Insulin Regulation of Malic Enzyme Gene Expression in Rat Liver: Evidence for Nuclear Proteins That Bind to Two Putative Insulin Response Elements

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Diabetes in rats is characterized by insulin deficiency accompanied by a decrease in lipogenic enzymes. The malic enzyme (ME) gene, which encodes an important lipogenic enzyme, was used to investigate insulin regulation of gene expression. ME mRNA levels were reduced by more than 90% in the liver of diabetic rats. The administration of insulin (3 U/15 days) to either control or diabetic rats increased ME mRNA by 2- to 10-fold, respectively. Since diabetes reduces circulating T_3 and the levels of nuclear T_3-receptors, the potential role of thyroid hormone on insulin regulation of ME gene expression was also evaluated in thyroidectomized-diabetic rats. In these animals the levels of ME mRNA were undetectable but were increased by insulin even in the absence of thyroid hormones. These *in vivo* effects of insulin and T_3 were not additive. The transcription rate of the gene was also reduced in the diabetic liver and recovered after insulin therapy.

By computer analyses we have identified two different putative insulin response elements (IREs) in the ME gene promoter, hereafter referred to as IRE-I (−683 to −692), which is similar to the phosphoenol pyruvate carboxy kinase promoter IRE and IRE-II (−161 to −170), which is similar to the glyceraldehyde phosphate dehydrogenase gene promoter IRE-A. Results from gel retardation assays suggest that a single nuclear protein binds to IRE-I whereas two different nuclear proteins bind to IRE-II. The protein/IRE-I complex increased in liver nuclear extracts from diabetic rats and decreased after insulin administration. In contrast, the protein/IRE-II complex decreased in liver nuclear extracts from diabetic rats and increased after insulin administration. Analysis of the IRE-II sequence revealed a GC-rich motif similar to the Sp1 element. These results suggest that insulin increases ME gene transcription by modulating the levels of the above mentioned DNA nuclear protein complexes. (Molecular Endocrinology 8: 1361–1369, 1994)

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INTRODUCTION

Diabetes in the rat is accompanied by a decrease in the activity of lipogenic enzymes (1, 2). It has been reported that streptozotozin (STZ)-induced diabetes is associated with a decrease in hepatic malic enzyme (ME; EC 1.1.1.40) activity (3, 4). The levels of this important lipogenic enzyme strongly correlate with *de novo* fatty acid synthesis and are regulated by complex hormonal (3–6) and dietary (7–10) interactions. ME gene is transcribed into two mRNAs of 3.1 kilobases (kb) and 2.1 kb, respectively. Although both mRNAs can be effectively translated into the functional protein, they are differentially expressed in a tissue-specific manner; in liver, the 2.1 kb form is preferentially expressed while the opposite is true in almost all other mammalian tissues. However, there is no evidence up to now for a selective transcriptional regulation, and it has been suggested that different tissue-specific polyadenylation factors are involved (11). It is well known that thyroid hormone T_3 is one of the main regulators of ME gene expression (11, 12), and hepatic ME gene transcription decreases in thyroidectomized (Tx) rats (13). Diabetes is associated with a deficiency of circulating thyroid hormones (T_3 and T_4) (14) and a decrease in their nuclear receptor concentration and occupancy (15, 16). This observation suggests that the effect of diabetes on ME may be related to a decrease in nuclear thyroid hormone receptor and/or T_3 and T_4 levels in these animals, rather than to reduced circulating insulin levels. The results obtained in the present work show that in diabetic rats the decrease in ME activity is due to a reduction in ME gene expression and that insulin by itself is able to increase ME mRNA levels and the rate of ME gene transcription. However, administration of T_3 to diabetic rats does not increase ME mRNA levels.

To analyze the interactions between thyroid hormones and insulin in the regulation of ME gene expression, we have studied the ME response to T_3 in Tx diabetic (TxD) rats and have found that the transcriptional response to T_3 is profoundly blocked in diabetic hypothyroid rats. The effect of insulin and T_3 was not additive.

Although insulin is a very important hormone that controls the expression of many genes, its molecular mechanisms of action are still poorly understood (17). The finding of *cis*-acting insulin response elements (IREs) is a fundamental step toward elucidation of the mechanisms involved in insulin action at the molecular level. In recent years different *cis*-acting DNA sequences that mediate insulin-induced modification of gene transcription have been reported for c-*fos* (18), phosphoenolpyruvate carboxykinase (PEPCK) (19, 20), amylase (21), glyceraldehyde phosphate dehydrogenase (GAPDH) (22), and glucagon (23). Since ME gene transcription is positively regulated by insulin (6), expression of this gene in diabetic rats provides a good model for studying the molecular mechanisms responsible for insulin regulation of gene expression. The ME promoter has been cloned (24) and two putative different IREs have been identified in this promoter by computer analysis. One of them, hereafter referred to as IRE-I (−683 to −692), is similar to the PEPCK IRE (19, 20), and the other, IRE-II (−161 to −170), resembles the GAPDH IRE-A (22). Gel retardation assays have identified specific binding of nuclear proteins to both IRE-I and IRE-II. The protein/IRE-I complex increases in nuclear extracts from livers of diabetic rats and decreases after insulin administration. In contrast, the protein/IRE-II complex decreases in nuclear extracts from livers of diabetic rats and increases after insulin administration. Analysis of the IRE-II sequence reveals a GC-rich motif similar to the Sp1 element (25). An Sp1-DNA binding motif in the δ1-crystallin gene promoter has previously been defined as a positive IRE (26). Thus, these results suggest that the ability of insulin to regulate ME gene expression could be mediated by at least two types of nuclear protein complexes that bind to a negative regulatory domain (IRE-I) and to a positive regulatory domain (IRE-II), the latter being an Sp1-like element in the ME gene promoter.

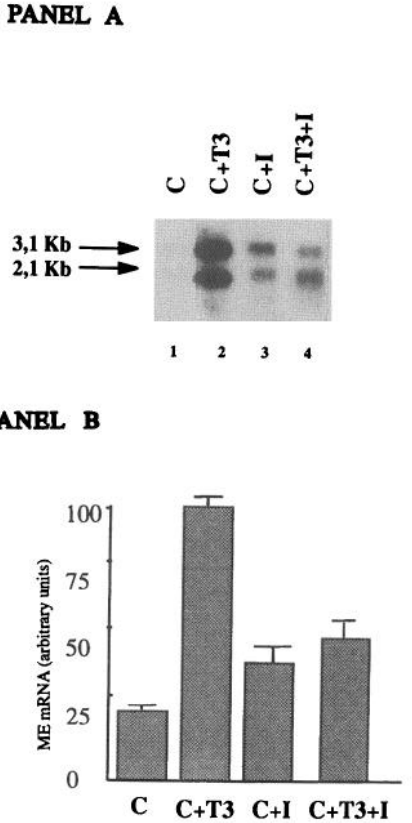

Fig. 1. Insulin and T_3 Increase ME mRNA Levels in Liver of Control Rats

Insulin (3 U/100 g BW) or a receptor-saturating dose of T_3 (250 μg/100 g BW) were administered separately or together to control (C) rats as described in *Materials and Methods*. Panel A shows a representative Northern blot with 50 μg total RNA isolated from the liver of each experimental group and hybridized with ME cDNA. The sizes of the two ME mRNAs are indicated. Panel B shows the average of three independent experiments. The densitometric values are corrected with those obtained by hybridization with the cDNA of β-actin to correct differences in the total RNA quantity applied in each lane. The standard deviation is shown. Control levels can be detected and measured after longer exposures.

RESULTS

Insulin Induces ME Gene Expression in the Liver of Control Rats

In the present work we demonstrate a direct role for insulin, rather than simply a permissive role for T_3 action, in the regulation of ME gene expression *in vivo* in rat liver. The levels of ME mRNA in control rats and their regulation by a receptor-saturating dose of T_3 and insulin were studied. As Fig. 1 shows, the levels of ME mRNA in the liver of control rats were low (lane 1). As previously described by others (11, 13), a single saturating dose of T_3 [250 μg/body weight (BW)] given to control rats increased ME mRNA levels (lane 2). Insulin (3 U/15 days) also increased ME mRNA levels. In control rats the effect of T_3 was greater than that of insulin (6-fold *vs*. 2-fold). The effect of both hormones together was not additive. Control rats used here were age-paired with the experimental animals; their levels of ME mRNA were always very low, which agrees with previously reported low ME activity in old rats (27).

Insulin Is a Potent Inducer of ME Gene Expression in the Liver of Diabetic Rats

The ability of insulin and T_3 to increase ME mRNA levels described above was observed in control rats in which there was circulating insulin and T_3. Since hepatic ME activity has previously been reported to decrease in diabetes (3), the liver levels of ME mRNA and their

regulation by T_3 and insulin were studied in diabetic rats. As shown in Fig. 2, the levels of ME mRNA were reduced to almost undetectable values in diabetic rats (lane 2) as compared with controls (lane 1). A dose of insulin (3 U/15 days) given to diabetic rats increased ME mRNA levels 20-fold (lane 3). In contrast to the effect observed in control rats, a saturating dose of T_3 did not increase the very low levels of ME mRNA (lane 5) in diabetic rats. This agrees with the hypothesis that the presence of insulin is necessary to obtain a T_3 response (3). Administration of both hormones together to diabetic rats increased ME mRNA levels by 5-fold, which was less than the effect of insulin alone.

This experiment included a group of food restricted (FR) nondiabetic animals, so that their catabolic state remained as close as possible to that of diabetic rats. In the liver of the FR rats (lane 7) the levels of ME mRNA were intermediate between those of controls and diabetic rats, and their response to T_3 was larger (lane 6) than in diabetic rats. This shows that the lack of response in the diabetic animals cannot simply be attributed to their catabolic state.

Insulin Induces Higher Increases in ME Gene Expression in the Liver of TxD Rats Than Does T_3

In the preceding experiments it is difficult to separate the effects of thyroid hormone deficiency from other changes occurring in diabetic rats. Moreover, the problem of determining whether the response to insulin in diabetic rats was due to an increase in T_3 levels and not merely an effect of insulin alone, prompted the use of Tx and TxD rats to analyze the ME mRNA response to insulin and T_3. As expected, the levels of ME mRNA were undetectable in Tx rats (Fig. 3 line 4). A single and saturating dose of T_3 given to control (lane 3) or Tx rats (lane 2) increased ME mRNA levels by 5- and 20-fold respectively. Insulin alone was able to increase ME mRNA levels when given to Tx rats (lane 5), and this increase was higher (30-fold) than that obtained with T_3. Administration of both hormones together did not result in any additional increase (lane 6). TxD rats

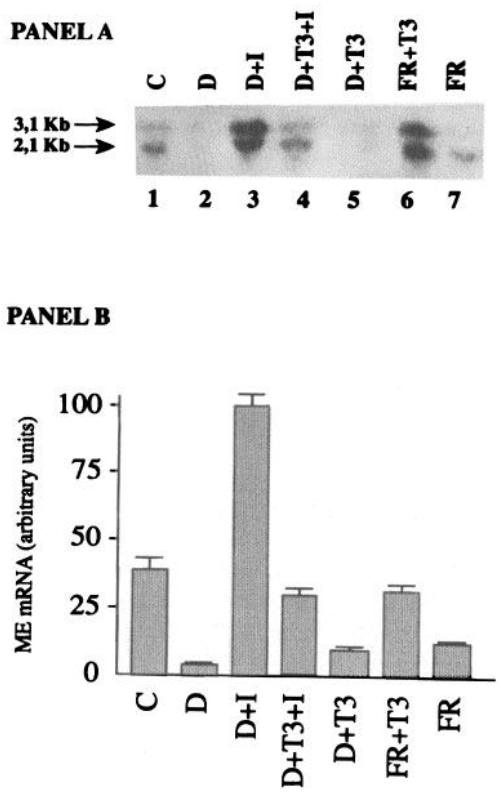

Fig. 2. Insulin Is a Potent Inducer of ME Gene Expression in the Liver of Diabetic Rats

Insulin (3 U/100 g BW) or a receptor-saturating dose of T_3 (250 μg/100 g BW) were administered separately or together to diabetic (D) or food restricted (FR) rats as described in *Materials and Methods*. Panel A shows a representative Northern blot with 30 μg of total RNA isolated from the liver of each experimental group and hybridized with the ME cDNA. The sizes of the two ME mRNA are indicated. Panel B shows the quantification of the ME mRNAs, after correction with β-actin mRNA, by densitometer scanning of autoradiograms of Northern blots from three independent experiments. The data are media and standard deviation. Control levels can be detected and measured after longer exposures.

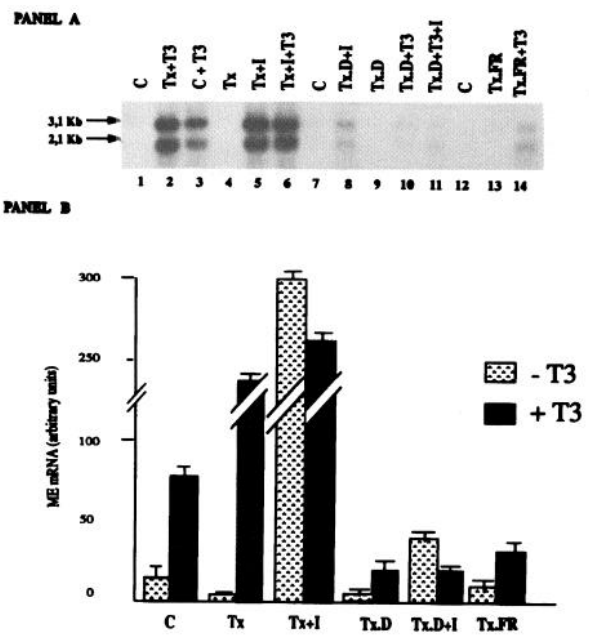

Fig. 3. T_3 and Insulin Increase ME mRNA Levels in Liver of Tx Rats

Insulin (3 U/100 g BW) or a receptor-saturating dose of T_3 (250 μg/100 g BW) was administered separately or together to Tx rats, subjected or not to diabetes (TxD) or food restriction (TxFR) as described in *Materials and Methods*. Panel A shows a representative Northern blot with 10 μg polyA+ RNA isolated from the liver of each experimental group and hybridized with the ME cDNA. The sizes of the two ME mRNA are indicated. Panel B shows the quantification of the ME mRNAs, after correction with β-actin mRNA, by densitometer scanning of autoradiograms of Northern blots from three independent experiments. The data are media and standard deviation. Control levels can be detected and measured after longer exposures.

showed a weak response to T_3 or insulin, but insulin (lane 8) remained a stronger inducer than T_3 (lane 10). Administration of T_3 to Tx-FR rats (lane 14), resulted in a 2.5-fold increase of ME mRNA levels, a larger effect than that found when T_3 was administered to D rats. This indicates that the low levels of ME mRNA and the low response to T_3 when diabetes is present are due to something more than just the catabolic state.

Insulin Stimulates Malic Enzyme Gene Transcription

The next question was whether the insulin-induced increase in ME mRNA levels could be due to an increase in the stability of mRNA, an increase in the relative transcription rate, or both. To determine whether insulin specifically affected the ME gene transcription rate, run-on experiments with liver nuclei isolated from rats subjected to different treatments were used. Figure 4 shows that diabetes decreased the transcription rate of the ME gene to almost undetectable values. Insulin increased the transcription rate in control rats 2-fold, and this effect was more dramatic (8- to 10-fold) in diabetic rats. As expected, a receptor-saturating dose of T_3 increased the transcription rate of ME gene in control but not in diabetic rats. The effect observed when both hormones were administered together was not additive, which agrees with the nonadditive effect on ME mRNA levels observed above.

Proteins Mediating the Effect of Insulin on the ME Gene Promoter

The results obtained here clearly show that insulin alone can regulate ME gene transcription and that it does not act simply as a permissive hormone for T_3. The ME gene is thus a good model for the study of the molecular mechanisms involved in insulin regulation of gene expression. Since the ME promoter has been extensively studied (24), we first analyzed by computer whether any of the previously described IREs were present. Two different putative IREs were detected one IRE-I between −692 and −682 (TATTGTTTTG), which was similar to the PEPCK IRE (19, 20), and another IRE-II between −169 and −162, (CCCGCCTC), which was similar to the GAPDH IRE-A (22). Synthetic oligonucleotides containing these sequences were made and analyzed by gel retardation assays to determine whether they could bind nuclear proteins and if so whether this was regulated by insulin.

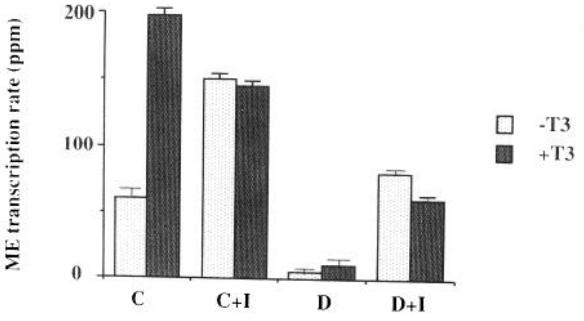

Fig. 4. Insulin Induces ME Gene Transcription

Nuclei from control (C) or diabetic (D) rats treated or not with insulin (3 U/100 g BW) and/or a receptor-saturating dose of T_3 (250 μg/100 g BW) were isolated. The nuclei were incubated under run-on transcription conditions to allow elongation of gene transcripts initiated in control rats. Specific transcription of the ME gene was determined as described in *Materials and Methods*. The values represent the mean transcriptional activity of three independent experiments and the standard deviation.

Liver nuclear extracts (5 μg) from control, FR, diabetic, or diabetic plus insulin rats were tested for their ability to bind a 20-base pair (bp) oligonucleotide (5′-GGCTGTATTGTTTTGTTTTA-3′) corresponding to the IRE-I region (−697 to −678) of the ME gene promoter. A retarded band was detected in gel shift assays in control rats (Fig. 5, lane 2). This protein/DNA complex increased 2- to 4-fold over the control when nuclear extracts from diabetic (lane 4) or FR rats (lane 3) were used. The administration of insulin to diabetic rats decreased the protein/DNA complex 4-fold (lane 5). The specificity of complex formation was demonstrated in competition experiments, in which the complex was competed by a 100-fold excess of the same unlabeled oligonucleotide IRE-I (lane 6), but not by an unrelated oligonucleotide (lane 7).

The same experimental approach was followed with IRE-II. Liver nuclear extracts (5 μg) from control, FR, diabetic, or diabetic plus insulin rats were tested for their ability to bind a 20-bp oligonucleotide (5′-CCCGCCCCCGCCTCCTCGCA-3′) corresponding to the IRE-II region (−175 to −156) of the ME gene promoter. Two different retarded bands were detected in gel shift assays in control rats (Fig. 6, lane 2). The two protein/DNA complexes decreased to almost undetectable levels when nuclear extracts from food restricted (lane 3) or diabetic (lane 4) rats were used. Insulin administration to diabetic rats restored both protein/DNA complexes (lane 5) to control levels. As with IRE-I the complexes could be competed by a 100-fold excess of the same unlabeled oligonucleotide IRE-II (lane 6), but not by an unrelated oligonucleotide (lane 7).

These results suggest that insulin regulates ME gene transcription by controlling the ratio between trans-acting proteins that bind to either a putative negative regulatory element (IRE-I) or to a putative positive regulatory element (IRE-II).

DISCUSSION

The present work confirms and extends earlier studies that showed that insulin is an important hormone for ME gene expression in rat liver (3–6). Since diabetes in rats leads to depressed thyroid function (14–16), the low levels of ME activity were traditionally explained by

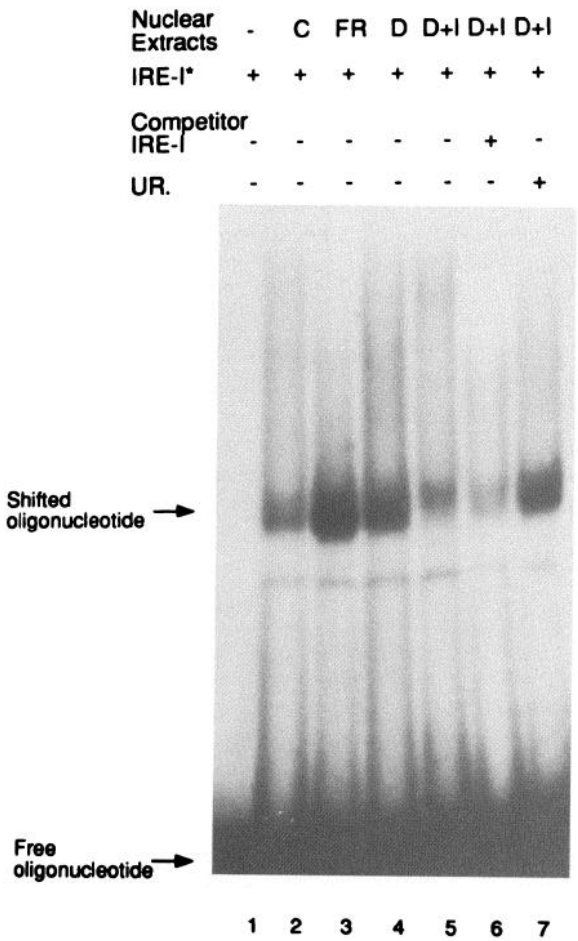

Fig. 5. Insulin Inhibits the Protein/DNA Complex Formed on IRE-I

Nuclear extracts (5 μg) from livers of control (C), food restricted (FR), diabetic (D), or diabetic plus insulin (D+I) rats were incubated with the labeled oligonucleotide IRE-I for 30 min at room temperature. Free and bound DNA were separated as described in *Materials and Methods*. For competition a 100-fold excess of unlabeled related (IRE-I) or unrelated oligonucleotides, was used. The IRE-I has a decamer sequence equivalent to the IRE described of PEPCK (19, 20).

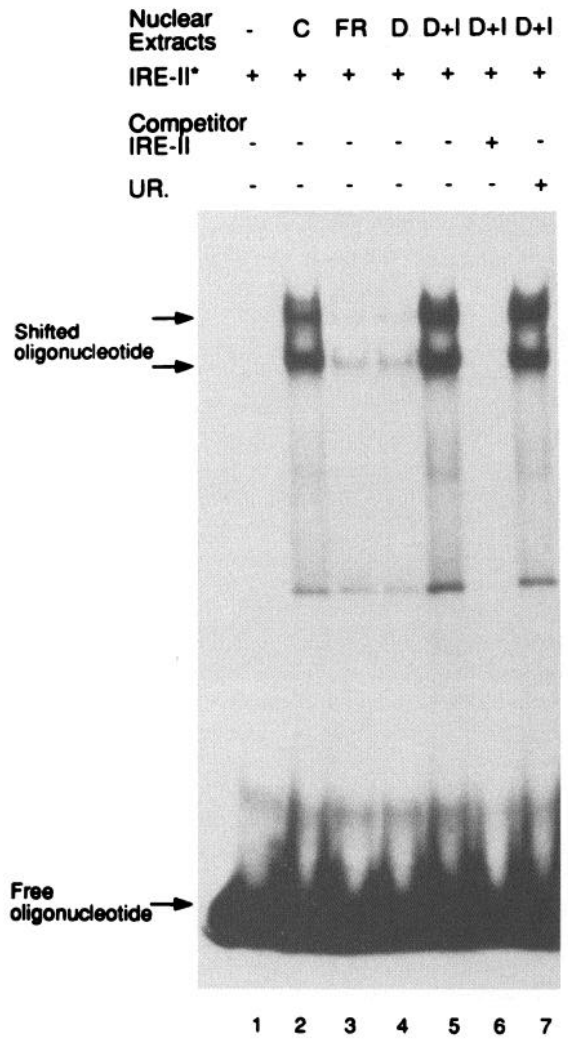

Fig. 6. Insulin Induces the Protein/DNA Complex Formed on IRE-II

Nuclear extracts (5 μg) from livers of control (C), food restricted (FR), diabetic (D), or diabetic plus insulin (D+I) rats were incubated with the labeled oligonucleotide IRE-II for 30 min at room temperature. Free and bound DNA were separated as described in *Materials and Methods*. For competition a 100-fold excess of unlabeled related (IRE-II) or unrelated oligonucleotides, was used. The IRE-II has an octamer sequence equivalent to the IRE of GAPDH (22).

the decreased levels of nuclear thyroid hormone receptors and/or circulating T_4 and T_3, and not to their low insulin levels. First, this study has shown that insulin increases ME mRNA levels even in control rats. Dramatically lower levels of ME mRNA were found in diabetic rats and recovered after insulin injection but not after T_3 injection (Fig. 2). These data suggest that innulin increases ME mRNA levels independently of T_3, while T_3 seems to need the presence of insulin to increase ME mRNA levels. In hepatocytes from chicken embryos (28) or from rats (4) insulin has a limited effect on ME mRNA. This difference from the clear action of insulin presented here could be due to the differences between species, or between primary cultures and our *in vivo* study in the whole animal.

In order to deepen our understanding of the insulin effect, as separate from that of T_3 on ME gene expression, experiments were done with Tx and TxD rats. In both groups thyroid hormone levels were almost undetectable and any effect found after insulin administration would be due to insulin alone. As expected, administration of T_3 to either control or Tx rats significantly increased ME mRNA levels. The administration of insulin to Tx rats increased the ME mRNA levels to a greater extent than occurred with T_3. We do not observe a differential regulation of both transcripts in the experiments. Our results confirm that the predominant form present in liver is the 2.1 kb mRNA and insulin and/or T_3 do not increase specifically one of the messengers, but homogenize the levels of both of them.

This is a biologically reproducible phenomenon and cannot be attributed to a poor efficiency of hybridization.

The reduction in ME mRNA levels in diabetic rats could be due to a shortening of the mRNA half-life and/or a decrease in the rate of transcription. Our results clearly demonstrate that diabetes decreases ME gene transcription as determined by run-on analysis. Treatment of both control and diabetic rats with insulin increased the ME transcription rate. However, since insulin-induced increases in ME transcription rate cannot totally explain the larger increase observed in ME mRNA levels, we think that insulin exerts its action at both levels, *i.e.* by 1) increasing the transcription rate and 2) stabilizing messengers, as has been shown for T_3 (29).

It is important to comment on the nonadditive nature of the effect of T_3 and insulin. Our previous work in primary brown adipocyte cultures showed a clearly additive effect of insulin and T_3 in increasing ME mRNA levels. However, in the whole animal study reported here, both these hormones appear to inhibit each other's effect on ME mRNA levels, something that was also observed in the transcription rate. These results are difficult to interpret. One possible explanation is that certain factors, which are present in the whole animal and absent in primary culture, block the additive effect. The simplest explanation could be the secondary effects of altering the hormonal equilibrium in the whole animal. Perhaps the levels of other hormones that act as negative regulators are increased by these treatments. An example would be pancreatic somatostatin, which raises intracellular cAMP levels thereby blocking ME gene expression (30, 31).

Another possibility is that at these doses, T_3 and insulin compete in the whole animal by similar mechanisms at the level of the ME gene promoter. Transcriptional interference, as described previously (32, 33), between proteins binding to the putative IRE-II and the thyroid hormone response elements might also explain the nonadditive effect of both hormones and the superinduction found in Tx rats. However the elements are 86 bp apart, a distance that may be too great for such competitive effects, unless conformational changes in the chromatin structure contribute to looping interferences, which place the elements in closer contact. This possibility will be studied in the future in relation to recent information about thyroid response elements (34, 35) and the observations reported here about *cis* elements and trans-acting factors that mediate insulin action.

Animals subjected to food restriction were used as a model reflecting the catabolic state of diabetic animals. This allowed us to separate direct insulin effects from others mediated by negative metabolic balance. The levels of ME mRNA in FR rats and their response to T_3 are intermediate between those found in control and diabetic rats. Thus the strong reduction in ME gene expression found in diabetes is not totally mediated by the catabolic state of the animals. Moreover in TxFR rats ME mRNA levels were undetectable, as expected, but the response to T_3 was again intermediate between that found in Tx and TxD rats. The insulin induction of ME mRNA levels and gene transcription was observed after 15 days of insulin administration to diabetic rats. The fact that insulin increases the ME transcription rate makes this gene a good model to study the molecular mechanisms by which insulin mediates its nuclear effects. Although insulin is a very important hormone that controls the expression of many genes, its molecular mechanism of action is still poorly understood (17). The identification of *cis*-acting sequences working as IREs is a fundamental step toward the understanding of the mechanisms by which insulin regulates gene expression. In recent years different *cis*-acting DNA sequences mediating insulin-induced modification of gene transcription have been reported for c-*fos*, PEPCK, amylase, GAPDH, glucagon (18–23), and δ-crystallin (26). In contrast to most sequences regulated by hormones or second messengers, the IREs identified so far do not share any homology. Computer analysis was used to check the existence of previously identified IREs on the ME gene promoter. Two different putative IREs were found that share homology with previously defined IREs. The so called IRE-I is homologous with the IRE of PEPCK gene promoter while the putative IRE-II is homologous with one of the IREs of GAPDH. Gel shift assays with IRE-I suggest that this sequence could mediate negative regulation by insulin as has been reported with PEPCK (19, 20). The increased complex formation between a nuclear protein and the IRE-I observed in diabetic rats was decreased by insulin administration. The sequence homologous to the hexamer core motif of the GAPDH IRE-A sequence (22) binds two different nuclear proteins that are clearly insulin induced. In diabetic rats the formation of complexes between both nuclear proteins and the IRE-II is almost undetectable. Although no consensus sequence has been identified as an IRE, one of the characteristics so far identified as common to several sequences is a GC-rich sequence. The core motif CGCCTC found in the ME gene promoter very closely matches the consensus sequence present in various genes regulated by insulin, including glucagon (23) and GAPDH (22). This sequence is reminiscent of the motif of the Sp1 element. The existence of a GC in the middle of the hexamer is important for the binding of Sp1 proteins (25). Alemany *et al.* (26) have previously reported that in the δ1-crystallin gene promoter formation of the Sp1 protein/DNA complexes is positively regulated by insulin. Sp1 consists of two species of 95 and 105 kilodaltons (36). One possibility is that insulin regulates ME gene expression through an Sp1 element by inducing the binding of two Sp1 protein species. This hypothesis needs to be tested either by gel shift assays using the Sp1 consensus sequence for competition or by supershift assays using antibodies against the Sp1 protein. The construction of heterologous promoters containing the putative IREs identified in the ME gene

for use in transfection studies should show clearly whether these sequences act as real IREs.

MATERIALS AND METHODS

Animals and Treatments

Male Wistar rats bred in our colony, weighing approximately 150–200 g at the onset of each experiment, were used. They were kept under constant temperature (22 C) and a 12-h light, 12-h dark cycle (lights on at 0700). Diabetes was induced as previously described (15), by a single injection of STZ (7.5 mg/100 g BW; Upjohn Laboratories, Kalamazoo, MI). Four days after the STZ injection, diabetic rats received either saline or 3 U/100 g BW bovine insulin injection (Novo Lente, Copenhagen, Denmark), sc, twice daily for 15 days until death. The effect of T_3 was assessed by a single injection of a saturating dose of 250 μg/100 g BW during the last 24 h. Groups of rats fed for 15 days on 50% of the diet of the controls were also used.

In other experiments the effects of diabetes and thyroid hormone were analyzed in hypothyroid rats. The animals were surgically thyroidectomized when weighing 80–100 g, and 1 week later received an ip injection of 100 μCi ^{131}I to eliminate vestiges of thyroid tissue. The criteria for total thyroidectomy was BW stasis and low plasma T_4 and T_3 levels (<3.5 and 0.15 ng/ml respectively), and only animals that stopped growing and had low circulating thyroid hormones were used. Two and a half months after thyroidectomy, a group of animals received STZ 18 days before death. The insulin treatment was a daily injection of 3 U/100 g BW for 15 days and a TxD insulin-treated group was obtained. Half of the animals from each group were treated with 250 μg T_3 for the last 48 h before death. An additional group of Tx rats was submitted to 50% food restriction for the last 18 days, and the response to T_3 was also evaluated in these animals.

In all cases six to eight animals per group were used, and in each experiment there was a group of intact age-matched controls.

At the end of each experiment the animals were killed by exsanguination under light ether anesthesia, and the livers were perfused *in situ* with Dulbecco's PBS containing 3 U heparin/ml. The blood was collected, and the livers were rapidly removed and immediately frozen in dry ice and stored at −80 C.

Plasma glucose was determined by the glucose oxidase method (37) and the plasma T_3 by specific RIAs (38). The criterion for diabetes was hyperglycemia (705 ± 8 mg/dl) that increased almost 6-fold over control levels (117 ± 6 mg/dl). Insulin treatment restored the glucose levels (150 ± 4 mg/dl) to normal. The Tx rats showed low levels of plasma T_3 (12.4 ± 0.9 ng/dl) as compared with control rats (69.2 ± 5.8 ng/dl).

RNA Extraction and Hybridization

Total RNA was extracted from liver homogenates by the guanidinium thiocyanate method (39). In some cases poly $(A)^+$ RNA was obtained by oligo(dT)-cellulose chromatography (40). Samples of total RNA or poly $(A)^+$ were electrophoresed in 1% agarose gels containing 2.2 M formaldehyde (41). For total RNA, ethidium bromide staining of the gels revealed the presence of equal amounts of RNA. RNA was blotted on nylon membranes (Nytran, Schleicher & Schuell, Keene, NH) as described by the manufacturer. The filters were hybridized using the ME cDNA probe (3.1 kb) (29). This probe was a generous gift from Dr. Vera M. Nikodem and was prepared by digesting the plasmid pMEB (42), which contains full-length ME cDNA, with *Bam*HI and *Bgl*II. Both ME cDNA sequences (1250 and 1627 bp) were isolated and labeled by random oligopriming to a specific activity of 1×10^9 cpm/μg DNA. cDNA from β- actin was used to correct for the amount of RNA applied. The levels of ME mRNA and β-actin mRNA were determined by autoradiogram and quantified by densitometric scanning. The results were expressed as an intensity percentage compared to the maximum expression in each experiment. The final values represent the sum of the 3.1 and the 2.1 kb ME mRNAs. The data are media and standard deviation of three independent experiments. Control levels were detected and measured after longer exposures of the films.

In Vitro Run-on Transcription Assay

Nuclei from perfused livers were obtained as described (43). *In vitro* transcription assays were performed by the method of McKnight and Palmiter (44), following modifications described by Santisteban *et al.* (45). Quantification of RNA-DNA hybrids was performed by scintillation counting of the filters, using a plasmid containing the cDNA for β-actin as a nonregulated control and the plasmid pUC19 as a control for nonspecific hybridization. The hybridization solution also contained 9000 cpm ^{3}H-labeled ME mRNA in order to evaluate hybridization efficiency. This mRNA was prepared as we described previously (6). The ME gene transcription rate is expressed as parts per million. These values were obtained after subtraction of the radioactivity bound to the nonspecific DNA and corrected for the small differences in the efficiency of the individual hybridization, which varied between 15–20%.

Preparation of Nuclear Extracts and Electrophoresis Mobility Shift Assay

Nuclear extracts were prepared following the method of Gorski *et al.* (46) with the modifications introduced by Petty *et al.* (47). Proteins were quantified in the extracts by the method of Bradford (48) with the Bio-Rad kit (Bio-Rad, Richmond, CA), using BSA as standard. The final protein concentration was 2–5 mg/ml. The electrophoresis mobility shift assay was performed using two different oligonucleotides, corresponding to the sequences previously described as IREs for PEPCK (IRE-I) and for GAPDH (IRE-II) (20–22). Oligonucleotides were labeled by phosphorylation with T_4 polynucleotide kinase (γ^{-32}P)ATP and annealed as described (49). Binding reactions were carried out as follows: 5 μg nuclear proteins were preincubated in a binding reaction mixture containing 40 mM HEPES, pH 7.9, 200 mM KCl, 0.5 mM dithiothreitol, 0.2 mM EDTA, 5% Ficoll, and 3 μg poly (dI-dC) for 15 min on ice. In competition experiments, the unlabeled competitor oligonucleotides were added in excess (100×). Then 50 pg labeled oligonucleotide were added to the mixture and incubated for 30 min at room temperature. The resulting DNA-protein complexes were separated from free DNA on a 5% polyacrylamide gel (30:0.8, acrylamide-bisacrylamide). Gels were run at 20 A in a cold room in 0.5× TBE (1× TBE is 90 mM Tris, 90 mM boric acid, and 1 mM EDTA, pH 8) before being vacuum dried and exposed to a x-ray film at −70 C.

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