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Identification of Transcription Factor in the Promoter Region of Rat Regucalcin Gene: Binding of Nuclear Factor I-A1 to TTGGC Motif

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Abstract Hepatic nuclear protein has been reported to bind specifically to the TTGGC sequence of the rat regucalcin gene promoter region in stimulating the promoter activity (Misawa and Yamaguchi [2000] Biochem. Biophys. Res. Commun. 279: 275–281). The present study was undertaken to identify transcription factor, which binds to TTGGC motif in the rat regucalcin gene promoter, using the yeast one-hybrid system. The sequence between −525 and −504, which has been defined as a functional promoter element II-b, was used as bait to screen a rat liver cDNA library. Two cDNA clones were identified as a nuclear factor I-A1 (NF1-A1). The results of gel mobility shift assay and mutation analysis using recombinant NF1-A1 protein showed that this protein could specifically bind to TTGGC motif of the II-b oligonucleotide in promoter region. The expression of NF1-A1 mRNA was found in the liver, kidney, heart, spleen, and brain of rats. This study demonstrates that NF1-A1 is a transcription factor in stimulating the rat regucalcin gene promoter activity. J. Cell. Biochem. 84: 795–802, 2002. © 2002 Wiley-Liss, Inc.

Key words: regucalcin; nuclear factor I; transcription factor; rat liver

Calcium ion (Ca^{2+}) plays an important role in the regulation of many cell functions. Ca^{2+} signal is partly transmitted to intracellular responses, which are mediated through a family of Ca^{2+}-binding proteins [Wasserman, 1991]. Regucalcin, which is found as a novel Ca^{2+}-binding protein not including the EF-hand motif [Yamaguchi and Yamamoto, 1978; Yamaguchi, 1988; Shimokawa and Yamaguchi, 1993], has been demonstrated to play a role as a regulatory protein for Ca^{2+} signaling in the liver, kidney, and brain of rats [Yamaguchi, 2000a,b in Review].

The rat regucalcin gene is localized on chromosome Xq11.1-12 proximal end [Shimokawa et al., 1995]. The organization of rat regucalcin gene consists of seven exons and six introns [Yamaguchi et al., 1996]. Rat regucalcin mRNA is mainly expressed in the liver and only to a small extent in the kidney as assayed by Northern blot analysis [Shimokawa and Yamaguchi, 1992], suggesting that it is expressed in a highly tissue-specific manner. The expression of hepatic regucalcin mRNA has been shown to be stimulated by various factors; the expression is raised by the administration of $CaCl_2$ [Shimokawa and Yamaguchi, 1992], insulin [Yamaguchi et al., 1995], and 17β-estradiol [Yamaguchi and Oishi, 1995] to rats.

The promoter characterization of the 5′-flanking region in the regulation of the rat regucalcin gene and its transcription by various signaling factors has been shown [Murata and Yamaguchi, 1998, 1999; Yamaguchi and Nakajima, 1999]. Recently, we have determined the nuclear factor binding site, which contains a nuclear factor I (NF1)-like motif TTGGC $(N)_6$CC, in the promoter region of the rat regucalcin gene [Misawa and Yamaguchi,

Abbreviations used: bp, base pair(s); NF1, nuclear factor I; GST, glutathione S-transferase; DIG, digoxigenin; ORF, open reading frame.

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2000a]. The binding of nuclear protein to the TTGGC sequence of regucalcin gene has been shown to be enhanced by intracellular signaling factors, and the enhancement is partly involved in the phosphorylation of hepatic nuclear protein which is mediated through various protein kinases [Misawa and Yamaguchi, 2000b]. Hepatic nuclear factor which binds to the TTGGC sequence of regucalcin gene promoter region, however, remains to be identified.

The present study was undertaken to determine transcription factor which binds to the TTGGC sequence in the promoter region of rat regucalcin gene. We used a yeast one-hybrid system to isolate a clone encoding specific transcription factor from a rat liver cDNA library. We found that the cDNA encoding NF1-A1 was isolated, and that the recombinant NF1-A1 protein could specifically bind to the TTGGC sequence of rat regucalcin gene promoter region.

MATERIALS AND METHODS

Chemicals

Adenosine 5′-[γ-^{32}P]triphosphate ([γ-^{32}P] ATP; 111 TBq/mmol) was purchased from New England Nuclear (Boston, MA). Fetal bovine serum (FBS) was purchased from Sigma (St. Louis, MO). Molecular size standards (0.28~6.6 kb RNA ladder) for electrophoresis of RNA, pGL3-Basic vector and pRL-TK vector were purchased from Promega (Madison, WI). pGEX-4T-1 fusion protein expression vector and glutathione-Sepharose 4B were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). DIG RNA labeling kit was obtained from Roche Molecular Biochemical, Inc. (Indianapolis, IN). Other reagents and chemicals used were of the highest grade of purity commercially available.

Yeast One-Hybrid Screening

A rat liver cDNA/GAL4 activation domain fusion library was purchased from Clontech (MATCHMAKER pACT2 vector). Yeast one-hybrid screening was carried out according to the MATCHMAKER one-hybrid protocol (Clontech). Briefly, five tandem repeats of the −525 to −504 bp sequence (CAGTTTGGCAGGGAGCCTTGAA, II-b) from the rat regucalcin gene [Misawa and Yamaguchi, 2000a] were ligated into yeast integration and reporter vector pHISi or pLacZi to generate pHISi-II-b or pLacZi-II-b, respectively. Each pHISi-II-b and pLacZi-II-b reporter construct was linearized and integrated into the genome of competent yeast YM4271, sequentially. The resulting yeast cells with the integrated pHISi-II-b and pLacZi-II-b were used for one-hybrid screening with a rat liver cDNA library. Positive colonies were selected on SD/-His/-Leu plates with 45 mM 3-amino-1,2,4-triazole (3-AT). To exclude false positive clones, we perfomed the β-galactosidase filter lift assay according to manufacturer's instruction (Clontech). Plasmids were rescued from selected blue yeast colonies, and retransformed into *E. coli* DH5α. Each cDNA insert was sequenced and compared with known sequences in the DDBJ/EMBL/GenBank.

Expression and Purification of Recombinant GST Fusion Protein

A 1,557-bp (64−1620) cDNA containing the coding region of NF1-A1 was generated by PCR using a set of primers with the *Eco* RI site and *Sal* I site for 5′ and 3′, respectively. The DNA fragment was subcloned into the pGEX-4T-1 fusion protein expression vector (Amersham Pharmacia Biotech). The correct orientation, sequence, and in-frame of the fragment in the vector (GST-NF1-A1) were confirmed by direct DNA sequencing. *E. coli* carrying this plasmid was cultured at 30°C, and GST fusion protein was induced by addition of 0.2 mM isopropyl β-D-thiogalactopyranoside for 12 h. Bacteria were sonicated in phosphate-buffered saline containing 5 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride, and the GST fusion proteins were purified using glutathione-Sepharose 4B (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The purified proteins were stocked −80°C.

Oligonucleotides for Gel Mobility Shift Assays

A 22-bp double-stranded oligonucleotide probe corresponding to the promoter region [−525 to −504, termed as II-b, which contains a NF1-like motif TTGGC$(N)_6$CC] of the rat regucalcin gene was synthesized and labeled with T4 polynucleotide kinase and [γ-^{32}P]-ATP. Sequences of oligonucleotides used for competition assay were as follows: II-b; 5′-CAGTTTGGCAGGGAGCCTTGAA-3′, II-b-m1; 5′-CAGTTTGGCAGGGATAATTGAA-3′, II-b-m2; 5′-CAGTTACCCAGGGAGCCTTGAA-3′, and II-a; 5′-AAGCATAGAATGTTCTTTGCCT-3′[Misawa and Yamaguchi, 2000a].

Gel Mobility Shift Assays

Gel mobility shift assays were performed as previously described [Misawa and Yamaguchi, 2000a]. For the competition experiments, pre-incubation was performed in the presence of unlabeled competitor DNA fragment at 100-fold molar excess.

Construction of the Reporter Gene Plasmids

The reporter gene plasmids were generated as previously described [Misawa and Yamaguchi, 2000a]. Briefly, the DNA fragment −710/+18 of the rat regucalcin gene was ligated into the pGL3-Basic promoterless plasmid containing the firefly luciferase gene. This wild-type promoter construct was named −710/+18 LUC (WT). A mutant of −710/+18 LUC (WT) was constructed by site-directed mutagenesis. This mutant plasmid −710/+18 LUC (mut) contains a 3-bp substitution mutation (CAGTTACC-CAGGGAGCCTTGAA) in the region of II-b [Misawa and Yamaguchi, 2000a].

Cell Culture and Transfection

The cloned rat hepatoma H4-II-E cells were cultured as previously described [Misawa and Yamaguchi, 2000b]. 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, a Tfx-20 reagent, according to the manufacturer'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 analysis of regulation of the regucalcin promoter by FBS, the transfected cells were maintained for 24 h in serum-free α-MEM, and then cultured for 24 h in the same medium containing either vehicle or 10% FBS before harvesting. After culture, the transfectants were lysed, and the luciferase activity in the cell lysates was measured by dual-luciferase reporter assay system (Promega).

Northern Blot Analysis

Total RNA extracted from five different tissues of rat (male Wistar strain of 5 weeks old; Japan SLC, Hamamatsu, Japan). Ten micrograms of total RNA was subjected to Northern blotting as described previously [Murata et al., 1997]. Northern blots were probed with DIG-labeled NF1-A1, regucalcin, and G3PDH probe, respectively. The blots were detected with alkaline phosphatase-conjugated anti-DIG antibody and a chemiluminescent substrate according to the manufacturer's instructions (Roche). The size of mRNA was determined using an RNA ladder (Promega).

Sequence Analysis

DNA sequencing was performed in both directions on double-stranded plasmid DNA using an Applied Biosystems 373A automated DNA sequencer and Taq dye terminators.

RESULTS

Isolation of Transcription Factors That Interact With the II-b Region in the Rat Regucalcin Gene Promoter

Screening of 1×10^6 clones from rat liver cDNA library using a yeast one-hybrid system generated 15 positive clones. These candidate clones were partially sequenced and analyzed by a BLAST search of the DDBJ/EMBL/GenBank database. Two of the clones were highly identical to a NF1 gene [Xu et al., 1997]. The third clones did not have a significant homology for a known sequence as found in the DDBJ/EMBL/GenBank. Other twelve clones were highly identical to be already known sequences which were not characterized as nuclear transcription factor. Our focus was directed first to NF1.

Sequence Analysis and Characterization of a NF1 Gene

Two of the positive clones for NF1 contained 3,150- and 2,650-bp inserts, respectively. The nucleotide sequence of these clones contained an open reading frame (ORF) of 1,527 bp with the first ATG surrounded by an appropriate Kozak consensus sequence [Kozak, 1987], as shown in Figure 1. The shorter clone was 3′-untranslated region as a truncated version of the longer one. The predicted ORF encoded a protein of 509 amino acid residues with a calculated molecular mass of 55,975 Da. From the result with BLAST search of this entire sequence, our NF1 contained an identical ORF to the cDNAs previously isolated as rat NFI-A1

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   1: cacactcacgcacacctccagcccgcacacagacgtgcacgcaccccccgccgcccggcagttatgtattctccgctctgtctcacccaggatgagtttcatcctttcattgaagcactt
   1:                                                                 M  Y  S  P  L  C  L  T  Q  D  E  F  H  P  F  I  E  A  L

 121: ctgccccatgtccgcgccttcgcctacacatggttcaacctgcaggcccgaaagcggaaatacttcaaaaagcatgagaagcgcatgtcgaaagaagaggagagggctgtgaaggatgaa
  20: L  P  H  V  R  A  F  A  Y  T  W  F  N  L  Q  A  R  K  R  K  Y  F  K  K  H  E  K  R  M  S  K  E  E  E  R  A  V  K  D  E

 241: ctgctaagtgagaagcctgaggtcaagcaaaagtgggcttcccgacttctggccaagttacggaaagatatccgacccgagtaccgagaggattttgttcttacagttacagggaaaaaa
  60: L  L  S  E  K  P  E  V  K  Q  K  W  A  S  R  L  L  A  K  L  R  K  D  I  R  P  E  Y  R  E  D  F  V  L  T  V  T  G  K  K

 361: cctccatgctgtgttctttccaacccagaccagaaaggcaagatgcggagaattgactgcctccgccaggcagataaagtatggaggttggacctcgtcatggtgatcttgttcaaaggt
 100: P  P  C  C  V  L  S  N  P  D  Q  K  G  K  M  R  R  I  D  C  L  R  Q  A  D  K  V  W  R  L  D  L  V  M  V  I  L  F  K  G

 481: attccgctggaaagtactgatggtgaacgccttgtgaagtccccacagtgctcgaatccagggctctgtgtccagccccatcacataggggtttctgtaaaggaactcgatttatatttg
 140: I  P  L  E  S  T  D  G  E  R  L  V  K  S  P  Q  C  S  N  P  G  L  C  V  Q  P  H  H  I  G  V  S  V  K  E  L  D  L  Y  L

 601: gcatactttgtacatgcagcagattcaagtcaatctgaaagtcccagccaaccaagtgatgctgacattaaggaccagccagaaaatggacatttgggcttccaggacagcttcgtcaca
 180: A  Y  F  V  H  A  A  D  S  S  Q  S  E  S  P  S  Q  P  S  D  A  D  I  K  D  Q  P  E  N  G  H  L  G  F  Q  D  S  F  V  T

 721: tcaggtgttttcagtgtgactgagctagtaagagtgtcacaaacaccaatagctgcaggaaccggccccaatttttctctctctgatttggaaagttcctcatactacagcatgagtcca
 220: S  G  V  F  S  V  T  E  L  V  R  V  S  Q  T  P  I  A  A  G  T  G  P  N  F  S  L  S  D  L  E  S  S  S  Y  Y  S  M  S  P

 841: ggagcaatgaggaggtctctacccagcacatcctctaccagctctaccaagcgcctcaagtctgtggaggacgaaatggacagtcctggtgaagaaccattttacacaggccaagggcgc
 260: G  A  M  R  R  S  L  P  S  T  S  S  T  S  S  T  K  R  L  K  S  V  E  D  E  M  D  S  P  G  E  E  P  F  Y  T  G  Q  G  R

 961: tccccagggagtggcagccagtccagcggatggcatgaagtagagccaggcatgccatctccaaccactctgaagaagtctgagaagtctggtttcagcagcccctccccttcgcagacc
 300: S  P  G  S  G  S  Q  S  S  G  W  H  E  V  E  P  G  M  P  S  P  T  T  L  K  K  S  E  K  S  G  F  S  S  P  S  P  S  Q  T

1081: tcctccctcggaacagcattcacacagcatcaccgacctgtcataacaggaccgagagcaagcccacatgcgacgccatcgactctccactttccaacgtcacctatcatccagcagcct
 340: S  S  L  G  T  A  F  T  Q  H  H  R  P  V  I  T  G  P  R  A  S  P  H  A  T  P  S  T  L  H  F  P  T  S  P  I  I  Q  Q  P

1201: gggccttatttctcacacccagccatccgttaccaccctcaggagacgctgaaggagtttgtccaacttgtctgccccgatgctggtcagcaagctggacaggtggggttcctcaatccc
 380: G  P  Y  F  S  H  P  A  I  R  Y  H  P  Q  E  T  L  K  E  F  V  Q  L  V  C  P  D  A  G  Q  Q  A  G  Q  V  G  F  L  N  P

1321: aatggaagcagtcaaggcaaggtgcacaacccattcctccccaccccaatgttgccgccgccaccgccaccaccgatggccaggcctgtgcctctgcccatgccagacaccaagcctcca
 420: N  G  S  S  Q  G  K  V  H  N  P  F  L  P  T  P  M  L  P  P  P  P  P  P  P  M  A  R  P  V  P  L  P  M  P  D  T  K  P  P

1441: accacatcaacagaaggcggtgcagcctcccccacctcaccgacctactcgacacccagcacctcccccgcaaaccgattcgtcagtgttggaccacgggatccaagctttgtaaatatc
 460: T  T  S  T  E  G  G  A  A  S  P  T  S  P  T  Y  S  T  P  S  T  S  P  A  N  R  F  V  S  V  G  P  R  D  P  S  F  V  N  I

1561: cctcaacagacacagtcctggtacctgggataaaagttgcagcgtcccaccatcctccagacagaccacctgaccccttctcaactctgtaacatggacgcaacctcaacccggcgcagt
 500: P  Q  Q  T  Q  S  W  Y  L  G  *

1681: tacaacttcactgtcagtggaaggggagagacaccaactcgaatcaacttgtacatggaaacagcgaacgtcgtggtcaaacagcaaaggccataacttttcgggattctttttttttt
1801: ttaatactttagggactgtcgtaatttctcatatggtgctggaaatggttgggctgtgtgacatttgaagtgttccagtggtggtgtgagcagtaggtgaagcggctggtaggggatgag
1921: ccttgctcacgaacttcctgttgtaacacttgtgagcctggccttttcacagtatttcaagaatttgcccgcgcaggtgtgtgaccctcccggagcacttggagacacctggagaatgaa
2041: atctttcgtagtagctaagatctgtgatctgtaacttacaggacactcaaagggcaatgtttttgggtttcttttttttctgtaacatattagaaaagcaaatgcagttaagttgat
2161: ctttcttttagtttggtttggttcagcagtcagcagttaaatacataacacaaccctcaaggacagtgaacccactcatgttgcagagcaaatccaaaaagacagcaaagactactacta
2281: ctactctcagttcggttgtttgttttttttttttttgtttgtttttttattaagttttgaaatgctgcacttacattttaaaaaaaatttttcaacaatttcaacaatgacacaaaa
2401: gttcacatggaaatggggaagatggtctgttttgacagaaactgacaggaatcaaaacaatcgaattttgaattgagtgaagtgcaatttcattggatagctaagtatctttgtaagata
2521: gagattgttgaaaattctattttgtttttcaagtcctcccaccccaggactctaaattattggggttaaaaaaaaacagccttgcaagaaaaaggggagctattttttgctttttatgtt
2641: ttttattgttaaacttgtatccctttaaaaaaaaaaaccgaaggaaaatttaaaaaaaaacaaaaaaaaaaaatacaaatctaatggtgcttttaccacaatatgttaactacattaaat
2761: gctaattaattattttctgttatcaaagcacataactcaaatgaaatcatggtatctgttaattttataagctagaaatcactataatgaattatgccaattctggaaaattttacacat
2881: gtatggcaacaccagatctatcggttttcaaacgcctcgtcgtaaccagacattgtccagaagtttcaaagacccttcgcaaaccctgaactgggctacggggaaataatactaagactg
3001: ataaggacataactgtgtgagcccattcagatcaaagtacttgttagggaaaataaacaaacaaacaaaaacaaaatgttcacacacacacacacccccaaatgtcttgtatcttatgtt
3121: taaggaaaaaagaaaagacaaaaaaaaaaa
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Fig. 1. The nucleotide and deduced amino acid sequences of the rat NF1-A1. The ATG translational start site is highlighted in black. The asterisk indicates the stop codon. The DDBJ/EMBL/GenBank accession number for this sequence is AB060652.

(D78017) [Xu et al., 1997] or rat NF1L21 (X84209) [Monaci et al., 1995]. Rat NF1-A mRNA is classified into four alternative splicing isoforms, and the mRNA has been well characterized [Xu et al., 1997; Imagawa et al., 2000]. From this, our clone for NF1 was termed as NF1-A1.

Tissue Distribution of the NF1-A1 mRNA

Figure 2 shows the results of the Northern blot analysis for the NF1-A1 mRNA. Total RNAs obtained from several rat tissues (including liver, kidney, heart, spleen, and brain) were hybridized with DIG-labeled NF1-A1 probe (panel A). Multiple RNA species were present in a characteristic combination and relative abundance in the tissues. The expression of regucalcin mRNA was detected as double bands of approximately 1.8 and 1.6 kb in the liver and kidney as reported previously [Shimokawa and Yamaguchi, 1992] (panel B). The expression of G3PDH mRNA was found in the used all tissues (panel C).

Binding of a Recombinant GST-NF1-A1 Protein to TTGGC Sequence in the II-b Oligonucleotide

To determine whether the cloned NF1-A1 can directly bind to II-b oligonucleotide, we generated a recombinant NF1-A1 as fusion protein with GST and subjected to gel mobility shift

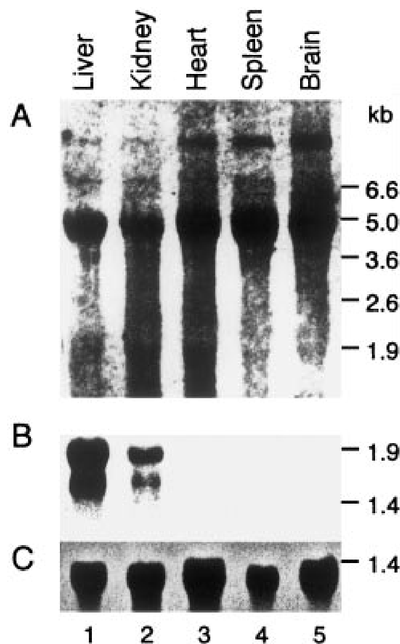

Fig. 2. Northern blot analysis of NF1-A1 mRNA expression in rat tissues. The total RNAs (10 μg) extracted from rat tissues were subjected to Northern blot analysis. Blots were hybridized to the probes for NF1-A1 (**A**), regucalcin (**B**), and G3PDH (**C**) mRNAs, respectively. The positions of the markers are indicated alongside the RNA lanes. **Lane 1**, liver; **lane 2**, kidney; **lane 3**, heart; **lane 4**, spleen; **lane 5**, brain.

assay. As shown in Figure 3A, GST-NF1-A1 was bound to radiolabeled II-b oligonucleotide in a dose-dependent manner, whereas the control GST protein was not bound to II-b.

We reported previously an existence of rat liver nuclear protein, which was specifically bound to the TTGGC sequence in the II-b oligonucleotide by competition gel mobility shift assay using II-b mutant oligonucleotides [Misawa and Yamaguchi, 2000a]. To determine the binding region of GST-NF1-A1 the radiolabeled II-b oligonucleotide was incubated with the GST-NF1-A1 and unlabeled II-b mutant oligonucleotides (Fig. 3B). The presence of 100-fold molar excess of the unlabeled II-b prevented completely the formation of the indicated complex (lane 7). The prevention was not seen by using nonspecific oligonucleotide (lane 10). Moreover, two II-b mutant oligonucleotides, which contain three different bp substitutions in the NF1 consensus motif, were used as competitors for gel mobility shift assay. The mutant oligonucleotide II-b-m1 inhibited the binding of GST-NF1-A1 to radiolabeled II-b (lane 9). The II-b-m2 did not compete (lane 8). Similar result was observed by using the nuclear extracts obtained from rat liver (lanes 1–5). GST-NF1-A1 protein could specifically recognize TTGGC in the sequence of II-b.

Involvement of TTGGC Sequence in the Regucalcin Gene Expression in H4-II-E Cells

Whether the endogenous protein in the liver nuclei binds to TTGGC sequence and has a role in the stimulation of regucalcin gene expression was examined. The reporter genes, which ligated the promoter region including either TTGGC (WT) or T<u>ACC</u>C (mut) with 3-bp substitution mutation of TTGGC in rat regucalcin gene, were transiently transfected into H4-II-E cells to assay luciferase activity. Cell culture with FBS (10%) caused a significant increase in luciferase activity in the cells transfected with the −710/+18 LUC (WT) construct (Fig. 4A; FBS showed 6.26 ± 0.39-fold, as compared with Basic LUC, respectively). However, the effect of FBS in increasing luciferase activity was not seen in the cells with the −710/+18 LUC (mut) construct (Fig. 4B).

DISCUSSION

The nuclear proteins obtained from rat liver and cloned rat H4-II-E hepatoma cells have been demonstrated to bind specifically to TTGGC sequence in the NF1-like motif [TTGGC$(N)_6$CC] in the rat regucalcin gene promoter region [Misawa and Yamaguchi, 2000a,b]. The present study was undertaken to identify transcription factor which binds to TTGGC sequence. We isolated the cDNA which encoded a NF1-A1 from a rat liver cDNA library using a yeast one-hybrid system.

Nuclear factor 1 (NF1) is originally identified as a DNA-binding protein that stimulates the replication of adenovirus DNA in HeLa cells [Nagata et al., 1982]. NF1-like elements containing the highly conserved TGG motif have been found in the promoters of many genes. NF1 plays a role as an enhancer for gene expression. A number of NF1 factors have been found as transcriptional activator in the gene expression of rat CYP1A2 [Zhang et al., 2000], human p53 [Furlong et al., 1996], rat albumin [Paonessa et al., 1988], human brain fatty acid-binding protein [Bisgrove et al., 2000], and human and mouse 5HT3 serotonin receptor [Bedford et al., 1998]. Meanwhile, NF1 factors are also found as negative regulators in the promoter activity of

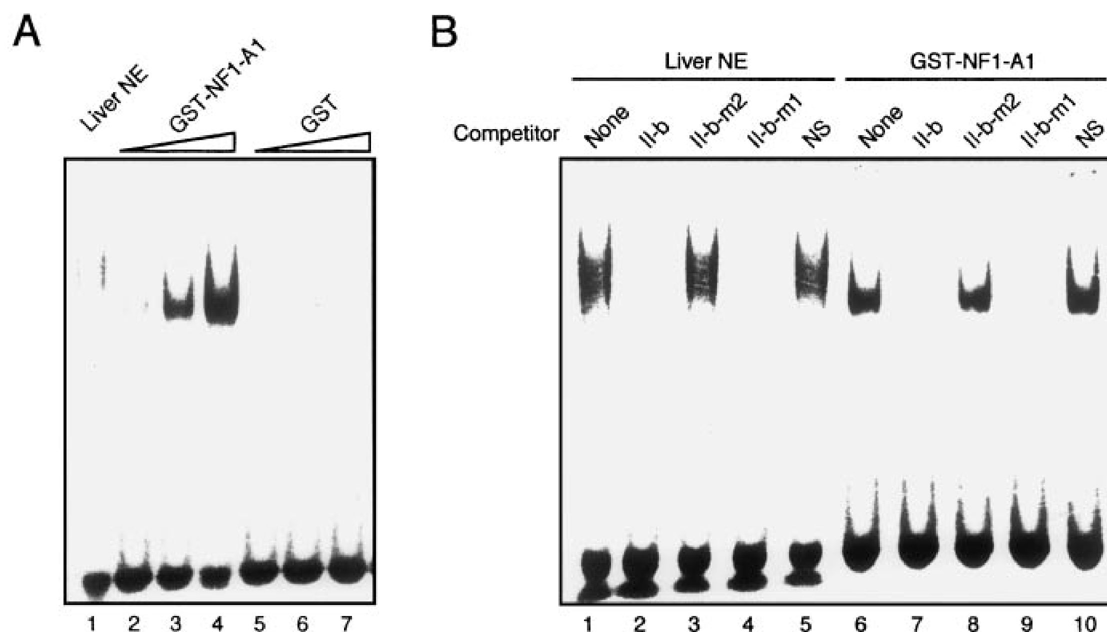

Fig. 3. Binding of the GST-NF1-A1 to TTGGC sequence of the II-b oligonucleotide. Gel mobility shift assays were performed using rat liver nuclear extract, recombinant GST-NF1-A1, and GST protein with a radiolabeled II-b oligonucleotide as a probe. Competition assays were performed in the presence of 100-fold molar excess of the double stranded oligonucleotides. **A**: Binding profile of rat liver nuclear extract (3 μg of protein, **lane 1**), GST-NF1-A1 (1, 2, and 4 ng of protein, **lanes 2–4**), and GST (1, 2, and 4 ng of protein, **lanes 5–7**) to II-b. **B**: Competition assay with oligonucleotide II-b and its mutants using rat liver nuclear extract (3 μg of protein, **lanes 1–5**) and GST-NF1-A1 (1 ng of protein, **lanes 6–10**). Lanes 1 and 6, no competitor; lanes 2 and 7, II-b as competitor; lanes 3 and 8, II-b-m2; lanes 4 and 9, II-b-m1; lanes 5 and 10, II-a (a 22-bp regucalcin gene fragment in Reference of Misawa and Yamaguchi, 2000a) as nonspecific competitor.

rat GST-P [Osada et al., 1997], mouse hepatocyte growth factor [Jiang et al., 2000], and growth arrest and DNA damage inducible gene 153 (gadd153) [Nakamura et al., 2001]. NF1s may constitute a family consisting of at least four genes, i.e., NF1-A, NF1-B, NF1-C, and NF1-X [Rupp et al., 1990; Fletcher et al., 1999]. In addition, each subtype possesses different isoforms which are generated by alternative splicing [Kruse and Sippel, 1994; Osada et al., 1997]. The rat NF1-A gene consists of four splicing isoforms (NF1-A1, NF1-A2, NF1-A3, and NF1-A4) [Xu et al., 1997]. NF1-A1 has a complete ORF of NF1-A. On the other hand, NF1-A2, NF1-A3, and NF1-A4 lacked some exons. Our NF1-A1 from rat liver had a complete ORF.

To clarify whether a cloned NF1-A1 is the TTGGC sequence-binding protein, the isolated recombinant GST-fusion protein of NF1-A1 was incubated with the radiolabeled TTGGC oligonucleotide. The results of gel mobility shift assay and competition assay showed that GST-NF1-A1 could specifically bind to TTGGC element of the regucalcin gene promoter. This finding demonstrates that NF1-A1 is a TTGGC-binding protein in the promoter region of rat regucalcin gene. Moreover, the result with mutation analysis for the luciferase reporter assay showed that TTGGC sequence was necessary for the stimulation of regucalcin gene promoter activity in H4-II-E cells (Fig. 4). From this result, it is assumed that endogenous NF1-A1 in the liver nucleus may play a role as an enhancer in the regulation of regucalcin gene promoter activity.

The expression of NF1-A1 mRNA in the various tissues of rats was examined by Northern blot analysis. NF1 has been reported as a ubiquitous gene. However, the multiple transcripts of NF1 gene have been shown in a characteristic combination and a relative abundance in each tissue [Paonessa et al., 1988; Osada et al., 1999]. Our result showed that some NF1-A1 mRNA species were present and that their expression pattern was different in the various tissues of rats. Regucalcin mRNA was mainly expressed in liver and kidney [Shimokawa and Yamaguchi, 1992]. Also, regucalcin mRNA was expressed only slightly in the heart and brain neuron of rats [Yamaguchi et al., 1999, 2000].

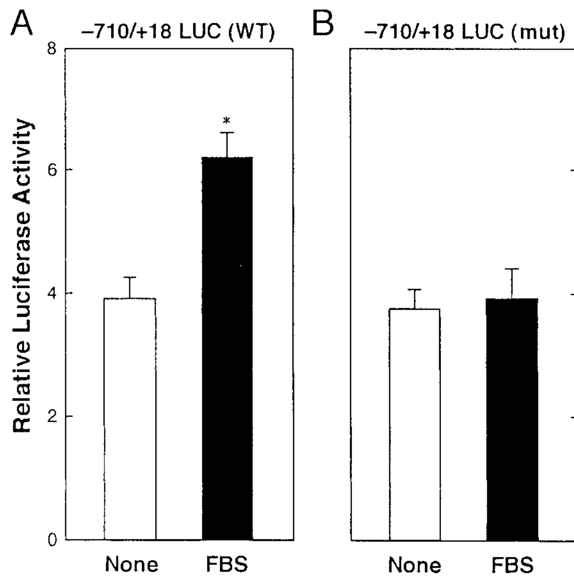

Fig. 4. Effect of FBS on promoter activities from −710/+18 LUC (WT) or (mut) in H4-II-E cells. A DNA fragment −710/+18 was ligated into the pGL3-Basic promoterless plasmid (Basic LUC). A mutant reporter gene plasmid was generated by site-directed mutagenesis as described under Materials and Methods. H4-II-E cells were transiently co-transfected with test plasmid and pRL-TK internal control plasmid, and cultured for 24 h in a serum-free medium. Then the cells were incubated for 24 h in a medium containing either vehicle or 10% FBS. 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 a fold-stimulation in comparison with the luciferase activity measured after transfection with Basic LUC, which was set as 1.0. Each value is the mean ± SEM of three separate experiments. **A**: Wild type −710/+18 LUC. **B**: Mutant −710/+18 LUC. $^*P < 0.01$, compared with the control value without stimulation.

The expression of NF1-A1 mRNA was seen in the liver, kidney, heart, spleen, and brain of rats. This suggests that NF1-A1 is related to the tissue-specific expression of regucalcin mRNA in the liver and kidney. It has been reported that calcium administration stimulates the binding of AP-1 to the region −710/−575 in the rat regucalcin gene, suggesting that this factor participates in the regulation of regucalcin gene expression [Murata and Yamaguchi, 1998]. Presumably, a multi-transcription factor is related to the tissue specific expression of the regucalcin gene in rats.

In conclusion, it has been demonstrated that NF1-A1 can specifically bind to the TTGGC sequence in the promoter region of rat regucalcin gene. NF1-A1 may be a transcription factor for the enhancement of regucalcin gene promoter activity.

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