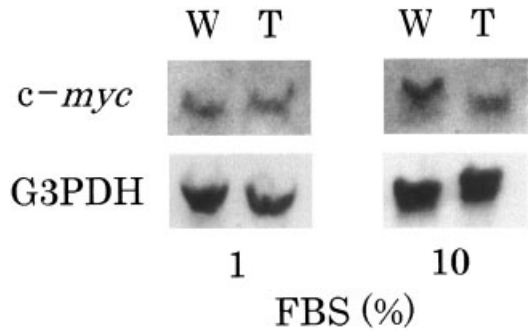

Fig. 5. Change in c-*myc* mRNA expression in the cloned rat hepatoma H4-II-E cells (wild-type; W) or stable RC/pCXN2 transfectants (T) with different concentration of FBS. Cells were cultured for 72 h in the presence of 1 or 10% FBS. Total RNAs (30 μg) extracted from the cells were analyzed by Northern blot. The figure shows one of four experiments with separate samples. The densitometric data for c-*myc* mRNA band in the transfectants cultured with 1 or 10% FBS was 105 ± 8.9 or 61 ± 5.3 (% of wild-type (W); mean ± SEM for four experiments), respectively.

rat hepatoma cells has been shown to be reduced in normal rat liver [Inagaki et al., 2000]. Regucalcin has a suppressive effect on the enhancement of Ca^{2+}-dependent protein kinase activity [Inagaki and Yamaguchi, 2001a], protein tyrosine phosphatase activity [Inagaki and Yamaguchi, 2000], nitric oxide synthase activity [Izumi et al., 2003], and DNA synthesis activity [Inagaki and Yamaguchi, 2001b] with proliferation of the cloned rat hepatoma H4-II-E cells. Moreover, regucalcin has been shown to suppress cell proliferation and DNA synthesis in the cloned rat hepatoma H4-II-E cells overexpressing regucalcin [Misawa et al., 2002]. Regucalcin may have a suppressive effect on proliferation of the cloned rat hepatoma H4-II-E cells.

This study demonstrates novel finding that the expression of c-*myc* or Ha-*ras* mRNAs is suppressed in the cloned rat hepatoma H4-II-E cells overexpressing regucalcin as compared with that of wild-type cells, and that the expression of *p53* mRNA is enhanced in the transfectants.

The proliferation of transfectants overexpressing regucalcin was suppressed as compared with that of the cloned rat hepatoma cells (wild-type) or pCXN2-transfected cells (mock-type), supporting the view that endogenous regucalcin has a suppressive effect on hepatoma cell proliferation. It is known that c-*myc*, c-*fos*, c-*jun*, and Ha-*ras* are tumor stimulator genes. The expression of c-*myc* or Ha-*ras* mRNAs is suppressed in transfectants overexpressing regucalcin. The expression of the tumor suppressor gene *p53* mRNA [Hulla and Schneider, 1993] was found to be enhanced markedly in transfectants overexpression regucalcin. Presumably, the suppression of c-*myc* or Ha-*ras* mRNA expression and the enhancement of *p53* mRNA expression in transfectants overexpressing regucalcin is partly involved in the retardation of proliferation of hepatoma cells.

The mechanism by which regucalcin regulates the expression of genes related to tumor is

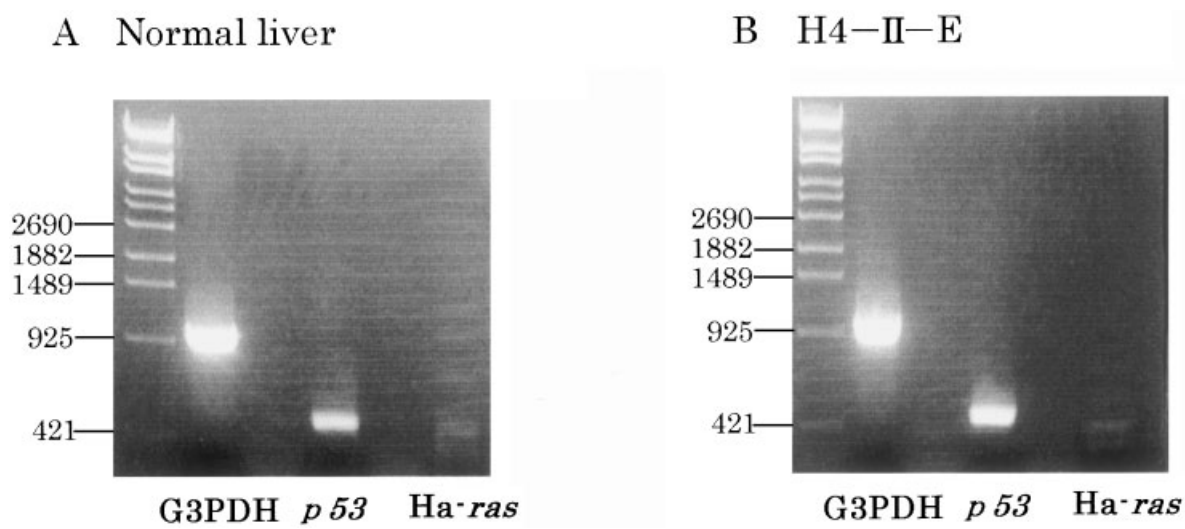

Fig. 6. Expression of *p53* or Ha-*ras* mRNAs in normal rat liver or the cloned rat hepatoma H4-II-E cells (wild-type). Cells were cultured for 72 h in the presence of 10% FBS. Total RNAs (1 μg) extracted from normal rat liver or hepatoma cells were analyzed by RT-PCR using specific primers. The figure shows one of four experiments with separate samples.

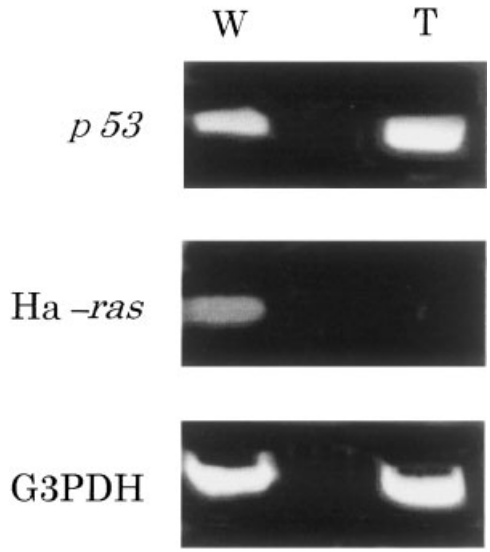

Fig. 7. Change in *p53* or Ha-*ras* mRNAs expression in the cloned rat hepatoma H4-II-E cells (wild-type; W) or stable RC/pCXN2 transfectants (T). Cells were cultured for 72 h in the presence of 10% FBS. Total RNAs (1 μg) extracted from the cells were analyzed by RT-PCR using specific primers. The figure shows one of four experiments with separate samples. The densitometric data for *p53* or Ha-*ras* mRNAs expression in transfectants (T) was 203 ± 11.5 or 6 ± 1.0 (% of wild-type cells; mean ± SEM for four experiments), respectively. These data were significant ($P < 0.01$) as compared with that of wild-type cells.

unknown. Regucalcin has been shown to translocate into the nucleus of rat liver [Tsurusaki et al., 2000], and the protein has an inhibitory effect on RNA synthesis in isolated rat liver nucleus [Tsurusaki and Yamaguchi, 2002b]. It is speculated that regucalcin may bind to promoter region of tumor-related genes, and that the protein may suppress the expression of tumor stimulator gene and stimulate the expression of tumor suppressor gene in the cloned rat hepatoma H4-II-E cells overexpressing regucalcin. As the result, the proliferation of hepatoma cells overexpressing regucalcin may be suppressed. The result of RT-PCR analysis showed that the expression of c-*myc*, c-*fos*, or c-*jun* mRNAs was not seen in the liver of normal rats. However, these hepatic gene expressions were markedly stimulated by a single intraperitoneal administration of calcium chloride to normal rats. Calcium administration has been shown to enhance regucalcin mRNA expression in rat liver which is partly mediated through AP-1 [Murata and Yamaguchi, 1998]. In many cells, AP-1, which consists of homo- and/or heterodimers of the c-*jun* and c-*fos* gene products, regulates the expression of genes that contain specific AP-1-binding sites, named PMA-responsive elements [Curran, 1991]. The present result may support the view that calcium administration-induced enhancement of regucalcin mRNA expression is partly involved in Fos/Jun proteins in rat liver cells.

The expression of c-*myc* mRNA in the cloned rat hepatoma H4-II-E cells (wild-type) was enhanced by culture with 10% FBS as compared with that of 1% FBS. The enhancement was significantly suppressed in hepatoma cells overexpressing regucalcin. This result suggests that endogenous regucalcin has a suppressive effect on the enhancement of c-*myc* mRNA expression by culture with 10% FBS including various hormones and growth factors. It is speculated that regucalcin may partly inhibit the action of intracellular signaling factors that are related to gene expression in hepatoma cells [Nahajima et al., 1999].

In conclusion, it has been demonstrated that the proliferation of hepatoma cells is suppressed in the cloned rat hepatoma H4-II-E cells overexpressing regucalcin, and that the tumor stimulator c-*myc* and Ha-*ras* mRNA expression is suppressed and the tumor suppressor gene *p53* mRNA expression is enhanced in the transfectants. The finding suggests that regucalcin has a role as the suppressor in proliferation of hepatoma cells.

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