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Hepatic Nuclear Factor 3 and High Mobility Group I/Y Proteins Bind the Insulin Response Element of the Insulin-Like Growth Factor-Binding Protein-1 Promoter*

SUSANNE V. ALLANDER, SUSAN K. DURHAM, ANN O. SCHEIMANN, RICHARD M. WASSERMAN, ADISAK SUWANICHKUL, AND DAVID R. POWELL

Department of Pediatrics (S.K.D., A.O.S., R.M.W., A.S., D.R.P.), Baylor College of Medicine, Houston, Texas 77030; and Department of Clinical Genetics (S.V.A.), Karolinska Institute, S-171 76 Stockholm, Sweden

ABSTRACT

The insulin response element (IRE) of the human insulin-like growth factor-binding protein-1 (IGFBP-1) promoter contains a palindrome of the T(A/G)TTT sequence crucial to hormonal regulation of many genes. In initial studies of how this IRE participates in hormonal regulation, the electromobility shift assay was used under a variety of conditions to identify IRE-binding proteins. An exhaustive search identified five proteins that specifically bind this IRE; purified proteins were used to show that all five are related to either the high mobility group I/Y (HMGI/Y) or hepatic nuclear factor 3 (HNF3) protein families. Further studies used purified HNF3 and HMGI proteins to show: 1) each protects the IGFBP-1 IRE from deoxyribonuclease I (DNaseI) digestion; and 2) HNF3 but not HMGI/Y binds to the related phosphoenolpyruvate carboxykinase and Apo CIII IREs. A series of IRE mutants with variable responsiveness to insulin were used to show that the presence of a TGTTT sequence in the mutants did parallel, but HMGI/Y and HNF3 binding to the mutants did not parallel, the ability of the mutants to confer the inhibitory effect of insulin. In contrast, HNF3 binding to these IRE mutants roughly correlates with response of the mutants to glucocorticoids. The way by which HNF3 and/or other as yet unidentified IRE-binding proteins confer insulin inhibition to IGFBP-1 transcription and the role of HMGI/Y in IRE function have yet to be established. (*Endocrinology* **138:** 4291–4300, 1997)

SERUM LEVELS of insulin-like growth factor binding protein-1 (IGFBP-1), a soluble 25-kDa protein that plays a role in glucose homeostasis, are regulated by multiple hormones. Insulin inhibits, while glucocorticoids stimulate, serum IGFBP-1 levels, and this regulation takes place primarily at the level of hepatic transcription (reviewed in Refs. 1 and 2–6). In HEP G2 human hepatoma cells, two glucocorticoid response elements in the proximal human IGFBP-1 (hIGFBP-1) promoter act cooperatively to confer dexamethasone stimulation, while an insulin response element (IRE) located between the two glucocorticoid response elements confers the entire inhibitory effect of insulin on both basal and glucocorticoid-stimulated promoter activity. In addition to conferring insulin inhibition, the IRE is essential for maximal glucocorticoid stimulation. The IRE motif CAAAACAAACTTATTTTG, located from −118 to −101 bp 5′ to the transcription start site, is an inverted palindrome made up of A (CAAAACA) and B (TATTTTG) elements. Although the A element is more important than the B element in conferring insulin inhibition and glucocorticoid stimulation of IGFBP-1 promoter activity, both elements are required for maximal effect of these hormones (5, 6). The IRE sequence, location, and function are highly conserved in the rat and mouse IGFBP-1 promoters, further emphasizing the importance of this element (7–9). Hepatic expression of phosphoenolpyruvate carboxykinase (PEPCK), a key enzyme in gluconeogenesis, is also inhibited by insulin and stimulated by glucocorticoids, and the PEPCK promoter also contains an element that is necessary for these hormones to have their full effect on transcription (10–12). The sequence of this element, TGTTTTG, has seven of seven and six of seven nucleotides in common with the A and B elements, respectively, of the IGFBP-1 IRE. The closely related IRE sequence ACAAACA in the tyrosine aminotransferase (TAT) gene promoter appears to confer the same glucocorticoid and insulin effects to hepatic TAT transcription (12–15). This suggests that the same hepatic protein(s), or proteins sharing a common function, may bind the T(G/A)TTT sequence to confer insulin and glucocorticoid responsiveness to these, and perhaps other, genes. In addition, the inhibitory effect of phorbol esters on PEPCK gene transcription is also conferred through the TGTTTTG sequence in the PEPCK promoter (16), suggesting that additional modulators may act through this crucial sequence to regulate transcription of each of these genes. Thus, identifying the proteins that bind the IGFBP-1 IRE is an important first step toward unraveling the role of this element in the regulated hepatic expression of the IGFBP-1, and probably many other, genes.

The hepatic nuclear factor 3 (HNF3) family of proteins play a role in hepatic development (17, 18). One class of HNF3-binding sites contains the sequence TGTTT (19), identical to the sequence shared in the above IREs. Early studies found that HNF3 forms do indeed bind to the IGFBP-1, PEPCK, and

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Address all correspondence and requests for reprints to: David R. Powell, Texas Children's Hospital, Clinical Care Center, MC 3–2482, 6621 Fannin, Houston, Texas 77030. E-mail: dpowell@bcm.tmc.edu.

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TAT IRE regions, and later studies suggested that HNF3 forms act through these IREs to confer glucocorticoid stimulation (13, 14, 20–23). Although initial studies presented some indirect evidence to suggest that insulin does not work through HNF3 proteins to inhibit glucocorticoid-stimulated promoter activity (20, 21), a role for HNF3 proteins in this process must still be considered as they are the only known proteins to bind the IRE in each of these genes.

The high mobility group (HMG) I/Y family of proteins enhance transcription from some promoters by acting as accessory factors that enhance the DNA binding and activity of neighboring transcription factors (24). HMGI and the smaller HMGY proteins, which differ by only 11 amino acids due to alternative splicing of a single primary transcript (25), bind preferentially to A-T-rich sequences (26) similar to those present in the IGFBP-1 IRE, but binding of HMGI/Y forms to this IRE has not been evaluated.

The present study describes a search for proteins contained in hepatocyte extracts that bind specifically to the IGFBP-1 IRE. Additional studies identify these proteins and test their ability to bind: 1) an IRE from the Apo CIII promoter, which contains the T(G/A)TTT motif and confers insulin inhibition (12, 27); and 2) a series of IGFBP-1 IRE mutants that have variable insulin responsiveness.

Materials and Methods

Plasmid constructs

A 1.3-kb fragment of human genomic DNA, which contains sequence spanning from −1205 (5′) to +68 (3′) bp relative to the hIGFBP-1 transcription start site, was inserted into the promoterless pCAT(An) vector to create p1205CAT (28). Construction of plasmids pAmBm and pAm2Bm2 used in transfections, and plasmid p207CCAAT used in deoxyribonuclease I (DNase I) protection assays, has been described (5, 6, 28). Plasmid pRShGRα (29), which expresses human glucocorticoid receptor (hGR) under the control of the Rous Sarcoma Virus long terminal repeat (RSV LTR), was kindly provided by Dr. Ronald M. Evans (The Salk Institute for Biological Studies, La Jolla, CA). Plasmid pGST-HNF3β, used for expression of glutathione-*S*-transferase (GST)-mHNF3β fusion protein in *Escherichia coli*, was constructed by digesting murine HNF-3β cDNA (kindly provided by Dr. Brigid L. M. Hogan, Vanderbilt University School of Medicine, Nashville, TN) (30) and pGEX2T (Pharmacia, Piscataway, NJ) with *Eco*RI, filling in with Klenow polymerase, and then joining by blunt-end ligation.

Site-directed mutagenesis

The 1.3-kb hIGFBP-1 promoter fragment present in the M13-based vector M13 mp18 was mutated by the Kunkel method using synthetic oligonucleotides and the Muta-Gene kit (Bio-Rad, Hercules, CA). The sequence of all mutations and orientation of all constructs was confirmed by DNA sequence analysis using Sequenase (U.S. Biochemicals, Cleveland, OH) in the dideoxy chain termination method (28, 31).

The putative HNF3-binding element AAACAAACTTAT spanning bp −116 to −105 of the IGFBP-1 IRE was mutated as follows. Oligonucleotide 5′-GCACTAGCAAAAGAAACTTCTTTTGAACACTC-3′ mutated the C and A nucleotides at bp −113 and −106 to G and C, respectively, creating pG/C-A/C. Oligonucleotide 5′-ACTAGCAAAACACCGGTATTTTGAACAC-3′ mutated bp −111 to −108 from AACT to CCGG, creating pCCGG. Oligonucleotide 5′-ACTAGCAAAACAGGATTATTTTGAACA-3′ mutated bp −111 to −109 from AAC to GGA, creating pGGA. Oligonucleotide 5′-TGCACTAGCAAAGTCAATAATCTTTGAACACTCA-3′ mutated bp −114 to −105 from ACAAACTTAT to GTCAATAATC, creating pTTR. Oligonucleotide 5′-GCACTAG CAAAAACAACAAACTTTGAACACTCA-3′ mutated bp −113 to −105 from CAAACTTAT to ACAACAAAC, creating pHFH2–7. These mutant sequences are presented in alignment with the native IRE as part of Fig. 6.

Cell culture and DNA transfection

Maintenance and transfection of HEP G2 human hepatoma cells have been described (2, 28). Cells were transfected with 5 μg chloramphenicol acetyltransferase (CAT) plasmid and, in some experiments, with 1 μg hGR expression vector pRShGRα. One microgram of pRSVL plasmid, which contains the RSV LTR upstream to the luciferase reporter gene (32), was cotransfected to control for transfection efficiency. Transfected cells were washed three times in PBS and then incubated with serum-free medium (DMEM supplemented with 5 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin) ± 100 nM dexamethasone (Sigma Chemical Co., St. Louis, MO) and/or 100 nM insulin (kindly provided by Eli Lilly Co., Indianapolis, IN).

Chloramphenicol acetyltransferase and luciferase assays

CAT and luciferase assays were performed by standard methods (32, 33).

Preparation of cellular extracts

Whole cell extracts (WCE) were prepared as described previously (34). HEP G2 cells (~5×10^7 cells) were washed with PBS, harvested, and centrifuged. Cells were frozen at −80 C and thawed by adding 3 volumes of lysis buffer [20 mM HEPES, pH 7.9, 0.2 mM EDTA, 0.2 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine, 1.0 mM dithiothreitol, 10% glycerol (vol/vol), 0.5 mM phenylmethylsulfonylfluoride, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 50 μM L-1-tosylamide-2-phenylethyl/chloromethyl/ketone, 25 μM *N*-α-p-tosyl-L-lysine chloromethylketone, 0.5 mM benzamidine, 10 mM Na molybdate, 2 mM Na pyrophosphate, 2 mM Na_3VO_4] and 1 volume of 2 M KCl. The lysate was mixed for 60 min and centrifuged at 80,000 × *g* for 30 min at 4 C. Supernatant was collected and diluted with lysis buffer to a final KCl concentration of 0.15 M. Precipitate was removed by centrifugation, and the extract was aliquoted and stored at −80 C.

Perchloric acid extracts (PAE) were prepared from HEP G2 cells and from human liver obtained with permission at autopsy; these extracts are rich in HMG proteins (35). Briefly, HEP G2 cells (~5×10^7 cells) were washed with PBS, harvested, and centrifuged. Liver was frozen at −80 C, ground to fine pieces in a cold mortar, and transferred to 100 ml hypotonic buffer (10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4). The cell mixture was passed through cheese cloth and the hepatocytes were collected by centrifugation. Cell pellets were resuspended in 2 volumes of ice-cold 5% perchloric acid. After centrifugation, the supernatant was made 0.3 M in HCl, mixed with 9 volumes of acetone, and then kept at −20 C overnight. The precipitate was recovered by centrifugation at 4 C, washed with acetone, aliquoted, air-dried at room temperature, and stored at −80 C.

Protein expression

GST-mHNF3β fusion protein expressed in *E. coli* (BL21(DE3)pLysE) was purified on a glutathione Sepharose 4B affinity column following the recommendations of the manufacturer (Pharmacia). GST-mHNF3β eluted from the column was concentrated 10-fold at 4 C using a Centricon-30 Microconcentrator (Amicon, Beverly, MA).

Electrophoretic mobility shift assay (EMSA)

Proteins studied by EMSA included HEP G2 and liver WCE and PAE described above; GST-mHNF3β fusion protein described above; purified recombinant human HMGI (hHMGI) kindly provided by Dr. Raymond Reeves, Washington State University (Pullman, WA) (36); and recombinant full-length rat His(6)-HNF3α (His(6)-rHNF3α) kindly provided by Dr. Kenneth Zaret, Brown University (Providence, RI) (37). These studies also used anti- and preimmune sera to HMGI/Y, kindly provided by Dr. Reeves (38), and antisera to rat HNF3α and rat HNF3β, kindly provided by Dr. James Darnell, Jr., Rockefeller University (New York, NY) (17, 39).

Standard binding assay. Complementary 33-bp oligonucleotides encoding either native or mutant IRE sequences within the −124 to −96 bp region of the IGFBP-1 promoter were annealed and labeled as described previously (5). In addition, six other pairs of complementary 33-bp

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oligonucleotides were annealed and labeled as above: 1) the Apo CIII probe, 5′-CTAGTGTGCCTTTACTCCAAACATCCCCCAGCC-3′ and 5′-CTAGGGCTGGGGGATGTTTGGAGTAAAGGCACA-3′, spanning from −474 to −446 bp of the human Apo CIII promoter and containing an IRE; 2) the PEPCK probe, 5′-CTAGACCTCACAGCTGTGGTGTTT-TGACAACCA-3′ and 5′-CTAGTGGTTGTCAAAACACCACAGCTGT-GAGGT-3′, spanning from −428 to −400 bp of the PEPCK promoter and containing a well characterized IRE; 3) the PEPCKm probe, 5′-CTA-GACCTCACAGCTGTGGTGGGGGTACAACCA-3′ and 5′-CTAGTG-GTTGTACCCCCACCACAGCTGTGAGGT-3′, which spans the identical region of the PEPCK promoter but contains the M2 mutation, which blocks the ability of the IRE to confer insulin effect (21); 4) the Am2B probe, 5′-CTAGCACTAGCAACCATGACTTATTTTGAACAC-3′ and 5′-CTAGGTGTTCAAAATAAGTCATGGTTGCTAGTG-3′, which contains a mutation of the IRE A element; 5) the ABm2 probe 5′-CTAG-CACTAGCAAAACAAACCATGGTTGAACAC-3′ and 5′-CTAGGT-GTTCAACCATGGTTTGTTTTGCTAGTG-3′, which contains a mutation of the IRE B element; and 6) the Am2Bm2 probe 5′-CTAGCAC-TAGCAACCATGACCATGGTTGAACAC-3′ and 5′-CTAGGTGT-TCAACCATGGTCATGGTTGCTAGTG-3′, which contains both the A and B element mutations (6, 10, 12, 27). The Am2B, ABm2, and Am2Bm2 sequences are presented in alignment with the native IRE as part of Fig. 4. Labeled probe (~2–5 fmol) was incubated at 4 C with the protein of interest in 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5% glycerol (vol/vol) in a final volume of 20 μl; 1 μg poly (dG-dC) was also added as nonspecific competitor except when purified hHMGI and GST-HNF3 fusion proteins were included. After a 15-min incubation, the mixture was separated at 4 C and 190–210 V over 2–3.5 h on a 5% nondenaturing polyacrylamide gel using a low ionic strength gel buffer system (5).

Competition studies. Competition studies were performed as described previously (21). A graded excess of nonconcatamerized and unlabeled competitor DNA was mixed with ~2 fmol labeled IRE probe before addition of the protein of interest. Binding was analyzed by EMSA. Dried gels were exposed to a Storage Phosphor Screen for ~1–24 h and then quantification of relevant protein/DNA probe complexes was performed with a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA) using ImageQuant software.

DNase I protection assays

The plasmid p207CCAAT (28), containing a hIGFBP-1 promoter fragment spanning from −207 to +15 bp relative to the transcription start site, was digested at the 5′-end with *Hin*dIII, labeled with [α-^{32}P]deoxycytidine triphosphate (CTP), and then digested at the 3′-end with *Eco*RI to release a 249-bp fragment labeled on the antisense strand. This hIGFBP-1 promoter fragment was incubated in the presence or absence of hHMGI and then digested with DNase I (Worthington Biochemicals, Freehold, NJ) as described previously (31). Plasmids p1205CAT, pTTR, and pHFH2–7 were digested at the 3′-end with *Xho*I, labeled with [α-^{32}P]dCTP, and then digested at the 5′-end with *Pvu*II to release 320-bp fragments, labeled on the sense strand, which contained from −246 to +68 bp of the hIGFBP-1 promoter. These hIGFBP-1 promoter fragments were incubated in the presence or absence of His(6)-rHNF3α and then digested with DNase I (Worthington Biochemicals). Specific nucleotides protected from DNase I digestion were determined using sequence ladders derived from the appropriate hIGFBP-1 promoter probes (40).

Results

Multiple proteins bind the IRE in the IGFBP-1 promoter

As shown in Fig. 1A, multiple proteins present in WCE from HEP G2 cells bind the native, but not the AmBm mutant, IRE probe by EMSA. These proteins migrate in two groups, one with low mobility (proteins 1 and 2) and the other with high mobility (proteins 3–5). Additional studies using a variety of gel shift conditions (heparin, salmon sperm DNA, poly (dA-dT), poly (dI-dC), poly (dG-dC) acid, poly (dAG-dCT) as nonspecific competitors; presence of ATP; presence of excess oligonucleotides that specifically bind HNF3 with high affinity; absence of phosphatase inhibitors; high ionic strength gels) failed to identify additional proteins that shift the native IRE probe but not the AmBm mutant probe (data not shown).

HNF3α and HNF3β are IRE-binding proteins 1 and 2

HNF3 proteins bind specifically to the IGFBP-1 IRE (20, 21). To determine whether any of these five proteins in HEP G2 WCE are HNF3α or -β, antisera to these HNF3 forms were incubated with HEP G2 WCE and then separated by EMSA.

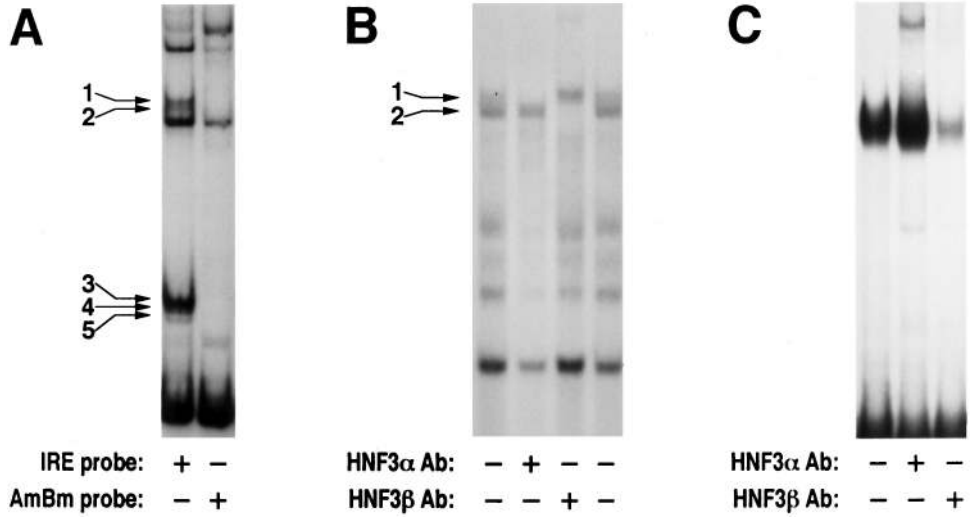

FIG. 1. EMSA studies of IRE-binding proteins in HEP G2 WCE. Complementary 33-bp oligonucleotides encoding either native (IRE) or mutant (AmBm) IGFBP-1 IRE sequences were annealed and labeled with ^{32}P. Labeled probe (2 fmol) was incubated at 4 C with 3.4 μg HEP G2 WCE and with or without rat HNF3α and HNF3β antisera, in a buffer described in *Materials and Methods*. After a 15-min incubation, the mixture was separated by electrophoresis on a 5% nondenaturing polyacrylamide gel using a low ionic strength Tris-borate buffer as described in *Materials and Methods*. A, Lanes marked + contained IRE or AmBm probes. On the *left*, proteins that bound to the IRE but not the AmBm probe were designated proteins 1 and 2 (low mobility) and 3, 4, and 5 (high mobility). B, Lanes marked + contained 2 μl HNF3α or HNF3β antisera. Proteins 1 and 2, which interacted with these antisera, are identified on the *left*. C, Lanes marked + contained 2 μl HNF3α or HNF3β antisera. Instead of HEP G2 WCE, 10 ng GST-mHNF3β were incubated with the IRE probe in each lane.

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Figure 1B shows that HNF3α antiserum blocked binding of protein 1 while HNF3β antiserum blocked binding of protein 2, suggesting that proteins 1 and 2 are probably HNF3α and HNF3β, respectively. Binding of HNF3 forms to the IGFBP-1 IRE was confirmed by expressing mHNF3β as a GST fusion and then incubating it with native and AmBm mutant IRE probes. GST-mHNF3β, purified on a glutathione Sepharose 4B column, did not bind the AmBm probe (not shown) but did bind the native IRE probe, and this binding was competed most strongly by HNF3β antiserum (Fig. 1C).

HMGI/Y proteins bind the IGFBP-1 IRE

HMGI/Y proteins are small basic proteins that migrate rapidly during electrophoresis and preferentially bind AT-rich stretches of double-stranded DNA (24, 26, 36, 41), suggesting that they might be related to the high mobility proteins 3–5, which bind the IRE during EMSA (Fig. 1A). Since PAE of cells are rich in HMG proteins, such extracts were prepared from HEP G2 cells and from adult human liver. As shown in Fig. 2, HEP G2 PAE contains the same three high mobility proteins as WCE. The presence of HMGI antiserum, but not preimmune antiserum, blocks binding of the two fastest migrating species. Thus, proteins 4 and 5 are HMGI and -Y, respectively, while protein 3, previously named IREBP (5), is not recognized by HMGI antiserum. In contrast, PAE from adult human liver contains HMGI and -Y but does not contain IREBP. Purified hHMGI protein migrates as two bands recognized by HMGI antiserum; the slower migrating band is probably intact HMGI, whereas the faster migrating band is probably an HMGI fragment.

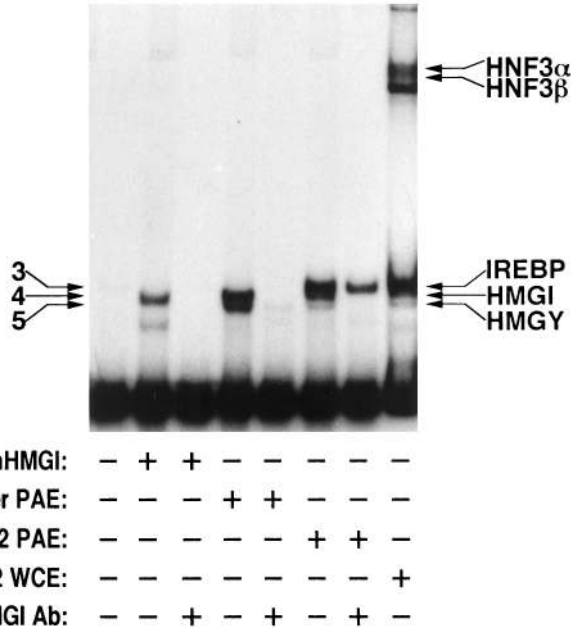

FIG. 2. HMGI/Y proteins bind the IGFBP-1 IRE. Two femtomoles of IRE probe were incubated with (+) or without (−) additives and then analyzed by EMSA. From *left* to *right*, lanes have: no additives (lane 1); 4 ng hHMGI protein (lanes 2 and 3); 390 ng human liver PAE (lanes 4 and 5); 160 ng HEP G2 PAE (lanes 6 and 7); and 3.4 μg HEP G2 WCE (lane 8). Lanes 3, 5, and 7 contained 1 μl HMGI antiserum, while lanes 2, 4, and 6 contained 1 μl preimmune serum. Proteins 3, 4, and 5 from Fig. 1 are labeled on the *left* and identified on the *right* as IREBP, HMGI, and HMGY. HNF3α and -β are also identified on the *right*.

Localization of HNF3α and HMGI/Y binding within the IRE

Recombinant rHNF3α and hHMGI proteins footprinted the IRE. As shown in Fig. 3A, 1 and 4 ng His(6)-rHNF3α protected the A element (−118 to −112 bp) and the B element (−107 to −101 bp) of the IGFBP-1 IRE from DNAse I digestion, and a new hypersensitive site appeared in the A element at bp −114. As shown in Fig. 3B, hHMGI protected the IGFBP-1 IRE from DNAse I digestion. Interestingly, hHMGI also protected the AT-rich region from −71 to −61 bp, which binds HNF-1 and is essential for basal activity of the IGFBP-1 promoter (28).

The binding of HMGI/Y proteins to the native IRE probe and to IRE probes mutated in the A element (Am2B), the B element (ABm2), and both elements (Am2Bm2) was investigated by EMSA (Fig. 4). HMGI/Y binds strongly only to the native IRE probe; HMGI/Y binds weakly when the A element is mutated, and binding is difficult to detect when either the B element is mutated alone or the A and B elements are mutated together. This suggests that HMGI binds more strongly to the B element than to the A element.

HNF3β, but not HMGI/Y, binds to IREs from many genes

As shown in Fig. 5, GST-mHNF3β binds to the native IGFBP-1 IRE and to the Apo CIII and PEPCK IREs, but not to the well characterized M2 mutant of the PEPCK IRE that is unresponsive to insulin. In contrast, HMGI/Y proteins bind to the native IGFBP-1 IRE, but not to the native PEPCK and Apo CIII IREs.

Specific IRE mutations have different abilities to confer insulin inhibition

The A and B elements of the IGFBP-1 IRE form an inverted palindrome containing two copies of the T(G/A)TTT IRE motif. These two elements, and the four nucleotides that separate them, also contribute to the AAACAAACTTAT sequence, which has 10 of 12 bp in common with the consensus HNF3-binding domain (42). As shown in Fig. 6, a series of mutations targeted 1) the G and A nucleotides of the IRE A and B elements, respectively; 2) the four nucleotides between the IRE A and B elements; or 3) the HNF3 sequence itself. Activity of the G/C-A/C mutant was increased by dexamethasone, and this dexamethasone-mediated increase in activity was not inhibited by insulin. Similar to the G/C-A/C mutant, the CCGG and GGA mutants were responsive to dexamethasone; however, the dexamethasone-mediated increase in activity of the CCGG and GGA mutants was clearly inhibited by insulin, in contrast to the G/C-A/C mutant. In the HFH2–7 plasmid, the HNF3 site in the IGFBP-1 IRE was mutated to a sequence reported to bind HNF3/fork head 2 (HFH2) but not HNF3 forms despite the presence of the TGTTT motif. In the TTR plasmid, the HNF3 site in the IGFBP-1 IRE was mutated to the sequence spanning −106 to −96 bp of the TTR promoter, which binds HNF3 with high

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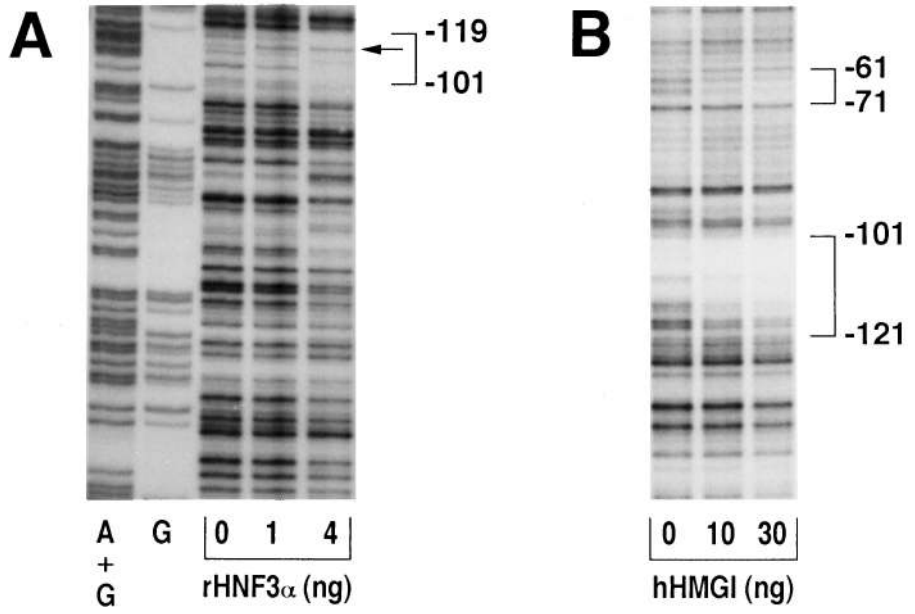

FIG. 3. HNF3 and HMGI proteins protect the IGFBP-1 IRE from DNase I digestion. A, A 320-bp DNA fragment containing hIGFBP-1 promoter sequence spanning from −246 to +68 bp relative to the transcription start site was labeled with ^{32}P. Two femtomoles of fragment labeled on the sense strand were incubated with 0.03 U DNase I and either 0, 1, or 4 ng His(6)-rHNF3α. After incubation, mixtures were electrophoresed on a 6.5% sequencing gel, dried, and autoradiographed. The protected region spanning from −119 to −101 bp is shown on the *right*, and the hypersensitive site at −114 bp is indicated with an *arrow*. B, A 249-bp DNA fragment containing hIGFBP-1 promoter sequence spanning from −207 to +15 bp relative to the transcription start site was labeled with ^{32}P. Two femtomoles of fragment labeled on the antisense strand were first incubated with 0.03 U DNase I and either 0, 10, or 30 ng hHMGI and then treated as described above. The protected regions, spanning from −61 to −71 bp and from −101 to −121 bp relative to the IGFBP-1 transcription start site, are shown on the *right*.

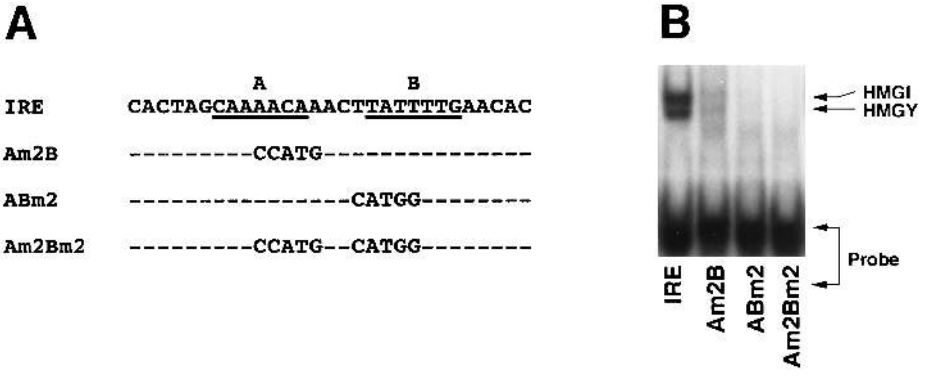

FIG. 4. HMGI/Y binding to native and mutated A and B elements of the IGFBP-1 IRE. A, Sequence of the native IRE probe (A and B elements are *underlined*) and of IRE probes containing mutations of the A and/or B elements. Conserved nucleotides are indicated by −. B, Two femtomoles of labeled native IRE probe and mutant Am2B, ABm2, and Am2Bm2 probes were incubated with 780 ng liver PAE and then analyzed by EMSA; HMGI/Y bands are identified with *arrows*.

affinity but does not contain the T(G/A)TTT motif (42). Although both plasmids were responsive to dexamethasone, insulin inhibited the dexamethasone-mediated increase in activity of only the HFH2–7 construct.

As shown in Fig. 7, insulin clearly inhibited the basal activity of plasmids containing the native IRE and the CCGG, GGA, and HFH2–7 mutants but not those containing the G/C-A/C, TTR, and Am2Bm2 mutants, consistent with the effect of insulin on dexamethasone-stimulated activity of these plasmids.

HNF3 binding to native IRE and IRE mutants

Previous studies found that HNF3α and HNF3β bound to the native IRE with higher affinity than to the G/C-A/C and CCGG IRE mutants (21). As shown in Fig. 8A, HNF3α and HNF3β (proteins 1 and 2) present in HEP G2 WCE were competed from labeled IRE probe by HFH2–7, TTR, and native IRE oligonucleotides, but not AmBm oligonucleotide, during EMSA; TTR competed much more efficiently than did IRE or HFH2–7 oligonucleotides. Labeled HFH2–7, TTR, and native IRE probes, but not AmBm probe, also directly bound GST-mHNF3β, His(6)-rHNF3α, and HNF3 forms in HEP G2 WCE (Fig. 8B); in these studies, HNF3α and HNF3β forms bound to the TTR probe as well as or better than they bound to the IRE or HFH2–7 probes.

Because the TTR mutant did not confer insulin effect but bound His(6)-rHNF3α at least as well as did the insulin-

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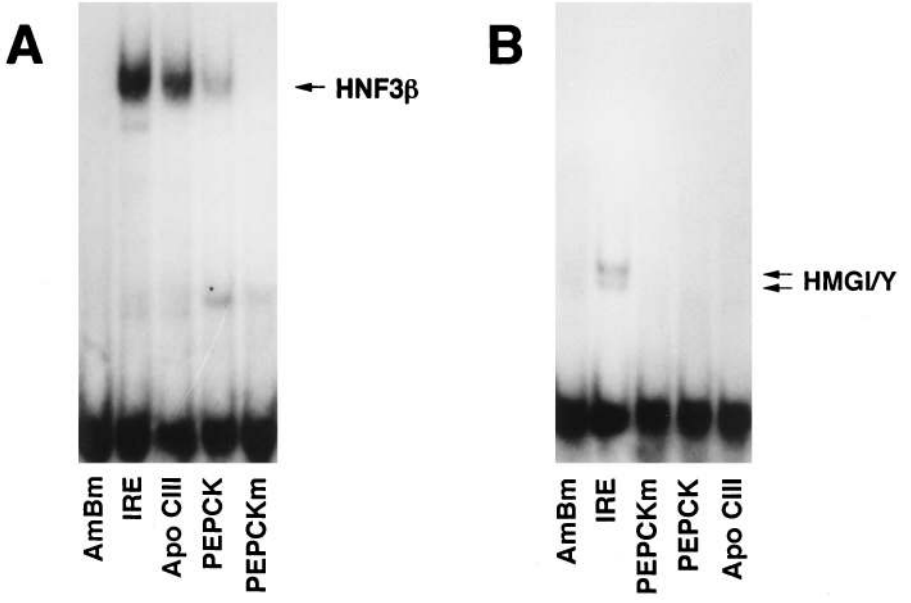

FIG. 5. Comparison of HNF3β and HMGI/Y binding to IREs from various genes. A, Two femtomoles of labeled AmBm, IRE, Apo CIII, PEPCK, and PEPCKm probes were incubated with 5 ng GST-mHNF3β and then analyzed by EMSA. Location of the GST-mHNF3β-IRE complex is shown on the *right* with an *arrow*. B, Two femtomoles of labeled AmBm, IRE, PEPCKm, PEPCK, and Apo CIII probes were incubated with 780 ng liver PAE and then analyzed by EMSA. Location of the hHMGI/Y-IRE complexes are shown on the *right* with *arrows*.

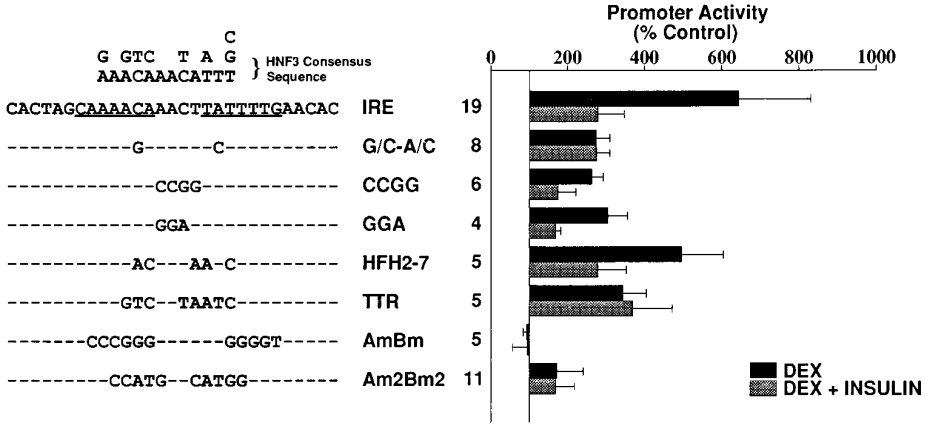

FIG. 6. Ability of insulin to inhibit dexamethasone-stimulated activity of IGFBP-1 IRE mutants. *Left*, sequences of the native IRE and a series of seven IRE mutants. Conserved nucleotides are indicated by −. The CAAAACA and TATTTTG sequences of the IRE A and B elements, respectively, are *underlined*. The degenerate HNF3 consensus sequence (42) is aligned *above* the IRE sequence. *Right*, HEP G2 cells were transfected with hIGFBP-1 promoter construct p1205CAT containing either native or mutant IRE sequences and cotransfected with the hGR expression vector pRShGRα; cells were then incubated with no additives (control value) or with 100 nM dexamethasone ± 100 nM insulin. After 18 h, hIGFBP-1 promoter activity was estimated by CAT assay. The effect of hormones is shown as % control (no additives = line at 100%). Promoter activity for each experimental condition = mean ± SD of n independent experiments, with n presented to the *right* of the construct name.

responsive HFH2–7 mutant and the native IRE, it is possible that His(6)-rHNF3α binds to the TTR mutation in a different way than it binds to the HFH2–7 mutation or to the native IRE. As shown in Fig. 9, identical regions of the native IRE, the TTR mutation, and the HFH2–7 mutation were protected from DNase I digestion by His(6)-rHNF3α. In addition, His(6)-rHNF3α induced the appearance of the same hypersensitive site at −110 bp in the TTR and HFH2–7 mutants; thus, if His(6)-rHNF3α binds these two IRE mutants in a fundamentally different way, it could not be demonstrated by DNase I digestion.

HMGI/Y binding to native IRE and IRE mutants

As shown in the representative EMSA of Fig. 10, recombinant hHMGI, and also HMGI/Y proteins present in liver PAE, bound to labeled G/C-A/C, CCGG, HFH2–7, TTR, and native IRE probes, but not AmBm probe. HMGI/Y proteins bound more tightly to the native IRE sequence than to the G/C-A/C, CCGG, HFH2–7, and TTR mutant sequences, while hHMGI bound more uniformly to the IRE and the mutant sequences; the reason for this is unclear. Of greater significance, however, hHMGI bound comparably to CCGG

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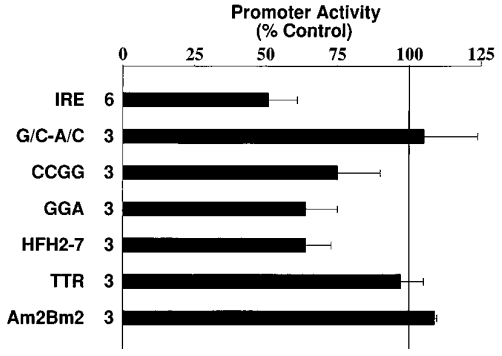

FIG. 7. Ability of insulin to inhibit basal activity of IGFBP-1 IRE mutants. HEP G2 cells were transfected with the identical IGFBP-1 promoter constructs used in Fig. 6; cells were then incubated with or without 100 nM insulin. After 18 h, hIGFBP-1 promoter activity was estimated by CAT assay. The effect of hormones is shown as % control (no additives = line at 100%). Promoter activity for each experimental condition = mean ± SD of n independent experiments, with n presented to the *right* of the construct name.

and G/C-A/C mutants, and all HMGI/Y proteins appeared to bind comparably to TTR and HFH2–7 mutants.

Discussion

Some circumstantial evidence suggests that HNF3 proteins confer insulin inhibition of gene transcription. First, HNF3 proteins bind the IREs in the IGFBP-1, PEPCK, and TAT promoters, which confer insulin inhibition to these genes (Refs. 15, 20, and 21, and present study). Second, an IRE that confers insulin inhibition to the Apo CIII gene (12, 27) was shown in the present study to bind HNF3β. Third, HNF3 amino acid sequences contain potential phosphorylation sites for cdc2 kinase and extracellular signal-regulated kinases (erks), all of which are activated by insulin in hepatocytes (43–46). Although HNF3 proteins are important regulators of embryonic development consistent with their membership in the fork head superfamily of transcription factors, HNF3 proteins also participate in the function of differentiated hepatocytes during extrauterine life (17, 18, 47).

Despite the above evidence implicating HNF3 in insulin effect, initial studies suggested that HNF3 is not involved. Unterman *et al* (20) described an IRE mutation that disrupted HNF3 binding but conferred a weak insulin effect (20). Also, the CCGG and G/C-A/C mutants were used previously to explore this issue; the CCGG mutation targets the HNF3-binding site between the A and B IRE elements, while the G/C-A/C mutation targets the HNF3-binding site within the A and B elements. These mutations demonstrated comparable affinity for HNF3 forms, but the CCGG mutation allowed insulin response while the G/C-A/C mutation did not (Table 1 and Ref. 21). Nevertheless, the Unterman and G/C-A/C mutants show a decrease both in insulin response and in affinity for HNF3. In the present study the IRE was replaced with a high-affinity HNF3- binding site from the TTR promoter. Despite the fact that HNF3α and HNF3β forms bound to the TTR mutation as well as or better than they bound to the native IRE or the HFH2–7 mutation, the TTR mutation did not confer insulin inhibition, in contrast to the native IRE and the HFH2–7 mutation. Table 1 clearly shows that HNF3 binding to IRE mutants does not correlate with the responsiveness of the mutants to insulin.

In contrast to HNF3 binding, the presence of the T(G/A)TTT motif in IRE mutants directly correlates with the ability of the mutants to confer insulin inhibition (Table 1). Thus the IGFBP-1, PEPCK, TAT, and Apo CIII IREs, and all IGFBP-1 and PEPCK IRE mutants that confer insulin inhibition, contain the T(G/A)TTT motif, whereas insulin-unresponsive IRE mutants do not contain this motif (Refs. 5, 6, 10, 20, and 21 and present study). At present, it is unclear what protein binds the T(G/A)TTT motif to confer insulin effect. HNF3 proteins may be responsible if they bind native IREs and IRE mutants that contain the T(G/A)TTT motif (such as HFH2–7) in a different spatial orientation than IRE mutants lacking the T(G/A)TTT motif (such as TTR); in this scenario, insulin would activate only the HNF3 bound to T(G/A)TTT-containing IREs. Although there is reason to suspect that HNF3 forms may bind differently to T(G/A)TTT-containing elements than to the TTR element (48), nevertheless HNF3α footprints the TTR and HFH2–7 mutations over the same span of nucleotides, and HNF3α binding induces the same hypersensitive site in footprints of the TTR and HFH2–7 IRE mutants. This suggests that HNF3α binds these two IRE mutants in a very similar spatial orientation, making it unlikely that insulin can selectively activate T(G/A)TTT-bound HNF3 alone. Alternatively, HNF3 could participate in insulin inhibition by binding to the IRE as a complex with other proteins; as part of the complex, HNF3 would have an affinity for the IRE and IRE mutants that parallels insulin response. Finally, a protein(s) other than HNF3 may confer insulin effect to the IGFBP-1 IRE and related IREs. One candidate is HFH2, a member of the fork head protein superfamily; HFH2 is expressed in liver and other tissues during extrauterine life and should bind the insulin-responsive HFH2–7 mutation (42, 49). To date, an exhaustive search using HEP G2 extracts and a wide variety of EMSA conditions has failed to identify additional bands that might represent IRE binding to HFH2, other candidate proteins, or HNF3 as part of a protein complex.

This study identifies HMGI and -Y as proteins present in normal human liver that bind the IGFBP-1 IRE. HMGI/Y proteins bind in the minor groove of A-T-rich DNA and upon binding may bend the DNA helix; they may play a role in nucleosome positioning and also act as accessory factors that bend DNA to allow enhanced binding and activity of transcription factors (24, 26, 36, 41). Present and past (5) studies also identify a protein, designated IREBP, which 1) is expressed in HEP G2 cells but not normal liver; 2) binds the hIGFBP-1 IRE; and 3) shares many characteristics with HMGI/Y proteins. Based on electrophoretic mobility and expression in hepatoma cells rather than normal liver, IREBP is probably HMGI-C, which is highly expressed in HEP G2 cells and is a close relative of HMGI/Y (35). IREBP/HMGI-C were not studied further due to their lack of expression in normal liver tissue.

HMGI/Y proteins appeared initially to be candidate proteins for conferring insulin effect. HMGI/Y proteins are likely targets of insulin-regulated erks and cdc2 kinase in liver (43–46). In fact, a motif present in the DNA-binding

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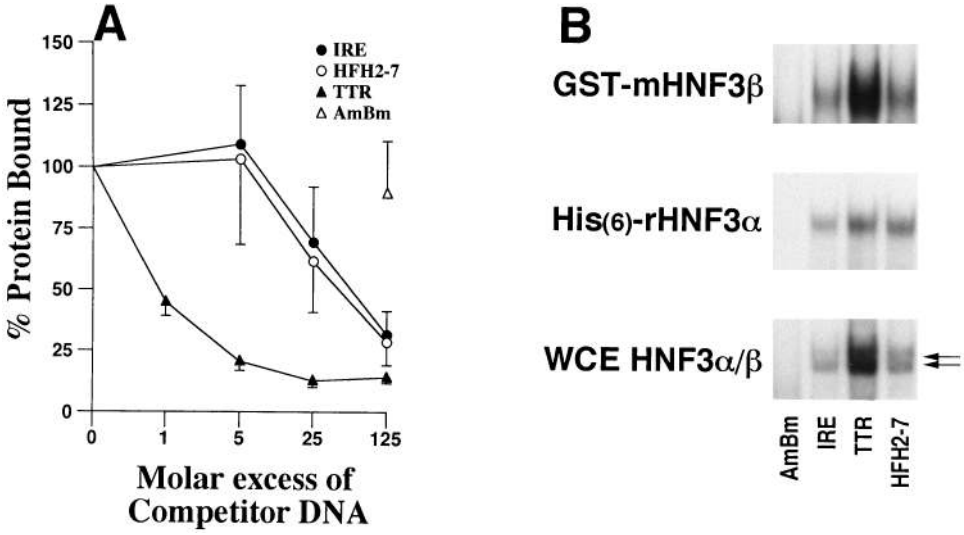

FIG. 8. HNF3α and HNF3β binding to IRE and IRE mutants. A, 6.8 μg HEP G2 WCE were added to 2 fmol IRE probe and the indicated molar excess of unlabeled IRE, TTR, HFH2–7, and AmBm oligonucleotides and then analyzed by EMSA. After dried gels were exposed to a Storage Phosphor Screen, HNF3α and HNF3β bound to labeled probe were quantitated with a PhosphorImager. B, Two femtomoles of labeled AmBm, IRE, TTR, and HFH2–7 probes were incubated with 5 ng GST-mHNF3β, 10 ng His(6)-rHNF3α, or 6.8 μg HEP G2 WCE (WCE HNF3α/β) and then analyzed by EMSA. The location of the HNF3α and HNF3β bands in the HEP G2 WCE are shown on the *right* with *arrows*.

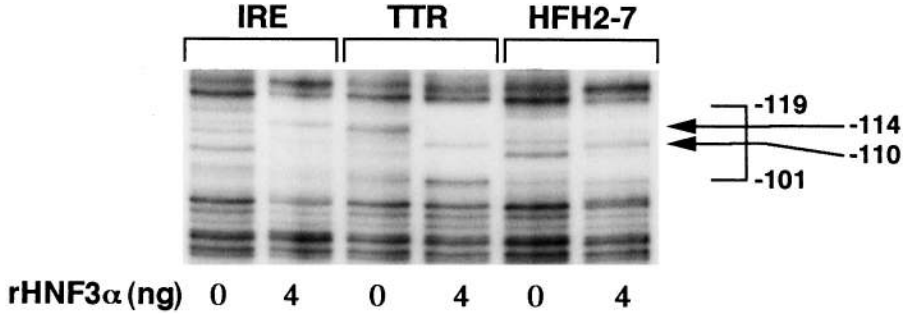

FIG. 9. HNF3 protects similar regions of native and mutant IREs from DNase I digestion. Fragments (320-bp) of plasmids p1205CAT, pTTR, and pHFH2–7, spanning from −246 to +68 bp relative to the transcription start site, were labeled with ^{32}P as described in *Materials and Methods*. Two femtomoles of each fragment were incubated with 0.03 U DNase I and either 0 or 4 ng His(6)-rHNF3α. After incubation, mixtures were electrophoresed on a 6.5% sequencing gel, dried, and autoradiographed. The protected region spanning from −119 to −101 bp is shown on the *right*. The hypersensitive site at −114 bp in the native IRE and the hypersensitive site at −110 bp in the TTR and HFH2–7 mutant IREs are indicated by *arrows*.

domain of HMGI can be phosphorylated by cdc2 kinase both *in vitro* and *in vivo*, resulting in a 20-fold decrease in affinity of HMGI for DNA (36). Also, IRE-ABP, which likely bends DNA upon binding to the 3′-region of the upstream IRE present in the glyceraldehyde-3-phosphate dehydrogenase promoter, is a member of the HMG superfamily (50, 51). Nevertheless, the studies presented here suggest that HMGI/Y does not confer insulin response: 1) HMGI/Y proteins do not bind the PEPCK or Apo CIII IREs, which makes a role for HMGI/Y unlikely if it is assumed that these structurally related IREs confer insulin effect by a common mechanism; 2) hHMGI bound as well to the insulin responsive CCGG mutant as to the insulin unresponsive G/C-A/C mutant, and all HMGI/Y proteins bound as well to the insulin-responsive HFH2–7 mutant as to the insulin-unresponsive TTR mutant. Thus, there is no correlation between ability of IRE mutants to confer insulin effect and their ability to bind HMGI (Table 1); and 3) HMGI/Y proteins bind strongly to the combined A and B elements of the native IRE, bind weakly to the B element alone, and bind very weakly, if at all, to the A element alone; in contrast, the IRE A element alone is more responsive to insulin than is the B element and is almost as responsive to insulin as the combined A and B elements (5, 6).

The IREs in the IGFBP-1, PEPCK, and TAT genes are also accessory sites necessary for glucocorticoids to exert their full stimulatory effect on gene transcription (6, 8, 11, 13, 14). Prior studies found that the ability of glucocorticoids to stimulate activity of IGFBP-1 promoter constructs containing either the native IRE or the CCGG and G/C-A/C IRE mutants correlated directly with ability of these sequences to bind HNF3 (21), suggesting an association between HNF3 binding and glucocorticoid responsiveness. Indeed, HNF3 proteins appear to augment the glucocorticoid effect on TAT and PEPCK gene transcription by binding to their respective IREs (22, 23). The present study was not designed to examine the role of HNF3 and HMGI/Y proteins in conferring glucocorticoid effect; glucocorticoids were used to amplify the inhibitory

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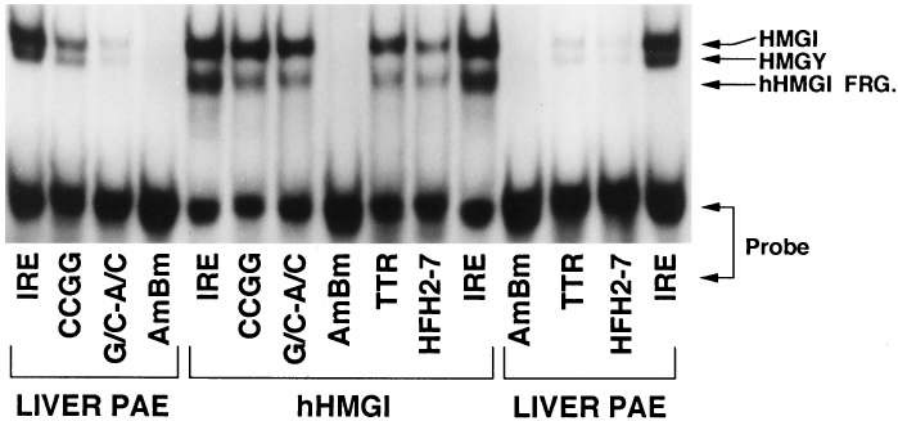

FIG. 10. HMGI/Y binding to IRE and IRE mutants. Two femtomoles labeled G/C-A/C, CCGG, HFH2–7, TTR, AmBm, and native IRE probes were incubated with 780 ng liver PAE or 27 ng hHMGI and then analyzed by EMSA. Location of HMGI and HMGY bands is shown on the *right*, as is the location of a hHMGI fragment (hHMGI FRG.).

TABLE 1. Structural and functional analysis of native and mutant IRE sequences

IRE construct	Effect of insulin on basal CAT expression[a]	T(G/A)TTT motif	HNF3 binding	HMGI binding
IGFBP-1				
Native IRE	−49 ± 10%	Yes	++	++
G/C-A/C	+5 ± 19%	No	+[b]	+
CCGG	−25 ± 15%	Yes	+[b]	+
GGA	−36 ± 11%	Yes		
HFH2-7	−36 ± 9%	Yes	++	+
TTR	−3 ± 8%	No	++++	+
AmBm	+37 ± 13%[c]	No	−	−
Am2Bm2	+9 ± 1%	No		−
PEPCK				
Native IRE[b]	−38%[b]	Yes[b]	++[b]	−
Mutant M2[b]	+6%[b]	No[b]	−[b]	−

[a] % change; − signifies inhibition.
[b] From Ref. 21.
[c] From Ref. 5.

effect of insulin on IGFBP-1 promoter activity. Nevertheless, data from this study show that HNF3 forms bind the native IRE, the TTR mutant, and HFH2–7 mutant, which confer glucocorticoid effect, but do not bind the AmBm mutant, which does not confer glucocorticoid effect, suggesting that HNF3 forms may augment glucocorticoid stimulation of the IGFBP-1 promoter. However, these and past correlations do not prove an association; studies designed to show a direct interaction between HNF3 and GR pathways are needed and are underway in many laboratories. In contrast to HNF3 forms, HMGI/Y proteins are unlikely to confer glucocorticoid effect to the IGFBP-1 IRE. First, HMGI/Y proteins do not bind the A element in the ABm2 mutation of the IGFBP-1 IRE despite the fact that the A element in the ABm2 mutation is very responsive to glucocorticoids (6). Second, HMGI/Y proteins do not bind the PEPCK IRE, which makes a role for HMGI/Y unlikely if it is assumed that the structurally related IGFBP-1 and PEPCK IREs confer glucocorticoid effect by a common mechanism.

Most past studies examined the ability of IGFBP-1, PEPCK, and TAT IREs to confer insulin inhibition of glucocorticoid-stimulated promoter activity (6, 10, 15, 21), but it is unlikely that insulin inhibits IGFBP-1 transcription solely by inhibiting the IRE-binding protein that potentiates glucocorticoid effect: 1) the G/C-A/C and TTR mutants augment glucocorticoid stimulation but are totally unresponsive to insulin, suggesting that each hormone effect is conferred through a separate protein pathway; and 2) insulin inhibits basal activity of each IRE mutant examined in this study to roughly the same degree that it inhibits glucocorticoid-stimulated activity of the same mutant, suggesting that the mechanism of insulin inhibition is the same in the presence and absence of glucocorticoid stimulation. It is conceivable, then, that HNF3 confers the effects of glucocorticoids but not insulin on IGFBP-1 transcription.

The binding of HMGI/Y proteins to the IGFBP-1 IRE is similar to the binding of C/EBP proteins to the PEPCK IRE (12, 21, 52). HMGI/Y proteins bind only the IGFBP-1 IRE, while C/EBP proteins bind only the PEPCK IRE. Also, neither protein family appears to play a role in the insulin inhibition conferred through these IREs. Nevertheless, important and perhaps complementary roles for these proteins in the function of their respective IREs may ultimately be recognized; thus, a role for each protein family should be considered in any newly described IRE function.

In summary, both HNF3 and HMGI/Y proteins bind the IGFBP-1 IRE, but the role these proteins play while bound to the IRE is unclear. The inability of HMGI/Y proteins to bind other IREs suggests they do not play a role in insulin or glucocorticoid effects. The data presented here are compatible with, but not proof of, a role for HNF3 in conferring glucocorticoid stimulation of IGFBP-1 transcription. Although HNF3 forms are the only proteins found to specifically bind the related IGFBP-1, PEPCK, TAT, and Apo CIII IREs, any future hypothesis implicating HNF3 proteins in insulin inhibition must account for the dissociation of their binding to IRE mutants and the ability of those mutants to confer insulin effect. Ultimately, the protein(s) responsible for the insulin effect will be found to interact with the T(G/A)TTT sequence, and in particular with the G or A nucleotide, of the IRE motif to confer the inhibitory effect of insulin on IGFBP-1 gene transcription.

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