Biochemical and Biophysical Research Communications **279,** 275–281 (2000)
doi:10.1006/bbrc.2000.3934, available online at http://www.idealibrary.com on IDEAL®

Intracellular Signaling Factors—Enhanced Hepatic Nuclear Protein Binding to TTGGC Sequence in the Rat Regucalcin Gene Promoter: Involvement of Protein Phosphorylation

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Received November 4, 2000

The transcriptional mechanism of regucalcin gene expression was determined using gel mobility shift assay with TTGGC oligonucleotide (II-b) which is located between position −523 and −506 in the promoter region, containing a nuclear factor I (NF1) consensus motif TTGGC(N)$_6$CC. The mutation analysis in this motif showed that TTGGC sequence was a specific binding region of the nuclear protein in rat liver and the cloned rat hepatoma cells (H4-II-E). When liver nuclei were incubated with ATP (1 mM), the nuclear protein binding to TTGGC sequence was increased. This binding was also increased in the nuclei of H4-II-E cells cultured with 10% FBS. Such an increase was also seen by culture with vanadate (100 μM), a potent inhibitor of protein tyrosine phosphatase. Serum-enhanced nuclear protein binding to TTGGC sequence was decreased in the presence of TFP (10 μM), staurosporine (100 nM), genistein (10 μM), PD98059 (10 μM), or wortmannin (10 nM), which are inhibitors of various protein kinases. Treatment of a monoclonal phosphotyrosine antibody (4G10) caused an alteration in the TTGGC oligonucleotide–nuclear protein complex formation, indicating that tyrosine phosphorylation of nuclear protein is partly involved in the binding to TTGGC sequence. Moreover, when H4-II-E cells were cultured with FBS (10%), Bay K 8644 (5 μM), PMA (1 μM), or insulin (20 nM), the protein binding to TTGGC sequence in the nuclei was increased, while it was reduced in the cytoplasm, indicating a nuclear localization of the TTGGC sequence-binding protein. This study demonstrates that hepatic nuclear protein can specifically bind to the TTGGC sequence in rat regucalcin gene promoter region, and that this binding is enhanced by intracellular signaling factors which are partly mediated through protein phosphorylation.

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

Calcium ion (Ca^{2+}) plays a critical role in the regulation of many cell functions. The Ca^{2+} signals are transmitted to intracellular responses partly via a family of Ca^{2+}-binding proteins (1). A Ca^{2+}-binding protein regucalcin, which is mainly distributed in the cytoplasm of liver and kidney cells, has been demonstrated to regulate the Ca^{2+}-dependent signaling system; the protein inhibits Ca^{2+}/calmodulin-dependent protein kinase, protein kinase C, protein phosphatase, and Ca^{2+}-activated DNA fragmentation (2–5). Regucalcin may play a role as an inhibitory protein in the process of signal transduction in cells.

The rat regucalcin gene is localized on chromosome Xq11.1-12 proximal end (6), and it has been demonstrated in human, mouse, bovine, monkey, dog, rabbit, and chicken but not yeast (7). Comparison of the nucleotide sequences of regucalcin from vertebrate species was highly conserved in their coding region, and they were greatly conserved throughout evolution (8–12). 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 (13).

The expression of hepatic regucalcin mRNA is stimulated by various physiologic and pathophysiologic factors (14, 15). Regucalcin mRNA level in the liver has been shown to be raised markedly by the administration of calcium chloride to rats; the expression may be partly mediated through Ca^{2+}/calmodulin-dependent pathway (16–18). Regucalcin mRNA expression has been stimulated by the addition of fetal bovine serum

Abbreviations used: bp, base pair(s); NF1, nuclear factor I; PMA, phorbol 12-myristate 13-acetate; TFP, trifluoperazine; FBS, fetal bovine serum.

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0006-291X/00 $35.00

(unknown) or Bay K 8644, an antagonist of Ca^{2+} influx, in the rat H4-II-E hepatoma cells (19). The promoter characterization of the 5′-flanking region of the rat regucalcin gene and its transcriptional regulation by various signaling factors has been shown (18, 20, 21). More 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 (22). However, the cellular signaling mechanism for the transcriptional regulation of regucalcin gene expression has not been clarified.

The present study was undertaken to determine the signaling mechanism by which the nuclear protein binds to a NF1-like motif TTGGC$(N)_6$CC in the promoter region of the rat regucalcin gene, using the gel mobility shift assay with TTGGC oligonucleotide as a probe. We found that the binding of nuclear protein to the TTGGC sequence of regucalcin gene is enhanced by intracellular signaling factors, and that this enhancement is partly involved in phosphorylation of hepatic nuclear protein which is mediated through various protein kinases.

MATERIALS AND METHODS

Chemicals. Fetal bovine serum (FBS), insulin, phorbol 12-myristate 13-acetate (PMA), trifluoperazine (TFP), genistein, and sodium orthovanadate were purchased from Sigma (St. Louis, MO). S(−)-Bay K 8644 was obtained from Research Biochemicals International (Natick, MA). Staurosporine, okadaic acid, PD98059, and wortmannin were obtained from Wako Pure Chemical Co. (Osaka, Japan). 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 removed after perfusion with ice-cold 0.25 M sucrose solution and minced for the preparation of nucleus.

Oligonucleotides for gel mobility shift assays. A 22-bp double-stranded oligonucleotide probe corresponding to the promoter region [−525 to −504, termed as TTGGC sequence (II-b)] 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′ (22).

Construction of the reporter gene plasmids. The reporter gene plasmids were generated as previously described (22). 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 (CAGTTACCCAGGGAGCCTTGAA) in the region of II-b (22).

Cell culture and transfection. The cloned rat hepatoma H4-II-E cells were cultured as previously described (21). 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 signaling factors, 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 in the absence or presence of 50 μM vanadate 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).

Preparation of nuclear extracts. The nuclei were prepared from the rat liver as previously described (18). For analysis of regulation by signaling factors, the nuclei were incubated for 30 min at 37°C in buffer A (40 mM Hepes, pH 7.4, 10 mM $MgCl_2$, and 3 mM $MnCl_2$) without or with 10 μM TFP, 10 μM genistein, 100 nM staurosporine, 50 nM okadaic acid, 100 μM vanadate or 1 mM ATP. Meanwhile, H4-II-E cells were grown on 35-mm dishes to approximately 70% confluence and washed once with serum-free α-MEM. The cells were maintained for 24 h in serum-free α-MEM, and then cultured for 16 h in the same medium containing either vehicle or 10% FBS in the absence or presence of signaling factors before harvesting. Nuclear extracts were prepared from rat liver (18) and H4-II-E cell (21) as previously described. Nuclear extracts were divided into aliquots, and stored at −80°C until use. Protein concentration was determined by the method of Bradford (23), using a kit from Bio-Rad and BSA as a standard.

Preparation of cytosolic fractions from H4-II-E cells. The cytosolic fraction from H4-II-E cells was obtained from the supernatant of the nuclear preparation (21). Briefly, the cells were homogenized, and the nuclei were separated by centrifugation at 1000*g* for 5 min. The 1000*g* supernatants were further centrifuged at 15,000*g* for 15 min. Resulting supernatants were used as cytosolic fractions.

Gel mobility shift assays. Gel mobility shift assays were performed as previously described (21). For the competition experiments, preincubation was performed in the presence of unlabeled competitor DNA fragment at 100-fold molar excess. For analysis of the effect of signaling factors on the nuclear protein binding, the reaction mixtures were contained the factors. For antibody supershift experiments, the nuclear extracts were preincubated with either nonimmune rabbit IgG or monoclonal phosphotyrosine antibody (4G10).

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

The binding region of liver nuclear factor to the fragment A of regucalcin gene. We found previously nuclear proteins, which specifically bind to the promoter region [−525 to −504; a NF1-like motif TTGGC$(N)_6$CC] of rat regucalcin gene by competition gel mobility shift assay using fragment A (330 bp; −709 to −380) as a probe (22). This binding protein recognized TTGGC in the NF1 con-

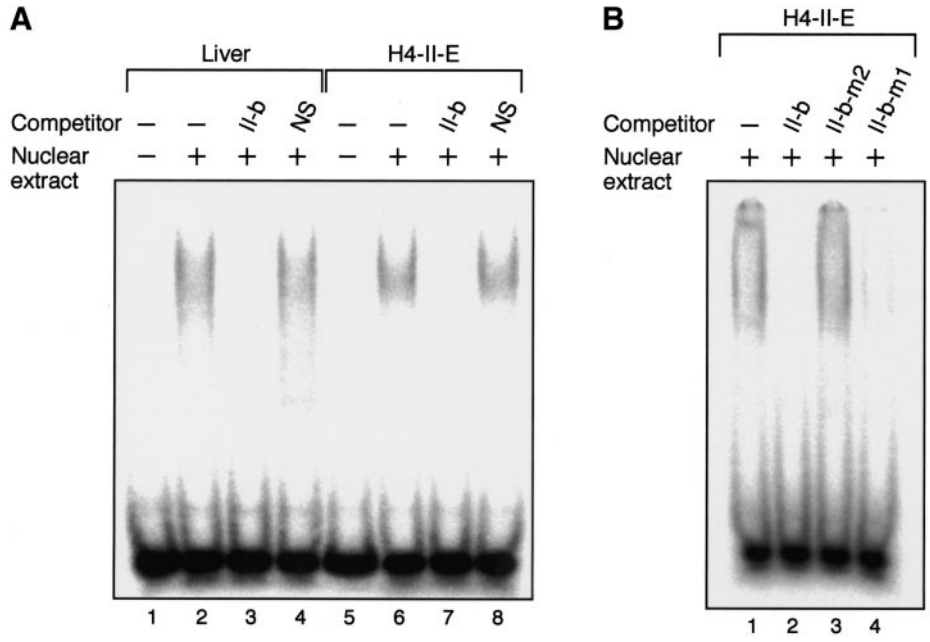

FIG. 1. Gel mobility shift analysis of nuclear factors bound to TTGGC oligonucleotide (II-b) of the rat regucalcin gene promoter. Gel mobility shift assays were performed using nuclear extracts (3 μg of protein) from rat liver and H4-II-E cells, and a ^{32}P-labeled II-b as a probe. Competition assays were performed in the presence of 100-fold molar excess of the double-stranded oligonucleotide. (A) Binding profile of nuclear proteins from rat liver (lanes 1–4) and H4-II-E cells (lanes 5–8) to II-b. Lanes 1 and 5, no extracts; lanes 2 and 6, no competitor; lanes 3 and 7, II-b as competitor; lanes 4 and 8, II-a (a 22-bp regucalcin gene fragment in Ref. 22) as nonspecific competitor. (B) Competition assay with oligonucleotide II-b and its mutant using nuclear extracts from H4-II-E cells. Lane 1, no competitor; lane 2, II-b as competitor; lane 3, II-b-m2; lane 4, II-b-m1. The figure shows representative results of four separate experiments.

sensus sequence of promoter region (22). To determine the binding region of nuclear protein, furthermore, radiolabeled TTGGC oligonucleotide (II-b) was incubated with the nuclear extract from rat liver. The result of gel mobility shift assay showed a single major band which was shifted upwards from the free DNA probe (Fig. 1A, lane 2). The presence of 100-fold molar excess of unlabeled II-b prevented completely the formation of the indicated complex (Fig. 1A, lane 3), but this prevention was not seen using nonspecific oligonucleotide (Fig. 1A, lane 4). Similar result was observed using the nuclear extracts obtained from the cloned rat hepatoma H4-II-E cells (Fig. 1A, lanes 5–8). Moreover, two II-b mutant oligonucleotides, which contain three different bp substitutions in the NF1 consensus sequence, were used as competitors for gel mobility shift assay. In H4-II-E cells, II-b and II-b-m1 inhibited the binding of nuclear factor to radiolabeled II-b (Fig. 1B). However, II-b-m2 did not compete. Thus, we found that the nuclear binding protein recognizes TTGGC in the NF1 consensus sequence of II-b.

To determine whether the binding of nuclear factor to the TTGGC sequence of rat regucalcin gene promoter region is altered by various inhibitors for protein kinases or protein phosphatases, the isolated rat liver nuclei were incubated with TFP (10 μM), genistein (10 μM), staurosporine (100 nM), okadaic acid (50 nM) or vanadate (100 μM). Nuclear extracts prepared from the nuclei were used for gel mobility shift assays (Fig. 2). The formation of the protein–oligonucleotide complex with the binding of nuclear extracts to TTGGC sequence was enhanced by the treatment of vanadate, a potent inhibitor of protein tyrosine phosphatase (Fig. 2, lane 7). Treatment with TFP, genistein, or staurosporine, which is an inhibitor of protein kinases, did not cause an appreciable alteration in the formation of the complex. However, when liver nuclei were incubated with ATP (1 mM), as the substrate of protein kinases, the nuclear factor binding to TTGGC sequence was markedly increased (Fig. 2, lane 8), indicating an involvement of protein phosphorylation.

Effects of serum stimulation on the nuclear protein binding to TTGGC sequence in H4-II-E cells. H4-II-E cells were cultured in a medium containing either vehicle or 10% FBS in the absence or presence of vanadate (100 μM). The nuclear extracts obtained from H4-II-E cells were incubated with the radiolabeled TTGGC oligonucleotide (II-b). The formation of the nuclear protein-TTGGC oligonucleotide binding complex was increased by the treatment of vanadate or FBS (Fig. 3, lanes 3 or 5). The synergistic effect with vanadate and FBS was not observed (Fig. 3, lane 7). Moreover, a direct addition of vanadate in the gel shift binding mixture had no effect on complex formation (Fig. 3, lanes 2, 4, 6, and 8). Thus, treatment with FBS in H4-II-E cells enhanced the binding of nuclear pro-

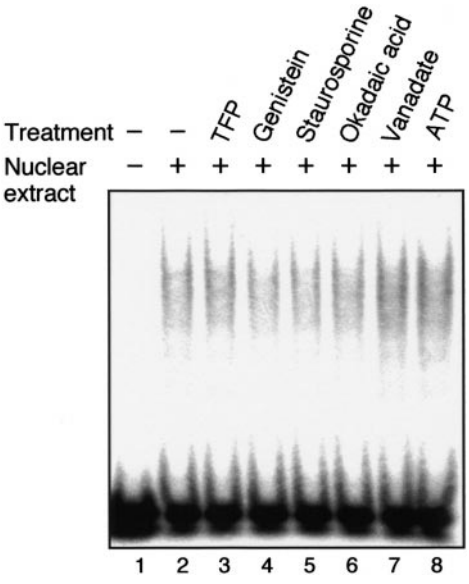

FIG. 2. Binding of the nuclear proteins obtained from control or inhibitor-treated rat liver nuclei to TTGGC sequence of the rat regucalcin gene promoter. Nuclear extracts (3 μg of protein) from rat liver nuclei treated with or without various inhibitors were incubated with ^{32}P-labeled TTGGC oligonucleotide (II-b) as a probe. Lane 1, no extracts; lane 2, control; lane 3, 10 μM TFP; lane 4, 10 μM genistein; lane 5, 100 nM staurosporine; lane 6, 50 nM okadaic acid; lane 7, 100 μM vanadate; lane 8, 1 mM ATP. The figure shows representative results of four separate experiments.

tein to TTGGC sequence of the regucalcin gene promoter region.

The effect of various signaling inhibitors on the serum-enhanced nuclear protein binding to TTGGC sequence is shown in Fig. 4. Gel mobility shift assays were performed using the nuclear extracts from H4-II-E cells. The effect of FBS in increasing nuclear protein binding was reduced by TFP (10 μM), staurosporine (100 nM), genistein (10 μM), PD98059 (10 μM), or wortmannin (10 nM). This result indicates that the effect of serum stimulation is partly mediated through signaling pathway for various protein kinases.

Whether protein tyrosine phosphorylation was involved in the serum-enhanced formation of the nuclear protein-TTGGC sequence complex was determined using gel mobility shift assay with 4G10, a monoclonal phosphotyrosine antibody (Fig. 5). The effect of serum in increasing the formation of nuclear protein-binding TTGGC sequence (lane 1) was altered in the presence of 4G10. This effect was dose-dependent. IgG alone had no effect (lane 6). The result indicates that the tyrosine phosphorylation of nuclear protein is partly involved in the complex formation of nuclear protein-binding TTGGC sequence.

Moreover, whether serum or vanadate induces an increase in the regucalcin gene expression was examined (Fig. 6). Reporter genes which ligated the promoter region of rat regucalcin gene were transiently transfected into H4-II-E cells to assay luciferase activity. The cell culture with vanadate (50 μM) or FBS (10%) caused a significant increase in luciferase activity in the cells transfected with the −710/+18 LUC (WT) construct (Fig. 6A; vanadate or FBS showed 5.67 ± 0.26- or 6.52 ± 0.25-fold, as compared with Basic LUC, respectively). The effect of vanadate or FBS in increasing luciferase activity was not seen in the cells with the −710/+18 LUC (mut) construct (Fig. 6B).

Intracellular localization of TTGGC sequence-binding protein. The intracellular localization of TTGGC sequence-binding protein following the stimulation of various signaling factors in H4-II-E cells was determined. The cells were cultured in a medium containing either vehicle, FBS (10%), Bay K 8644 (5 μM), insulin (20 nM), or PMA (1 μM). The nuclear extracts were incubated with radiolabeled TTGGC oligonucleotide (II-b). The binding of protein to TTGGC sequence in the nuclei was raised by the treatment of FBS, Bay K 8644, insulin or PMA (Fig. 7, lanes 1–5). Meanwhile, TTGGC sequence-binding protein in the cytosol was reduced by the treatment of all signaling factors used (Fig. 7, lanes 6–10).

DISCUSSION

The previous study demonstrated that the specific DNA sequence in the promoter region of regucalcin

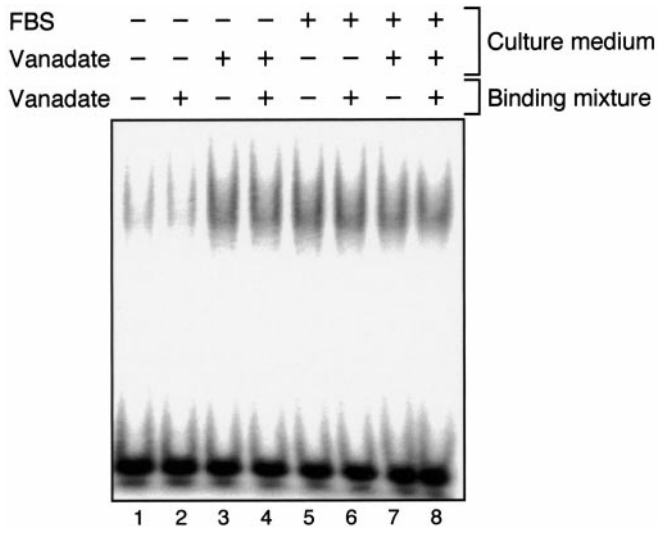

FIG. 3. Binding of the nuclear proteins obtained from control or FBS and vanadate-treated H4-II-E cells to TTGGC sequence of the rat regucalcin gene promoter. H4-II-E cells were cultured in a medium containing either vehicle (lanes 1 and 2), 100 μM vanadate (lanes 3 and 4), 10% FBS (lanes 5 and 6), or 10% FBS with 100 μM vanadate (lanes 7 and 8). Nuclear extracts (3 μg of protein) from H4-II-E cells were incubated with ^{32}P-labeled TTGGC oligonucleotide (II-b) as a probe. Gel mobility shift assays were performed in the presence (lanes 2, 4, 6, and 8) or absence (lanes 1, 3, 5, and 7) of 100 μM vanadate. The figure shows representative results of four separate experiments.

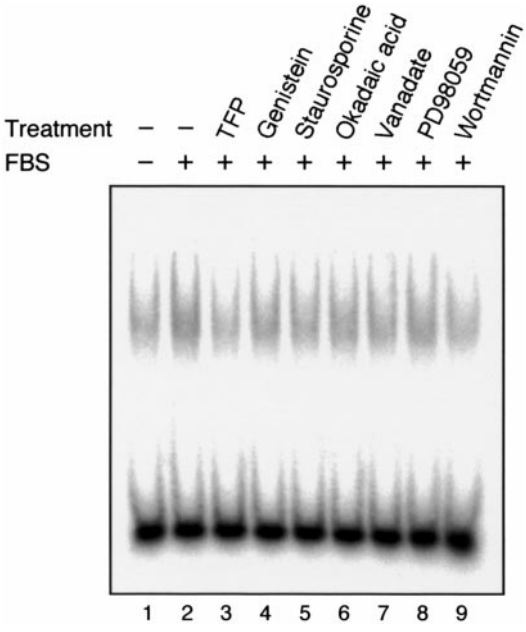

FIG. 4. Binding of the nuclear proteins obtained from control or various inhibitor-treated H4-II-E cells to TTGGC sequence of the rat regucalcin gene promoter. H4-II-E cells were cultured in a medium containing either vehicle (lane 1) or 10% FBS (lanes 2–9), without or with 10 μM TFP (lane 3), 10 μM genistein (lane 4), 100 nM staurosporine (lane 5), 50 nM okadaic acid (lane 6), 100 μM vanadate (lane 7), 10 μM PD98059 (lane 8), and 10 nM wortmannin (lane 9). Nuclear extracts (3 μg of protein) from H4-II-E cells were incubated with ^{32}P-labeled TTGGC oligonucleotide (II-b) as a probe. The figure shows representative results of four separate experiments.

gene was involved in the binding to the *trans*-acting factor using competition gel mobility shift assay with II-b oligonucleotide containing a nuclear factor I (NF1) consensus motif TTGGC$(N)_6$CC (22). In the present study, we found that the nuclear protein, which can specifically bind to the promoter region of regucalcin gene, recognized TTGGC in the NF1 consensus sequence of II-b. Also, the nuclear protein obtained from the cloned rat H4-II-E hepatoma cells was found to specifically bind to TTGGC sequence of the regucalcin gene.

The mutation of TTGGC in the NF1 consensus sequence TTGGC$(N)_6$CC caused a complete inhibition of the binding of liver nuclear protein to TTGGC sequence. Hepatic nuclear protein, which binds to TTGGC motif in the NF1 consensus sequence TTGGC$(N)_6$CC, seemed to be a member of NF1 family. Nuclear NF1-like sequence-binding proteins, which are distinct from NF1, have been reported in α1(I) collagen promoter (24), bone sialoprotein promoter (25), glial fibrillary acidic protein promoter (26), and rat p53 promoter (27–29). We reported previously that the binding of hepatic nuclear protein to the fragment A (330 bp; −709 to −380) in the promoter region of regucalcin gene was not affected by anti-NF1 antibody (22), suggesting the possibility that the nuclear protein, which can specifically bind to NF1-like sequence, differs from NF1. Presumably, liver nuclear protein (TTGGC sequence-binding protein), which binds to TTGGC sequence of the regucalcin gene promoter region, is a novel protein.

To clarify a signaling regulation of TTGGC sequence-binding protein, the isolated hepatic nuclei were incubated with various inhibitors for protein kinases and protein phosphatases. In the gel mobility shift assay, the binding of nuclear protein to TTGGC oligonucleotide was clearly enhanced by the treatment of vanadate, an inhibitor of protein tyrosine phosphatases (30, 31), and ATP, a substrate of protein phosphorylation. This finding suggests that the binding of nuclear protein to the TTGGC sequence of regucalcin gene promoter is involved in protein phosphorylation. Additionally, the TTGGC sequence binding of nuclear protein from H4-II-E cells was enhanced by the culture with FBS or vanadate. The regucalcin mRNA expression in H4-II-E cells has been demonstrated to be increased by serum addition (19). Thus, nuclear protein binding to TTGGC sequence may mediate up-regulation of regucalcin mRNA by serum stimulation in H4-II-E cells. The serum-enhanced nuclear protein binding to TTGGC sequence was clearly reduced by the treatment of TFP or staurosporine, which can inhibit Ca^{2+}/calmodulin-dependent protein kinase or protein kinase C. Such a reduction was also seen in the case of genistein, an inhibitor of protein tyrosine kinase, PD98059, an inhibitor of MAP kinase, or wortmannin, an inhibitor of PI3 kinase. The stimulatory effect of serum

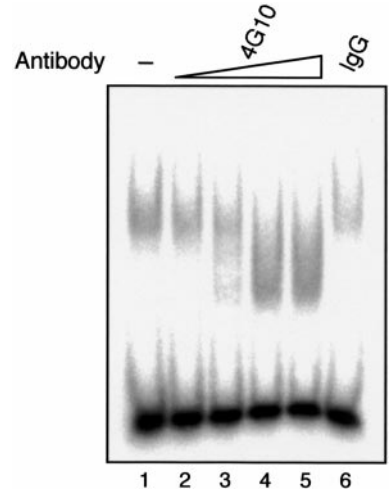

FIG. 5. Effect of 4G10, a monoclonal phosphotyrosine antibody, on the binding of nuclear proteins obtained from H4-II-E cells to TTGGC sequence of the rat regucalcin gene promoter. Gel mobility shift assays were performed using nuclear extracts (3 μg of protein) from 10% FBS-cultured H4-II-E cells with a ^{32}P-labeled TTGGC oligonucleotide (II-b) as a probe. Nuclear extracts were incubated without (lane 1) or with 4G10 (lanes 2–5; 0.05, 0.5, 5, and 10 μg, respectively), and nonimmune rabbit IgG (lane 6; 5 μg). The figure shows representative results of four separate experiments.

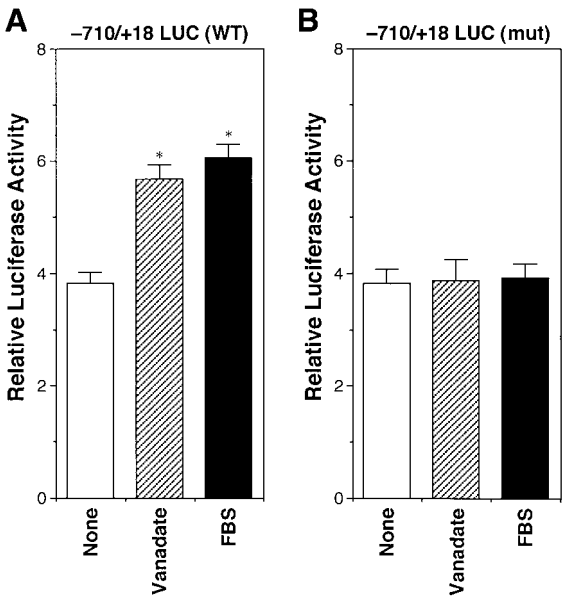

FIG. 6. Effect of vanadate and FBS on promoter activities from −710/+18 LUC (WT) or (mut) in H4-II-E cells. A DNA fragment −710/+18 was ligated into the pGL3-Basic promoterless plasmid (Basic LUC). A mutant reporter gene plasmid was generated by site-directed mutagenesis as described under Materials and Methods. H4-II-E cells were transiently cotransfected 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, 50 μM vanadate, 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 five separate experiments. (A) Wild type −710/+18 LUC. (B) Mutant −710/+18 LUC. *$P < 0.01$, compared with the control value without stimulation.

on the nuclear protein binding to TTGGC sequence may be mediated through various protein kinases.

The result of gel mobility shift assay using a monoclonal phosphotyrosine antibody (4G10), that the formation of DNA (TTGGC sequence)-nuclear protein complex was decreased by the treatment of 4G10, suggests the possibility that the nuclear protein bound to TTGGC sequence forms homo- or heterodimeric complex. The phosphorylation of tyrosine residues of TTGGC sequence-binding protein may be partly involved in the complex formation. By the way, NF1 family members have been reported to bind to DNA with both homo- and heterodimers (32). The threonine residues of NF1 protein are phosphorylated by the cdc2 protein kinase (33). However, the tyrosine residues of NF1 protein are not phosphorylated (34). Thus, the present result may further support the view that the nuclear protein, which binds to TTGGC sequence in the promoter region of regucalcin gene, differs from NF1 protein.

Moreover, the result with mutation analysis for the luciferase reporter assay demonstrates that TTGGC sequence is a necessary for the stimulation of regucalcin gene promoter activity in H4-II-E cells. Regucalcin gene promoter activity was clearly increased by culture with FBS or vanadate, suggesting that this increase is partly involved in protein kinase activation and protein tyrosine phosphatase inhibition. Such an increase was not seen in the cells with the mutant construct. This finding supports that the *cis*-acting DNA sequence, which mediates the effect of serum or vanadate in H4-II-E cells, is located in TTGGC sequence of the regucalcin gene promoter region. Regucalcin gene promoter activity may be enhanced by an augmentation of TTGGC sequence-binding protein phosphorylated in the nucleus. Nuclear TTGGC sequence-binding protein may play an important role in the stimulation of regucalcin gene expression in liver cells.

Some transcriptional factors have been shown to be translocated from cytoplasm to nucleus by hormone signals in cells (35). TTGGC sequence-binding protein was greatly present in the nuclei of H4-II-E cells cultured with FBS, Bay K 8644, insulin, and PMA, an activator of protein kinase C, while it was decreased in the cytoplasm. This finding demonstrates that TTGGC sequence-binding protein in the cytoplasm is translo-

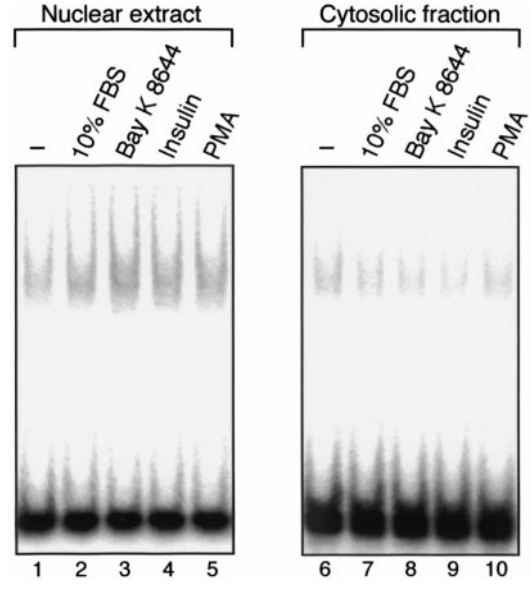

FIG. 7. Binding profile of the nuclear or cytosolic proteins obtained from the signaling factor-stimulated H4-II-E cells to TTGGC sequence of the rat regucalcin gene promoter. Nuclear extracts (3 μg of protein, lanes 1–5) and cytosolic fractions (3 μg of protein, lanes 6–10) from H4-II-E cells treated with or without signaling factors were incubated with ^{32}P-labeled TTGGC oligonucleotide (II-b) as a probe. H4-II-E cells were cultured in a medium containing either vehicle (lanes 1 and 6), 10% FBS (lanes 2 and 7), 5 μM Bay K 8644 (lanes 3 and 8), 20 nM insulin (lanes 4 and 9) or 1 μM PMA (lanes 5 and 10). The figure shows representative results of four separate experiments. Lanes 6 to 10 were exposed to imaging plate longer than lanes 1 to 5.

cated to the nucleus of cells following signaling factor stimulation.

In conclusion, it has been demonstrated that hepatic nuclear protein can specifically bind to the TTGGC sequence of the promoter region of rat regucalcin gene. Further studies on cDNA cloning for TTGGC sequence-binding protein are in progress.

ACKNOWLEDGMENT

This work was supported in part by a Grant-in Aid for Scientific Research (C) 10672048 from the Ministry of Education, Science, Sports, and Culture, Japan.

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