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An insulin response element in the glyceraldehyde-3-phosphate dehydrogenase gene binds a nuclear protein induced by insulin in cultured cells and by nutritional manipulations *in vivo*

(gene transcription/trans-acting factor)

NARGIS NASRIN, LOUIS ERCOLANI*, MARIA DENARO, X. F. KONG, INWHA KANG, AND MARIA ALEXANDER†

Howard Hughes Medical Institute, Diabetes Unit and Medical Services, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114

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ABSTRACT **Two independent cis-acting insulin response elements (IREs) in the gene encoding glyceraldehyde-3-phosphate dehydrogenase [D-glyceraldehyde-3-phosphate: NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12], designated IRE-A and IRE-B, are sufficient to direct insulin-inducible gene expression. Using the electrophoretic mobility shift assay, a 4-fold increase in the amount of IRE-A DNA bound to nuclear proteins was detected when extracts isolated from insulin-stimulated differentiated 3T3-L1 cells or from the liver of rats refed a high-carbohydrate/low-fat diet after a 72-hr fast were compared to control nuclear extracts. The points of contact between protein and IRE-A DNA may represent a sequence recognized by at least one class of insulin-sensitive transcription factor(s).**

The interaction of insulin with its cell surface receptor initiates changes in the activity and cellular content of metabolic enzymes that promote energy storage and cell growth. Although many genes have been described recently to be regulated by insulin at the level of transcription, the molecular mechanism by which insulin mediates its effects is unknown. In an ongoing attempt to elucidate the signal transduction pathway of insulin action on gene expression, we have attempted to define cis-acting sequences that mediate the effect of insulin on gene transcription and work backward to define the mechanism by which the trans-acting factors that interact with these sequences are regulated.

We previously isolated an insulin-responsive gene for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1) and showed that the expression of a hybrid reporter gene containing bases −488 to +21 of the human gene for GAPDH and the coding region of the gene encoding chloramphenicol acetyltransferase (CAT), designated plasmid HGAPDH-CAT, was increased 3-fold in insulin-treated differentiated 3T3-L1 cells and 5-fold in H35 hepatoma cells that were stably transfected with this hybrid gene (2). An increase in HGAPDH-CAT mRNA was detected within 4 hr after insulin exposure and was maintained overnight, a pattern that parallels the time course of GAPDH mRNA induction (3).

These results indicated that the human gene for GAPDH contained a cis-acting element capable of conferring insulin inducibility to a marker gene. As it appears that binding of cellular transcription factors to inducible cis-acting elements is required for function, we used the gel shift assay to detect nuclear factors that bind this element in a specific manner. This paper describes the regulation of an insulin-inducible factor detected *in vitro* with the differentiated 3T3-L1 cultured cell line and *in vivo* in the liver of fasted rats refed a high-carbohydrate/low-fat diet, a model in which circulating insulin levels are high and many metabolic enzymes required to promote energy storage are induced.

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MATERIALS AND METHODS

Cell Culture. H35 hepatoma cells were obtained from John Koontz (University of Tennessee).

Differentiated 3T3-L1 cells were exposed to medium containing 1% fetal bovine serum buffered with 10 mM Hepes (pH 7.4) ≈16 hr prior to isolation of nuclear extracts as described (3).

Cellular Transfection Assays. H35 hepatoma cells were transfected by using the calcium phosphate-mediated DNA transfer procedure as described (2, 4, 5; see Fig. 2 and Table 1 legends for details).

Plasmids and Probes. BAL-31 deletion mutants of the plasmid HGAPDH-CAT (2), which contains nucleotides −488 to +21 of the human GAPDH gene promoter (see Fig. 1), were analyzed by sequencing to ascertain the extent of the 5′ deletions. A synthetic oligonucleotide that contained nucleotides −480 to −435 of the human GAPDH gene, AAGTTCCCCAACTTTCCCGCCTCTCAGCCTTTGAAAGAAAGAAAGG, referred to as the insulin response element A (IRE-A), was subcloned into the *Hin*dIII site of a truncated HGAPDH-CAT plasmid that contained nucleotides −268 to +21 of the human GAPDH gene promoter and was not regulated by insulin.

Extraction of Nuclear Proteins and Binding Assay. Nuclear extracts were isolated from untreated differentiated 3T3-L1 cells or cells exposed to insulin for 1 hr by a modification of the procedure of Dignam *et al.* (6). Differentiated 3T3-L1 cells were lysed in buffer A (10 mM Hepes, pH 7.9 at 4°C/1.5 mM $MgCl_2$/10 mM KCl/0.5 mM dithiothreitol/20 μM leupeptin/20 μM pepstatin/100 kallikrein units of Trasylol per ml/50 mM NaF/2 mM sodium vanadate/0.5 mM phenylmethylsulfonyl fluoride (PMSF). The nuclear extraction buffer contained 20 mM Hepes (pH 7.9), 25% (vol/vol) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20 μM leupeptin, 20 μM pepstatin, 100 kallikrein units of Trasylol per ml, 50 mM NaF, 2 mM sodium vanadate, and 0.5 mM PMSF. NaF and sodium vanadate were included to inhibit endogenous phosphatase activity, but these additions did not appear to be critical. In some experiments, the dialyzed differentiated 3T3-L1 nuclear extracts were concentrated by precipitation with polyethylene glycol and resuspended in Buffer D (20

Abbreviations: IRE, insulin response element; CAT, chloramphenicol acetyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RSV, Rous sarcoma virus; SRE, serum regulatory element; GH, growth hormone.

*Present address: Renal Unit, Massachusetts General Hospital, Boston, MA 02114.

†To whom reprint requests should be addressed at: Wellman 306, Blossom Street, Howard Hughes Medical Institute, Massachusetts General Hospital, Boston, MA 02114.

mM Hepes/20% glycerol/0.1 M KCl/0.2 mM EDTA/0.5 mM PMSF/0.5 mM dithiothreitol/0.02 mM leupeptin/0.02 mM pepstatin/0.02 mM Trasylol/0.02 mM diisopropyl fluorophosphate. Nuclear extracts were isolated from rats fasted for 48–72 hr or rats fasted and then refed a high-carbohydrate/low-fat diet exactly as described by Gorski *et al.* (7); no additional proteolysis or phosphatase inhibitors were used. Protein concentration was determined by the Bio-Rad protein assay.

Nuclear extract (4 μg) was incubated at room temperature for 30 min with 0.5–1 ng of ^{32}P-labeled IRE-A in 20 μl of binding buffer containing 20 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA, 20% glycerol, 0.5 mM PMSF, 2 μg of poly(dI)·poly(dC), and 1 μg of pUC DNA. When the incubation was complete, separation of the bound complexes from free DNA was accomplished by electrophoresis on a 5% polyacrylamide gel that had been preelectrophoresed in 50 mM Tris·HCl, pH 8.4/380 mM glycine/2 mM EDTA for 60–90 min at 11 V/cm at 4°C.

Interference of Binding by Premethylation. A partially methylated probe, end-labeled on the antisense strand of the IRE-A, was bound to crude nuclear extract (8), and the protein-bound complex was eluted from a 5% nondenaturing polyacrylamide gel into 1 × 89 mM Tris/89 mM sodium borate/25 mM EDTA by using a Bio-Rad electroelution apparatus. The labeled DNA was captured on DE 81 paper and eluted by incubation in the presence of 20 mM Tris·HCl, pH 8/1 mM EDTA/1 M NaCl/0.5% SDS for 60 min at 70°C. The DNA was extracted with phenol, and cleavage was completed with piperidine as described (8). The band pattern of free and protein-bound DNA was compared on a 6% urea/polyacrylamide sequencing gel.

RNA Isolation. Sprague–Dawley rats were fasted for 48 to 72 hr and refed overnight with a high-carbohydrate/low-fat diet obtained from Bio-Serve (Frenchtown, NJ). Total liver RNA was isolated as described (9) and analyzed (10 μg) for GAPDH mRNA content by synthesis of a complementary DNA strand as described (2).

RESULTS AND DISCUSSION

The sequences required for insulin to increase human GAPDH gene transcription were mapped by using a series of plasmids with nested deletions from −488 to −269 of a HGAPDH-CAT construct, which contained nucleotides −488 to +21 of the human GAPDH gene promoter (Fig. 1). Insulin-stimulated expression of CAT activity was examined in transiently transfected H35 hepatoma cells; a metallothionein–GH construct, PXGH5, was cotransfected to provide an internal measure of transfection efficiency (2, 4, 5). Insulin treatment of confluent H35 hepatoma cell lines transiently transfected with a plasmid containing human GAPDH gene sequences (−488 to +21) fused to the gene for CAT resulted in a 3-fold increase in CAT expression under conditions where RSV-CAT was not regulated by insulin (Fig. 2 and Table 1, experiments I–IV). Deletion of bases −488 to −270 from the HGAPDH-CAT construct completely eliminated the ability of insulin to stimulate CAT gene expression in confluent H35 hepatoma cells (Fig. 2). Analysis of experiments in which insulin-stimulated HGAPDH-CAT 4-fold or more suggested that deletion of nucleotides −488 to −409 eliminated half the stimulatory effect of insulin, whereas deletion of the next 100 bases, −408 to −309, had no further effect on insulin inducibility. Deletion of the next 39 bases, −308 to −269, eliminated an element that conferred a 2-fold effect of insulin on HGAPDH-CAT expression. Deletion of nucleotides −488 to −269 did not significantly affect basal promoter activity in these experiments (data not shown). In aggregate, analysis of this deletion series suggested that two physically separate DNA sequences, IRE-A and IRE-B, independently mediated the stimulatory effect of insulin on GAPDH gene transcription.

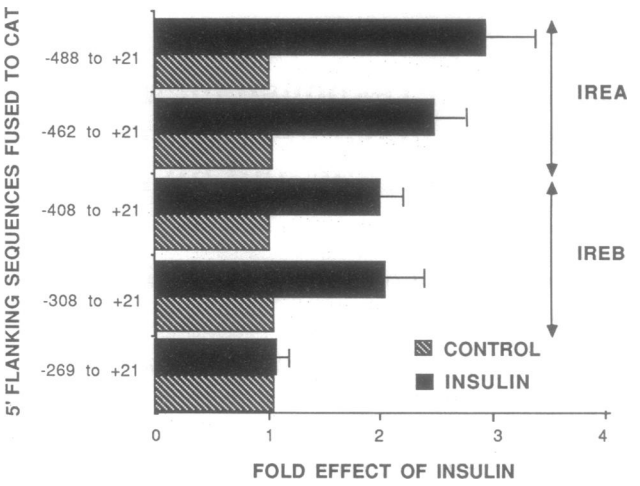

FIG. 2. Insulin regulation of BAL-31 deletion mutants of the human GAPDH-CAT fusion gene in transiently transfected H35 hepatoma cell lines. The HGAPDH-CAT construct (10 μg) or BAL-31 deletion mutants thereof were cotransfected with a human growth hormone (GH) construct PXGH5 (5 μg) into H35 hepatoma cells by using a calcium phosphate batch transfection protocol (2, 4, 5). Under the following conditions we found that (*i*) RSV-CAT activity was not regulated by insulin, a criterion we use to monitor the specificity of the insulin response; (*ii*) endogenous GAPDH mRNA was regulated 3-fold; and (*iii*) CAT activity was at least 1–2% per 4 hr/100 μg of protein. Freshly trypsinized H35 hepatoma cells (0.5×10^7) were resuspended with 1 ml of the calcium phosphate precipitate containing the plasmids described for 15 min at room temperature, then diluted into 11 ml of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, and plated in 60-mm dishes. The next day the cells were washed twice with phosphate-buffered saline and grown to confluence in 0.1% bovine serum albumin/DMEM (usually 24 hr). The medium was changed, and no hormone was added or insulin was added at a final concentration of 1 milliunit/ml for the last 16 hr of the incubation. The medium was removed, and the expression of GH was detected by using a Nichols RIA kit. Cellular CAT activity was measured as described (2). The percent of total [^{14}C]chloramphenicol acetylated per 4 hr/100 μg of extract protein was calculated and normalized for expression of GH in the medium. The fold effect of insulin on each construct was calculated, and the results from four independent studies were averaged. The error bars represent the standard error of four independent measurements.

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                |----------IREA----------|----------
-488  TCGAGGAGAAGTTCCCCAACTTTCCGCCTCTCAGCCTTTGAAAGAAAGAAAGGGGAGGGGGCAGGCCGCGTGCAGCCGCGAGCGGTGCTGGGCTCCGGCTCCAATTCCC
      **********************************************
      ----------IREB----------|
-378  CATCTCAGTCGTTCCCAAAGTCCTCCTGTTTCATCCAAGCGTGTAAGGGTCCCCGTCCTTGACTCCCTAGTGTCCTGCTGCCCACAGTCCAGTCCTGGGAACCAGCACCG
-268  ATCACCTCCCATCGGGCCAATCTCAGTCCCTTCCCCCTACGTCGGGGCCCACACGCTCGGTGCGTGCCCAGTTGAACCAGGCGGCTGCGGAAAAAAAAAAGCGGGGAGAA
-158  AGTAGGGCCCCGGCTACTAGCGGTTTTACGGGCGCACGTAGCTCAGGCCTCAAGACCTTGGGCTGGGACTGGCTGAGCCTGGCGGGAGGCGGGGTCCGAGTCACCGCCTGC
 -48  CGCCGCGCCCCCGGTTTCTATAAATTGAGCCCGCAGCCTCCCGCTTCG/          |----IREA'-----|-----|
 + 1                                                  [CTCTCTGCTCCTCCTGTTCGA
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FIG. 1. Sequence of nucleotides −488 to +21 of the human GAPDH gene promoter (1). One correction has been made, an insertion of C at nucleotide −260 (boldface type underlined).

The existence of two independent cis-acting elements in this gene was established by demonstrating that the element IRE-A could independently confer insulin-responsive gene expression to a truncated HGAPDH-CAT reporter gene. A portion of the upstream element IRE-A (−480 to −435) that contained a putative insulin-inducible binding motif (see Fig. 3 and Table 2) was fused to a truncated HGAPDH-CAT (−268 to +21) construct that lacked both insulin regulatory elements; insulin responsiveness of this construct was compared with truncated HGAPDH-CAT (−408 to +21) that contained IRE-B. In three separate experiments (Table 1, experiments V–VII), CAT expression from a construct devoid of both elements was stimulated 1.2 ± 0.1-fold by insulin, whereas constructs that contained either the IRE-A or IRE-B alone were stimulated 2.1 ± 0.3 and 2.3 ± 0.42-fold, respectively. CAT activity driven by the RSV promoter was stimulated 1.1-fold in these experiments.

The electrophoretic mobility shift assay was used to examine the interaction of DNA regulatory sequences with nuclear proteins. Nuclear extracts from differentiated 3T3-L1 cells incubated with a ^{32}P-labeled probe containing both IRE-A and IRE-B (bases −488 to −269) generated a complex pattern of shifted ^{32}P bands; the amount of DNA–protein complex in the uppermost band was increased substantially if the differentiated 3T3-L1 cells were treated with insulin prior to extraction (data not shown). Sequential deletion of bases from each end of this DNA fragment simplified the pattern and indicated that an insulin-induced DNA-binding protein interacted with nucleotides located between −488 and −423, the region that contained the transcriptionally active IRE-A motif (−480 to −435).‡ Treatment of differentiated 3T3-L1 cells with insulin for 1 hr prior to preparation of nuclear extract gave a 4-fold increase in the ^{32}P content of the retarded band (Fig. 3). Formation of the insulin-induced complex was inhibited by a 50- to 100-fold excess of unlabeled IRE-A DNA. The specificity of this interaction is indicated by the observation that a 100-fold excess of an oligonucleotide corresponding to the c-*fos* SRE, which binds a transcription factor implicated in mediating the effect of serum (8, 10, 11) and insulin (12) on c-*fos* gene transcription, did not inhibit the formation of this complex. An oligonucleotide containing the Sp1 consensus sequence does not inhibit the formation of this complex (Table 2), nor does pure Sp1 bind the sequence (data not shown). The bands migrating more rapidly than the insulin-induced complex are variably present, are not eliminated by excess unlabeled IRE-A fragment, and are not protected in the methylation interference assay.

The physiologic relevance of insulin regulation of GAPDH gene expression and induction of the IRE-A DNA–protein complex in differentiated 3T3-L1 cells was assessed by determining whether analogous regulation of the GAPDH gene and the corresponding DNA–protein complex could be detected by using hepatic nuclear extracts prepared from animals shifted from a low-insulin to a high-insulin state *in vivo*. The process of fasting and refeeding is a nutritional manipulation known to induce metabolic enzymes necessary for the conversion of glucose to glycogen and fat in the lipogenic organs of rats. The cellular content of GAPDH mRNA was 10-fold higher in the liver of rats refed a high-carbohydrate/low-fat diet after a 72-hr fast as compared with fasted rats (Fig. 4). Thus, GAPDH gene expression is stimulated in a second insulin-sensitive tissue *in vivo* under conditions where glucose and insulin levels are known to be elevated, indicating that the phenomenon is a potentially important part of the physiologic program of insulin action *in vivo*.

‡Although we have detected an insulin-induced DNA-binding protein that interacts with IRE-B, the boundary of the binding motif and the specificity of the interaction have not been confirmed by footprint analysis and will not be discussed in this report.

Table 1. Regulation of HGAPDH-CAT constructs by insulin in transiently transfected H35 hepatoma cell lines

HGAPDH-CAT		Fold effect of insulin		
IRE-A/IRE-B		Exp. I–IV	Exp. V–VII	Exp. VIII–X
−	−	1.0 ± 0.3	1.2 ± 0.1	2.1 ± 0.5
+	−	—	2.1 ± 0.3	3.5 ± 0.5
G → T	−	—	—	2.1 ± 0.1
−	+	2.0 ± 0.4	2.3 ± 0.4	—
+	+	3.1 ± 1.2	—	3.3 ± 0.2
RSV-CAT		1.0 ± 0.2	1.1 ± 0.1	1.6 ± 0.4

H35 hepatoma cells were trypsinized 24 hr prior to transfection, plated at a density of 500,000 cells per 60-mm plate, and cotransfected with calcium phosphate precipitates containing 5 μg of PXGH5 and 20 μg of a truncated HGAPDH-CAT construct that contained bases −269 to +21 of the HGAPDH gene (− IRE-A/− IRE-B) or this construct fused to wild-type sequence from −480 to −435 (+ IRE-A/− IRE-B), or IRE-A with a G → T mutation at position −462 (see Table 2), or IRE-B from −408 to +21 (− IRE-A/+ IRE-B) or bases −488 to +21 (+ IRE-A/+ IRE-B), or RSV-CAT as described (2). CAT activity was >1% conversion per 4 hr/100 μg of protein for each data point, but GH activity was below 0.5 ng/ml in some. To include all data points, the fold effect of insulin is reported without normalization to GH expression. The data in Fig. 2 are presented in the first column without normalization to GH expression for comparison.

Nuclear proteins extracted from the livers of fasted and fasted/refed rats form specific retarded bands with IRE-A DNA that migrate as a doublet; the upper band of this doublet comigrates with the insulin-induced band present in differentiated 3T3-L1 nuclear extract (Fig. 3). The amount of this DNA–protein complex was increased 8-fold when nuclear extracts from carbohydrate-refed rats were compared with

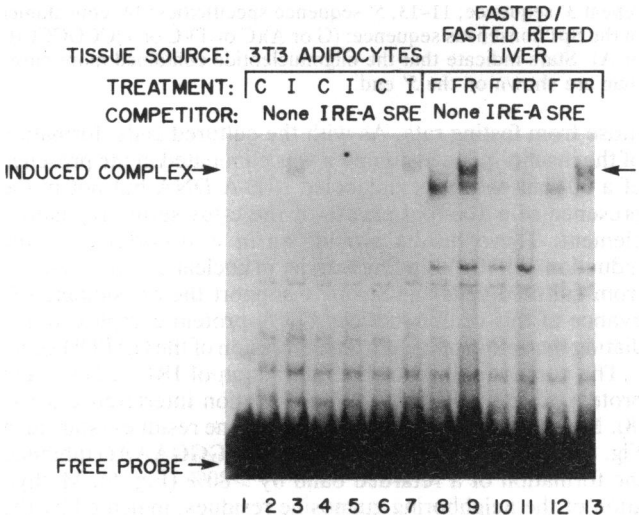

FIG. 3. Detection of an insulin-sensitive IRE-A DNA binding protein in differentiated 3T3-L1 cells and rat liver nuclear extracts. Nuclear extracts (4 μg) were isolated from untreated (lanes C) 3T3-L1 adipocytes or adipocytes exposed to insulin for 1 hr (lanes I) or from fasted (lanes F) or fasted/refed (lanes F/FR) rat liver and were incubated with 0.5–1 ng of ^{32}P-labeled IRE-A under the conditions described. Parallel binding reactions were performed in the presence of a 50-fold excess of unlabeled IRE-A DNA or a 50-fold excess of unlabeled c-*fos* serum response element (SRE). The autoradiograph of a dried 5% polyacrylamide gel is shown. Lanes: 1, IRE-A alone (no nuclear extract); 2, differentiated 3T3-L1 cell nuclear extract (without insulin); 3, differentiated 3T3-L1 cell nuclear extract (with insulin); 4 and 5, lanes 2 and 3 with unlabeled IRE-A DNA; 6 and 7, lanes 2 and 3 with unlabeled *fos* SRE DNA; 8, fasted liver nuclear extract (lane F); 9, fasted/refed liver nuclear extract (lane FR); 10 and 11, lanes 8 and 9 with unlabeled IRE-A DNA; 12 and 13, lanes 8 and 9 with unlabeled *fos* SRE DNA.

Table 2. Competition of IRE-A binding by a ×100 excess of mutant binding motifs

Description	Competitor oligonucleotide	% of Control
1. Footprint	CTTTCCCGCC T CTCAGCC	100
2. Length	AACTTTCCCGCC T CTCAGCCTTTGAAAG	11
3. G → T	AACTTTCCC*T*CC T CTCAGCCTTTGAAAG	97
4. C → T	AACTTTCC*T*GCC T CTCAGCCTTTGAAAG	99
5. GC → TA	AACTTTCCC*TA*C T CTCAGCCTTTGAAAG	104
6. 3′ length	*AACTTTCCCGCC T CTCAGCCTTTG	61
7. Insertion	AACTTTCCCGCC*AGG*CTCAGCCTTTGAAAG	87
8. Repeat	*CCCGCCTCCCGCC*AGG*CTCAGCCAGTCCCAG	127
9. Specificity	**GA*GTTTCCCGCC T CTCAGCC*CTCGAG*	60
10. 3′ specificity	AACTTTCCCGCC T CTCAGCC*CAAGTCCC*	110
11. 5′ specificity	AACTT*G*CCCGCC T CTCAGCCTTTGAAAG	97
12. 5′ specificity	AACTT*A*CCCGCC T CTCAGCCTTTGAAAG	38
13. 5′ specificity	AAC*A*TTCCCGCC T CTCAGCCTTTGAAAG	21
14. Sp1 × 3	GCCCCGCC *C* C	100

Nuclear extract (10 μg) from differentiated 3T3-L1 cells was incubated with 0.5 pmol of the unlabeled competitor oligonucleotide and 5 fmol of labeled IRE-A essentially as described in Fig. 3, except that 3.0 pmol of the 18-base motif CTTTCCCGCCTCTCAGCC, 0.2 μg of Bluescript DNA, and 0.2 μg of poly(dI)·poly(dC) were used in each reaction to inhibit nonspecific binding, and the incubation was allowed to proceed at 4°C for 90 min. The binding reactions were subjected to electrophoresis, and the radioautograph was scanned. The data are presented as the percent of the control value. Description of sequences: 1, the protected sequence and region of homology with phosphoribosyltransferase; 2, the minimal length required to fully support IRE-A binding; 3–5, mutation of one contact point on the sense (G → T) and antisense (C → T) strand and mutation to completely inhibit binding (GC → TA); 6, shortened 3′ sequence; 7, IRE-A with substitution of AGG for T; 8, downstream repeat of IRE-A; 9, 5′ and 3′ sequence specificity; 10, IRE-A with IRE-A repeat 3′ sequence; 11–13, 5′ sequence specificities; 14, concatamer of the Sp1 consensus sequence: (G or A)(C or T)(C or T)CCGCCC(C or A). Stars indicate that the oligonucleotide contained more bases than are shown on the 5′ end.

those from fasting rats. As with the cultured cells, formation of the insulin-induced complex was eliminated in the presence of a 50-fold excess of unlabeled IRE-A DNA but not in the presence of a 100-fold excess of the c-*fos* serum regulatory element. These results provide an *in vivo* correlate of the induction of IRE-A binding activity in nuclear extracts isolated from cultured cells and strongly support the physiologic relevance of this insulin-induced DNA–protein complex in mediating the action of insulin on expression of the GAPDH gene.

The contact points for the interaction of IRE-A DNA and protein were mapped by the methylation interference assay (8). Methylation of the boldface guanosine residues (starred in Fig. 5) in the sequence *GCTGAGAGGCGGGAAAG* inhibited the formation of a retarded band by >80% (Fig. 5). Methylation of the neighboring guanosine residues, indicated by the italicized *G*, inhibited formation of the complex by ≈50%. Control- and insulin-stimulated adipocyte nuclear extracts, fasted and fasted/refed rat liver nuclear extracts, and H35 hepatoma nuclear extracts all gave an interference pattern indicating complete interference of binding due to modification of the six guanosine nucleotides GAGGCGGG (data not shown). Inasmuch as both components of the doublet observed in the nuclear extracts from livers of fasted/refed rats gave a comparable footprint, it is likely that each retarded band contacts a protein with specificity for the same nucleotide sequence. The duplex band pattern may result from posttranslational modification of the IRE-A binding protein, dimerization of the protein, or a protein–protein interaction.

The methylation interference pattern led us to predict that the central G (boldface; starred in Fig. 5) on the sense strand of the protected IRE-A sequence CTTTCCCGCCTCTCAGC

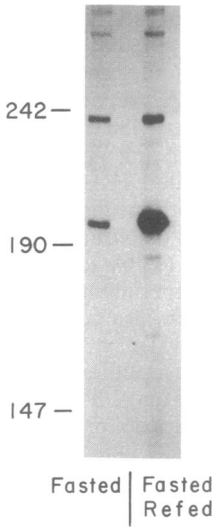

FIG. 4. GAPDH mRNA regulation in the liver of rats fasted and refed a high-carbohydrate/low-fat diet. Total RNA was isolated from the liver of fasted or fasted/refed rats, and the content of GAPDH mRNA was analyzed by synthesis of a specifically primed cDNA strand. As described (2), the extension products were subjected to electrophoresis on a denaturing 8% polyacrylamide gel; the autoradiograph is shown.

might be critical to the binding interaction. This view is supported by the finding that a 100-fold excess of the mutant IRE-A (G → T) does not displace the adipocyte insulin-induced DNA binding protein from labeled IRE-A (Table 2). When the ^{32}P-labeled mutant oligonucleotide (G → T) was incubated with nuclear extracts under conditions where the probe was saturating, the intensity of the shifted complex was reduced by a factor of 10 compared with that achieved with the wild-type binding motif (data not shown). We correlated the effect of this mutation on binding affinity with the ability

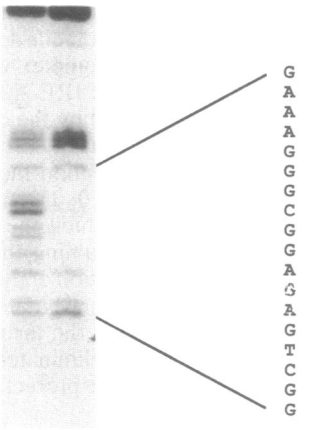

FIG. 5. Methylation interference of binding to the IRE-A motif. A ^{32}P-labeled partially methylated (8) probe from the antisense strand of the IRE-A was bound to crude nuclear extract from fasted refed liver as described in Fig. 4. Protein-bound and free DNA were recovered as described. The band pattern of free ^{32}P-labeled methylated DNA (left lane) was compared to that of protein-bound DNA (right lane) on a urea/8% polyacrylamide sequencing gel loaded with equal radioactivity in each lane. Methylation of the starred guanosine nucleotides in GAGGCGGG interfered with the formation of a retarded band. Scans of the radioautograph were performed, and the ratio of bound to free cpm were calculated for each nucleotide. The following values correspond to the vertical sequence (from the bottom up) and to the lower horizontal (antisense) sequence minus five nucleotides on the left and eight nucleotides on the right: G (1); G (0.5); C,T, and G (0.44); A and G (0.2); A and G (0); G (0); C and G (0.05); G (0.06); G (0); AAA and G (0.55); T,T,G,G,G and G (1.1) each. The fact that the intensity of neighboring guanosine nucleotides (italicized below) is decreased 50% when the data are normalized, suggests that the contacts for IRE-A protein may span the sequence *GCTGAGAGGCGGGAAAG*.

to confer insulin responsiveness to a truncated HGAPDH-CAT construct (Table 1, experiments VIII–X). In three experiments in which RSV-CAT was stimulated 1.6 ± 0.4-fold and truncated HGAPDH-CAT was stimulated 2.1 ± 0.5-fold by insulin, the mutant IRE-A conferred a 2.1 ± 0.1-fold effect of insulin to truncated HGAPDH-CAT, whereas the wild-type IRE-A conferred a 3.5 ± 0.5-fold effect. When normalized to RSV-CAT, these results confirm that the wild-type IRE-A confers a specific 2-fold effect of insulin to HGAPDH-CAT gene expression, while the truncated construct and mutant IRE-A confer a 1.3-fold effect.

We compared the minimal sequence contacted by the IRE-A DNA-binding protein, CCCGCCTC, to an assembled file of sequences from genes reported to be regulated by insulin and/or nutritional manipulations and found that these eight bases are perfectly conserved in the 5′ flanking region of several genes including c-*fos* (12, 13), c-*myc* (13), and genes encoding phosphoribosyltransferase (14), ornithine decarboxylase (15), tyrosine amino transferase (16), glycerophosphate dehydrogenase (17), and P33 (18). The most striking example of identity was the perfect match found between the 14-nucleotide motif of the IRE-A and the gene for phosphoribosyltransferase (CCCGCCTCTCAGCC), a gene recently reported to be regulated by insulin (14).

The contact points of the IRE-A are repeated twice on the antisense strand of the GAPDH gene in a region just upstream from the promoter (see Fig. 1). One repeat, CCCGCC*AG*-*G*CTCAGCC, was similar to a 14-nucleotide segment of the IRE-A, CCCGCC*T*CTCAGCC, which contained the contacted site except for an insertion of *AGG* for *T*.§ Although substitution of *AGG* for *T* in the IRE-A impaired the ability of a 100-fold excess of mutant IRE to competitively block wild-type binding, this mutation did not eliminate binding by high concentrations of oligonucleotide. In contrast, substitution of the 5′ and 3′ sequences in the IRE-A with sequences from the IRE-A repeat completely eliminated insulin-sensitive binding. Thus, although some substitutions are tolerated, the 28-base motif required to support insulin-sensitive binding appears to represent more than a length requirement. A more complete analysis of the 5′ and 3′ sequence requirements of the IRE-A binding motif is shown in Table 2.

The sequence we have proposed for an insulin response element, based on functional analysis, is different from the potential consensus sequence recently proposed by Osborn *et al.* (19) on the basis of sequence comparison alone. Although these sequence similarities may yet prove of interest when functional analysis is complete, the regions of the phospho*enol*pyruvate carboxykinase and amylase genes that confer insulin responsiveness have been preliminarily mapped (20–23) to areas distinct from the regions that contain the proposed consensus sequence (19).

In summary, we have identified two physically separate IREs in the 5′ flanking region of the human GAPDH gene, each of which can independently confer insulin responsiveness to a CAT reporter gene; together they function in an additive fashion. We have shown that exposure of differentiated 3T3-L1 cells to insulin induces a 4-fold increase in the functional level of a novel DNA binding protein selective for one of these two IREs. An analogous rat liver DNA binding protein with identical sequence specificity binds the IRE-A DNA sequence, and its activity is increased *in vivo* by refeeding starved rats a high-carbohydrate diet. The methylation interference data coupled with the competition data show that the binding interaction is sequence specific. Functional analysis of the mutant binding motif confirms that the interaction of the IRE-A protein with the wild-type element correlates with the ability of the cis-acting element to confer insulin responsiveness. Based on the analysis of the critical DNA elements required for formation of the insulin-sensitive complex, we propose a minimal structural motif AACTTT*C*-*CCGCCTC*TCAGCCGAAAG that may characterize at least one class of IREs.

§Cotransfection of a plasmid containing the IRE-A and a plasmid containing the truncated human GAPDH gene promoter results in marked stimulation of promoter activity *in vivo*, which suggests that the sequence similarities between the IRE-A and its repeat may be important.

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