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Involvement of Hepatic Nuclear Factor I Binding Motif in Transcriptional Regulation of Ca^{2+}-Binding Protein Regucalcin Gene

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The characterization of the binding of nuclear protein on the 5′-flanking region of the rat regucalcin gene was investigated. Nuclear extracts from rat liver and H4-II-E hepatoma cells were used for oligonucleotide competition gel mobility shift assay. An oligonucleotide between position −523 and −506 in the 5′-flanking region of the rat regucalcin gene, which contains a nuclear factor I (NF1) consensus motif TTGGC(N)$_6$CC, competed with the probe for the binding of the nuclear proteins from rat liver and H4-II-E cells. The mutation of TTGGC in the consensus sequence caused an inhibition of the binding of nuclear factors. The presence of Bay K 8644, insulin, and phorbol esters could stimulate the binding of the nuclear factors to the TTGGC region of the rat regucalcin gene in H4-II-E cells. The specific mutation introduced in this region, which was ligated to a luciferase reporter gene, reduced significantly the effects of Bay K 8644, insulin, and phorbol esters in stimulating the regucalcin gene transcriptional activity in H4-II-E cells. These results suggest that the specific nuclear factor binds to the NF1-like sequence, which can stimulate the transcriptional activity, in the promoter region of regucalcin gene in liver cells.

Key Words: **regucalcin; nuclear factor I; rat liver.**

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). A novel Ca^{2+}-binding protein regucalcin, which is mainly distributed in the cytoplasm of liver and kidney cells, has been shown to have an inhibitory effect on the activation of Ca^{2+}/calmodulin-dependent enzymes and protein kinase C by Ca^{2+} or diacylglycerol in the cells (2–4). Regucalcin may play a crucial role in the regulation of cell functions related to Ca^{2+} signaling.

The rat regucalcin gene is localized on chromosome Xq11.1-12 proximal end (5), and it has been demonstrated in human, mouse, bovine, monkey, dog, rabbit, and chicken but not yeast (6). The organization of rat regucalcin gene consists of seven exons and six introns, and several consensus regulatory elements exist in the upstream of the 5′-flanking region of the gene (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.

The expression of hepatic regucalcin mRNA is stimulated by various factors. Regucalcin mRNA level in the liver has been shown to be raised markedly by the administration of $CaCl_2$ to rats; the expression may be partly mediated through Ca^{2+}/calmodulin-dependent pathway (8–10). Also, regucalcin mRNA expression is significantly stimulated by the administration of insulin (11) and 17β-estradiol (12) in the liver of rats. In addition, regucalcin mRNA is expressed in the cloned human Hep G2 hepatoma cells (13) and the rat H4-II-E hepatoma cells (14). Regucalcin mRNA expression in the H4-II-E cells has been reported to be stimulated by the addition of Bay K 8644, a Ca^{2+}-channel agonist (14, 15). Presumably, the promoter of regucalcin gene contains sequence elements that are targets for regulatory transcription factors related to Ca^{2+} signaling. The transcriptional mechanism for the expression of regucalcin gene, however, has not been fully clarified.

The present study was undertaken to determine the nuclear factors, which can bind to the 5′-flanking region of the rat regucalcin gene, using an oligonucleotide competition gel mobility shift assay. The nucleo-

The nucleotide sequence reported in this paper has been submitted to the GenBank/EBI Data Bank with Accession No. D67071.

Abbreviations used: bp, base pair(s); NF1, nuclear factor I; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride; HSF, heat shock transcription factor.

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tide sequences between position −523 and −506 of rat regucalcin gene contain a nuclear factor I (NF1)-like motif TTGGC(N)$_6$CC. This motif has been reported in several genes which are included in α1(I) collagen promoter (16, 17), bone sialoprotein promoter (18), glial fibrillary acidic protein promoter (19), and rat p53 promoter (20, 21). The hepatic nuclear protein that binds to the NF1-like sites, however, has not been identified so far. In this study, we found an involvement of specific nuclear factor which can bind to the NF1-like sequence in the 5′-flanking region of the regucalcin gene in liver cells.

MATERIALS AND METHODS

Chemicals. Fetal bovine serum, N^6,2′-dibutyryl cyclic adenosine 3′,5′-monophosphate (dibutyryl cAMP), insulin, and PMA were purchased from Sigma (St. Louis, MO). S(−)-Bay K 8644 was obtained from Research Biochemicals International (Natick, MA). pGEM-T vector (TA vector), pGL3-Basic vector, and pRL-TK vector were purchased from Promega (Madison, WI). Adenosine 5′-[γ-^{32}P]triphosphate ([γ-^{32}P]ATP; 111 TBq/mmol) was purchased from New England Nuclear (Boston, MA).

Animals and tissues. 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 rats were sacrificed by bleeding. The liver was dissected and minced from rats for the preparation of nuclear extracts.

DNA fragments for gel mobility shift assays. The radiolabeled probes and competitor fragments used in the binding assays are shown in Fig. 1. To obtain DNA fragment −710/+18 of the rat regucalcin gene, PCR was performed using pBluescript II SK$^+$ containing the 5.5-kb *Eco* RI-*Xho* I fragment of genomic λRCB2 (7) as the substrate using the pair of primer 5′-ACAGGTACCGAATTCCTGACTGATCTTT-3′ and 5′-ACACTCGAGGGTTGTAATGACTCCTGGC-3′. A PCR product was subcloned into TA vector, and sequenced. Fragment A was prepared by digesting −710/+18 TA vector with *Eco*RI and *Dra*I. Fragment I, II, and III were prepared by digesting −710/+18 TA vector with *Eco*RI and *Bst*XI, *Bst*XI, and *Bst*XI and *Dra*I, respectively. Fragment A was end-labeled with [γ-^{32}P]-ATP and T4 polynucleotide kinase. The labeled DNA fragments were separated by the electrophoresis through 4% nondenaturing polyacrylamide gels (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.

Synthetic oligonucleotides. The single-stranded oligonucleotides used for gel mobility shift assay and site-directed mutagenesis were synthesized by International Reagent Co. (Kobe, Japan), and these oligonucleotides are shown in Table 1. These synthetic oligonucleotides were annealed with their complementary oligonucleotides to produce double-stranded DNA. The bases changed are underlined in the mutated oligonucleotides.

Construction of the reporter gene plasmids. The reporter gene plasmid was generated by subcloning restriction fragment from −710/+18 TA vector. The DNA fragment −710/+18 was prepared from a vector by *Kpn*I/*Xho*I restriction digestion, and then cloned into the pGL3-Basic promoterless plasmid containing the firefly luciferase gene (15). This wild-type promoter construct was named −710/+18 LUC (WT). A mutant of −710/+18 LUC (WT) was constructed by mutant oligonucleotide II-b-m3 (NF1 mut3) (Table 1) and its complementary oligonucleotide using a QuickChange Site-Directed Mutagenesis Kit (Stratagene). This mutant plasmid −710/+18 LUC (mut) contains a 3-bp substitution mutation (CAGTTACC-CAGGGAGCCTTGAA) in the region of II-b.

Cell culture and transfection. Rat H4-II-E hepatoma cells were cultured as previously described (15). For the transfection experiments, the cells were grown on 35-mm dishes to approximately 70% confluence and washed once with serum-free α-MEM. Either 2 μg of pGL3-Basic plasmid or an 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, according to the manufacture's instructions (Promega). The pRL-TK vector containing the *Renilla* luciferase gene under control of the herpes simplex virus thymidine kinase promoter was used as an internal control for differences in transfection efficiency and cell number. For functional analysis of the basal promoter region of the rat regucalcin gene, the transfected cells were maintained for 48 h in serum-supplemented medium before harvesting. For analysis of regulation of the regucalcin promoter by signaling factors, the transfected cells were maintained for 24 h in serum-supplemented medium and preincubated for 14 h in serum-free α-MEM supplemented with 0.1% BSA, 50 units/ml penicillin, and 50 μg/ml streptomycin. After preincubation, the transfectants were incubated for 16–20 h in the same medium supplemented with or without Bay K 8644 (2.5 μM), dibutyryl cAMP (0.5 mM), insulin (10 nM), and PMA (1 μM) before harvesting. At the end of the culture period, the transfectants were lysed, and the luciferase activity in the cell lysates was measured by dual-luciferase reporter assay system (Promega).

Preparation of nuclear extracts. Nuclear extracts were prepared from the rat liver (10) and H4-II-E cells (15) as previously described. Nuclear extracts were divided into aliquots, and stored at −80°C. The protein concentration was determined by the method of Bradford (22), using a kit from Bio-Rad and BSA as a standard.

Gel mobility shift assays. Gel mobility shift assays were performed as previously described (15). For the competition experiments, preincubation was performed in the presence of unlabeled competitor DNA fragment at 100-fold molar excess. For antibody supershift experiments, nuclear extracts were preincubated with 5 μg of either nonimmune rabbit IgG or anti-NF1 polyclonal antibody (Santa Cruz Biotechnology, Inc., CA).

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 significant. Also, we used a multiway ANOVA and Tukey-Kramer multiple comparison test to compare the treatment groups.

RESULTS

Identification of specific binding region of liver nuclear factor to the fragment II of regucalcin gene. We reported previously a nuclear protein component which specifically binds to the fragment II (*Bst*XI-*Bst*XI) containing the upstream region from the first exon of rat regucalcin gene using the gel mobility shift assays (23). To determine the element for its binding protein, we analyzed the fragment II sequence with TRANSFAC database (24) using TFSEARCH program. We found the existence of potential binding sites for heat shock transcription factor 1 (HSF1), HSF2, NF1, c-Myb, GATA-2, and HSF in the fragment II of the 5′-flanking region of the rat regucalcin gene (Fig. 1). To identify the nuclear factors which can bind to these motifs, a competition gel mobility shift assay was performed using oligonucleotides corresponding to these motifs (Table 1). When radiolabeled fragment A was incu-

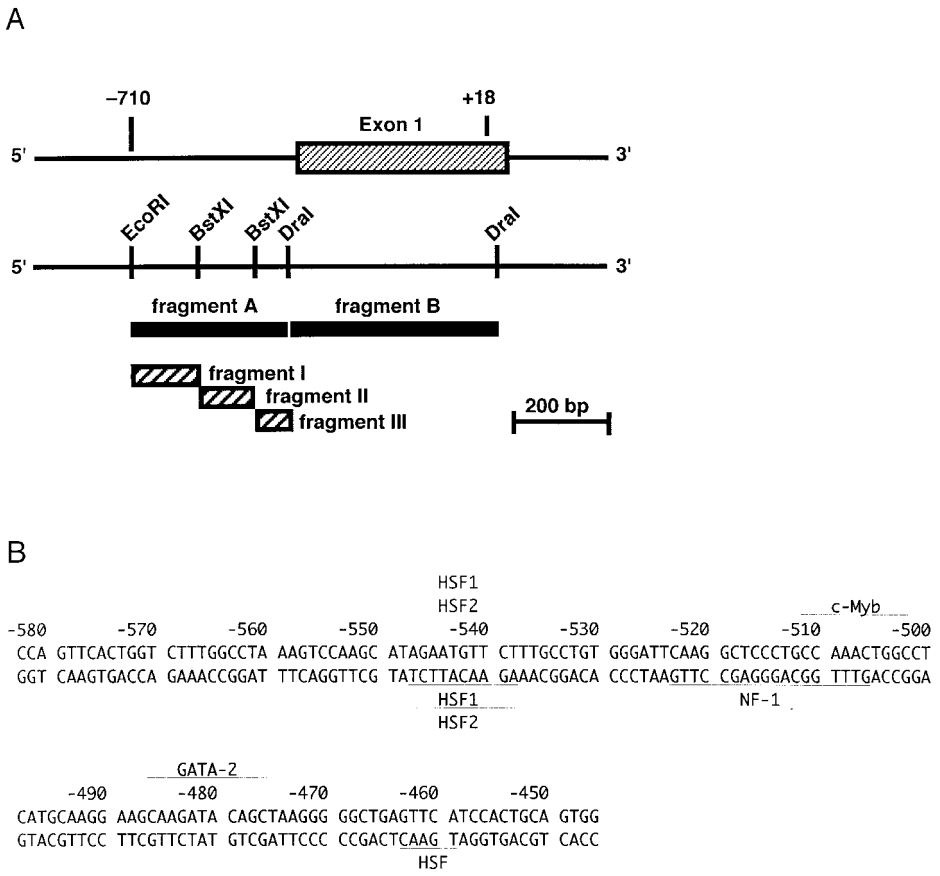

FIG. 1. The 5′-flanking region of the rat regucalcin gene. (A) DNA fragments of the rat regucalcin gene used in gel mobility shift assays. Each DNA fragment was produced from the 5′-flanking region of the rat regucalcin gene as described under Materials and Methods. Fragments A (*Eco*RI-*Dra*I) and B (*Dra*I-*Dra*I) were 330 bp and 437 bp. Fragments I (*Eco*RI-*Bst*XI), II (*Bst*XI-*Bst*XI), and III (*Bst*XI-*Dra*I) were 135 bp, 124 bp, and 71 bp, respectively. (B) Nucleotide sequence from −582 bp to −446 bp (fragment II). Sequence data analysis was done with TRANSFAC database (24) using TFSEARCH program, the threshold score of greater than 85.0 point. Potential binding sites by HSF1, HSF2, NF1, c-Myb, GATA-2, and HSF are marked by solid lines above (sense) or below (antisense). This genomic sequence has been deposited in the GenBank data base under Accession No. D67071.

bated with the nuclear extract from rat liver, the gel mobility shift assay revealed a single major band which was shifted upward from the free DNA probe (Fig. 2A, lane 2). The presence of 100-fold molar excess of unlabeled fragment A or fragment II prevented the formation of the indicated complex (Fig. 2A, lanes 3 and 4). Moreover, when oligonucleotides toward the II-a (HSF1+2), II-b (NF1), II-c (c-Myb), II-d (GATA-2), and II-e (HSF) were used as competitor, the II-b (NF1) oligonucleotide only was specifically competed with fragment A (Fig. 2, lane 6) for the binding protein. This result suggested that the protein which bound to this motif is a NF1 family protein.

To determine the exact DNA sequence for the II-b (NF1) that is involved in the formation of the DNA-protein complex, we carried out mutation analysis for this region. Mutation sites were selected from the conserved nucleotide sequence in the NF1 consensus sequence (Table 1). Two double-stranded oligonucleotides were contained three different bp substitutions, and those were used as competitors for gel mobility shift assay. Figure 2B showed that II-b (NF1) and II-b-m1 (NF1 mut1) inhibited the binding of nuclear factor to fragment A, but II-b-m2 (NF1 mut2) did not compete, indicating that the binding protein recognizes TTGGC in the NF1 consensus sequence.

A supershift assay with the anti-NF1 antibody was carried out (Fig. 2C). The formation of DNA–protein complex was not supershifted or decreased by the presence of anti-NF1 antibody which can recognize all forms of NF1 protein (19, 20, 25).

Identification of specific binding region of hepatoma cell nuclear factor to the fragment A of rat regucalcin gene. In rat hepatoma cells (H4-II-E), the binding of the nuclear proteins to fragment A of the 5′-flanking region of the rat regucalcin gene was examined by the gel mobility shift assays (15). When radiolabeled fragment A was incubated with the nuclear extracts from H4-II-E cells, the gel mobility shift assay revealed the

TABLE 1

Oligonucleotide Sequences Used in This Study

Oligonucleotide	Sequence[a]	Position[b]
HSF1 consensus	5′-RGAANRTTCN-3′	
HSF2 consensus	5′-NGAANNWTCK-3′	
II-a (HSF1+2)	5′-AAGCATAGAATGTTCTTTGCCT-3′	−553 to −532 (±)
HSF1 inverse consensus	5′-NGAAYNTTCY-3′	
HSF2 inverse consensus	5′-MGAWNNTTCN-3′	
NF1 consensus	5′-NNTTGGCNNNNNNCCNNN-3′	
II-b (NF1)	5′-CAGTTTGGCAGGGAGCCTTGAA-3′	−525 to −504 (−)
II-b-m1 (NF1 mut1)	5′-CAGTTTGGCAGGGA<u>TAA</u>TTGAA-3′	
II-b-m2 (NF1 mut2)	5′-CAGTT<u>ACC</u>CAGGGAGCCTTGAA-3′	
II-b-m3 (NF1 mut3)	5′-CCTTGCATGAGGCCAGTT<u>ACC</u>CAGGGAGCCTTGAATCC-3′	
c-Myb consensus	5′-NNNAACKGNC-3′	
II-c (c-Myb)	5′-TGCCAAACTGGCCT-3′	−513 to −500 (+)
GATA-2 consensus	5′-NNGATRNNN-3′	
II-d (GATA-2)	5′-AAGCAAGATACAGCT-3′	−490 to −476 (+)
HSF consensus	5′-AGAAN-3′	
II-e (HSF)	5′-TGGATGAACTCAG-3′	−468 to −456 (−)

[a] Mutated sequences are underlined. Single letter code: W = A or T; S = C or G; R = A or G; Y = C or T; K = G or T; M = A or C; N = A, C, G, or T.

[b] −, antisense; +, sense; ±, both sense and antisense.

formation of complexes 1, 2, and 3 which were shifted upward from the free DNA (Fig. 3A, lane 2). The presence of a 100-fold molar excess of unlabeled fragment A prevented the formation of all complexes (Fig. 3A, lane 3), indicating that the formation of these complexes is specific to the fragment A region. When competition experiments were performed with a 100-fold molar excess of unlabeled fragment I or fragment II, the presence of fragment I prevented the formation of complex 3 (Fig. 3A, lane 4), and unlabeled fragment II inhibited the formation of complex 1 and 2 (Fig. 3A, lane 5). However, the presence of unlabeled fragment III did not abolish the formation of complex 1, 2, and 3 (Fig. 3A, lane 6).

To determine whether the binding components in the nuclear extracts from rat liver and H4-II-E cells are identical to fragment A, both oligonucleotide competition assays and supershift assays were examined (Figs. 3B, 3C, and 3D). The presence of an oligonucleotide II-b (NF1) inhibited the formation of complex 1 and 2 (Fig. 3B, lane 6). This pattern of competition was the same as fragment II (Fig. 3B, lane 4). II-a (HSF1+2), II-c (c-Myb), II-d (GATA-2), and II-e (HSF) had no effect on the formation of complex 1-3 (Fig. 3B, lanes 5 and 7–9). Moreover, the presence of II-b-m2 (NF1 mut 2) did not inhibit the formation of complex 1-3 (Fig. 3C, lane 4). In supershift experiment (Fig. 3D), anti-NF1 antibody did not prevent the formation of the nuclear factor-DNA complexes.

Effect of mutation on transcriptional activity of the promoter of rat regucalcin gene in H4-II-E cells. The 5′-flanking region of the rat regucalcin gene ligated to the luciferase reporter plasmid possesses promoter activity in H4-II-E hepatoma cells, and the region −102/+18 is essential for basal functional promoter activity of the rat regucalcin gene (15). To determine whether the mutation of the II-b (NF1) region can alter the rat regucalcin gene promoter activity, we constructed a wild-type −710/+18 region of the rat regucalcin gene and its three bp substitutions (−511 to −509, TGG to ACC) which ligated to the luciferase reporter gene. Those plasmids were transiently transfected into H4-II-E cells to assay luciferase activity. The cells were cultured in the presence of 10% fetal bovine serum. The relative luciferase activity of the −710/+18 LUC (WT) plasmid showed an increase in 8.0-fold as compared with Basic LUC, whereas that of the −710/+18 LUC (mut) plasmid increased 5.8-fold; the relative activity was significantly ($P < 0.01$) decreased from 8.0 ± 0.1 in WT to 5.8 ± 0.1 in mut (Fig. 4). Thus, the mutation at the site of II-b (NF1) caused a significant decrease in basal promoter activity of the rat regucalcin gene.

Effects of Bay K 8644, dibutyryl cAMP, insulin, and PMA on the nuclear protein binding to the 5′-flanking region of the rat regucalcin gene. The radiolabeled fragment A was incubated with the nuclear extracts obtained from H4-II-E cells which are cultured in the presence of Bay K 8644 (2.5 μM), dibutyryl cAMP (0.5 mM), insulin (10 nM) or PMA (1 μM). The formation of complex 1 with binding of nuclear extracts to fragment A was clearly increased by the presence of Bay K 8644, insulin, and PMA (Fig. 5, lanes 2, 4, and 5). However, the treatment with dibutyryl cAMP did not cause a significant change in the formation of complex 1 (Fig. 5, lane 3). In contrast, the formation of complex 2 and 3 were not altered by the treatment of all mediators used (Fig. 5, lanes 1–5). Moreover, whether II-b (NF1) region is related to the formation of complexes 1 which

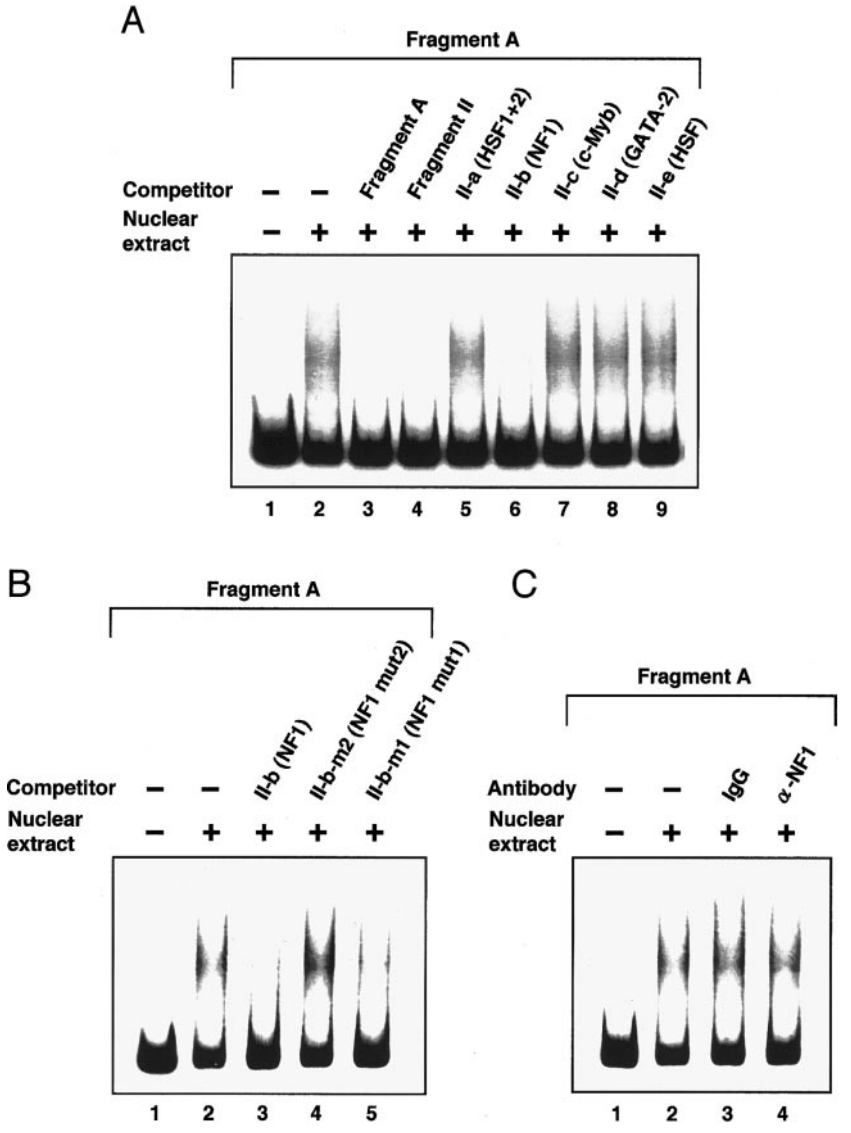

FIG. 2. Gel mobility shift analysis of nuclear factors bound to fragment A of the rat regucalcin promoter. Gel mobility shift assays were performed using nuclear extracts from rat liver (3.5 μg of protein) and a ^{32}P-labeled fragment A as a probe. Competition assays were performed in the presence of 100-fold molar excess of the competitor DNA fragment or double-stranded oligonucleotide. (A) Identification of specific binding region of nuclear protein from rat liver to fragment II of rat regucalcin gene. Lane 1, no extracts; lane 2, no competitor; lane 3, fragment A as competitor; lane 4, fragment II; lane 5, II-a (HSF1+2); lane 6, II-b (NF1); lane 7, II-c (c-Myb); lane 8, II-d (GATA-2); lane 9, II-e (HSF). (B) Competition assay with oligonucleotide II-b (NF1) and its mutants. Lane 1, no extracts; lane 2, no competitor; lane 3, II-b (NF1) as competitor; lane 4, II-b-m2 (NF1 mut2); lane 5, II-b-m1 (NF1 mut1). (C) Gel mobility shift assay with anti-NF1 antibody. Lane 1, no extracts; lane 2, no antibody; lane 3, nonimmune rabbit IgG (5 μg); lane 4, anti-NF1 polyclonal antibody (5 μg).

was increased by Bay K 8644, insulin, and PMA was examined by competition gel mobility shift assay with II-b (NF1) oligonucleotides. The presence of II-b (NF1) completely prevented the formation of complex 1 (Fig. 5, lanes 6–10).

Regulation of regucalcin promoter by Bay K 8644, dibutyryl cAMP, insulin, and PMA. To determine whether the mutation of the II-b (NF1) region induces a change in the regucalcin gene expression, H4-II-E cells were transfected with either −710/+18 LUC (WT) construct or −710/+18 LUC (mut) construct. In the absence of fetal bovine serum, culture with Bay K 8644 (2.5 μM), insulin (10 nM), and PMA (1 μM) caused a significant increase in luciferase activity in the cells transfected with the −710/+18 LUC (WT) construct (Bay K 8644, insulin, and PMA showed an increase in: 8.7 ± 0.2-, 11.0 ± 0.8-, and 10.4 ± 0.3-fold, respectively; $P < 0.01$ as compared with the control value) (Fig. 6A). However, the cells with the −710/+18 LUC (mut) construct weakened the effect of Bay K 8644, insulin, and PMA in elevating the luciferase activity (Bay K 8644, insulin, and PMA showed an elevation of:

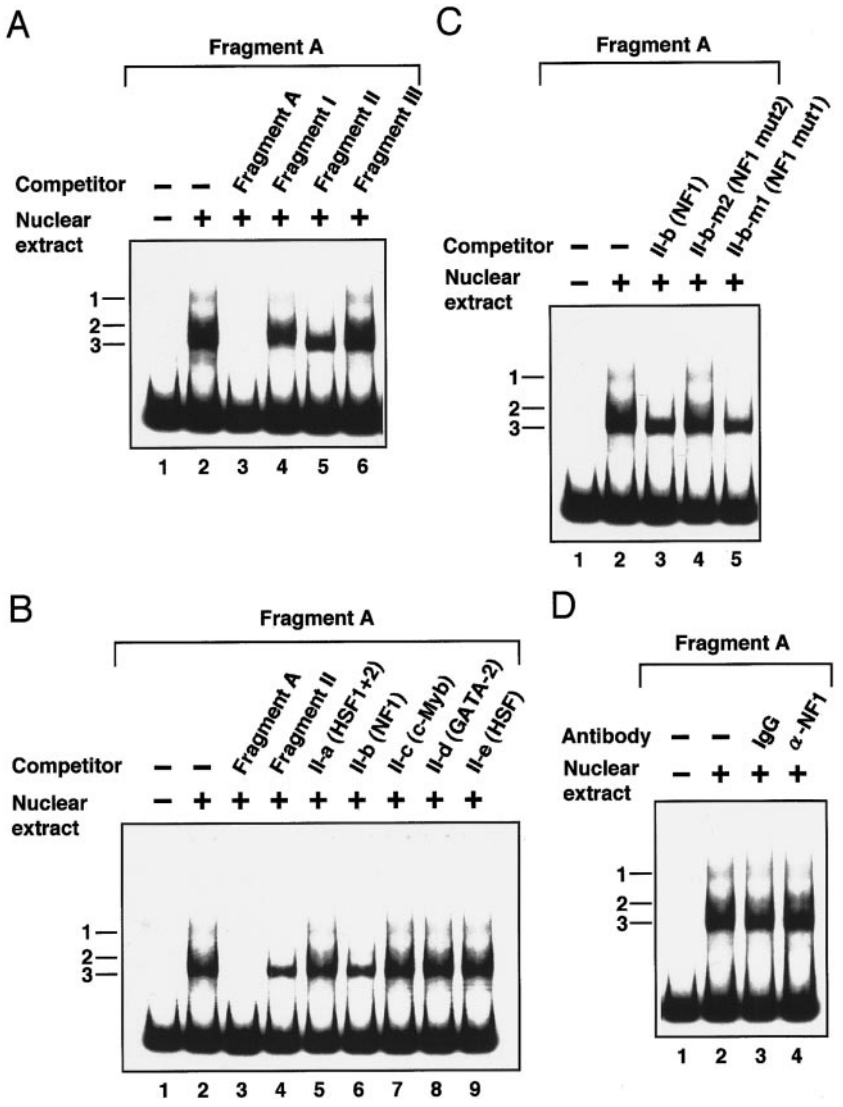

FIG. 3. Binding profile of nuclear proteins from H4-II-E cells to fragment A of the rat regucalcin promoter. Gel mobility shift assays were performed using nuclear extracts from H4-II-E cells (3 μg of protein) and a ^{32}P -labeled fragment A as a probe. Competition assays were performed in the presence of 100-fold molar excess of the competitor DNA fragment or double stranded oligonucleotide. (A) Identification of specific binding region by competition with gel mobility shift experiments. Lane 1, no extracts; lane 2, no competitor; lane 3, fragment A as competitor; lane 4, fragment I; lane 5, fragment II; lane 6, fragment III. (B) Identification of specific binding region of the nuclear protein from H4-II-E cells to fragment II of rat regucalcin gene. Lane 1, no extracts; lane 2, no competitor; lane 3, fragment A as competitor; lane 4, fragment II; lane 5, II-a (HSF1+2); lane 6, II-b (NF1); lane 7, II-c (c-Myb); lane 8, II-d (GATA-2); lane 9, II-e (HSF). (C) Competition assay with oligonucleotide II-b (NF1) and its mutants. Lane 1, no extracts; lane 2, no competitor; lane 3, II-b (NF1) as competitor; lane 4, II-b-m2 (NF1 mut2); lane 5, II-b-m1 (NF1 mut1). (D) Gel mobility shift assay with anti-NF1 antibody. Lane 1, no extracts; lane 2, no antibody; lane 3, nonimmune rabbit IgG (5 μg); lane 4, anti-NF1 polyclonal antibody (5 μg).

6.0 $\pm$ 0.1-, 6.1 $\pm$ 0.2-, and 7.3 $\pm$ 0.1-fold, respectively; $p < 0.01$ as compared with the control value) (Fig. 6B). Thus, the *cis*-acting DNA sequences which mediate the effect of Bay K 8644, insulin, and PMA in H4-II-E cells were located in the region II-b of the rat regucalcin gene. Meanwhile, luciferase activity in H4-II-E cells transfected with the $-710/+18$ LUC (WT) construct was significantly ($p < 0.01$) increased (6.9 $\pm$ 0.2-fold) by the treatment with dibutyryl cAMP (0.5 mM) (Fig. 6A). Such an increase was also seen in the cells transfected with the $-710/+18$ LUC (mut) (5.9 $\pm$ 0.1-fold). The difference in luciferase activity in the cells with both constructs was negligible. II-b (NF1) region was not related to dibutyryl cAMP stimulation in H4-II-E cells.

DISCUSSION

The present study demonstrates that the specific DNA sequence in the promoter region of regucalcin gene is involved in the binding to the *trans*-acting

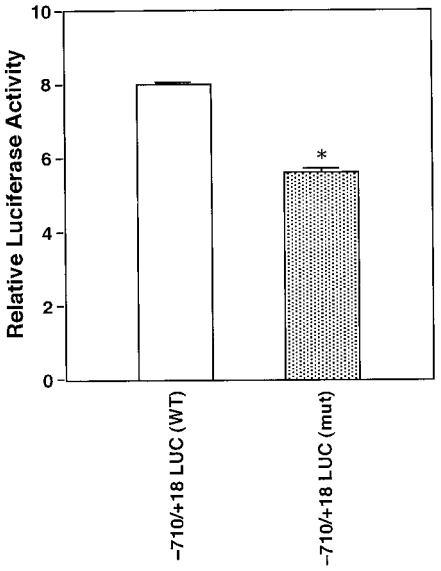

FIG. 4. Basal expression of wild-type and mutant derivative of the rat regucalcin promoter in H4-II-E hepatoma cells. A DNA fragment −710/+18 was ligated into the pGL3-Basic promoterless plasmid (Basic LUC). A mutant reporter gene plasmid was generated by site-directed mutagenesis as described under Materials and Methods. H4-II-E hepatoma cells were transiently cotransfected with test plasmid and pRL-TK internal control plasmid, and the cells were cultured for 48 h in a medium containing 10% fetal bovine serum. After 48 h, luciferase activity in the cell lysates was measured. The firefly luciferase activity of the test plasmid was corrected for *Renilla* luciferase activity of the pRL-TK plasmid. The results are expressed as -fold stimulation compared to the luciferase activity measured after transfection with Basic LUC, which was set as 1.0. Each value is the mean ± S.E.M. of eight separate experiments. $*p < 0.01$, compared with the value obtained from wild-type.

factor by using competition gel mobility shift assay with II-b (NF1) oligonucleotide. Element II-b contains the region which has a homology to NF1 consensus sequence TTGGC$(N)_6$CC. The mutation of TTGGC in the consensus sequence caused an inhibition of the binding of nuclear factors by a competition gel mobility shift assay with mutant oligonucleotides. Thus, nuclear protein, which binds to fragment II, may be a member of NF1 family.

On the other hand, NF1-like sequence-binding proteins, which are distinct from NF1, have been reported in α1(I) collagen promoter (16, 17), bone sialoprotein promoter (18), glial fibrillary acidic protein promoter (19), and rat p53 promoter (20, 21). Now, the binding of hepatic nuclear protein to the fragment II in the promoter region of regucalcin gene was not supershifted or decreased by gel mobility shift assay with an anti-NF1 antibody (19, 20, 25). From this result, it can not exclude the possibility that the nuclear protein, which can specifically bind to NF1-like sequence in the promoter region of regucalcin gene, differs from NF1.

The region from bases −710 to −343 in the 5′-flanking sequence of the regucalcin gene contains a *cis*-element, which mediates the induction of the gene by Bay K 8644, insulin, and PMA in rat H4-II-E hepatoma cells (15). The region −710/−343 is mostly consistent with fragment A (−710/−380). To determine the specific DNA sequence, which is involved in binding to the *trans*-acting factor in H4-II-E cells, we performed gel mobility shift assays by the same strategy using rat liver. The formation of complexes 1 and 2 by the binding of nuclear proteins to the fragment II in fragment A was related to the NF1-like sequence (II-b) (Fig. 3). The nuclear proteins, which bind to the NF1-like sequence (II-b), in H4-II-E cells may be identical to that of normal rat liver, although their binding profiles were different.

Interestingly, we found that the formation of complex 3 was specific in the fragment I by using the nuclear extracts from H4-II-E cells. This complex 3 was not seen in the nuclear extracts from rat liver. The putative *cis*-acting elements, which contain AP-1 and GATA-2, are located in the fragment I region (15). However, AP-1 is not involved in the formation of complexes 1–3 in the region −710/−343 in mobility shift experiments by using a consensus oligonucleotide for this factor (15). The identification of this complex in rat H4-II-E hepatoma cells remains to be elucidated.

The 5′-flanking region of the rat regucalcin gene has been shown to have a functional promoter activity by the gene transfection experiments using a promoter-

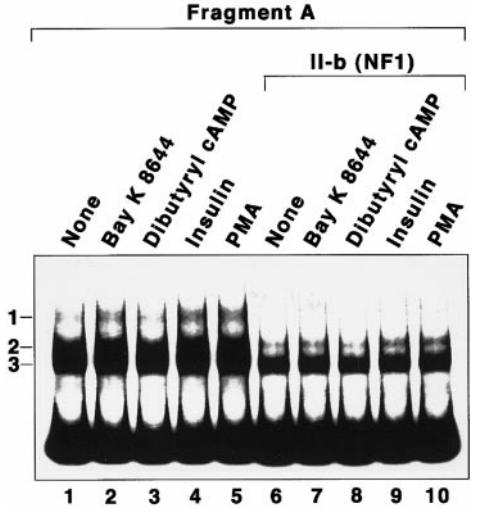

FIG. 5. Binding profile of nuclear proteins from the mediator-stimulated H4-II-E cells to fragment A of the rat regucalcin promoter. H4-II-E cells were grown on 35-mm dishes to approximately 70% confluence in serum-supplemented medium, and the cells were deprived of serum for 14 h and then incubated for 16 h in a medium containing vehicle (none), 2.5 μM Bay K 8644, 0.5 mM dibutyryl cAMP, 10 nM insulin, or 1 μM PMA. In the gel mobility shift experiments, end-labeled fragment A was incubated with nuclear extracts (3 μg of protein) from the cells cultured without (control; lanes 1 and 6), or with Bay K 8644 (lanes 2 and 7), dibutyryl cAMP (lanes 3 and 8), insulin (lanes 4 and 9), and PMA (lanes 5 and 10). Competition assays were performed in the presence of 100-fold molar excess of II-b (NF1) as competitor (lanes 6 to 10).

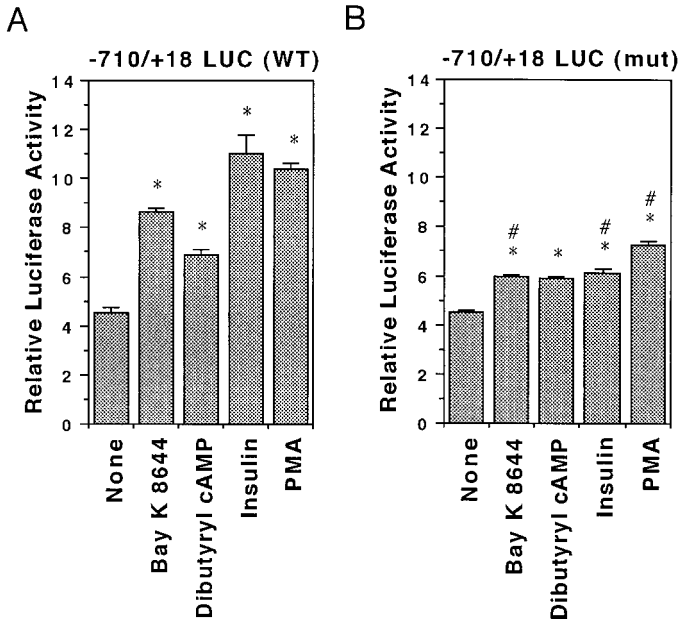

FIG. 6. Effect of Bay K 8644, dibutyryl cAMP, insulin, and PMA on promoter activities from −710/+18 LUC (WT) or (mut) in H4-II-E hepatoma cells. Fragment −710/+18 was ligated into the pGL3-Basic promoterless plasmid (Basic LUC), and the mutant was generated by site-directed mutagenesis as described under Materials and Methods. H4-II-E hepatoma cells were transiently cotransfected with test plasmid and pRL-TK internal control plasmid, and maintained in serum-supplemented medium for 24 h. The cells were preincubated in serum-free medium for 14 h, and then incubated for 20 h in the same medium supplemented with or without 2.5 μM Bay K 8644, 0.5 mM dibutyryl cAMP, 10 nM insulin, and 1 μM PMA. Luciferase activity was measured by the dual-luciferase reporter assay system. The firefly luciferase activity of the test plasmid was corrected for *Renilla* luciferase activity of the pRL-TK plasmid. The results are expressed as -fold stimulation compared to the luciferase activity measured after transfection with Basic LUC, which was set as 1.0. Each value is the mean ± S.E.M. of six separate experiments. (A) Wild-type −710/+18 LUC. (B) Mutant −710/+18 LUC. *$p < 0.01$, compared with the control value without signal mediator addition. #$p < 0.01$, compared with the value obtained from WT.

less luciferase reporter plasmid in the cloned rat H4-II-E hepatoma cells (15). The present study with mutation analysis shows that TTGGC sequence in the II-b (NF1) is necessary for the binding of nuclear proteins, from normal rat liver and H4-II-E cells, to the promoter region of regucalcin gene. Moreover, the mutation of the II-b (NF1) region produced a significant decrease in the rat regucalcin gene promoter activity in H4-II-E cells. These results suggest that the expression of regucalcin mRNA is partly regulated through the NF1-like element.

Hepatic regucalcin promoter activity has been shown to be up-regulated by Bay K 8644, insulin, and PMA (15). The 5′-flanking region of the regucalcin gene, which is regulated by these mediators, is located in −710 to −343 (15). Treatment with Bay K 8644, dibutyryl cAMP, insulin, and PMA caused a significant increase in luciferase activity in H4-II-E cells transfected with the −710/+18 LUC (WT) construct. However, in H4-II-E cells transfected with −710/+18 LUC (mut) construct, the stimulatory effect of Bay K 8644, insulin, and PMA was weakened significantly. These findings may further support the view that the *cis*-acting DNA sequence, which mediates the effect of Bay K 8644, insulin, and PMA in increasing the regucalcin gene promoter activity, is located in the NF1-like sequence (TTGGC) of the regucalcin gene in H4-II-E cells. Meanwhile, treatment with dibutyryl cAMP induced an increase in luciferase activity in H4-II-E cells transfected with either −710/+18 LUC (WT) construct or −710/+18 LUC (mut) construct. This result suggests that II-b (NF1) region was not related to the stimulatory effect of dibutyryl cAMP in H4-II-E cells. In addition, the formation of complex 1 with the binding of nuclear protein to the fragment A was increased by the treatment of Bay K 8644, insulin, and PMA in H4-II-E cells, although treatment of dibutyryl cAMP had no effect on complex 1 formation. Bay K 8644-, insulin-, and PMA-inducible nuclear factors may be involved in the positive regulation of regucalcin gene transcription due to its binding to the NF1-like sequence in H4-II-E cells.

Bay K 8644, a Ca^{2+}-channel agonist, produces the formation of Ca^{2+}/calmodulin complex, and it induces an activation of Ca^{2+}/calmodulin-dependent protein kinases. The expression of regucalcin mRNA is partly mediated through Ca^{2+}/calmodulin-dependent protein kinase in the liver of rats (8, 10). The activation of protein kinase C by PMA can initiate a cascade of events which enhances the action of specific transcription factors (26). 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 some genes including specific AP-1 binding sites which are PMA-responsive elements (27, 28). However, AP-1 is not related to the PMA-induced formation of complex 1, which is seen in the region −710/−343 of the regucalcin gene (15), in H4-II-E cells. NF1-like sequence binding protein may play an important role in the stimulation of regucalcin gene expression in liver cells. Presumably, this binding is modulated by signaling pathways, which are mediated through Ca^{2+}/calmodulin-dependent protein kinase and protein kinase C.

In conclusion, it has been demonstrated that hepatic nuclear protein can specifically bind to the NF1-like sequence of the 5′-flanking region of rat regucalcin gene. The expression of regucalcin gene through this *cis*-acting element in the NF1-like sequence may be stimulated by various signaling factors which are related to protein kinases.

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