Transcriptional regulation of human insulin receptor gene by the high-mobility group protein HMGI(Y)

ANTONIO BRUNETTI,*,[1] GUIDALBERTO MANFIOLETTI,† EUSEBIO CHIEFARI,* IRA D. GOLDFINE,‡ AND DANIELA FOTI§

*Dipartimento di Medicina Sperimentale e Clinica 'G. Salvatore', Cattedra di Endocrinologia, Università degli Studi di Catanzaro 'Magna Græcia', Catanzaro, Italy; †Dipartimento di Biochimica, Biofisica e Chimica delle Macromolecole, Università degli Studi di Trieste, Trieste, Italy; ‡Department of Medicine, Division of Diabetes and Endocrine Research, Mount Zion Medical Center, University of California San Francisco, San Francisco, California 94120, USA; and §Dipartimento di Medicina Sperimentale e Clinica 'G. Salvatore', Cattedra di Medicina Interna, Università degli Studi di Catanzaro 'Magna Græcia', Catanzaro, Italy

ABSTRACT **We have previously identified two closely related nuclear binding proteins that specifically interact with two unique functional AT-rich sequences of the 5′ regulatory region of the human insulin receptor gene. Expression of these nuclear binding proteins increases during myocyte and adipocyte differentiation, and in other tissues appears to correlate with insulin receptor content. We have hypothesized, therefore, that insulin receptor expression in the insulin target tissues is regulated at least in part by these nuclear proteins. Here we show data on purification and biochemical characterization of these DNA binding proteins. Using a conventional chromatographic purification procedure combined with electrophoresis mobility shift assay and immunoblot analyses, a unique ~15 kDa protein, either identical to or highly related to the architectural transcription factor HMGI(Y), has now been identified, suggesting an essential role for HMGI(Y) in regulating insulin receptor gene transcription. Direct evidence of HMGI(Y) insulin receptor promoter interactions is provided by functional analysis with the CAT reporter gene and by hormone binding studies in cells expressing HMGI(Y) antisense RNA. In these experiments, antisense HMGI(Y) specifically inhibits insulin receptor promoter function and insulin receptor protein expression, indicating that HMGI(Y) is required for proper transcription of insulin receptor gene. Moreover, our data consistently support the hypothesis that a putative defect in this nuclear binding protein may cause insulin receptor dysfunction with subsequent impairment of insulin signaling and action.—Brunetti, A., Manfioletti, G., Chiefari, E., Goldfine, I. D., Foti, D. Transcriptional regulation of human insulin receptor gene by the high-mobility group protein HMGI(Y). *FASEB J.* 15, 492–500 (2001)**

Key Words: insulin receptor promoter · transcription factors · CAT reporter gene

The interaction of insulin with target cells is mediated by a specific receptor located on the plasma membrane (1, 2). The insulin receptor serves a critical role both in directing insulin to specific target tissues and in initiating the response of these tissues to the hormone (3). Although insulin receptors are present on the surface of virtually all cells, data suggest that the insulin receptor can be regulated by a wide variety of factors and under different environmental conditions (4–6). For example, glucocorticoids enhance transcription of the insulin receptor gene, whereas insulin down-regulates its own receptor (7, 8). Also, insulin receptor mRNA levels increase after the differentiation of BC3H-1 mouse myoblasts and 3T3-L1 fatty fibroblasts (4, 9, 10). In addition, developmental regulation of insulin receptor gene expression has been documented in *Drosophila* (11).

The insulin receptor is usually expressed at low levels in all cell types but at higher levels in the classical insulin target tissues muscle, liver, and fat (2). However, little is known about the regulatory mechanisms controlling insulin receptor at the level of gene expression. The insulin receptor is of major importance in certain states of insulin resistance in humans, in which qualitative and quantitative abnormalities of the receptor may lead to defective transmembrane signaling (12–16). Moreover, even though it is an open question whether insulin receptor plays a critical role in aging and longevity in mammals as well as in *Caenorhabditis elegans* (17), inhibition of the neuronal insulin receptor causes Alzheimer-like disturbances in rats (18). Involvement of insulin receptor has been documented in human neoplasias in which abnormalities of insulin receptor expression and/or function have been reported (19–22). Thus, regulation of insulin receptor gene expression seems to be important from both biological and clinical aspects.

In eukaryotes, the binding of proteins to specific DNA sequences is critical for the regulation of many

[1] Correspondence: Cattedra di Endocrinologia, Policlinico 'Mater Domini', Via T. Campanella 115, 88100 Catanzaro, Italy. E-mail: antonio.brunetti@tin.it

0892-6638/01/0015-0492 © FASEB

nuclear events, such as replication and transcription. Initiation of mRNA synthesis is a major control point in gene expression. Gene expression in eukaryotic cells is controlled by nuclear regulatory proteins (*trans*-acting factors) that modulate the transcription of genes and gene networks (23–25). During the last decades, unique DNA sequences that are involved in gene regulation (*cis* elements) have been identified, and this process has led to the detection and characterization of DNA regulatory proteins (25). As a step toward understanding the molecular basis of regulation of insulin receptor gene expression, the promoter region of the human insulin receptor gene has been identified and analyzed by several groups (26–34). This region extends over 1800 bases 5′ upstream from the insulin receptor gene ATG codon and is extremely GC-rich, containing a series of GGGCGG repeats that are putative binding sites for the mammalian transcription factor Sp1 (25). It has neither a TATA box nor a CAAT box, reflecting the common features for the promoters of constitutively expressed genes (so-called housekeeping genes).

Previously we identified two similar or identical DNA nuclear binding proteins that specifically interacted with two unique AT-rich sequences of the insulin receptor gene promoter, C2 and E3 (between nucleotides −782 to −800 and −1740 to −1775, respectively), which had *in vivo* transcriptional activity (32). These nuclear binding proteins were readily detected in cells containing high levels of insulin receptor, but were almost undetectable in cells with low insulin receptor content (32). Thus, we have hypothesized that these DNA binding proteins interacting with these AT-rich sequences of the insulin receptor gene promoter may have major importance in regulating the expression of the insulin receptor in target tissues.

We show data on purification and molecular characterization of these nuclear binding proteins, using a cell line that readily expresses both insulin receptor DNA binding proteins: IM-9 human lymphocytes. Analyses of protein–DNA interactions with the positive-acting *cis* elements (C2 and E3), combined with immunoblot analyses of nuclear protein from IM-9 cells, identified a unique insulin receptor DNA binding protein (IR-DBP) highly related to the high-mobility group (HMG) protein: HMGI(Y). HMGI(Y) is a distinct member of the HMG protein family that has been demonstrated to specifically interact with AT-rich regions of DNA and to contribute to the transcriptional regulation of many mammalian genes by interacting with different transcription factors (35, 36). Here we demonstrate for the first time that IR-DBP and HMGI(Y) are indistinguishable in their DNA binding properties, immunological reactivity, and *in vivo* activation of transcription. We show that inhibition of HMGI(Y) in cells naturally expressing relatively high insulin receptor levels significantly reduces cell surface expression of the insulin receptor. Conversely, overexpression of HMGI(Y) in transfected cultured cells with relatively low insulin receptor content increases insulin receptor protein expression, indicating that HMGI(Y) may play an essential role in regulating the transcriptional activity of the insulin receptor gene.

MATERIALS AND METHODS

Cells and protein extracts

IM-9 human lymphocytes (8) were cultivated in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco Laboratories, Grand Island, N.Y.), 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified 5% CO_2 atmosphere at 37°C. Nuclear extracts from IM-9 cells were prepared, with only minor modification, according to Dignam et al. (37). Briefly, cells were washed with phosphate-buffered saline (PBS) at 4°C and harvested into buffer A [(10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT)]. After centrifugation (1200 rpm for 10 min), cells were resuspended in buffer A and kept on ice for 10 min. Cells were then homogenized with a glass Dounce (10 strokes with a type B pestle) and centrifuged at 2000 rpm to pellet nuclei. Nuclei were resuspended in buffer B [(20 mM HEPES, pH 7.9, 0.55 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml antipain, 1 μg/ml leupeptin)], and homogenized as above. The suspension was centrifuged for 1 h at 35,000 rpm and the supernatant dialyzed against 100 volumes of buffer C (15 mM HEPES, pH 7.9, 20% glycerol, 1 mM EDTA, 40 mM KCl, 0.5 mM DTT, 0.5 mM PMSF) for at least 12 h at 4°C. The dialysate was centrifuged at 35,000 rpm for 30 min and the resulting supernatant, designated the nuclear extract, was frozen in liquid nitrogen and stored at −80°C. Final protein concentration in the extract was determined by the Bradford protein assay (Bio-Rad Laboratories, Hercules, Calif.). Typically, 20 liters of IM-9 cells yielded ~200 mg of nuclear protein. HepG2 human hepatoma cells (38) and HTC rat hepatoma cells (39, 40) were cultivated in DME medium H-21 with 10% FBS. Chinese hamster ovary (CHO) cells were maintained in Ham's F-12 medium containing 10% FBS (40).

Oligonucleotides and EMSA

45-mer double-stranded oligonucleotides corresponding to C2 (5′-TATGAACAAAATAGCAAAATGGTAGAGAAAGGATCTG-TGCCGCTG-3′) and E3 (5′-GAGAAAAACTCCATCTAAAAAA-AAAAAAAAAAAAAAAAAAAAACA-3′) sequences of the 5′ regulatory region of the human insulin receptor gene were synthesized chemically (BioTeZ, Berlin, Germany), end-labeled with (^{32}P)dATP using DNA polymerase I (41) and used in electrophoresis mobility shift assay (EMSA) as described previously (32). 27-mer double-stranded oligonucleotides containing wild-type (5′-GGGAAATTCCGTGGGAAATTCCGAGCT-3′) and mutated (5′-GGGA*G*ATTCCGTGGGA*G*ATTCCGAGCT-3′) PRDII element motif of the β-interferon promoter (42) were 5′ end labeled with (γ-^{32}P)ATP and T4 polynucleotide kinase (Sigma-Aldrich, Milwaukee, Wis.) and used in EMSA. Unlabeled 22-mer double-stranded oligonucleotide containing consensus binding site for Sp1 transcription factor (Santa Cruz Biotechnology, Santa Cruz, Calif.) was used in competition studies.

Purification of IR-DBP and SDS-PAGE

Prepacked HiTrap heparin-Sepharose column (1 ml) was equilibrated with start buffer (0.01 M sodium phosphate with a neutral pH ~7) and used under conditions suggested by the

supplier (Pharmacia, Piscataway, N.J.). IM-9 nuclear protein (2 mg) was applied, washed with start buffer, and eluted with a 10 ml gradient from 0.1 to 1.0 M NaCl in start buffer. Eluted samples were desalted using a prepacked HiTrap Desalting column (Pharmacia) and analyzed by EMSA for protein–DNA binding activity. Fractions containing the protein(s) of interest were pooled and subjected to further purification by DNA affinity chromatography, according to Kadonaga and Tijan (43). Double-stranded oligonucleotides corresponding to C2 and E3 sequences of the insulin receptor gene promoter with TCGA 5′ overhanging ends were coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia) at a concentration of 1.0 nmol of DNA/ml of Sepharose gel and chromatography was carried out in binding buffer (15 mM HEPES, pH 7.9, 1 mM EDTA, 40 mM KCl, 0.5 mM DTT, 5% glycerol). The load was mixed with nonspecific competitor calf thymus DNA (100 μg) (Sigma, St. Louis, Mo.) and applied dropwise to the affinity resin. The column was washed with binding buffer, step eluted with the same buffer containing successively 0.1 M, 0.2 M, 0.6 M NaCl; protein–DNA complexes were visualized by EMSA.

IM-9 affinity-purified IR-DBP was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using the Tricine SDS-discontinuous buffer system of Schagger and Von Jagov (44), and visualized by the PLUSONE Silver-Staining Kit Protein (Pharmacia), according to the manufacturer's instructions.

Western transfers

Aliquots of IM-9 cell nuclear extracts and purified protein were resolved on 16.5% SDS-PAGE, electro-transferred onto 0.2 μm PVDF membrane (Immun-Blot, Bio-Rad Laboratories) using a mini electrophoretic transfer cell (Trans-blot, Bio-Rad Laboratories), and detected by DNA and antibody probes. Southwestern blotting experiments were performed on proteins transferred onto membranes that were denatured/renatured as described by Celenza and Carlson (45). This treatment increased the recovery of active molecules. To assay for DNA binding activity, membranes were first immersed for 12 h in blocking solution (2.5% non-fat dry milk, 25 mM HEPES, pH 8.0, 1 mM DTT, 10% glycerol, 50 mM NaCl, 1 mM EDTA) at 4°C, then incubated in TNE-50 (10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT) containing ^{32}P-DNA binding site probe (either C2 or E3) (2×10^6 cpm/ml) and poly(dI-dC) (1.0 mg/ml). After 1 h at room temperature, the filters were washed with TNE-50, patted dry and exposed to Kodak X-ray film.

For Western blot analysis, membranes from Southwestern blots were first immersed for 2 h in blocking solution (5% non-fat dry milk in PBS), then probed with an antibody raised against a peptide specific for HMGI(Y) (46). After 2 h at room temperature, goat anti-rabbit IgG horseradish peroxidase conjugate (Amersham, Arlington Heights, Ill.) was added (1 h at room temperature) and immune complexes were visualized by enhanced chemiluminescence (ECL, Amersham).

For the immunoprecipitation studies, 10 μl of anti-HMGI(Y) antibody were incubated with ~200 ng of affinity-purified IR-DBP for 2 h nutating at 4°C. Protein A beads (50 μl; Pharmacia) were added to the mixture and incubated overnight nutating at 4°C. Complexes were recovered, and the beads were washed twice with 0.2 M NaCl wash buffer (15 mM HEPES, pH 7.9; 1 mM EDTA; 40 mM KCl; 5% glycerol; 5 mM DTT) and once with 0.1 M NaCl wash buffer. Specifically bound material and supernatant fractions were analyzed by SDS-PAGE and immunoblotting.

Transfection studies

For the chloramphenicol acetyltransferase (CAT) assay, recombinant vectors containing C2 (pCAT-C2) or E3 (pCAT-E3) insulin receptor promoter sequence, both upstream from the bacterial CAT reporter gene, were transiently transfected into HepG2 cells and CAT activity assayed as described previously (32). Briefly, 16 h before transfection, HepG2 cells were plated in 60 mm dishes at a density of 5×10^5. Plasmid constructs were then transfected into cells by the calcium phosphate precipitation method (47). Forty-eight hours after transfection, cell extracts were prepared by three cycles of freezing-thawing, heated to 65°C for 10 min to inactivate endogenous deacetylases, then assayed for CAT activity with 0.25 mCi [^{14}C] chloramphenicol in a 125-μl reaction. Quantitation of CAT activity was done by liquid scintillation counting (LSC) of the two major acetylated forms of ^{14}C-labeled chloramphenicol, as described previously (32). As internal control of transfection efficiency, β-galactosidase activity was measured (41). For antisense HMGI(Y) experiments, C2- or E3-containing vectors were cotransfected into HepG2 cells with increasing concentrations of expression plasmid pcDNA1 containing the HMGI(Y) cDNA either in the sense (s) or antisense (as) orientation (48).

For ^{125}I-insulin binding studies, HepG2, HTC, and CHO cells were plated in 60 mm dishes and were ~50%-60% confluent at the time of transfection. The cells were refed with fresh media 3 h before transfection. Increasing concentrations of the expression plasmid containing the HMGI(Y) cDNA, either in the sense (s) or antisense (as) orientation, were then transfected into cells as described above. Seventy-two hours after transfection, the cells were washed three times with PBS and ^{125}I-labeled insulin binding was measured as described previously (4). IM-9 cells (4×10^6 cells per plate) were transfected by the DEAE dextran method (49) in 60 mm dishes using 5 μg of HMGI(Y) expression plasmid pcDNA1 or vector DNA and 1 μg of β-galactosidase control plasmid. The cells were harvested 72 h after transfection and ^{125}I-insulin binding was measured, as described previously (50).

RESULTS

Affinity purification of the IR-DBP

IM-9 human lymphocytes have been widely used for insulin receptor expression studies (7, 8). We have previously demonstrated that IR-DBP is readily expressed in these cells (32). Accordingly, nuclear extracts from this cell line were prepared and used for identification and purification of this nuclear binding protein. Initially, IM-9 nuclear extracts were loaded onto a heparin-Sepharose column and eluted with buffer containing increasing concentrations of NaCl (**Fig. 1*A***). DNA binding activity in salt-eluted fractions was characterized by EMSA. The bulk of DNA binding activity detectable with E3 probe eluted between 0.5 and 0.8 M NaCl. Identical results were obtained with the C2 probe (data not shown).

Next, fractions from the heparin-Sepharose column containing the protein(s) of interest were pooled and subjected to further purification with C2 or E3 site-specific DNA affinity column (Fig. 1*B*). In all preparations, sonicated calf thymus competitor DNA was combined with the sample and the DNA–protein mixture in

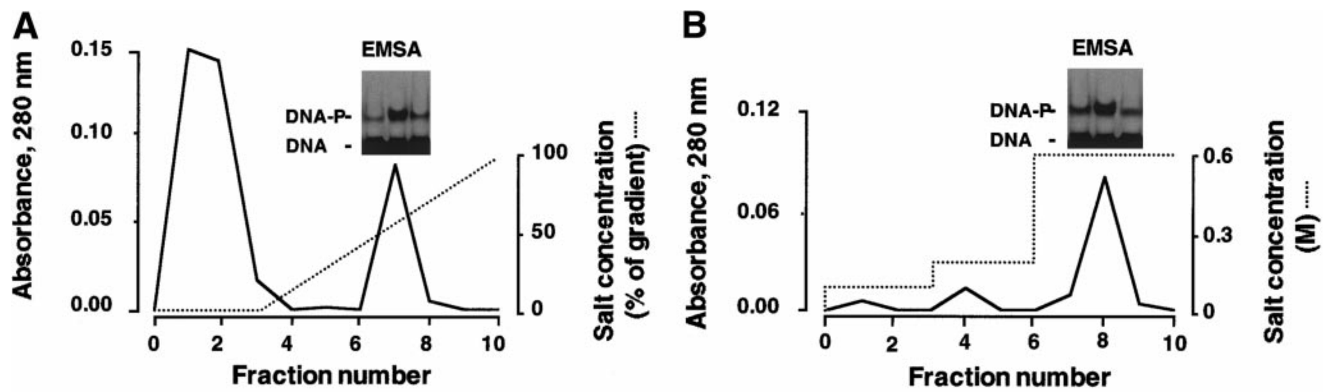

Figure 1. Purification of IR-DBP and SDS-PAGE. Profile of DNA binding activity eluted from heparin-Sepharose column (*A*) between 0.1 and 1.0 M NaCl (indicated as 0–100% of gradient) and from DNA affinity column (*B*) with a step gradient from 0.1 to 0.6 M NaCl. DNA binding activity in each fraction was assayed by EMSA, using 0.1 ng of E3 probe. Autoradiograms of DNA–protein complexes from active fractions are displayed in the inset. IM-9 affinity-purified IR-DBP was resolved by SDS-PAGE and visualized by silver staining (*C*). Lanes: M, molecular weight markers; 1, affinity column load; 2, affinity column flow-through; 3, 0.1 M NaCl eluate; 4, 0.2 M NaCl eluate; 5 and 6, 0.6 M NaCl eluate from C2- and E3-affinity columns, respectively. Molecular weight (mass) markers are labeled in kDa. The purified ~15 kDa protein species in lanes 5 and 6 is indicated by an arrowhead.

solution was applied to the affinity column (43). The protein–DNA mixture was eluted with steps of 0.1–0.6 M NaCl and binding activity was monitored by EMSA, in the absence of competitor DNA. The peak of sequence-specific DNA binding activity detected with E3 probe eluted at 0.6 M NaCl. Identical results were obtained with the C2 probe (data not shown).

Purification efficiency was analyzed by SDS-PAGE (Fig. 1*C*). An identical protein species with a mass of ~15 kDa was detected in silver-stained gel of active fractions from either C2- or E3-DNA affinity chromatography.

Because of the low molecular mass of affinity-purified IR-DBP (~15 kDa), in addition to its ability to specifically bind AT-rich regions of DNA, we investigated the relationship between the IR-DBP and HMGI(Y), a small basic protein that binds specifically to AT-rich sequences through the minor groove of DNA (35). Thus, DNA binding studies using the affinity-purified IR-DBP and the HMGI(Y) nuclear protein were conducted.

EMSA with affinity-purified IR-DBP and pure HMGI(Y)

Analyses of protein–DNA interactions with pure IR-DBP and HMGI(Y) with the positive-acting *cis* elements C2 and E3 of the insulin receptor promoter revealed similar electrophoretic migration characteristics (**Fig. 2**, lanes 2, 3, 8, 9). EMSA was performed using the well-characterized PRDII element of the β-interferon promoter as probe (42). It has been shown that HMGI(Y) protein binds specifically to this element (42, 48). Therefore, binding of IR-DBP and HMGI(Y) nuclear protein to PRDII was studied. As with C2 and E3 probes, binding of IR-DBP to PRDII probe produced a protein–DNA complex, whose mobility was identical to that of HMGI(Y) protein (Fig. 2, lanes 14, 15). Neither HMGI(Y) nor IR-DBP showed detectable retarded bands when a mutated PRDII element was used as probe (Fig. 2, lanes 17, 18). Similarly, no retarded bands were observed using either mutant C2 or E3

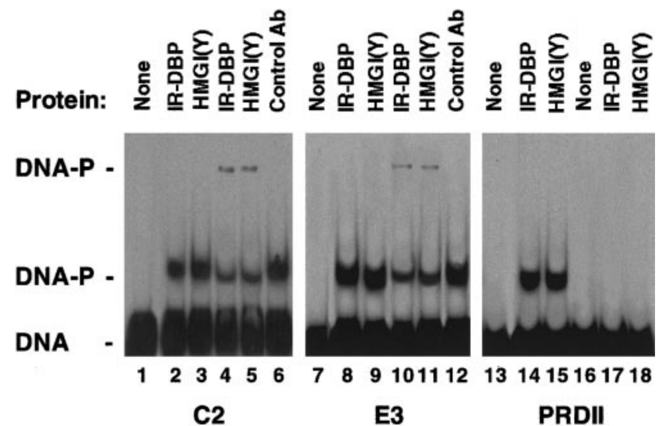

Figure 2. Comparison of C2, E3, and PRDII DNA binding activity to IR-DBP and pure recombinant HMGI(Y). Protein–DNA complexes were visualized by EMSA, using 0.1 ng of probe and 5 ng of each protein. Assays used no competitor DNA but included 2.0 μg of bovine serum albumin. In antibody supershift experiments, protein was preincubated with 0.5 μl of anti-HMGI(Y) antibody or control antibody before addition of the probe. Lanes: 1–6, C2 probe; 7–12, E3 probe; 13–15 wild-type PRDII probe; 16–18, mutated PRDII probe. Supershift assays are shown in lanes 4, 5, 10, 11. Dashes show the position of free (DNA) and bound (DNA-P) probe. A representative of three separate assays is shown.

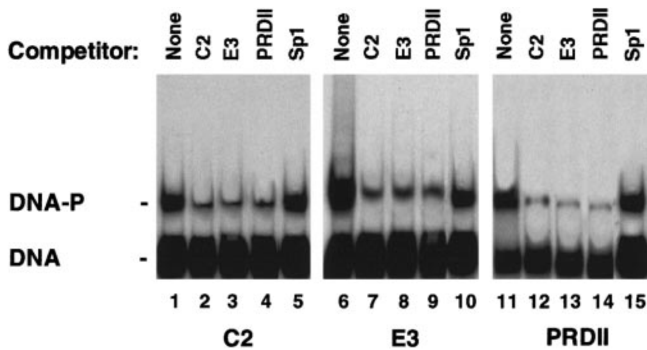

Figure 3. Specificity of DNA–protein binding. A 50-fold molar excess of either unlabeled C2, E3, PRDII, or Sp1 oligonucleotide was used in competition studies. Lanes: 1–5, C2 probe; 6–10, E3 probe; 11–15 PRDII probe. Dashes show the position of free (DNA) and bound (DNA-P) probe. A representative of three separate assays is shown.

probe (data not shown). To determine whether the IR-DBP is related to HMGI(Y), we carried out antibody supershift experiments with anti-HMGI(Y) antibody (46). As with pure HMGI(Y) protein, protein–DNA complexes from IR-DBP were recognized and supershifted to a slower migrating form by anti-HMGI(Y) antibody (Fig. 2, lanes 4, 5, 10, 11), indicating an antigenic similarity between HMGI(Y) and the IR-DBP detected with C2 and E3 site-specific DNA affinity columns. Control (unrelated rabbit serum IgG) antibody did not alter the mobility of the complex (Fig. 2, lanes 6, 12). Taken together, these results indicate that HMGI(Y) and IR-DBP are highly related proteins.

To determine the specificity of DNA–protein binding, competition assays were performed. A 50-fold molar excess of either unlabeled C2, E3, or PRDII oligonucleotide significantly reduced the binding of labeled C2, E3, or PRDII to DNA binding proteins, respectively (**Fig. 3**). Moreover, either unlabeled C2 or unlabeled E3 competitively inhibited the binding of PRDII to nuclear proteins. In concert with this observation, unlabeled PRDII inhibited the binding of labeled C2 or E3 to nuclear proteins (Fig. 3). A 50-fold molar excess of an unrelated oligonucleotide containing the consensus binding site for the transcription factor Sp1 did not compete for binding of nuclear proteins to oligonucleotides C2, E3, and PRDII (Fig. 3).

Western blot analysis of affinity-purified IR-DBP and pure HMGI(Y)

Direct evidence that the HMGI(Y) nuclear protein was responsible for the sequence-specific insulin receptor DNA binding activity was obtained by screening Western blots with DNA and antibody probes. For Southwestern blotting analysis, two equivalent transfers were probed with either labeled C2 or E3 element of the insulin receptor gene. **Figure 4*A*** shows the result obtained with the E3 probe, using crude (lane 1) and IM-9 affinity-purified (lane 2) nuclear protein and pure HMGI(Y) nuclear protein (lane 3). A major band labeled DNA-P was observed in all three lanes. An identical result was obtained with C2 probe (data not shown). The same transfers were then probed with anti-HMGI(Y) antibody. A ~15 kDa protein band corresponding to the species DNA-P detected with DNA probe was observed in each lane (Fig. 4*B*, lanes 4–6), indicating again an antigenic similarity between HMGI(Y) and the IR-DBP detected within C2 and E3 complexes.

These observations were supported further by tests of immunoprecipitation and immunodepletion, showing that the ~15 kDa IR-DBP detected in nuclear extracts from IM-9 cells and the HMGI(Y) nuclear factor did exhibit identical immunological properties. Figure 4*C* shows a Western blot analysis of C2 and E3 affinity-purified IR-DBP after immunoprecipitation and immunodepletion using anti-HMGI(Y) antibody. The ~15 kDa protein detected with C2 (lane 7) and E3 (lane 8) comigrated with pure recombinant HMGI(Y) protein (lane 13). Control antibody failed to precipitate the same protein species. In this latter case, an identical ~15 kDa protein species was detected in the supernatant of the same fractions (lanes 9 and 10), whereas it was barely detectable in the supernatant of fractions after depletion with anti-HMGI(Y) antibody (lanes 11 and 12).

Inhibition of CAT activity by antisense HMGI(Y) RNA

The results of DNA binding studies and Western blot analyses presented above indicate that the ~15 kDa

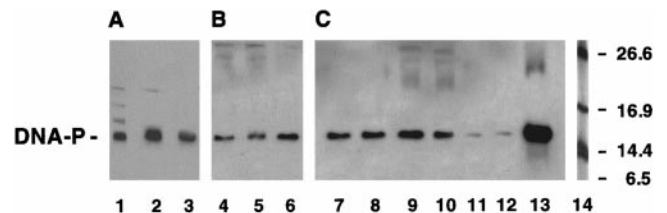

Figure 4. Analysis of the IR-DBP and HMGI(Y) by Western transfers. 20 μg of IM-9 cell nuclear extracts and ~200 ng of either affinity-purified IR-DBP or pure HMGI(Y) protein were resolved by SDS-PAGE, transferred to PVDF membranes, and detected by DNA and antibody probes. *A)* Southwestern analysis. Lanes: 1, crude IM-9 nuclear protein; 2, IM-9 affinity-purified IR-DBP; 3, pure HMGI(Y) protein. The largest band detected in all three lanes is labeled DNA-P. *B)* Western blot analysis. The ~15 kDa protein species detected in all three lanes comigrated with the largest DNA-P band detected with DNA probe. Lanes: 4, crude IM-9 nuclear protein; 5, IM-9 affinity-purified IR-DBP; 6, pure HMGI(Y) protein. *C)* Western blot of the immunoprecipitated and immunodepleted fractions produced using anti-HMGI(Y) antibody. Lanes: 7, 8, C2 and E3 affinity-purified IR-DBP, respectively; 9, 10, supernatant of the same fractions after depletion with control (unrelated rabbit serum IgG) antibody; 11, 12, supernatant of the same fractions after depletion with anti-HMGI(Y) antibody. Lane 13 is a Western blot of pure HMGI(Y) protein. The positions of molecular weight markers are indicated in lane 14.

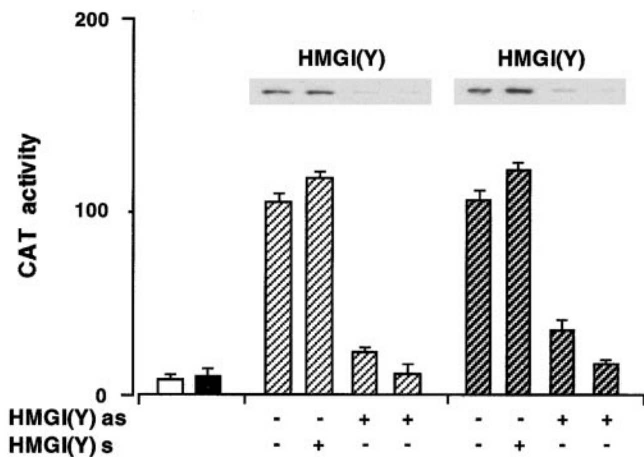

Figure 5. Inhibition of the insulin receptor promoter by antisense HMGI(Y). HepG2 cells were cotransfected with CAT reporter plasmids (2 μg) containing C2 (pCAT-C2) or E3 (pCAT-E3) insulin receptor promoter sequences and effector plasmids containing the HMGI(Y) cDNA either in the sense (s) (2 μg) or antisense (as) (0, 1, 2 μg) orientation. CAT activity is expressed as a percentage of the reporter activity in the absence of HMGI(Y) cDNA-containing plasmids. □, mock: no DNA; ■, pCAT-Basic: vector without an insert; ▨, pCAT-C2; ▨, pCAT-E3. Results are the mean ± SE for three separate transfections. Western blots of HMGI(Y) in each condition are shown in the autoradiograms.

IR-DBP and the HMGI(Y) nuclear protein are highly related, or identical, proteins. To test whether HMGI(Y) interacted with and regulated insulin receptor promoter function in intact cells, the ability of both C2 and E3 to drive the expression of the CAT gene was measured in HepG2 human hepatoma cells in which the expression of the HMGI(Y) protein was inhibited by antisense RNA (32, 48, 51). Increasing concentrations of the HMGI(Y) antisense expression plasmid inhibited the activity of C2 and E3 promoter elements as judged by the decrease in CAT activity in transfected cells (**Fig. 5**). Inhibition by the HMGI(Y) antisense plasmid was specific, since no inhibition of CAT activity was observed when C2- or E3-containing vectors were transfected into cells in the presence of expression vector containing HMGI(Y) cDNA in the sense orientation (Fig. 5). To ensure that inhibition by HMGI(Y) antisense plasmid was not related to a nonspecific effect, we transfected the pCAT-Promoter vector, a positive control having the simian virus 40 gene promoter upstream of the CAT coding region, into HepG2 and exposed the cells to HMGI(Y) antisense expression plasmid. No inhibition of this CAT reporter plasmid was observed (data not shown). As shown by Western blot analysis of cellular protein from HepG2 transfected cells, CAT activity correlated with HMGI(Y) expression levels (Fig. 5).

Insulin binding studies

The above results strongly suggest that HMGI(Y) plays an essential role in the regulation of the human insulin receptor gene. We obtained direct evidence for this conclusion by measuring ^{125}I-insulin binding to HepG2 and IM-9 cells, two cell lines with relatively high insulin receptor expression levels (32). Exposure of these cells to the HMGI(Y) antisense expression plasmid inhibited cell surface expression of the insulin receptor in a dose-dependent manner (**Fig. 6**). ^{125}I-labeled insulin binding to HepG2 and IM-9 transfected cells was decreased to ~30% and ~35% of untransfected control cells, respectively. The decrease in ^{125}I-labeled insulin binding paralleled the decrease in C2 and E3 promoter activity observed in HepG2 cells and was specific, since no inhibition of insulin binding was observed in cells transfected with HMGI(Y) cDNA in the sense orientation.

Next, we carried out transfection experiments in HTC rat hepatoma cells and CHO cells, two cell lines with relatively low insulin receptor expression (39, 40). Transfection of these cells with the expression vector containing HMGI(Y) cDNA in the sense orientation was followed by a six- to eightfold increase in specific ^{125}I-insulin binding (**Fig. 7**). Transfection efficiency was normalized to the expression of β-galactosidase from a separate reporter plasmid. In contrast, transfection of the antisense HMGI(Y) expression plasmid had no effect on cell surface expression of the insulin receptor (Fig. 7). These experiments provide direct evidence that HMGI(Y) protein is required for proper expression of the insulin receptor. Consistent with this, ^{125}I-labeled insulin binding to these cells correlated with HMGI(Y) protein expression, as shown by Western blot

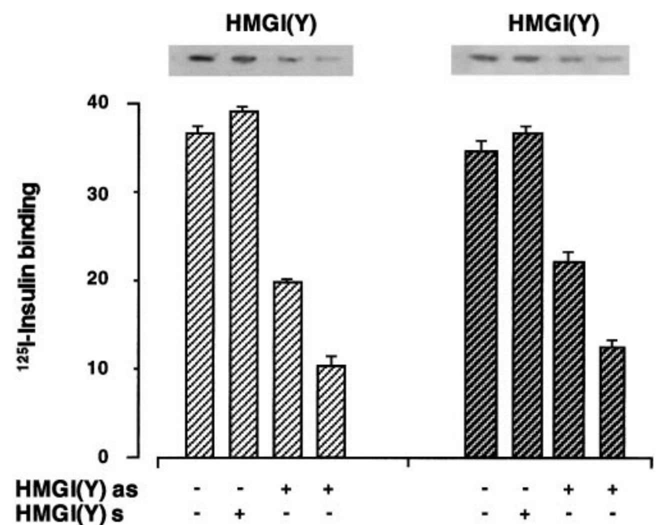

Figure 6. Effects of HMGI(Y) antisense on ^{125}I-labeled insulin binding to HepG2 and IM-9 cells. Effector plasmids containing the HMGI(Y) cDNA, either in the sense (5 μg) or antisense (0, 2.5, 5 μg) orientation, were transfected into HepG2 and IM-9 cells and specific ^{125}I-labeled insulin binding was measured 72 h after transfection. Binding is expressed as percentage of total/10^7 cells. Binding activity in the absence of HMGI(Y) cDNA-containing plasmids refers to untransfected control cells. ▨, HepG2 cells; ▨, IM-9 cells. Results are the mean ± SE for three separate experiments. Western blots of HMGI(Y) in each condition are shown in the autoradiograms.

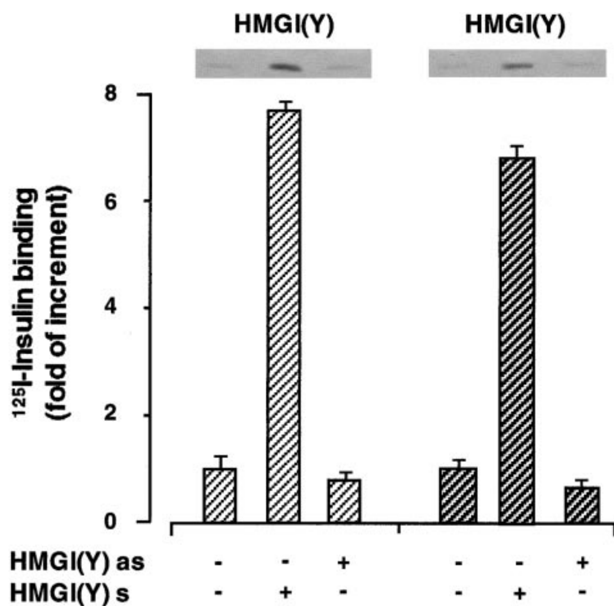

Figure 7. Effects of HMGI(Y) expression vector on ^{125}I-labeled insulin binding to HTC and CHO cells. Effector plasmids containing the HMGI(Y) cDNA, either in the sense (5 μg) or antisense (5 μg) orientation, were transfected into HTC and CHO cells and specific ^{125}I-labeled insulin binding was measured 72 h after transfection. Specific ^{125}I-insulin binding is expressed as fold of increment from the binding activity of control, vehicle-treated cells (cells transfected with expression vectors lacking the HMGI(Y) cDNA). Results are the mean ± SE for three separate experiments. Western blots of HMGI(Y) in each condition are shown in the autoradiograms.

analysis of cellular protein from either IM-9, HepG2, HTC, or CHO transfected cells (Figs. 6, 7).

DISCUSSION

DNA binding proteins recognize their DNA targets not only through the formation of specific contacts with the nucleotide bases, but also through inherent properties of the DNA sequence, including increased bendability and rigidity (52, 53). Several lines of evidence suggest that AT-rich sequences of DNA constitute a robust nucleation site that promotes transcription initiation in the absence of conventional promoter elements (54–58). It has been proposed that transcription activation at this site may be due to structural perturbation, which may result in bending of the DNA that promote the interaction between transcription factors and the basal transcriptional machinery (48). DNA binding protein interactions with AT-rich regions of promoters have been reported in mammalian genes (36, 59, 60).

We have previously identified two closely related DNA nuclear binding proteins that specifically interact with two unique functional AT-rich sequences of the 5′ regulatory region of the insulin receptor gene (32). In this report, the identities of both nuclear proteins have been determined. Analysis of protein–DNA interactions with the positive-acting *cis* elements, C2 and E3, combined with Western and Southwestern blotting analyses, identified a unique DNA binding protein highly related to the HMG family, which is composed of abundant and highly conserved nuclear proteins that are thought to play important roles in the transcriptional regulation of mammalian genes (35). An important member of this family is HMGI(Y), which refers to two proteins, HMGI and HMGY, closely related splice variants of the same gene (61). HMGI(Y) is an architectural transcription factor that binds to AT-rich regions in the minor groove of DNA via short basic domains, termed AT hooks, and functions mainly as a specific cofactor for gene activation (35, 48). HMGI(Y) by itself has no intrinsic transcriptional activity; rather, it has been shown to *trans*-activate promoters through mechanisms that facilitate the assembly and stability of stereospecific DNA–protein complexes (enhanceosomes) that drive gene transcription (35, 48). HMGI(Y) performs this task by modifying DNA conformation and by recruiting transcription factors to the transcription start site, facilitating DNA–protein and protein–protein interactions (35, 48).

Our data on molecular and biochemical characterization show unequivocally that the identified IR-DBP displays many properties common to HMG protein family. First, EMSA (using IM-9 affinity-purified IR-DBP) showed the same electrophoretic migration characteristics of pure recombinant HMGI(Y) protein with either the C2 or E3 element of the insulin receptor gene. Second, a similar behavior was observed when EMSA was performed using the PRDII element of the β-interferon promoter as probe. Third, antibody directed against HMGI(Y) significantly reduced the formation of DNA–protein complexes on the C2 and E3 oligonucleotides, suggesting that a protein antigenically related to HMGI(Y) binds to insulin receptor promoter *in vitro*.

The above findings were supported by Western and Southwestern blotting analyses. Anti-HMGI(Y) antibody specifically recognized a major protein species with mass of ~15 kDa in both unpurified and IM-9 affinity-purified nuclear protein. As in protein–DNA interaction analysis, in Western blot analysis this immunoreactive protein exhibited identical electrophoretic and immunological properties as recombinant HMGI(Y) protein. In addition, Southwestern studies revealed that the ~15 kDa protein species present in IM-9 cells binds either probe C2 or E3, as does recombinant HMGI(Y).

We have shown before that both C2 and E3 sequences of the regulatory region of the insulin receptor gene have functional activity and act as promoter elements driving transcription of the CAT reporter gene when transfected into mammalian cells (32). This observation agrees closely with the results reported in a previous work, in which deletional analysis of the human insulin receptor promoter confirms the contribution of C2- and E3-containing regions to overall promoter activity (29). Here, direct evidence of

HMGI(Y) insulin receptor promoter interactions is provided by reporter gene analysis with the CAT reporter gene and by hormone binding studies in cells in which the expression of the HMGI(Y) protein was inhibited by antisense RNA. We have shown that antisense HMGI(Y) RNA significantly decreased the level of C2 and E3 promoter function in intact cells, indicating that HMGI(Y) protein is required for proper transcription of insulin receptor gene. This result is consistent with our previous published data indicating that the ability of C2 and E3 to drive transcription in intact cells was almost completely abolished by preventing the binding of nuclear proteins to AT-rich sequences of DNA using the synthetic polynucleotide poly(dA-dT) (32). In concert with these findings, we have also observed that in HepG2 hepatocytes and IM-9 lymphocytes, two cell lines with relatively high insulin receptor expression, transfection of antisense HMGI(Y) significantly decreases insulin receptor binding. In contrast, in HTC and CHO cells, two cell lines with relatively low insulin receptor content, overexpression of HMGI(Y) significantly increases cell surface expression of the insulin receptor.

Taken together, these observations strongly support the conclusion that the identified nuclear binding protein for the 5′ regulatory region of the human insulin receptor gene is identical to the HMG protein, HMGI(Y). To our knowledge, the present finding that HMGI(Y) is specifically required for efficient expression of the insulin receptor gene, constitutes the first demonstration revealing the potential of this protein to modulate insulin receptor gene promoter. Studies are in progress to better define the role of HMGI(Y) during transcriptional regulation of the insulin receptor gene. We have preliminary evidence suggesting that this protein may act on the insulin receptor promoter as an architectural factor necessary for the formation of a transcriptionally active multiprotein–DNA complex involving the Sp1 nuclear transcription factor. A similar mechanism has already been described for the transcriptional activation of the human interferon-β gene (62). Thus, these studies add further insight into the molecular mechanisms that control insulin receptor gene expression. Moreover, they provide a paradigm to suggest another type of molecule, HMGI(Y), which should be searched for in investigations designed to evaluate the causes of impairment of insulin receptor function and/or expression. This observation has relevance to the pathophysiology of insulin-resistant syndromes and other pathological states in humans. FJ

We are most grateful to Dr. T. Maniatis for his generous gift of HMGI(Y) expression plasmids, pcDNA1. A very special thank to Drs. L. Levintow and V. Giancotti for helpful discussions and for critical reading of the manuscript. Sincere thanks to Dr. S. Filetti for his support. We also acknowledge helpful discussions with Drs. F. Trapasso and R. Iuliano. Thanks go to Mrs. A. Malta for secretarial help. The financial support of Telethon-Italy (Grant no. E.613) is gratefully acknowledged.

REFERENCES

1. Kahn, C. R. (1985) The molecular mechanisms of insulin action. *Annu. Rev. Med.* **36,** 429–451
2. Goldfine, I. D. (1987) The insulin receptor: molecular biology and transmembrane signalling. *Endocr. Rev.* **8,** 235–255
3. White, M. F., and Kahn, C. R. (1994) The insulin signaling system. *J. Biol. Chem.* **269,** 1–4
4. Brunetti, A., Maddux, B. A., Wong, K. Y., and Goldfine, I. D. (1989) Muscle cell differentiation is associated with increased insulin receptor biosynthesis and messenger RNA levels. *J. Clin. Invest.* **86,** 192–198
5. Brunetti, A., and Goldfine, I. D. (1990) Differential effects of fibroblast growth factor on insulin receptor and muscle specific protein gene expression in BC3H-1 myocytes. *Mol. Endocrinol.* **4,** 880–885
6. Mamula, P. W., McDonald, A. R., Brunetti, A., Okabayashi, Y., Wong, K. Y., Maddux, B. A., Logsdon, C., and Goldfine, I. D. (1990) Regulating insulin-receptor-gene expression by differentiation and hormones. *Diabetes Care* **13,** 288–301
7. McDonald, A. R., and Goldfine, I. D. (1988) Glucocorticoid regulation of insulin receptor gene transcription in IM-9 cultured lymphocytes. *J. Clin. Invest.* **81,** 499–504
8. Rouiller, D. G., McElduff, A., Hedo, J. A., and Gorden, P. (1985) Induction of the insulin proreceptor by hydrocortisone in cultured lymphocytes (IM-9 line). *J. Clin. Invest.* **76,** 645–649
9. Sibley, E., Kastelic, T., Kelly, T. J., and Lane, M. D. (1989) Characterization of the mouse insulin receptor gene promoter. *Proc. Natl. Acad. Sci. USA* **86,** 9732–9736
10. Standaert, M. L., Schimmel, S. D., and Pollet, R. J. (1984) The development of insulin receptors and responses in the differentiating nonfusing muscle cell line BC3H-1. *J. Biol. Chem.* **259,** 2337–2345
11. Petruzzelli, L., Herrera, R., Arenas Garcia, R., Fernandez, R., Birnbaum, M. J., and Rosen, O. M. (1986) Isolation of a *Drosophila* genomic sequence homologous to the kinase domain of the human insulin receptor and detection of the phosphorylated *Drosophila* receptor with an anti-peptide antibody. *Proc. Natl. Acad. Sci. USA* **83,** 4710–4714
12. Olefsky, J. M. (1980) Insulin resistance and insulin action. An in vitro and in vivo perspective. *Diabetes* **30,** 148–162
13. Taylor, S. I. (1992) Lilly lecture: molecular mechanisms of insulin resistance. Lesson from patients with mutations in the insulin-receptor gene. *Diabetes* **41,** 1473–1490
14. Polonsky, K. S., Sturis, J., and Bell, G. I. (1996) Non-insulin-dependent diabetes mellitus: a genetically programmed failure of the beta cell to compensate for insulin resistance. *N. Engl. J. Med.* **334,** 777–783
15. Taylor, S. I. (1999) Deconstructing type 2 diabetes. *Cell* **97,** 9–12
16. Virkamäki, A., Ueki, K., and Kahn, C. R. (1999) Protein–protein interaction in insulin signaling and the molecular mechanisms of insulin resistance. *J. Clin. Invest.* **103,** 931–943
17. Longo, V. D. (1999) Mutations in signal transduction proteins increase stress resistance and longevity in yeast, nematodes, fruit flies, and mammalian neuronal cells. *Neurobiol. Aging* **20,** 479–486
18. Hoyer, D., and Lannet, H. (1999) Inhibition of the neuronal insulin receptor causes Alzheimer-like disturbances in oxidative/energy brain metabolism and in behavior in adult rats. *Ann. N.Y. Acad. Sci.* **893,** 301–303
19. Papa, V., Pezzino, V., Costantino, A., Belfiore, A., Giuffrida, D., Frittitta, L., Vannelli, G. B., Brand, R., Goldfine, I. D., and Vigneri, R. (1990) Elevated insulin receptor content in human breast cancer. *J. Clin. Invest.* **86,** 1503–1510
20. Webster, N. J., Resnik, J. L., Reichart, D. B., Strauss, B., Haas, M., and Seely, B. L. (1996) Repression of the insulin receptor promoter by the tumor suppressor gene product p53: a possible mechanism for receptor overexpression in breast cancer. *Cancer Res.* **56,** 2781–2788
21. Bergmann, U., Funatomi, H., Kornmann, M., Beger, H. G., and Korc, M. (1996) Increased expression of insulin receptor substrate-1 in human pancreatic cancer. *Biochem. Biophys. Res. Commun.* **220,** 886–890
22. Amfo, K., Neyns, B., Teugels, E., Lissens, W., Bourgain, C., De Sutter, P., Vandamme, B., Vamos, E., and De Greve, J. (1995) Frequent deletion of chromosome 19 and a rare rearrangement

of 19p13.3 involving the insulin receptor gene in human ovarian cancer. *Oncogene* **11,** 351–358
23. Yamamoto, K. R. (1985) Steroid receptor regulated transcription of specific genes and gene networks. *Annu. Rev. Genet.* **19,** 209–215
24. Schleif, R. (1988) DNA binding by proteins. *Science* **241,** 1182–1187
25. Mitchell, P. J., and Tjian, R. (1989) Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* **245,** 371–378
26. Araki, E., Shimada, F., Uzawa, H., Mori, M., and Ebina, Y. (1987) Characterization of the promoter region of the human insulin receptor gene. *J. Biol. Chem.* **262,** 16186–16191
27. Mamula, P. W., Wong, K. Y., Maddux, B. A., McDonald, A. R., and Goldfine, I. D. (1989) Sequence and analysis of promoter region of human insulin-receptor gene. *Diabetes* **37,** 1241–1246
28. Seino, S., Seino, M., Nishi, S., and Bell, G. I. (1989) Structure of the human insulin receptor gene and characterization of its promoter. *Proc. Natl. Acad. Sci. USA* **86,** 114–118
29. Tewari, D. S., Cook, D. M., and Taub, R. (1989) Characterization of the promoter region and 3′-end of the human insulin receptor gene. *J. Biol. Chem.* **264,** 16238–16245
30. McKeon, C., Moncada, V., Pham, T., Salvatore, P., Kadowaki, T., Accili, D., and Taylor, S. I. (1990) Structural and functional analysis of the insulin receptor promoter. *Mol. Endocrinol.* **4,** 647–656
31. Lee, J. K., Tam, J. W. O., Tsai, M. J., and Tsai, S. Y. (1992) Identification of cis- and trans-acting factors regulating the expression of the human insulin receptor gene. *J. Biol. Chem.* **267,** 4638–4645
32. Brunetti, A., Foti, D., and Goldfine, I. D. (1993) Identification of unique nuclear regulatory proteins for the insulin receptor gene, which appear during myocyte and adipocyte differentiation. *J. Clin. Invest.* **92,** 1288–1295
33. Levy, J. R., and Hug, V. (1993) Nuclear protein-binding analysis of a GC-rich insulin-receptor promoter regulatory region. *Diabetes* **42,** 66–73
34. Webster, N. J. G., Kong, Y., Cameron, K. E., and Resnik, J. L. (1994) An upstream element from the human insulin receptor gene promoter contains binding sites for C/EBPb and NF-1. *Diabetes* **43,** 305–312
35. Bustin, M., and Reeves, R. (1996) High-mobility group proteins: architectural components that facilitates chromatin function. *Prog. Nucleic Acids Res.* **54,** 35–100
36. Pellecani, A., Chin, M. T., Wiesel, P., Ibanez, M., Patel, A., Yet, S-F., Hsieh C-M, Paulauskis, J. D., Reeves, R., Lee, M.-E., and Perrella, M. A. (1999) Induction of high mobility group-I(Y) protein by endotoxin and interleukin-1β in vascular smooth muscle cells. *J. Biol. Chem.* **274,** 1525–1532
37. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Accurate transcription by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11,** 1475–1489
38. Podskalny, J. M., Takeda, S., Silverman, R. E., Tran, D., Carpentier, J. L., Orci, L., and Gorden, P. (1985) Insulin receptors and bioresponses in a human liver cell line (HepG2). *Eur. J. Biochem.* **150,** 401–407
39. Hawley, D., Maddux, B. A., Patel, R. G., Wong, K. Y., Mamula, P. W., Firestone, G. L., Brunetti, A., Verspohl, E., and Goldfine, I. D. (1989) Insulin receptor monoclonal antibodies that mimic insulin action without activating tyrosine kinase. *J. Biol. Chem.* **264,** 2438–2444
40. Sung, C. K., Han, X-L, Brunetti, A., Maddux, B., Yamamoto-Honda, R., and Goldfine, I. D. (1992) Regulation of biological functions by an insulin receptor monoclonal antibody in insulin receptor β-subunit mutants. *Biochemistry* **31,** 168–174
41. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, pp. 9.31–9.58, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
42. Mantovani, F., Covaceuszach, S., Rustighi, A., Sgarra, R., Heath, C., Goodwin, G. H., and Manfioletti, G. (1998) NF-κB mediated transcriptional activation is enhanced by the architectural factor HMGI-C. *Nucleic Acids Res.* **26,** 1433–1439
43. Kadonaga, J. T., and Tjian, R. (1986) Affinity purification of sequence-specific DNA binding proteins. *Proc. Natl. Acad. Sci. USA* **83,** 5889–5893
44. Schagger, H., and Von Jagow, G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166,** 368–379
45. Celenza, J. L., and Carlson, M. (1986) A yeast gene that is essential for release from glucose repression encodes a protein kinase. *Science* **233,** 1175–1180
46. Chiappetta, G., Bandiera, A., Berlingeri, M. T., Visconti, R., Manfioletti, G., Battista, S., Martinez-Tello, F. J., Santoro, M., Giancotti, V., and Fusco, A. (1995) Expression of the high mobility group HMGI(Y) proteins correlates with the malignant phenotype of human thyroid neoplasias. *Oncogene* **10,** 1307–1314
47. Gorman, C., Moffat, L., and Howard, B. (1982) Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* **2,** 1044–1051
48. Thanos, D., and Maniatis, T. (1992) The high mobility group protein HMG I(Y) is required for NF-κB-dependent virus induction of the human IFN-β gene. *Cell* **71,** 777–789
49. Queen, C., and Baltimore, D. (1983) Immunoglobulin gene transcription is activated by downstream sequence elements. *Cell* **33,** 741–748
50. Trischitta, V., Wong, K. Y., Brunetti, A., Scalisi, R., Vigneri, R., and Goldfine, I. D. (1989) Endocytosis, recycling, and degradation of the insulin receptor. *J. Biol. Chem.* **264,** 5041–5046
51. Brunetti, A., and Goldfine, I. D. (1990) Role of myogenin in myoblast differentiation and its regulation by fibroblast growth factor. *J. Biol. Chem.* **265,** 5960–5963
52. Bianchi, M. E., Beltrame, M., and Paonessa, G. (1989) Specific recognition of cruciform DNA by nuclear protein HMG1. *Science* **243,** 1056–1059
53. Allemann, R. K., and Egli, M. (1997) DNA recognition and bending. *Chem. Biol.* **4,** 643–650
54. Shain, D. H., Zuber, M. X., and Neuman, T. (1998) Transcription initiation from a poly(dA) tract. *Nucleic Acids Res.* **26,** 1019–1025
55. Russel, D. W., Smith, M., Cox, D., Williamson, V. M., and Young, E. F. (1983) DNA sequences of two yeast promoter-up mutants. *Nature (London)* **304,** 652–654
56. Struhl, K. (1985) Naturally occurring poly(dA-dT) sequences are upstream promoter elements for constitutive transcription in yeast. *Proc. Natl. Acad. Sci. USA* **82,** 8419–8423
57. Nelson, H. C. M., Finch, J. T., Luisi, B. F., and Klug, A. (1987) The structure of an oligo(dA) oligo (dT) tract and its biological implications. *Nature (London)* **330,** 221–226
58. Gartenberg, M. R., and Crothers, D. M. (1991) Synthetic DNA bending sequences increase the rate of in vitro transcription initiation at the *Escherichia coli lac* promoter. *J. Mol. Biol.* **219,** 217–230
59. Bodner, M., Castrillo, J.-L., Theill, L. E., Deerinck, T., Ellisman, M., and Karin, M. (1988) The pituitary-specific transcription factor GHF-1 is a homeobox-containing protein. *Cell* **55,** 505–518
60. Fox, S. R., Jong, M. T. C., Casanova, J., Ye, Z-S., Stanley, F., and Samuels, H. H. (1990) The homeodomain protein, Pit-1/GHF-1, is capable of binding to and activating cell-specific elements of both the growth hormone and prolactin gene promoters. *Mol. Endocrinol.* **4,** 1069–1080
61. Johnson, K. R., Lehn, D. A., and Reeves, R. (1989) Alternative processing of mRNA's coding for human HMGI and HMGY proteins. *Mol. Cell. Biol.* **9,** 2114–2123
62. Thanos, D., and Maniatis, T. (1995) Virus induction of human IFNβ gene expression requires the assembly of an enhanceosome. *Cell* **83,** 1091–1100

Received for publication April 12, 2000.
Accepted for publication July 27, 2000.