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Suppression of Cell Proliferation and Deoxyribonucleic Acid Synthesis in Cloned Rat Hepatoma H4-II-E Cells Overexpressing Regucalcin

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Abstract The role of endogenous regucalcin (RC) in the regulation of cell proliferation was investigated in the cloned rat hepatoma H4-II-E cells overexpressing RC stably. H4-II-E cells were transfected with RC/pCXN2 vector and the multiple neomycin-resistant clones which overexpress stably RC were selected. The RC content of RC/pCXN2-transfected cells used in this study was 19.7-fold as compared with that of the parental wild type H4-II-E cells. Wild type H4-II-E cells, pCXN2 vector-transfected cells (mock type), and RC/pCXN2-transfected cells (transfectants) were cultured for 24, 48, and 72 h in the presence of fetal bovine serum (10% FBS). Cell numbers of wild and mock type were significantly increased with the time course of culture. Cell numbers of transfectants was significantly suppressed as compared with that of wild and mock type. Deoxyribonucleic acid (DNA) synthesis activity in the nuclear fraction of H4-II-E cells was significantly suppressed in transfectants with culture for 12–48 h. The presence of anti-RC monoclonal antibody (10–50 ng/ml) in the reaction mixture caused a significant increase in DNA synthesis activity in the nuclei of wild type and transfectants; this increase was remarkable in transfectants. The effect of anti-RC monoclonal antibody (50 ng/ml) in increasing DNA synthesis activity in transfectants was completely prevented by the addition of regucalcin (1 μM). This study demonstrates that cell proliferation is suppressed in the cloned rat hepatoma H4-II-E overexpressing RC stably. J. Cell. Biochem. 84: 143–149, 2002.

Key words: regucalcin; cell proliferation; DNA synthesis; cloned rat hepatoma H4-II-E cells; transfectant

Calcium ion (Ca^{2+}) plays a pivotal role in the regulation of many cell functions. The Ca^{2+} effect in cells is modulated by Ca^{2+}-binding proteins [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 in Ca^{2+}-related cell function [Yamaguchi, 2000a, b; in Review].

Regucalcin in mainly expressed in liver and kidney cortex [Shimokawa and Yamaguchi, 1992, 1993a; Yamaguchi and Isogai, 1993]. Regucalcin is translocated to the nucleus of liver [Tsurusaki et al., 2000]. In recent years, regucalcin has been demonstrated to play an important role in the regulation of nuclear function which is modulated by a signal transduction due to hormonal stimulation. Regucalcin has an inhibitory effect on protein kinase and protein phosphatase activities in the nucleus of regenerating rat liver [Katsumata and Yamaguchi, 1998; Omura and Yamaguchi, 1999]. Moreover, regucalcin can inhibit DNA and RNA synthesis in the nucleus of regenerating rat liver. Thus regucalcin may play a regulatory role in proliferative liver cells. This, however, remains to be elucidated.

The expression of regucalcin mRNA and its protein has been demonstrated in the cloned rat hepatoma H4-II-E cells and the expression is stimulated by Ca^{2+}-signaling mechanism [Murata and Yamaguchi, 1999; Yamaguchi and Nakajima, 1999; Misawa and Yamaguchi, 2000]. This cell may be a tool to study a role of regucalcin in the regulation of cell proliferation. More recently, it has been shown that regucalcin can inhibit protein kinase and protein

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phosphatase activities which are raised with a proliferation of hepatoma H4-II-E cells [Inagaki and Yamaguchi, 2000, 2001a], and that regucalcin has an inhibitory effect on nuclear DNA synthesis in the proliferation of H4-II-E [Inagaki and Yamaguchi, 2001b]. Endogenous regucalcin may have a suppressive effect on proliferation of the cloned rat hepatoma cells.

The present study, furthermore, was undertaken to determine the role of endogenous regucalcin in the regulation of cell prolliferation using the cloned rat hepatoma H4-II-E cells (transfectants) overexpressing regucalcin stably. We developed H4-II-E cells which were transfected with rat regucalcin cDNA and found that cell proliferation and DNA synthesis was suppressed in these 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) were purchased from Sigma (St. Louis, MO). [Methyl-^{3}H]deoxythymidine 5′-triphosphate ([^{3}H]-dTTP; 2.59 TBq/mmol) was obtained from New England Nuclear (Boston, MA). Other chemicals were obtained from Wako Pure Chemical Co. (Osaka, Japan). The reagents were dissolved in distilled water and ethanol solution.

Regucalcin Expression Construct

The cDNA encoding rat regucalcin was isolated and cloned into the pBluescript vector [Shimokawa and Yamaguchi, 1993]. 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 *Pst* I-*Pst* fragment was isolated (including the *Eco* RI site) and ligated into the cloning vector pBluscript II KS (+) in the corresponding restriction sites in order to introduce an extra *Eco* RI site downstream of the regucalcin cDNA. The resulting *Eco* RI fragment (containing the complete coding cDNA) was cloned into the *Eco* RI site of the pCXN2 expression vector [Niwa et al., 1991]. A construct was selected with the cDNA encoding regucalcin in the sense direction. The resultant plasmid was designated as regucalcin (RC)/pCXN2. The expression of regucalcin is regulated by the CMV immediate early enhancer-chicken β-actin hybrid promoter.

Isolation of Regucalcin

Male Wister rats, weighing 100–120 g, were obtained commercially from Japan SLC (Hamamatsu, Japan). Regucalcin is markedly expressed in rat liver cytosol [Shimokawa and Yamaguchi, 1992; Yamaguchi and Isogai, 1993]. Regucalcin was isolated from rat liver cytosol. The livers were perfused with Tris–HCl buffer (pH 7.4), containing 100 mM Tris, 120 mM NaCl, 4 mM KCl, cooled to 4°C. The livers were removed, cut into small pieces, suspended 1:4 (weight:volume) in Tris–HCl buffer (pH 7.4); the homogenate was spun at 5,500*g* in a refrigerated centrifuge for 10 min, and the supernatant was spun at 105,000*g* for 60 min. The resulting supernatant was heated at 60°C for 10 min and recentrifuged at 38,000*g* for 20 min. Regucalcin in the supernatant was purified to electrophoretic homogeneity by gel filtration on Sephadex G-75 and G-50 followed by ion-exchange chromatography on diethylaminoethyl (DEAE)-cellulose, as reported previous [Yamaguchi and Yamamoto, 1978].

Anti-Regucalcin Antibody

Anti-regucalcin antibody was a monoclonal antibody raised against regucalcin prepared using standard methods [Omura and Yamaguchi, 1999]. Mice (BALB/C, Japan SLC) were subcutaneously injected with 0.1 mg per animal of antigen (rat liver regucalcin) emulsified with Freund's complete adjuvant, and 19 days later, antigen (0.25 mg/animal) was intraperitoneally injected with Freund's incomplete adjuvant. Animals were killed by bleeding 3 days after the last injection. Spleen cells were prepared from immunized mouse and fused into myeloma cells. Anti-regucalcin monoclonal antibody-producing cells (hybridoma cells) were obtained by screening. The IgG from hybridoma cells was isolated through a protein A-agarose column (Sigma).

Cell Culture

Generation of the cloned rat hepatoma H4-II-E cells and the transfectant of H4-II-E cells 3.0×10^5) were maintained for 6–72 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]. 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 containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 μg/ml leupeptin, and disrupted for 60 s with an ultrasonic device. Scrapped cells were also homogenized in Potter-Elvehjem homogenizer with a Teflon pestle. The homogenates were spun at 100*g* in a refrigerated centrifuge for 5 min to obtain the nuclei. The 100*g* supernatant fraction was spun at 1,000*g* for 10 min and the precipitated fraction (containing nucleus) was pooled. DNA concentration in 1,000*g* precipitated fraction was determined by the method of Ceriotti [1955].

Selection of Stable Transfectants

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). At 48*g* after transfection, cells were harvested and used for subsequent experiment. 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.

Gel Electrophoresis and Immunoblotting

The cell cultures were washed twice with PBS and removed from the dish with a cell scraper. The cells were centrifuged and the pellet was homogenized by sonication in 0.1 ml of PBS. The homogenate was centrifuged for 10 min at 17,000*g*, and the protein concentration of supernatant was determined by the method of Lowry et al. [1951] using BSA as a standard. Ten or twenty micrograms of supernatant protein was loaded on a SDS–PAGE [Laemmli, 1970]. The proteins were electrophoretically transferred to a polyvinylidene difluoride membrane, and regucalcin was detected as described [Inagaki et al., 2000]. Quantification of the regucalcin levels on an immunoblot was performed by scanning with a Shimadzu CS-9000 densitometer (Shimadzu Co.).

Determination of Cell Numbers

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

Estimation of DNA Synthesis

DNA synthesis by the nuclear fraction of H4-II-E hepatoma cells was estimated by the procedure of Lynch et al. [1970] with a minor modification. DNA synthesis was measured for 30 min at 37°C in mixtures (0.5 ml) that contained 0.18 M Tris/HCl buffer (pH 8.2), 4 mM $MgCl_2$, 2 mM ATP, dGTP, dCTP, dATP (each 0.08 mM), 0.06 mM [^{3}H]dTTP, dextran (Type 100 C, 2%), 2.5 mM cadaverine, and the suspension of nuclei (0.1 ml containing 40–70 μg DNA). Regucalcin (1 μM) or anti-monoclonal antibody (10–50 ng/ml) was added as indicated. Reactions were stopped with 0.5 ml of NaOH and DNA was precipitated with addition (5 ml) of ice-cold trichloroacetic acid (10% TCA). The DNA was then dissolved (0.5 ml of 1 M NaOH) and precipitated (5 ml of 10% TCA), and the final precipitate dissolved in 0.5 ml of 1 M NaOH, was heated at 80°C for 15 min. Finally, the DNA was precipitated with 10% TCA and the precipitate was washed with acid, ethanol, and ether. The radioactivity in DNA was measured in a hyamine-toluene liquid scintillation mixture, and all data were corrected for the incorporation with control mixture that lacked the three unlabeled deoxynucleotides. DNA synthesis was expressed as disintegrations per minute (dpm) per milligram of DNA.

Statistical Analysis

Data were expressed as the mean ± SEM. The significance of the difference between the values was estimated by Student's *t*-test or by analysis of variance (ANOVA) for comparing multiple groups. A *P*-value of <0.05 was considered to indicate a statistically significant difference.

RESULTS

Generation of H4-II-E Cell Line Overexpressing Regucalcin

The cDNA-encoding rat regucalcin [Shimokawa and Yamaguchi, 1993] was cloned into the expression vector pCXN2 [Niwa et al., 1991]. H4-II-E cells were transiently transfected with the pCXN2 vector or the RC/pCXN2

construct by lipofection. At 48 h after transfection, cells were harvested and the overexpression of regucalcin was detected by immunoblotting using an anti-regucalcin antibody. As shown in Figure 1, the amount of expressed regucalcin in the RC/pCXN2 transfected cells (lanes 3 to 5) increased from 1.0- to 1.7-fold, respectively, when compared with the endogenous regucalcin level in control cells (lane 1).

To generate the transfectants which are stably overexpressed regucalcin in H4-II-E cells, pCXN2 vector- or RC/pCXN2-transfected H4-II-E cells were cultured in neomycin-containing medium. Multiple neomycin-resistant clones were selected and the regucalcin content of these clones was analyzed by immunoblotting with an anti-regucalcin antibody (Fig. 2). The regucalcin content of RC/pCXN2-transfected clones (A1, A2, A3, A5, A6, and A9) was increased 12.6–19.7-folds as compared with that of the parental wild type H4-II-E cells. Of these clones, the clone A1 expressed the highest regucalcin. This cell line (transfectants) and pCXN2-transfected cloned (mock) designated as Cl were used for subsequent experiments.

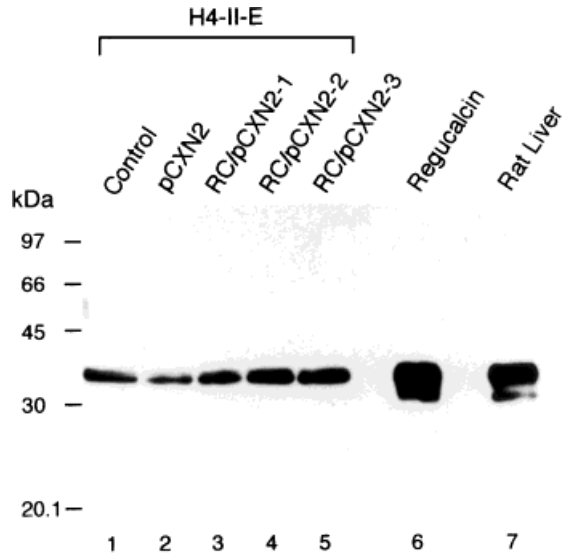

Fig. 1. Transient overexpression of regucalcin (RC) in the cloned rat hepatoma H4-II-E cells. The cells were transfected with the pCXN2 vector or the RC/pCXN2 construct by liprofection. At 48 h after transfection, the cells were harvested, and cell lysates were prepared and examined by immunoblotting using an anti-regucalcin antibody. **Lane 1**, H4-II-E cells (20 μg); **lane 2,** cells transfected with empty pCXN2 vector (20 μg); **lanes 3–5**, three different clones transfected with RC/pCXN2 (20 μg); **lane 6**, normal rat liver (2 μg); **lane 7**, regucalcin (0.1 μg) as the marker. The blot shown is representative of four experiments.

Change in Cell Proliferation of Transfectants

The cloned rat hepatoma H4-II-E cells (wild type), pCXN2-transfected H4-II-E cells (mock type), or RC/pCXN2-transfected H4-II-E cells

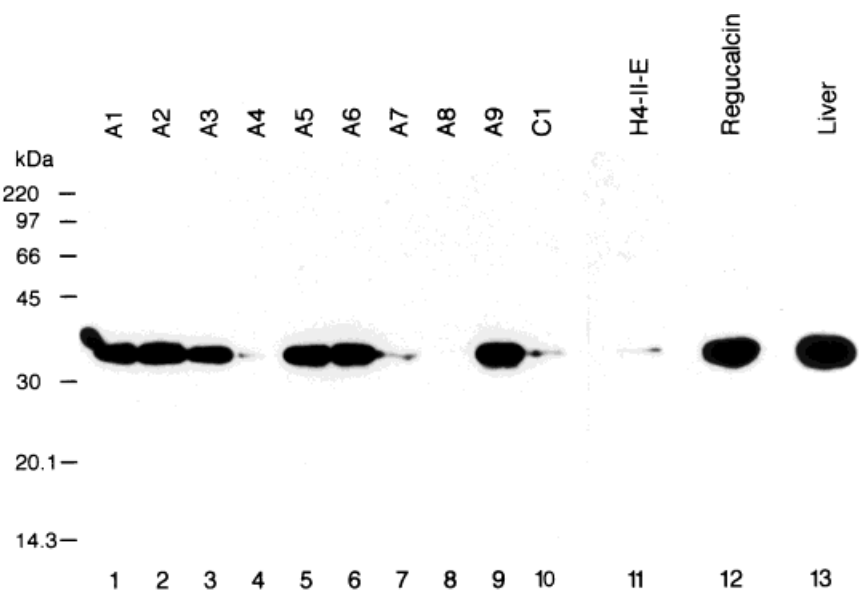

Fig. 2. Expression of regucalcin (RC) in stable RC/pCXN2 transfectants. RC content of multiple neomycin-resistant clones was analyzed by immunoblotting with an anti-regucalcin antibody. **Lanes 1–9**, RC/pCXN2 transfected clones (designated as A1–A9, 10 μg); **lane 10**, pCXN2-transfected clones (designated as C1, 10μg); **lane 11**, H4-II-E cells (10μg); **lane 12**, regucalcin (0.1 μg) as the marker; **lane 13**, normal rat-liver (2 μg). The blot shown is representative of four experiments.

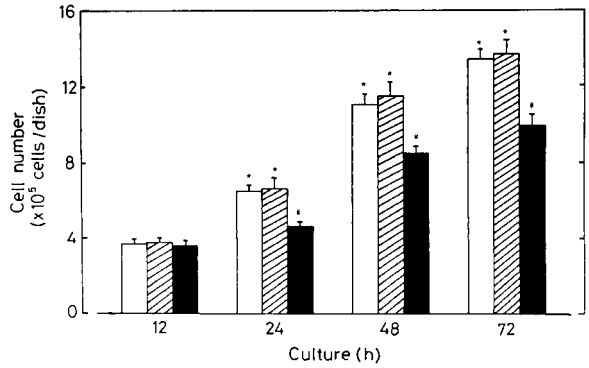

Fig. 3. Change in cell numbers of the cloned rat hepatoma H4-II-E cells (wild type), pCXN2-transfected cells (mock type), or stable RC/pCXN2 transfectants. Cells (3.0×10^5) were cultured for 12, 24, 48, and 72 h in the presence of FBS (10%). Each value is the mean ± SEM of five experiments. $*P < 0.01$, compared with the value obtained from 12 h-culture time. $\#P < 0.01$, compared with the value obtained by culture of wild or mock cells. Open bars, wild; hatched bars, mock; black bars, transfectant.

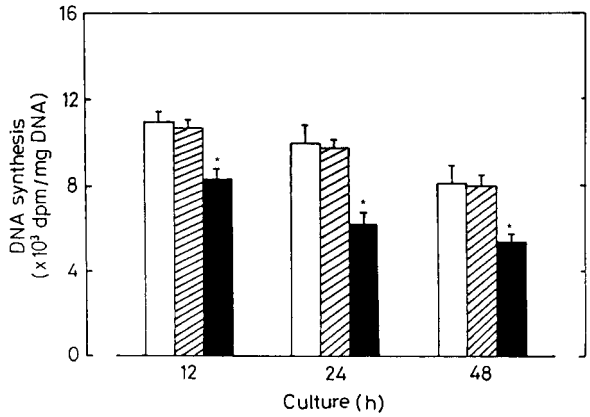

Fig. 4. Changes in DNA synthesis activity in the nuclei of the cloned rat hepatoma H4-II-E cells (wild type), pCXN-2 transfected cells (mock type), or stable RC/pCXN2 transfectant. Cell were cultured for 12, 24, and 48 h in the presence of FBS (10%). DNA synthesis activity was measured in a reaction mixture containing [^{3}H]-dTTP using the nuclear fraction of cell homogenate. Each value is the mean ± SEM of five experiments. $*P < 0.01$, compared with the value obtained by culture of wild or mock cells. Open bars, wild; hatched bars, mock; black bars, transfectant.

(transfectants) were cultured for 12, 24, 48, and 72 h in the presence of FBS (10%). The alteration in cell number is shown in Figure 3. Cell numbers were 3.0×10^5 at zero time of culture, and 12 h later those were not significantly raised. A significant increase in cell numbers of wild and mock type was seen at 24 h after culture. However, cell numbers of transfectants were not significantly raised by 24 h-culture. A remarkable elevation of cell numbers of wild and mock type was seen with culture of 48 and 72 h. Cell numbers of transfectants were significantly suppressed as compared with that of wild or mock type. The suppression of cell proliferation was found in transfectants which regucalcin stably overexpressed.

Change in DNA Synthesis of Transfectants

The change in DNA synthesis in the nuclear fraction of the cloned rat hepatoma H4-II-E cells (wild type), pCXN2-transfected H4-II-E cells (mock type), or RC/pCXN2-transfected H4-II-E cells (transfectants) is shown in Figure 4. Cells were cultured for 12, 24, and 48 h in the presence of FBS (10%). DNA synthesis activity in the nuclei of transfectants was significantly suppressed as compared with that of wild or mock type.

The effect of anti-regucalcin monoclonal antibody on DNA synthesis activity in the nuclei of H4-II-E cells (wild) or RC/pCXN2-transfected H4-II-E cells (transfectants) is shown in Figure 5. Cells were cultured for 24 h in the presence of FBS (10%). DNA synthesis activity in the nuclei of wild type was significantly

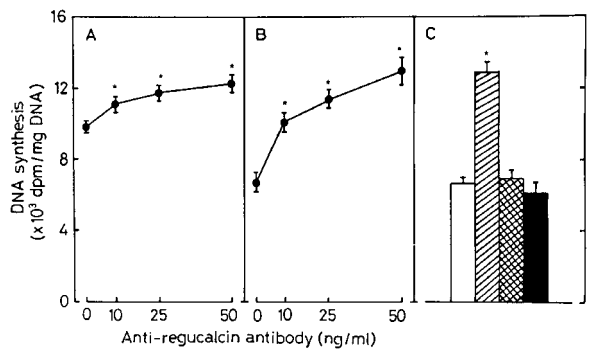

Fig. 5. Effect of anti-regucalcin monoclonal antibody on DNA synthesis activity in the nuclei of the cloned rat hepatoma H4-II-E cells (wild type) and stable RC/pCXN2 transfectant). Cells were cultured for 24 h in the presence of FBS (10%). DNA synthesis activity was measured in a reaction mixture containing either vehicle or anti-regucalcin monoclonal antibody (10, 25, and 50 ng/ml) using the nuclear fraction of cell homogenate from H4-II-E cells (**A**; wild) or tranfectants (**B**). **C:** DNA synthesis activity in the nuclei of transfectants was measured in a reaction mixture containing either vehicle, anti-regucalcin monoclonal antibody (50 ng/ml) plus regucalcin (1.0 μM). Each value is the mean ± SEM of five experiments. C: white bar, control (none); hatched bar, anti-regucalcin antibody; double hatched bar, none-immune IgG; black bar, anti-regucalcin antibody plus regucalcin.

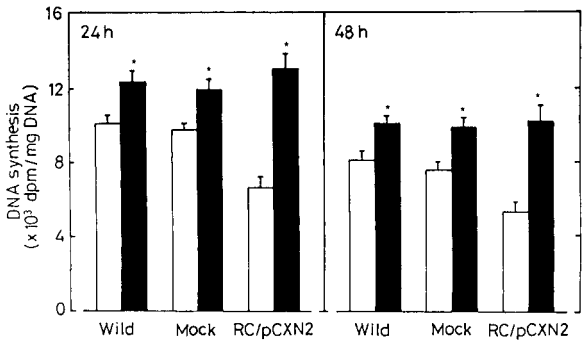

Fig. 6. Effect of anti-regucalcin monoclonal-antibody on DNA synthesis activity in the nuclei of the cloned rat hepatoma H4-II-E cells (wild type), pCXN2-transfected cells (mock type), and stable RC/pCXN2 transfectants with time course of culture. Cells were cultured for 24 or 48 h in the presence of FBS (10%). DNA synthesis activity was measured in a reaction mixture containing either vehicle or anti-RC monoclonal-antibody (50 ng/ml). Each value is the mean ± SEM of five experiments. White bars, control (none); black bars, anti-RC antibody.

increased in the presence of anti-regucalcin monoclonal antibody (10, 25, and 50 ng/ml) in the reaction mixture (Fig. 5A). Such an increase was also seen in the case of transfectants (Fig. 5B). The effect of antibody in increasing DNA synthesis was remarkable in transfectants. In the nuclei of transfectants, the effect of anti-regucalcin monoclonal antibody (50 ng/ml) in increasing DNA synthesis activity was completely prevented by the addition of regucalcin (1 μM) in the reaction mixture (Fig. 5C). Meanwhile, the presence of none-immune IgG (100 ng/ml) in the reaction mixture did not cause a significant alteration in DNA synthesis activity in nuclei of transfectants (Fig. 5C). In addition, the effect of anti-regucalcin monoclonal antibody (50 ng/ml) in increasing DNA synthesis activity in the nuclei of transfectanes was also seen when the cells were cultured for 48 h in the presence of 10% FBS (Fig. 6).

DISCUSSION

Regucalcin mRNA and its protein are expressed in the cloned rat hepatoma H4-II-E cells [Yamaguchi and Nakajima, 1999; Inagaki et al., 2000]. Endogenous regucalcin has been shown to have a suppressive effect on protein phosphatase, protein kinase, and DNA synthesis activities in proliferative H4-II-E cells following stimulation with FBS [Inagaki and Yamaguchi, 2000, 2001a, 2001b]. Regucalcin may play a suppressive role for overexpression of cell proliferation. This was further supported in the cloned rat hepatoma H4-II-E overexpressing regucalcin stably.

The regucalcin content of RC/pCXN2-transfected cells used in this study was 19.7-fold as compared with that of the parental wild type H4-II-E cells, pCXN2 vector-transfected cells (mock type), and RC/pCXN2 vector-transfected cells (transfectants) that were cultured for 72 h in the presence of FBS (10%). Cell numbers and DNA synthesis activity in the transfectants was found to be suppressed as compared with those of wild and mock type, suggesting that the overexpressed regucalcin has a suppressive effect on cell proliferation.

The presence of anti-regucalcin monoclonal antibody in the reaction mixture was found to induce a significant increase in DNA-synthesis activity in the nuclei obtained from wild type H4-II-E, mock type cells, and regucalcin-overexpressing transfectants, indicating that endogenous regucalcin reduces DNA synthesis activity. However, the augmentation of DNA synthesis activity was remarkable in the transfectants. This finding may support the view that endogenous regucalcin is overexpressed in the transfectants and that the protein reveals a great suppressive effect on DNA synthesis activity.

Cell numbers of wild type cells were progressively increased during 72 h of culture in the presence of FBS. Thus, DNA synthesis activity in the cell nuclei seemed to result from the replicative process of the cells. The increase in cell numbers was significantly suppressed in regucalcin-overexpressing transfectants. Presumably, endogenous regucalcin has an inhibitory effect on cell replication in the transfectants.

The expression of regucalcin mRNA in the cloned rat hepatoma H4-II-E cells has been shown to be stimulated by a Ca^{2+}-signaling mechanism [Murata and Yamaguchi, 1999; Yamaguchi and Nakajima, 1999]. Regucalcin is translocated to the nucleus of liver cells, and the protein can inhibit nuclear protein kinase phosphatase activities [Tsurusaki et al., 2000] which are involved in signal transduction to the nucleus [Hunter, 1995]. Regucalcin has an inhibitory effect of DNA synthesis in the nucleus of proliferative liver cells [Yamaguchi and Kanayama, 1996]. From these observations, it is assumed that regucalcin regulates a signaling system in the liver nucleus and that the protein directly inhibits DNA synthesis,

inducing suppression of cell proliferation. Regucalcin may play a physiologic role for the overexpression of proliferation of liver cells.

In conclusion, it has been shown that the suppression of cell proliferation and DNA synthesis is induced in the cloned rat hepatoma H4-II-E cells overexpressing regucalcin stably.

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