Ca^{2+} administration stimulates the binding of AP-1 factor to the 5′-flanking region of the rat gene for the Ca^{2+}-binding protein regucalcin

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mRNA of the Ca^{2+}-binding protein, regucalcin, is mainly expressed in the liver and only to a small extent in the kidney, and the expression of hepatic regucalcin mRNA is markedly stimulated by Ca^{2+} administration [Shimokawa and Yamaguchi (1992) FEBS Lett. **305**, 151–154]. The existence of nuclear factors that bind to the 5′-flanking region of the rat regucalcin gene was investigated. When nuclear proteins obtained from various rat tissues were used in gel mobility-shift assays, tissue-specific formation of a protein–DNA complex was found in the liver and kidney. An additional novel protein–DNA complex was formed when liver nuclear extracts obtained from Ca^{2+}-administered rats (10 mg of Ca^{2+}/100 g body weight) were used. Competition gel mobility-shift experiments using consensus and mutant oligonucleotides for AP-1 factor showed that the additional novel complex was formed from binding of the AP-1 factor to the regucalcin gene. Ca^{2+}-induced binding of the AP-1 factor to the regucalcin gene was completely inhibited by simultaneous administration of trifluoperazine, an antagonist of calmodulin, suggesting that the activation of nuclear AP-1 protein is partly mediated through a Ca^{2+}/calmodulin-dependent pathway. Moreover, the 5′-flanking region of the rat regucalcin gene ligated to a luciferase reporter gene possessed the promoter activity in H4-II-E hepatoma cells. This promoter activity was enhanced by treatment with Bay K 8644, a Ca^{2+}-channel agonist. The present study demonstrates that the Ca^{2+}-response sequences are located within the 5′-flanking region of the rat regucalcin gene.

INTRODUCTION

Ca^{2+} plays an important role in the regulation of many cell functions. Ca^{2+} signals are partly transmitted to intracellular responses which are mediated through a family of Ca^{2+}-binding proteins [1]. We have reported that a novel Ca^{2+}-binding protein, regucalcin, which differs from calmodulin, is distributed in the hepatic cytoplasm of rats [2]. This protein has a reversible effect on the activation and inhibition of various enzymes by Ca^{2+} in liver cells [2–4]. Regucalcin may play a regulatory role in liver cell functions related to Ca^{2+}.

The rat regucalcin gene consists of seven exons and six introns, and several consensus regulatory elements exist upstream of the 5′-flanking region [5]. The gene is localized on the proximal end of rat chromosome Xqll.1-12 [6], and has been demonstrated in human, mouse, cattle, monkey, dog, rabbit and chicken but not yeast [7]. Rat regucalcin mRNA is mainly present in the liver and only to a small extent in the kidney, as assayed by Northern-blot analysis [8], suggesting that it is expressed in a highly tissue-specific manner. Expression of hepatic regucalcin mRNA is stimulated by Ca^{2+} signals, which are partly involved in the regulation of functional events in liver cells [8]; the expression may be mediated through Ca^{2+}/calmodulin [9]. However, the regulatory mechanism by which Ca^{2+} administration stimulates the expression of hepatic regucalcin mRNA at the transcriptional level remains to be elucidated.

Regulation of gene expression at the transcription level is mediated by the interaction of a *trans*-acting factor with a *cis*-acting DNA sequence in genes [10]. Accordingly, interaction between a *trans*-acting regulatory factor and a *cis*-acting regulatory element may be important in regucalcin gene expression. However, the molecular mechanisms of regucalcin regulation, such as interaction of nuclear proteins with gene elements, have not so far been clarified. Therefore we investigated, by using a gel mobility-shift assay, the interaction of liver nuclear proteins with the regucalcin gene after a single intraperitoneal injection of $CaCl_2$ in rats. It was found that Ca^{2+} stimulates binding of nuclear AP-1 protein to the regucalcin gene in rat liver. To understand further the positive regulation of regucalcin gene expression by Ca^{2+}, we tested whether the 5′-flanking region of the gene can confer Ca^{2+}-responsiveness on a linked reporter gene in H4-II-E hepatoma cells. It was found that Ca^{2+} stimulates rat regucalcin gene expression through *cis*-acting elements in the 5′-flanking sequence of the gene.

MATERIALS AND METHODS

Materials

pBluescript II SK^+ vector was obtained from Stratagene (La Jolla, CA, U.S.A.) Leupeptin, aprotinin, dithiothreitol, PMSF, spermine, and spermidine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). TA Cloning vector, pCR™II, was obtained from Invitrogen (San Diego, CA, U.S.A.). Restriction enzymes and T4 polynucleotide kinase were purchased from Takara Shuzo Co. (Shiga, Japan). Poly(dI-dC)·poly(dI-dC) was obtained from Pharmacia Biotech (Uppsala, Sweden). The double-stranded oligonucleotides (AP-1 consensus, 5′-CGCTTGATGACTCAGCCGGAA-3′; AP-1 mutant, 5′-CGCTTGATGACTTGGCCGGAA-3′) containing the consensus sequence or mutant sequence of the AP-1-binding site were purchased from Santa Cruz Biotech Inc. (Santa Cruz, CA, U.S.A.). [γ-^{32}P]ATP (111 TBq/mmol) was obtained from Amersham International (Amersham, Bucks., U.K.). S (−)-Bay K 8644 from Research Biochemicals International (Natick, MA, U.S.A.) was dissolved in DMSO to a stock solution of 5 mM and then diluted

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in medium to give the desired drug concentration. pGL3-Basic vector and pRL-TK vector were purchased from Promega (Madison, WI, U.S.A.). Fetal bovine serum, α-minimum essential medium , penicillin and streptomycin were obtained from Gibco–BRL (Grand Island, NY, U.S.A.). Other reagents (analytical grade) were purchased from Sigma Chemical Co. and Wako Pure Chemical Co. (Osaka, Japan).

Administration procedure

Male Wistar rats, weighing 100–130 g, purchased from Japan SLC Inc. (Hamamatsu, Japan), were maintained on commercial laboratory chow (solid; Oriental Yeast Co., Tokyo, Japan), containing 57.5% carbohydrate, 1.1% calcium, and 1.1% phosphorus, and distilled water *ad libitum*. The following tissues were dissected and minced from rats for the preparation of nuclear extracts: liver, kidney, brain, spleen and heart.

$CaCl_2$ was dissolved in sterile distilled water at concentrations of 5, 10 and 20 mg of Ca^{2+}/ml. Trifluoperazine was dissolved in sterile distilled water containing 0.1% ethanol at a concentration of 5 mg/ml. These solutions were administered intraperitoneally to rats. At the indicated periods after the administration, the rats were killed by bleeding. Livers were perfused with ice-cold buffer A (10 mM Hepes/NaOH, pH 7.9, 0.35 mM sucrose, 10 mM KCl and 1.5 mM $MgCl_2$) and immediately removed for the preparation of liver nuclear extracts. Control animals received vehicle solution.

Preparation of nuclear extracts

All steps were carried out at 4 °C or on ice. The minced tissues (3–5 g) were rinsed twice with buffer B (10 mM Hepes/NaOH, pH 7.9, 0.35 M sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM dithiothreitol, 0.5 mM PMSF, 0.15 mM spermine, 0.5 mM spermidine, 2 μg/ml leupeptin and 2 μg/ml aprotinin), resuspended in 4 vol. of buffer B, and homogenized by 15 strokes in a Potter/Elvehjem glass homogenizer with a Teflon pestle. Nonidet P40 was then added to the homogenates to a concentration of 0.1%, and homogenization was performed by five strokes in a Potter/Elvehjem glass homogenizer. The homogenates were filtered through two layers of cheesecloth and centrifuged at 1000 ***g*** for 10 min. The pelleted nuclei were washed three times with buffer B.

Nuclear extracts were prepared by a modification of the method of Dignam et al. [11]. The nuclei were resuspended in 3 vol. of buffer C (10 mM Hepes/NaOH, pH 7.9, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM dithiothreitol, 5% glycerol and 1 mM PMSF), and then NaCl was added to the suspension to a concentration of 400 mM. After slow mixing for 30 min at 4 °C, the suspensions were centrifuged at 13000 ***g*** for 15 min. The supernatant was dialysed against 100 vol. of buffer D (20 mM Hepes/NaOH, pH 7.9, 75 mM NaCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, 5% glycerol, and 1 mM PMSF) for 3 h. The dialysate was then centrifuged at 13000 ***g*** for 15 min, divided into aliquots, and stored at −80 °C. The protein concentration was determined by the method of Bradford [12], using a kit from Bio-Rad (Richmond, CA, U.S.A.) and BSA as a standard.

DNA fragments for gel mobility-shift assays

Genomic DNA of rat regucalcin was prepared as described previously [5]. The DNA fragments for gel mobility-shift assays were amplified by PCR. PCR was performed using pBluescript SK^+ vector containing the 5.5 kb *Eco*RI–*Xho*I fragment of genomic λRCB2 as a substrate to obtain DNA fragments A, B, C and D. In the construction of fragment A, the pair of primers used were 5′-GAATTCCTGACTGATCTTTTT-3′ and 5′-ATTCGGACAATGGCAGGTGTG-3′. In the construction of fragment B, the pair of primers used were 5′-ACACCTGCCATTGTCCGAATT-3′ and 5′-AAGAAAGAGCTGATAAGACAT-3′. In the construction of fragment C, the pair of primers used were 5′-CAGGGTTGCTGTTAACCTGTG-3′ and 5′-GGATCCAGGGCAATGACAAAC-3′. In the construction of fragment D, the pair of primers used were 5′-CTAAAAATTACTGGATCCTTT-3′ and 5′-AGAAATCATACCAGCAAAGTA-3′. Samples were amplified for 30 cycles under the following conditions: denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C and extension for 1 min at 72 °C. A DNA fragment was then separated by electrophoresis on a 2% low-temperature-melting agarose gel, cloned into TA vector pCRII, and sequenced using a DNA sequencer (Applied Biosystems Inc.). The DNA fragments for gel mobility-shift assays were prepared from each vector by using appropriate restriction enzymes. The radiolabelled probes and competitor fragments used in the binding assays are depicted in Figure 1. The double-stranded DNA probe was end-labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The labelled DNA fragments were separated by electrophoresis using a 4% non-denaturing polyacrylamide gel (acrylamide/bisacrylamide ratio, 30:1), eluted with a high-salt buffer (10 mM Tris/HCl, pH 7.5, 1 mM EDTA and 1 M NaCl) overnight at room temperature, and purified.

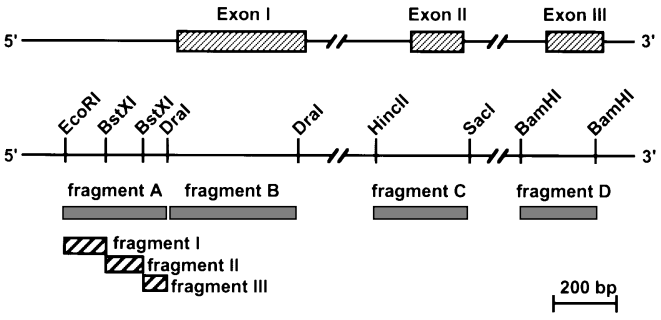

Figure 1 DNA fragments of the 5′-flanking region of the rat regucalcin gene used in gel mobility-shift assays

Each DNA fragment was produced from the 5′-flanking region of the rat regucalcin gene as described in the Materials and methods section. Fragments A (*Eco*RI–*Dra*I), B (*Dra*I–*Dra*I), C (*Hinc*II–*Sac*I) and D (*Bam*HI–*Bam*HI) were 330, 437, 314 and 243 bp respectively. Fragments I (*Eco*RI–*Bst*XI), II (*Bst*XI–*Bst*XI) and III (*Bst*XI–*Dra*I) were 135, 124 and 71 bp respectively.

Gel mobility-shift assays

Gel mobility-shift assays were carried out using the method of Garner and Revzin [13]. Nuclear extracts (3 μg of protein) were preincubated in 20 μl of binding buffer [10 mM Tris/HCl, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 50 μg/ml poly(dI-dC)·poly(dI-dC) and 5% glycerol] for 10 min at 24 °C. The labelled probe (0.15 ng) was then added and incubated at 24 °C for a further 30 min. The reaction mixture was loaded on to 4–6% non-denaturing polyacrylamide gels (acrylamide/bisacrylamide ratio, 30:1) and electrophoresed at 10 V/cm for 90 min in 0.5 × TBE (45 mM Tris, 45 mM boric acid and 1 mM EDTA). The gels were dried and analysed by autoradiography on X-ray film. For the competition experiments, the preincubation was performed in the presence of unlabelled competitor DNA fragment at the indicated fold molar excess.

Construction of the reporter gene plasmid

Reporter gene plasmids were created by cloning restriction fragments isolated from the 5′-flanking region of the rat regucalcin gene. PCR was performed using pBluescript SK$^+$ plasmid containing the 5.5 kb *Eco*RI–*Xho*I fragment of genomic λRCB2 as a substrate to obtain DNA fragments F1, F2 and F3. In the construction of fragment F1, the pair of primers used were 5′-ACAGGTACCGAATTCCTGACTGATCTTT-3′ and 5′-ACACTCGAGAAGAAAGAGCTGATAAGAC-3′. In the construction of fragment F2, the pair of primers used were 5′-ACAGGTACCCCAGTTCACTGGTCTTTGG-3′ and 5′-ACACTCGAGAAGAAAGAGCTGATAAGAC-3′. In the construction of fragment F3, the pair of primers used were 5′-ACAGGTACCTCATCCACTGCAGTGGAGC-3′ and 5′-ACACTCGAGAAGAAAGAGCTGATAAGAC-3′. Samples were amplified for 30 cycles under the following conditions: denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C, and extension for 1 min at 72 °C. A DNA fragment was then separated by electrophoresis on a 2 % low-temperature-melting agarose gel, cloned into TA vector pCRII, and sequenced using a DNA sequencer (Applied Biosystems Inc.). DNA fragments F1, F2 and F3 were prepared from each vector by *Kpn*I–*Xho*I restriction digestion. A series of DNA fragments with different 5′ ends was cloned into the pGL3-Basic promoterless plasmid containing firefly luciferase gene.

Cell culture and transfection

H4-II-E hepatoma cells were maintained in α-minimal essential medium supplemented with 5 mM glucose, 10 % heat-inactivated fetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin in humidified 5 % CO_2/95 % air at 37 °C. For the transfection experiments, the cells were grown on 35 mm dishes to approx. 50 % confluence and washed once with serum-free α-minimal essential medium. Either 2 μg of pGL3-Basic plasmid or the equivalent molar amount of test plasmid was co-transfected into H4-II-E cells along with 0.5 μg of pRL-TK plasmid using the synthetic cationic lipid component, Tfx™-20 reagent, following the manufacturer's instructions (Promega). The pRL-TK vector containing renilla luciferase gene under the control of the herpes simplex virus thymidine kinase promoter (Promega) was used as an internal control plasmid to allow for differences in transfection efficiency and cell number. After 24 h, the transfected cells were switched to medium supplemented without or with 2.5 μM Bay K 8644. Culture was then continued for a further 20 h. At the end of the culture period, the transfectants were lysed, and luciferase activity was measured using the dual-luciferase reporter assay system (Promega).

Statistical analysis

The significance of difference between values was estimated by Student's t test. A P value of less than 0.05 was considered to show statistically significant differences.

RESULTS

We prepared four different fragments, A, B, C and D, from the 5′-flanking region of the rat regucalcin gene, as shown in Figure 1. Each DNA fragment was assayed for nuclear protein binding by the gel mobility-shift experiments.

When radiolabelled fragment A was incubated with nuclear extract from rat liver, and gel mobility-shift assays revealed a single major band, which was shifted upwards from the free DNA (Figure 2, lane 1). The presence of unlabelled fragment II

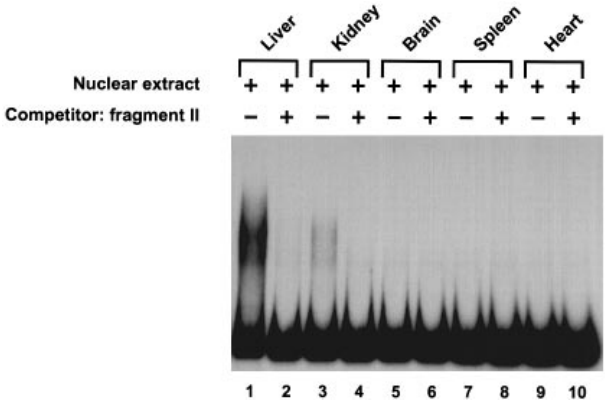

Figure 2 Tissue specificity of the binding of nuclear proteins to fragment A of rat regucalcin gene

End-labelled fragment A was incubated with nuclear extracts prepared from various tissues. Competition assays were performed in the presence of a 100-fold molar excess of unlabelled fragment II as a competitor. Lane 1, liver and no competitor; lane 2, liver and competitor; lane 3, kidney and no competitor; lane 4, kidney and competitor; lane 5, brain and no competitor; lane 6, brain and competitor; lane 7, spleen and no competitor; lane 8, spleen and competitor; lane 9, heart and no competitor; lane 10, heart and competitor.

prevented the formation of the indicated complex when competition reactions were performed in a 10 min preincubation with a 100-fold molar excess of unlabelled fragment II (Figure 2, lane 2). Incubation of rat liver nuclear extract with radiolabelled fragments, B, C and D failed to show specific protein–DNA binding (results not shown). To determine whether the binding factor is a general cellular protein or a tissue-specific protein, various nuclear extracts were prepared, and binding activities of the nuclear proteins to fragment A were examined. When nuclear extracts obtained from rat kidney, which possesses detectable amounts of regucalcin mRNA in Northern-blot analysis, were used in gel mobility-shift experiments, a protein–DNA complex with the same mobility was formed, similar to that seen with nuclear extract from rat liver (Figure 2, lanes 1 and 3). The nuclear protein–DNA complex was only formed to a small extent in the kidney. The formation of this complex in the kidney was also competitively inhibited by preincubation with a 100-fold molar excess of unlabelled fragment II (Figure 2, lane 4). Meanwhile, the protein–DNA complex was not formed when nuclear extracts from rat brain, spleen and heart, which do not possess detectable amounts of regucalcin mRNA in Northern-blot analysis, were used (Figure 2, lanes 5–10).

Binding of liver nuclear proteins to fragment A after a single intraperitoneal injection of $CaCl_2$ to rats is shown in Figure 3. When radiolabelled fragment A was incubated with liver nuclear extracts obtained from control rats, the gel mobility-shift assays showed the formation of complex I which was shifted upwards from the free DNA (Figure 3, lane 1). $CaCl_2$ was injected intraperitoneally into the rats (10 mg of Ca^{2+}/100 g body weight), and liver nuclear proteins were extracted 30, 60 or 90 min later. When radiolabelled fragment A was incubated with liver nuclear extracts obtained from Ca^{2+}-treated rats, an additional novel band (complex II) was found; this band was detected between 30 and 90 min after the administration of Ca^{2+} (Figure 3, lanes 2–4). However, the formation of complex I was not altered by Ca^{2+} administration (Figure 3, lanes 1–4). The additional novel band was not formed when Ca^{2+} (10^{-6} M) was added directly to the reaction mixture containing liver nuclear extract from normal

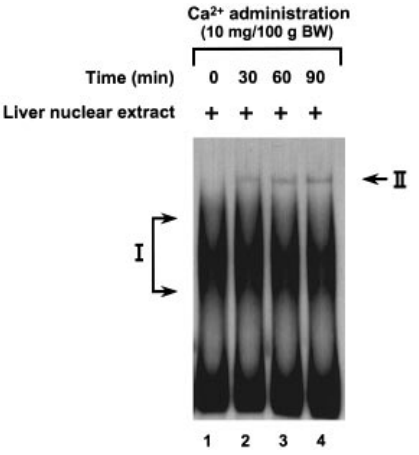

Figure 3 Effect of Ca^{2+} administration on the binding of liver nuclear proteins to fragment A of rat regucalcin gene

Rats received a single intraperitoneal injection of $CaCl_2$ [10 mg of Ca^{2+}/100 g body weight (BW)], and 30, 60 or 90 min later the animals were killed by bleeding. Liver nuclear extracts were prepared immediately. Control rats received an equivalent volume of distilled water. In the gel mobility-shift experiments, end-labelled fragment A was incubated with liver nuclear extracts obtained from rats 0 (lane 1), 30 (lane 2), 60 (lane 3) or 90 (lane 4) min after Ca^{2+} administration. The Figure shows one of four experiments with separate rats.

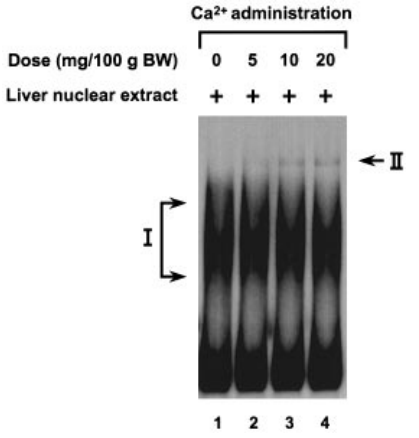

Figure 4 Effect of increasing doses of Ca^{2+} on the binding of liver nuclear proteins to fragment A of rat regucalcin gene

Rats received a single intraperitoneal injection of $CaCl_2$ [5, 10 or 20 mg of Ca^{2+}/100 g body weight (BW)], and 60 min later the animals were killed by bleeding. Liver nuclear extracts were prepared immediately. Control rats received an equivalent volume of distilled water. In the gel mobility-shift experiments, end-labelled fragment A was incubated with liver nuclear extracts obtained from control rats (lane 1) or rats given 5 (lane 2), 10 (lane 3) or 20 (lane 4) mg of Ca^{2+}/100 g body weight. The Figure shows one of four experiments with separate rats.

rats (results not shown). Meanwhile, incubation of liver nuclear extracts from control rats or Ca^{2+}-administered rats with radiolabelled fragments B, C or D failed to produce specific protein–DNA binding (results now shown).

The effect of increasing doses of Ca^{2+} on the formation of complexes I and II is shown in Figure 4. $CaCl_2$ was injected intraperitoneally into rats (5, 10 or 20 mg of Ca^{2+}/100 g body weight), and liver nuclear proteins were extracted 60 min later. When radiolabelled fragment A was incubated with liver nuclear extracts from Ca^{2+}-administered rats, formation of complex II clearly increased in a dose-dependent manner. However, formation of complex I was not affected by the dose of Ca^{2+}.

Competition gel mobility-shift analysis was performed on the complex formed between fragment A and liver nuclear extracts from Ca^{2+}-administered rats. The presence of a 100-fold molar excess of unlabelled fragment A prevented the formation of complexes I and II (Figure 5A, lane 2), indicating that the formation of these complexes is specific to the fragment-A region. When competition experiments were performed in the 10 min preincubation with a 100-fold molar excess of unlabelled fragment I, unlabelled fragment I clearly prevented formation of complex II (Figure 5A, lane 3). This result indicates that the binding site of nuclear protein components of Ca^{2+}-induced complex II may exist in the fragment-I region of rat regucalcin gene. As a potential AP-1-binding site was located on the fragment-I region of the regucalcin gene [5], gel mobility-shift competition assays were further performed using oligonucleotide containing the consensus sequence or mutant sequence of the AP-1-binding site as a competitor. Complex-II formation was completely abolished by the addition of a 100-fold molar excess of AP-1 consensus oligonucleotide, but was not affected by a 100-fold molar excess of mutated AP-1 oligonucleotide (Figure 5B, lanes 1–4). This finding suggests that complex II was formed by binding of AP-1 to fragment A. In the competition gel mobility-shift experiments using unlabelled fragment II as a competitor, complex-I formation was competitively inhibited by preincubation with a 100-fold molar excess of competitor (Figure 5A, lane 4). Thus the binding site of nuclear protein components of complex I may be in the region of fragment II. In addition, the presence of unlabelled fragment III did not affect the formation of complexes I and II (Figure 5A, lane 5).

We examined whether the formation of complexes I and II (AP-1 complex) is mediated through Ca^{2+}/calmodulin by using trifluoperazine, an antagonist of calmodulin. Administration of trifluoperazine (5 mg/100 g body weight) alone to rats caused a slight decrease in complex-I formation (Figure 6, lanes 1 and 3). When trifluoperazine (5 mg/100 g body weight) and $CaCl_2$ (10 mg of Ca^{2+}/100 g body weight) were simultaneously administered to rats, formation of the AP-1 complex induced by Ca^{2+} was completely abolished (Figure 6, lanes 2 and 4).

To examine whether the essential elements for the Ca^{2+} response are located within the 5′-flanking region of the rat regucalcin gene, chimeric constructs containing serial deletions of the 5′-flanking region of the rat regucalcin gene ligated to a luciferase reporter gene were prepared and transfected into H4-II-E hepatoma cells. The relative luciferase activity of the F1-LUC plasmid was increased 1.41-fold in Bay K 8644 (Ca^{2+} channel agonist)-treated transfectants as compared with untreated transfectants (Bay K 8644-treated, 1.57 ± 0.03 compared with untreated, 1.11 ± 0.06; $P < 0.05$; Figure 7). The relative luciferase activity of the F2-LUC plasmid, in which the fragment-I region was deleted, was increased 1.20 fold in Bay K 8644-treated transfectants as compared with untreated transfectants (Bay K 8644-treated, 1.65 ± 0.07 compared with untreated, 1.38 ± 0.06; $P < 0.05$; Figure 7). In contrast, the relative luciferase activity of the F3-LUC plasmid, in which fragment-I and fragment-II regions were deleted, was not affected by the drug treatment (Bay K 8644-treated, 1.02 ± 0.05 compared with untreated, 1.00 ± 0.06; not significant; Figure 7). The Bay K 8644-induced increase in luciferase activity was significantly ($P < 0.05$) lower in F2-LUC transfectants (1.20-fold increase) than in F1-LUC transfectants (1.41-fold increase). In other words, deletion of the fragment-I region significantly diminished the Ca^{2+} re-

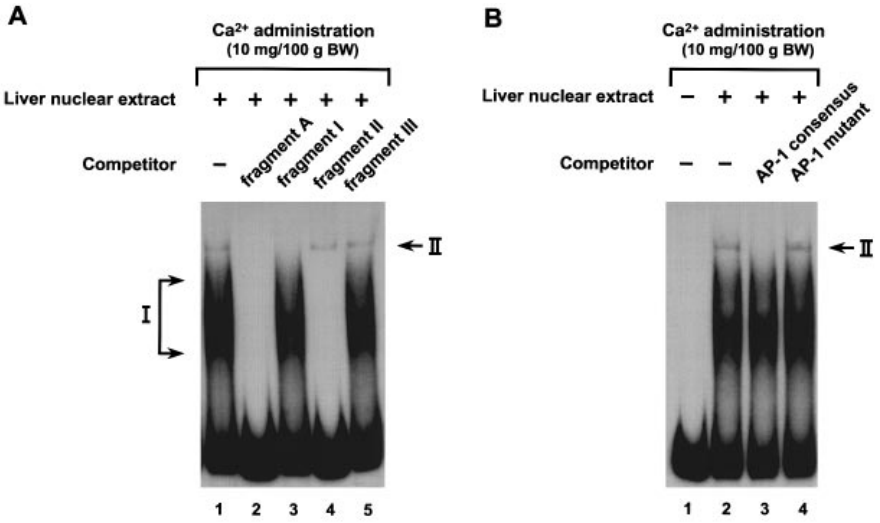

Figure 5 Binding profile of liver nuclear proteins from Ca^{2+}-administered rats to fragment A of rat regucalcin gene

(**A**) Identification of the specific binding region by competition gel mobility-shift experiments. End-labelled fragment A was incubated with liver nuclear extracts obtained from Ca^{2+}-treated rats. Competition assays were performed in the presence of a 100-fold molar excess of the competitor DNA fragment. Lane 1, no competitor; lane 2, fragment A as competitor; lane 3, fragment I as competitor; lane 4, fragment II as competitor; lane 5, fragment III as competitor. (**B**) Participation of AP-1 transcription factor in the formation of complex II. In the gel mobility-shift experiments, end-labelled fragment A was incubated with liver nuclear extracts obtained from Ca^{2+}-administered rats. Competition assays were performed in the presence of a 100-fold molar excess of the competitor oligonucleotide. Lane 1, no extracts; lane 2, no competitor; lane 3, AP-1 consensus oligonucleotide as competitor; lane 4, AP-1 mutant oligonucleotide as competitor. The Figure shows one of four experiments with separate rats.

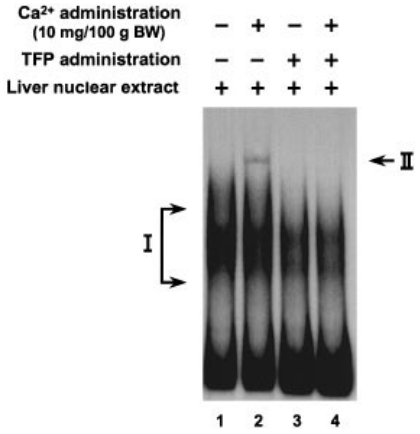

Figure 6 Effect of trifluoperazine administration on the binding of liver nuclear proteins to fragment A of rate regucalcin gene

Rats received a single intraperitoneal injection of $CaCl_2$ [10 mg of Ca^{2+}/100 g body weight (BW)], trifluoperazine (TFP; 5 mg/100 g body weight) or combined $CaCl_2$ and trifluoperazine, and 60 min later the animals were killed by bleeding. Liver nuclear extracts were prepared immediately. Control rats received an equivalent volume of distilled water. In the gel mobility-shift experiments, end-labelled fragment A was incubated with liver nuclear extracts obtained from control rats (lane 1), Ca^{2+}-administered rats (lane 2), trifluoperazine-administered rats (lane 3), or rats injected with both $CaCl_2$ and trifluoperazine (lane 4). The Figure shows one of four experiments with separate rats.

sponse by 14%, and deletion of the fragment-I and fragment-II regions abolished the Ca^{2+} response. Therefore, analysis of this deletion series suggests that the *cis*-acting Ca^{2+}-response elements are located within both fragment-I and fragment-II regions of the rat regucalcin gene.

DISCUSSION

The tissue distribution of rat regucalcin mRNA was previously investigated by Northern-blot analysis. Rat regucalcin mRNA was mainly expressed in the liver but only to a small extent in the kidney [8], suggesting that it is expressed in a highly tissue-specific manner. Sequence-specific DNA–protein interactions, which determine the degree of transcriptional activation for tissue-specific gene expression, occur in distinct regulatory regions [10]. Using gel mobility-shift assays, we found a nuclear protein component that specifically binds to the fragment-II (*Bst*XI–*Bst*XI) region of the rat regucalcin gene. This DNA-binding component was present in nuclear extracts from rat liver and kidney but not rat brain, spleen and heart. Therefore the nuclear proteins that bind to the fragment-II region may act as the positive *trans*-acting factors in the tissue-specific expression of the regucalcin gene.

It has been reported that expression of regucalcin mRNA in the liver is markedly stimulated by the administration of $CaCl_2$ to rats [8,9], and that the enhanced expression is completely inhibited by treatment with trifluoperazine, an antagonist of calmodulin [9]. Trifluoperazine is known to inhibit activation of various enzymes by calmodulin in the presence of Ca^{2+} [14]. Calmodulin has been shown to be present in rat liver nuclei [15]. It has been suggested that stimulation of hepatic regucalcin mRNA expression by Ca^{2+} administration is largely mediated through activation of a Ca^{2+}/calmodulin-dependent pathway [9]. In the present study, we demonstrated that Ca^{2+} administration enhanced the binding of AP-1 to the fragment-I region of the rat regucalcin gene, and that its activity was completely abolished by administration of trifluoperazine. In fact, a potential sequence of the AP-1-binding site is found in the fragment-I region of the rat regucalcin gene [5]. In many cells, AP-1, which consists of homo- and/or hetero-dimers of the c-*jun* and c-*fos* gene products [16,17], regulates the expression of genes that contain specific AP-1-binding sites, named PMA-responsive elements. Therefore

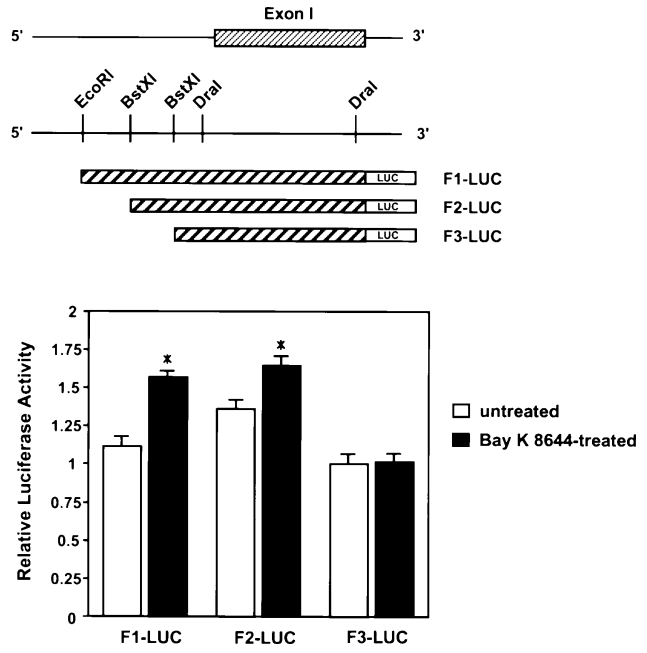

Figure 7 Effect of Bay K 8644, a Ca^{2+}-channel agonist, on the transcriptional activity of the rat regucalcin promoter in H4-II-E hepatoma cells

A series of DNA fragments with different 5′ ends and a common 3′ end were ligated into the pGL3-Basic promoterless plasmid. H4-II-E hepatoma cells were transiently co-transfected with test plasmid and pRL-TK internal control plasmid, and 24 h later switched to unsupplemented medium or medium supplemented with 2.5 μM Bay K 8644. Culture was then continued for a further 20 h. Luciferase activity was measured using the dual-luciferase reporter assay system (Promega). Firefly luciferase activity of the test plasmid was corrected for renilla luciferase activity of the pRL-TK Plasmid. Promoter activity values were calculated by subtracting the luciferase activity of the pGL3-Basic plasmid (0.23 ± 0.05 and 0.29 ± 0.06 in untreated transfectants and Bay K 8644-treated transfectants respectively) from the observed luciferase activity of the test plasmid. Results represent means $\pm$ S.E.M. from five independent experiments. $^{*}P < 0.05$ compared with untreated transfectants.

activation of the AP-1 factor through a Ca^{2+}/calmodulin-dependent pathway may be partly involved in the induction of hepatic regucalcin mRNA by Ca^{2+} administration. In the basal state, formation of complex I may be essential for tissue-specific expression of the regucalcin gene in the liver. In the Ca^{2+}-stimulated state, the additional formation of complex II (AP-1) factor activation) may co-operatively lead to induction of regucalcin gene expression.

There is interesting evidence that administration of trifluoperazine to normal rats causes an appreciable decrease in the hepatic regucalcin mRNA level [9]. This observation suggests that the basal expression of regucalcin mRNA in the liver is regulated, at least in part, through a Ca^{2+}/calmodulin-mediated process. The present study demonstrated that formation of nuclear protein–DNA complex I on the fragment-II region of the regucalcin gene was slightly reduced by the administration of trifluoperazine (Figure 6, lanes 1 and 3). Therefore complex I may include the nuclear proteins that act as the positive *trans*-acting factors in the basal expression of hepatic regucalcin mRNA through Ca^{2+}/calmodulin.

A previous study suggested that the induction of hepatic regucalcin mRNA expression by Ca^{2+} administration can be attributed to activation of a Ca^{2+}/calmodulin-dependent pathway [9]. The increase in intracellular Ca^{2+} may lead to formation of a Ca^{2+}/calmodulin complex and activation of Ca^{2+}/calmodulin-dependent protein kinases. Therefore there was the possibility that an increase in intracellular free Ca^{2+} concentration was likely to trigger the induction of hepatic regucalcin mRNA expression. To assess whether the DNA sequence of the 5′-flanking region of the rat regucalcin gene can functionally respond to increases in intracellular Ca^{2+}, we used reporter plasmids consisting of the 5′-deletion fragment linked to a luciferase reporter gene. As Northern-blot analysis showed detectable regucalcin mRNA levels in H4-II-E hepatoma cells (M. Yamaguchi and T. Murata, unpublished work), H4-II-E hepatoma cells were used in transfection experiments. Deletion of the fragment-I region diminished the Ca^{2+} response by 14%, and deletion of the fragment-I and fragment-II regions abolished the Ca^{2+} response. Therefore the analysis of this deletion series suggests that the *cis*-acting Ca^{2+}-response sequences are located within both fragment-I and fragment-II regions of the rat regucalcin gene. It has been reported that haloperidol stimulates the DNA-binding activity of the AP-1 factor in PC12 pheochromocytoma cells, and that its effect is dependent on Ca^{2+} influx [18], suggesting that the increase in intracellular Ca^{2+} leads to activation of the DNA-binding activity of AP-1. The sequence responsible for the increased intracellular Ca^{2+} was located within the fragment-I region of the rat regucalcin gene, and the fragment-I region contained the binding site for AP-1. Presumably, the AP-1 factor binds to the 5′-flanking region of the rat regucalcin gene to mediate the Ca^{2+} response.

Expression of regucalcin mRNA in liver cells is promoted by hormonal stimulation. Expression of hepatic regucalcin mRNA is clearly stimulated by a single subcutaneous injection of insulin into fasted rats [19]. Also, expression of regucalcin mRNA in human hepatoma cells (HepG2 cells) is stimulated by insulin treatment [20]. Insulin has been shown to stimulate AP-1-mediated gene expression and phosphorylation of AP-1 transcription factor and several Fos-related proteins, suggesting that phosphorylation of AP-1 by insulin plays an important role in hormonal regulation of gene expression [21]. It has recently been shown, using electrophoretic mobility-shift assays, that the DNA-binding activity of AP-1 is increased in insulin-stimulated cells [22]. Presumably, phosphorylation of AP-1 factor by insulin treatment is partly involved in the induction of hepatic regucalcin mRNA expression by hormonal stimulation. The signalling factor is not known. It is possible, however, that a Ca^{2+}/calmodulin-dependent pathway may participate in the induction of hepatic regucalcin mRNA expression by insulin treatment.

In conclusion, it has been demonstrated that Ca^{2+} administration stimulates the additional binding of AP-1 factor to the 5′-flanking region of the rat regucalcin gene, and that this binding may be mediated through a Ca^{2+}/calmodulin-dependent pathway. Moreover, it was found that Ca^{2+} stimulates rat regucalcin gene expression through the *cis*-acting elements in the 5′-flanking sequence of the gene.

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