Glucose and Insulin Function through Two Distinct Transcription Factors to Stimulate Expression of Lipogenic Enzyme Genes in Liver*

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Transcription of a number of genes involved in lipogenesis is stimulated by dietary carbohydrate in the mammalian liver. Both insulin and increased glucose metabolism have been proposed to be initiating signals for this process, but the pathways by which these effectors act to alter transcription have not been resolved. We have previously defined by electrophoretic mobility shift assay a factor in nuclear extracts from rat liver, designated the carbohydrate-responsive factor (ChoRF), that binds to liver-type pyruvate kinase and S\textsubscript{14} promoters at sites critical for regulation by carbohydrate. The sterol regulatory element binding protein-1c (SREBP-1c) has also emerged as a major transcription factor involved in this nutritional response. In this study, we examined the relationship between SREBP-1c and ChoRF in lipogenic gene induction. The two factors were found to possess distinct DNA binding specificities both in vitro and in hepatocytes. Reporter constructs containing binding sites for ChoRF were responsive to glucose but not directly to insulin. On the other hand, reporter constructs with an SREBP-1c site responded directly to insulin. The S\textsubscript{14} gene possesses binding sites for both ChoRF and SREBP, and both sites were found to be functionally important for the response of this promoter to glucose and insulin in hepatocytes. Consequently, we propose that SREBP-1c and ChoRF are independent transcription factors that mediate signals generated by insulin and glucose, respectively. For many lipogenic enzyme genes, these two factors may provide an integrated signaling system to support the overall nutritional response to dietary carbohydrate.

In mammals, the ingestion of carbohydrate in excess of that required to meet immediate energy needs triggers lipogenesis, the conversion of simple carbohydrates into triglycerides. Lipogenesis occurs predominantly in the liver and adipose tissue and its activation by carbohydrate diet is accompanied by the induction of many of the key enzymes involved in this metabolic conversion (for review see Refs. 1–3). Among these are enzymes of glycolysis, such as pyruvate kinase; of fatty acid synthesis, such as acetyl CoA carboxylase and fatty acid synthase (FAS); and of fatty acid maturation and packaging, such as stearoyl CoA desaturase. The increased production of these “lipogenic” enzymes results from induction of their specific mRNAs and in most cases correlates with increased transcription of the corresponding genes.

Two potential signaling pathways elicited in response to dietary carbohydrate could play a role in lipogenic enzyme induction. Increased insulin secretion by the β cell in response to elevated blood glucose could act as the primary signal. Alternatively, increased glucose metabolism itself might lead to alterations in gene expression. Attempts to sort out the respective roles of these pathways have been most effectively carried out in cultured primary hepatocytes. Treatment of hepatocytes with insulin and high glucose levels mimics the lipogenic response seen in the animal following dietary carbohydrate (4). However, neither signal alone is able to recapitulate the response. Treatment of hepatocytes with insulin alone (in low glucose conditions) does stimulate the expression of the glucokinase gene (5). Enhancement of pyruvate kinase gene expression, on the other hand, is mainly dependent on increases in glucose concentration. When glucokinase is expressed constitutively, induction of pyruvate kinase mRNA occurs in the absence of insulin (6). For most of the remaining lipogenic enzyme genes, however, both insulin and glucose are required for the induction process.

The intracellular signaling pathway responsible for the induction of lipogenic gene expression in response to glucose metabolism remains unresolved (for reviews see Refs. 2 and 3). Two genes, the liver-type pyruvate kinase (L-PK) and S\textsubscript{14} genes, have been extensively studied. The S\textsubscript{14} gene product, a 17-kDa nuclear protein, is hypothesized to play a regulatory role in lipogenesis (for review see Ref. 7). Transfection analyses of the S\textsubscript{14} and L-PK promoters in primary hepatocytes led to the identification of a conserved carbohydrate response element (ChoRE) that is necessary and sufficient for control by glucose (8–11). This sequence consists of two “half E box” motifs related to the sequence CACG (12). Definition of the ChoRE allowed the detection of a novel protein complex by EMSA that formed with active ChoREs but not with mutant ChoREs that failed to respond to glucose (12). The strong correlation between binding and function suggested that this complex, designated ChoRF, might be responsible for signaling through the ChoRE element. The identity of the protein(s) forming this complex is unknown.

The presence of E box-like sequences in the ChoRE suggests that a member of the basic/helix-loop-helix/leucine zipper fam-

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The abbreviations used are: FAS, fatty acid synthase; L-PK, liver-type pyruvate kinase; ChoRE, carbohydrate response element; ChoRF, carbohydrate-responsive factor; SREBP, sterol regulatory element-binding protein; SRE-1, sterol regulatory element; EMSA, electrophoretic mobility shift assay; PCR, polymerase chain reaction.
ily binds this site to mediate transcriptional responses to glu-
cose. Among this large family of factors, SREBP-1c has
emerged as a candidate for ChoRF. SREBP was first identified
as a transcription factor that regulates genes encoding en-
zymes involved in cholesterol uptake and biosynthesis (for re-
views see Refs. 13 and 14). SREBP has three isoforms: SREBP-
1a, -1c, and -2. SREBP-2 appears to be primarily involved in
regulating genes of cholesterol metabolism (13). SREBP-1c is
predominant in tissues active in lipogenesis such as liver and
adipose (15, 16). The expression of SREBP-1c itself was found
to be rapidly increased by dietary carbohydrate in the liver and
adipose tissue, suggesting a role for this factor in lipogenesis
(17–19). The effects of dietary carbohydrate were attributed to
a direct action of insulin in hepatocytes (19). Overexpression of
SREBP-1c induced lipogenic gene expression (e.g. FAS and
acetyl CoA carboxylase) in mouse liver and in cultured cells (17,
20, 21). Furthermore, carbohydrate-induced lipogenic gene ex-
pression was severely impaired in either SREBP-1 knockout
mice or in hepatocytes overexpressing a dominant negative
form of SREBP-1 (22, 23). Together these data provide a strong
case for the role of SREBP-1c in lipogenic enzyme induction;
however, they do not directly demonstrate that SREBP-1c is
the factor that also mediates the glucose signaling pathway. In
fact, overexpression of a constitutively active form of SREBP-1c
alone could not maximally induce lipogenic gene expression in
hepatocytes in low glucose conditions (19). In addition, overex-
pression of constitutively active SREBP-1c was unable to acti-
vate constructs containing the L-PK ChoRE either in COS cells
or in mhAT3F cells (24). Thus, although SREBP-1c might be
implicated in mediating insulin signaling, its direct involve-
ment in the regulation of ChoREs remains in question.
In this study, we have investigated the relationship between
ChoRF and SREBP-1c with respect to lipogenic gene induction.
We present evidence indicating that ChoRF is distinct from
SREBP-1c and acts through different response elements. These
data lead to a model in which both ChoRF activation by glucose
and SREBP activation by insulin play integral roles in carbo-
hydrate stimulation of gene expression.

Experimental Procedures

Primary Hepatocyte Culture and Transfections—Primary hepatocytes were isolated from male Harlan Sprague-Dawley rats (180–260 g) using the collagenase perfusion method as described previously (25). Following an attachment for 3–6 h, cells were transiently transfected using F1 reagent (Targeting Systems, San Diego, CA) in modified Williams’ E medium with 25 mM HEPES, 0.01 mM dexamethasone, 0.1 unit/ml insulin, 50 unit/ml penicillin, 50 µg/ml streptomycin, and 5.5 mM glucose for 12–14 h. Cells were then cultured in medium containing either 5.5 or 27.5 mM glucose for 30 h and harvested for luciferase assay. For experiments in Figs. 6 and 8, cells were transfected with medium containing 1.5 mM glucose and no insulin and subsequently cultured with medium in varying glucose concentrations in the absence or in the presence of 0.1 unit/ml insulin. In these experiments, 0.35 mg/ml of Matrigel (Collaborative Biomedical Products, Bedford, MA) was added to the medium after transfection. This treatment has been shown to enhance the maintenance of the differentiated phenotype in cultured hepatocytes (26). Results of luciferase assays are expressed as relative light units measured per µg protein.

Plasmid Constructs—Sequences of ChoREs from the rat L-PK and S14 genes, synthetic ChoREs derived from these sequences (mut 3/5 and m3–6), and the consensus SRE-1 are shown in Table I. Oligonucleotides containing these sequences (except mut3/5) were synthesized with BamHI and BgII sites at the 5’ and 3’ ends, respectively. Each oligonucleotide was ligated and treated with BamHI and BgII to isolate a DNA fragment with two copies in a head-to-tail orientation. Fragments containing two copies were then inserted into the BamHI site of a PK (–40) Luc construct. The PK (–40) Luc construct is based on the pGL3 basic vector (Promega, Madison, WI) with a modified polylinker region. The L-PK gene sequence from –40 to +12 that by itself exhibits basal promoter activity was inserted between PstI and XhoI sites. Two copies of mut3/5 sequences were excised from the 2Xmut3/5 PK (–96) CAT construct (25) with BamHI and PstI and inserted into PK (–40) Luc construct. The FAS (–150/–43) sequence was excised from the FAS promoter construct described previously (27) and inserted into KpnI and MluI sites of PK (–40) Luc construct.

The SRE site mutation of the rat S14 gene was generated by

| Oligonucleotide | Sequence |
|-----------------|----------|
| rS14 ChoRE (–1448/–1422) | GCCAGGTCTCAGCAGTGTCGCTGG | |
| L-PK ChoRE (–171/–142) | GGCAGGCGGATCCGTGCTCT | |
| mut 3/5 ChoRE | CAGTTCATGCCTGCTGCCATGTTG | |
| m3–6 ChoRE | GTCCATGGACCTGGAAGCCT | |
| SRE-1 | TGACTCCACCACTTGAGGAS | |

The half E box sites of each ChoRE are underlined, while the SRE element is shown in bold type. The half E box sites of each ChoRE are underlined, while the SRE element is shown in bold type. The half E box sites of each ChoRE are underlined, while the SRE element is shown in bold type.
of probe. For competition EMSA, a 10-, 25-, or 50-fold molar excess of unlabeled oligonucleotide was added together with radiolabeled probe into the sample prior to the incubation.

RESULTS

Glucose Does Not Influence the Transactivating Potential of SREBP-1c—As noted above, several lines of experimental evidence implicate SREBP-1c as the transcription factor responsible for mediating the nutritional response of lipogenic genes to carbohydrate feeding. SREBP-1c expression has been shown to respond rapidly to insulin treatment, providing a direct link between insulin signaling pathways and SREBP (17, 19). However, induction of most lipogenic enzyme genes requires both insulin and elevated glucose metabolism in the hepatocyte, and no effects of glucose on SREBP expression have been found in hepatocytes (23, 32). To test the possibility that SREBP transactivation potential might be regulated by glucose, as suggested by Foretz et al. (23), the following transfection experiment was performed. A reporter construct containing two copies of a consensus SREBP-binding site, SRE-1, linked to a basal TATA box promoter from the pyruvate kinase gene was prepared. This construct was cotransfected into primary hepatocytes with a eucaryotic expression vector for the nuclear form of rat SREBP-1c (amino acids 1–403). Cells were then incubated with either low (5.5 mM) or high (27.5 mM) glucose for 30 h. These conditions are identical to those used for measuring glucose induction of L-PK or S14 promoters in hepatocytes. Cotransfection of SREBP-1c expression vector resulted in robust induction of luciferase activity from the SRE-1-containing reporter construct both in low and high glucose, as expected (Fig. 1A). However, no significant differences in the extent of induction were observed between samples from low and high glucose conditions, suggesting that the transactivation potential of SREBP-1c is not affected in the presence of high glucose.

Because of the possibility that endogenous SREBPs might interfere with the binding of the transfected SREBP-1c, an additional experiment was performed using a recombinant SREBP-1c in which its DNA-binding domain was replaced with the GAL4 DNA-binding domain. An expression vector for this recombinant protein was cotransfected into hepatocytes with a basal TATA box promoter construct containing five copies of the GAL4-binding site. Again, expression of the SREBP fusion protein activated promoter activity, but no significant differences were found between low and high glucose conditions (Fig. 1B). These data argue against the possibility that SREBP-1c activity is enhanced by signals generated by glucose metabolism in primary hepatocytes.

ChoRF and SREBP Possess Distinct DNA Binding Specificities—We recently showed by EMSA that a series of glucose-responsive ChoREs, but not glucose-unresponsive oligonucleo-
tides, can form a novel protein complex, designated ChoRF, when incubated with partially purified rat liver nuclear proteins (12). Consequently, ChoRF was suggested to be responsible for mediating effects of glucose on genes containing the ChoRE site, such as S14 and L-PK. Among the glucose-unresponsive oligonucleotides used in that study was a consensus SRE-1, a strong binding site for SREBP. These data suggested that ChoRF is not identical with the well characterized SREBP dimer. To further verify this observation, competition EMSA was performed. In this experiment, liver nuclear proteins were incubated with a radiolabeled ChoRE probe from the L-PK promoter, and the abilities of various oligonucleotides to compete for the binding of the ChoRF were tested. As expected, all natural and modified oligonucleotides with functional ChoRE activity were able to compete for ChoRF binding with L-PK ChoRE with the degree of competition increasing as a greater excess of oligonucleotide was added (Fig. 2A). On the other hand, an oligonucleotide containing the consensus SRE-1 was unable to interfere with ChoRF binding when tested over the same range of concentrations. These data indicate that the ChoRF complex is not identical to the SREBP-1c homodimer.

Because it was still possible that SREBP-1c might be a part of a larger ChoRF complex together with other components, the ability of an antibody to SREBP-1c to disrupt the formation of ChoRF was tested. As a control, the binding of SREBP-1c to an SRE-1 oligonucleotide was compared. Attempts to detect SREBP-1c binding using nuclear extracts were unsuccessful because of its low abundance, as noted by others (33). To circumvent this problem, extracts were prepared from COS-1 cells that were transfected with a vector overexpressing the nuclear form of SREBP-1c. Using the resultant nuclear extracts, formation of a specific band was observed with the consensus SRE-1 (Fig. 2B). Addition of anti-SREBP-1 antibody completely disrupted the band formed between SREBP-1c and the SRE-1 oligonucleotide and led to the appearance of a slower migrating supershifted band. However, the intensity of the ChoRF complex on the L-PK ChoRE was undiminished in the presence of anti-SREBP-1 antibody. Thus, SREBP-1c is not likely to be a component of the ChoRF complex.

To complement this experiment, we were interested in testing whether oligonucleotides with ChoRE activity can compete with SREBP binding to its DNA recognition site. To test the binding affinity of various ChoREs to SREBP-1c, a competition EMSA was again performed. Although the SRE-1 oligonucleotide itself could compete for the binding of SREBP-1c to radiolabeled SRE-1 probe, several ChoREs were unable to disrupt the SREBP-1c band with SRE-1 over the same concentration range (Fig. 3). It is noteworthy that the wild type rat S14 ChoRE competes to some degree with SREBP-1c for binding. The rat S14 ChoRE contains one perfect CACGTG element, a common binding site for basic/helix-loop-helix/leucine zipper factors including SREBP (28). To verify that the presence of the CACGTG sequence could lead to competition for SREBP binding, the adenovirus major late promoter upstream stimulatory factor-binding site, which also contains a perfect CACGTG sequence but is glucose-unresponsive, was used for competition EMSA. As predicted, the adenovirus upstream stimulatory factor-binding site could compete for the SREBP-1c binding with SRE-1 to a similar degree to rat S14 ChoRE (data not shown). Consequently, the ability to compete for SREBP-1c binding does not correlate with the ability of various oligonucleotides containing ChoRE activity to support a glucose response.

To further exclude a role for SREBP-1c in the glucose regulation of ChoREs, it was important to confirm the conclusions of the in vitro binding experiments in the context of the hepatocyte. For this purpose, the ability of SREBP-1c to activate ChoRE-containing constructs was tested by cotransfection assays. As a control, the reporter construct containing two copies of the SRE-1 oligonucleotide was used. Transfection of this construct into hepatocytes gave a minimal level of luciferase activity only slightly higher than that obtained with the construct lacking SRE-1 sites. Cotransfection of a control overexpressing a nuclear form of SREBP-1c resulted in a robust increase in reporter activity. An approximately 25-fold induction of luciferase activity by SREBP-1c was achieved by adding only 2.5 ng of expression vector, and a maximal induction of 35-fold was observed with 10 ng of vector (Fig. 4). No further increase in luciferase activity was observed by adding 40 ng of expression vector. In contrast to the results seen with SRE-1, only a modest increase of reporter activity was observed when SREBP-1c expression vector was cotransfected with constructs containing two copies of various ChoREs. Unlike the SRE-1
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Fig. 5. Comparison of the sequence requirements for the glucose response and SREBP-1c-dependent activation in primary hepatocytes. Each construct shown in Fig. 4 was compared for its response to glucose and to exogenous SREBP-1c expression in primary hepatocytes. A, cells were cultured in 5.5 or 27.5 mM glucose for 30 h. Luciferase activity is shown as fold induction of the luciferase activity in cell extracts treated with high glucose compared with the value obtained for cells treated with low glucose. Values represent the means (± S.E.) of two independent experiments, each with duplicate transfections. B, the data shown in Fig. 4 with 2.5 ng of CMV4/SREBP-1c expression vector is presented for the comparison with A.

Construct, further induction of ChoREs was observed by adding up to 40 ng of SREBP-1c expression vectors. These data suggest that SREBP-1c has a much lower affinity for the ChoREs than for the SRE-1 sequence. It is noteworthy that the luciferase activity driven by the L-PK ChoRE was not induced by addition of SREBP-1c expression in primary hepatocytes, which is consistent with data observed in COS cells or in mhaT3F hepatoma cells (24). In fact, the relative induction of various ChoREs by SREBP-1c correlates with SREBP-1c binding affinity examined in EMSA, in which SREBP-1c showed the highest affinity for the rat S14 ChoRE and the lowest affinity for the rat L-PK ChoRE (Fig. 3).

The same ChoRE-containing constructs that were tested for their activation by SREBP-1c were evaluated for their ability to respond to glucose in primary hepatocytes. As shown in Fig. 5A, all ChoRE-containing constructs were induced at least 7-fold by high glucose. The SRE-1 construct, however, showed no significant induction by high glucose. The effects of high glucose are directly compared with the effects of SREBP-1c overexpression in Fig. 5B. There was no correlation between the relative induction by glucose with that by constitutively active SREBP-1c. Hence, it is unlikely that SREBP-1c is the factor that specifically recognizes ChoREs and mediates glucose signaling in hepatocytes.

Glucose and Insulin Differentially Activate through ChoRF and SREBP-binding Sites—The above data on the distinct nature of ChoRF and SREBP led us to speculate that both factors may both be involved in mediating the effects of carbohydrate feeding on lipogenic gene induction. We hypothesize that SREBP is primarily involved in mediating insulin effects and ChoRF in mediating the effects of glucose. To test this possibility, we compared reporter constructs that contained binding sites for only ChoRF or SREBP for their responses to glucose or insulin. One test construct contained two copies of the rat S14 ChoRE linked to a basal TATA box promoter. The other construct contained a region of the rat FAS promoter between −150 and −43 linked to the same basal promoter. This region of the FAS gene has been shown to bind to SREBP and together with two adjacent accessory factor-binding sites to mediate responses to the combined actions of insulin and glucose (27). Each construct was transfected into primary hepatocytes, and cells were incubated in varying glucose concentrations with or without insulin. As shown in Fig. 6A, the ChoRE-containing construct responded to a change in glucose concentrations between 5.5 and 27.5 mM even in the absence of insulin in the medium. Note that in these conditions sufficient glucokinase should remain in the cell following isolation of hepatocytes from normal animals to support this partial glucose response. However, this construct was unresponsive to insulin when glucose levels were at 1.5 or 5.5 mM. These data suggest that insulin is not required as a direct signal for mediating the carbohydrate response through the ChoRE.

The promoter activity of the ChoRE-containing construct in high glucose without insulin was less than that observed in the presence of both high glucose and insulin. Because glucokinase expression is known to be regulated by insulin and critical for effective glucose metabolism, the low level of glucokinase expression might in part limit the response to glucose when insulin is absent. Indeed, when a glucokinase expression vector was introduced together with the reporter gene, the level of reporter gene activity at 27.5 mM glucose in the absence of insulin was substantially increased. Interestingly, expression of glucokinase also increased reporter gene activity at 5.5 mM glucose. Under low glucose conditions, glucokinase is sequestered in the nucleus bound in an inactive form by the glucokinase regulatory protein (34). The overexpression of glucokinase likely saturated the amount of glucokinase regulatory protein, and the excess glucokinase was then able to use the available glucose for stimulating glycolysis and promoter activity from the ChoRE construct. This effect was not seen at 1.5 mM glucose, where substrate is limiting. These results thus support the conclusions that the ChoRE is primarily a glucose-responsive element.

The results with the SREBP-binding site shown in Fig. 6B are sharply different. In this case, insulin simulates promoter activity at each glucose concentration tested, even at 1.5 mM.
glucose where substrate is limiting. Changing glucose concentrations has little or no effect in the absence of insulin and only a modest effect in its presence. Moreover, overexpression of glucokinase in cells without insulin had no effect on the ability of the cells to respond to glucose. Hence, the SREBP-binding site present in the FAS promoter would appear to be predominantly an insulin-responsive element. These data suggest that glucose and insulin may function through distinct transcription factors to mediate their effects following carbohydrate ingestion.

**S14 Gene Expression Is Regulated by Glucose and Insulin through ChoRF and SREBP-binding Elements**—We previously reported that mutations in the ChoRE sequence located between −1448 and −1422 of the rat S14 gene promoter diminished the ability of this promoter to support a response to glucose in cells cultured in the presence of insulin (25). Recently, Mater et al. (35) reported on the presence of an SREBP-binding site in the proximal S14 promoter between −139 and −131 and demonstrated that this site was responsible for the suppression of S14 promoter activity by polyunsaturated fatty acids. These observations raised the possibility that both of these binding sites might be important for the regulation of S14 gene expression by carbohydrate diet. Previously, S14 mRNA had been shown to respond to increased glucose levels in primary hepatocyte cultures (36), but effects of insulin on S14 gene expression in cultured cells have not been reported. We therefore tested whether S14 mRNA expression is regulated by insulin in cultured hepatocytes. Cells were first incubated in the absence of insulin and low glucose for 18 h. Then cells were switched to medium with or without insulin under both low and high glucose conditions. When S14 mRNA levels were monitored in cells treated for 8 h with insulin, a striking increase was observed even in low glucose conditions (Fig. 7). This response occurred rapidly with changes being detectable as early as 1 h after insulin treatment (data not shown). Addition of glucose in the absence of insulin also led to a significant increase in S14 mRNA levels, although the action of glucose was clearly less dramatic than insulin. Again, we presume that this reflects at least in part the important role of insulin in stimulating glucose metabolism. When cells were treated with both insulin and high glucose, a further increase in S14 mRNA was detected that was more than additive of the independent ac-
tions of the two effectors. These data suggest that expression of the \( S\text{\textsubscript{14}} \) gene is regulated by two different pathways, one stimulated by insulin directly (as observed in low glucose conditions) and one by glucose metabolism.

To test the roles of ChoRE and SREBP-binding sites in the induction of \( S\text{\textsubscript{14}} \) gene expression, we prepared a construct that contained the ChoRE-containing region of the \( S\text{\textsubscript{14}} \) gene (−1467 to −1395) linked to \( S\text{\textsubscript{14}} \) proximal promoter sequences from −290 to +18, including the SREBP-binding site. Mutations were introduced into the ChoRE, the SREBP-binding site, or both sites in the context of this construct. These various plasmids were then tested for their ability to respond to glucose, insulin, or the combination of these factors. With the wild type construct, a significant induction was observed in either the transition from 5.5 to 27.5 mM in the absence of insulin or by the addition of insulin in cells maintained in 5.5 mM glucose, suggesting both effectors can act on this construct (Fig. 8). When cells were treated with both high glucose and insulin, promoter activity was enhanced synergistically compared with treatment with either component alone. Both the ChoRE and SREBP-binding sites appear to be critical for this synergistic action of glucose and insulin. A mutation that disrupted the ChoRE site resulted in a significant reduction in the presence of high glucose either in the absence or presence of insulin. On the other hand, the effects of insulin on this construct were not diminished at 5.5 mM glucose, suggesting that the insulin effect is mediated by sequences other than the ChoRE. The actions of insulin on this construct are clearly more pronounced in the presence of high glucose, suggesting that the \( S\text{\textsubscript{14}} \) proximal promoter (−290/+18) may contain additional sequences that mediate glucose responsiveness. A mutation that disrupted the SREBP-binding site had an even more dramatic effect, completely disrupting the effect of insulin and leaving a construct with a modest, but significant, response (−6-fold) to glucose. Combining the ChoRE and SREBP-binding site mutations resulted in a promoter that was no longer affected by either glucose or insulin. These data support the role of both ChoRE and SREBP-binding sites in the overall response to glucose and insulin and suggest that the factors binding to these sites function in a synergistic fashion to regulate \( S\text{\textsubscript{14}} \) promoter activity.

**DISCUSSION**

The ChoRE was defined as a regulatory sequence within the L-PK and \( S\text{\textsubscript{14}} \) promoter regions that conferred a response to changes in glucose metabolism in primary hepatocytes (8–11). By EMSA, we subsequently found a novel complex, ChoRF, that formed between this site and liver nuclear proteins (12). The ChoRF complex was detected with a variety of wild type and variant ChoREs that supported glucose responsiveness in transfection assays but not with mutants of these oligonucleotides that were unresponsive. Consequently, we proposed that ChoRF was responsible for the induction of L-PK and \( S\text{\textsubscript{14}} \) gene expression observed when animals are fed a high carbohydrate diet and that it might also mediate the induction of many other lipogenic enzyme genes following the nutritional stimulus. In this model, the role of insulin was suggested to be largely permissive in allowing enhanced glycolysis under conditions of elevated glucose levels. In particular, glucokinase expression is dependent on insulin levels, and its expression is critical for the ability of the hepatocyte to respond to elevated glucose levels (5, 37).

Concomitant with this work, SREBP-1c emerged as a major factor regulating production of lipogenic enzymes. SREBP-1c gene expression was found to increase rapidly in response to insulin in hepatocytes (23, 32). In addition, SREBP-binding sites were identified within the promoter regions of a number of lipogenic genes, including FAS, glycerol-3-phosphate acyltransferase, and stearoyl-CoA desaturase (38–40). Transgenic mice overexpressing SREBP-1c in liver exhibited elevated lipogenesis and induced the program of lipogenic enzyme expression observed with carbohydrate feeding (21). Furthermore, mice bearing a homozygous disruption of the SREBP-1 gene were impaired in their nutritional response to high carbohydrate diet (22). Together, these results indicated that SREBP is a major transcription factor regulating lipogenic genes in response to carbohydrate feeding and raised the possibility that ChoRF might be identical to SREBP-1c. Consequently, we undertook the present study to analyze this question.

The data from the current study support a model in which ChoRF and SREBP-1c are discrete transcription factors that both play a role in the induction of lipogenic enzyme genes and are primarily responsible for mediating signals generated by glucose and insulin, respectively. Although both recognize sequences related to the E box consensus CACGTG, the binding patterns of the two factors are distinct. ChoRF binding was not competed by a consensus SRE-1 site, and SREBP binding was not competed by most ChoRE sites. ChoRF binding requires two E box half-sites related to the sequence CACG (12). These two half-sites are found in either an inverted orientation with 9-base pair spacing or a direct orientation with 7-base pair spacing, and the spacing has been shown to be critical to ChoRF binding and function (11). ChoRF does not bind directly to oligonucleotides with a single CACGTG motif in which the half-sites are situated in an inverted orientation without spacing between them. SREBP can bind to the CACGTG motif in vitro (28), but in naturally occurring genes characterized to date, all SREBP-binding sites consist of E box half-sites related to the sequence (Py)CAC. In the consensus SRE-1 site, these half-sites are found as direct repeats with a 1-base pair spacing; however, much variation is found in the sequence and arrangement of naturally occurring SREBP-binding sites. The similarity in the nature of the binding sites suggests that ChoRF and SREBP may be related family members of the basic/helix-loop-helix/leucine zipper transcription factor family.

In addition to differences in binding specificities, a number of other lines of evidence suggest that ChoRF and SREBP are distinct factors. First, ChoRF migrates on EMSA more slowly than the SREBP-1c dimer formed on the consensus SRE-1 site. Second, ChoRF binding is strongly inhibited by poly(dI:dC) in EMSA, whereas SREBP is routinely assayed in the presence of this competitor. Third, SREBP was shown to bind tightly to a strong cation exchanger, S-Sepharose (33), whereas ChoRF did not bind to this resin at 0.1 M NaCl. Fourth, an antibody to SREBP did not interfere with ChoRF binding. Although it is possible that the epitope is precluded in the ChoRF complex, this same antibody efficiently competed for SREBP binding. Finally, SREBP expression is induced by feeding a carbohy-
Roles of ChoRF and SREBP in Lipogenic Gene Expression

Based on this information, we propose the following model for the induction of lipogenic genes by high carbohydrate diet (Fig. 9). Elevated blood glucose levels following a meal promote secretion of insulin from the pancreatic β cell. Insulin acts on the hepatocyte insulin receptor and leads to the induction of SREBP-1c mRNA and protein. SREBP binds to the promoter region of many lipogenic enzyme genes where it functions to stimulate transcription. Simultaneously, increased glucose levels following a carbohydrate meal lead to elevated glycolysis in the hepatocyte. This is mediated in part by the presence of the high K_m glucokinase, which allows glycolysis in the hepatocyte to be increased proportionately with postprandial glucose levels. Because glucokinase expression is induced by insulin, perhaps through SREBP-1c (23), the two pathways are partially coupled to each other. Increased glucose metabolism in the hepatocyte results in the generation of an unknown intracellular signal that activates the ChoRF. ChoRF binds to a distinct site from SREBP-1c on the promoter regions of many lipogenic enzyme genes to activate their expression. In many cases, ChoRF may function synergistically with SREBP to activate transcription of lipogenic enzyme genes and promote lipogenesis in the liver.

In support of this model, we have shown that the S14 gene product is induced directly by insulin, in addition to its stimulation by glucose. This possibility was suggested by earlier studies in which diabetic animals were shown to possess reduced levels of S14 mRNA, whereas insulin treatment normalized its expression (41). However, it is difficult to distinguish direct effects of insulin from those of glucose metabolism in such experiments. We have shown in this report that S14 gene expression is regulated independently and synergistically by glucose and insulin. Two regulatory sequences, the ChoRE at −1435 and an SREBP-binding site at −135, are required for the synergistic activation of S14 promoter activity. The critical roles of these two sites were demonstrated by mutations that disrupted either or both sites. Clearly, the S14 promoter construct with both sites mutated is completely incapable of responding to glucose and/or insulin. Mutation of the SREBP-binding site resulted in a complete loss in the ability of the S14 promoter to respond to insulin, consistent with the role of SREBP in mediating insulin action. Similarly, a construct that contained an SREBP-binding site from the FAS promoter also responded primarily to insulin. The SREBP-binding site mutant of the S14 promoter construct retained the ability to respond to glucose, but the overall activity of this construct was greatly reduced compared with the wild type construct. This effect presumably reflects the multiple roles of insulin in stimulating aspects of glucose metabolism as well as its direct action on SREBP. Mutations of the ChoRE site of the S14 gene, on the other hand, are reduced in their ability to respond to glucose but retain most of the insulin responsiveness. These data suggest that ChoRF is primarily mediating a glucose signal. The ability of the two copy ChoRE construct to respond to glucose in the absence of insulin also supports this model. However, the ChoRE mutant construct still retains significant glucose responsiveness. Several explanations could account for this observation. First, the S14 proximal promoter (−290/+18) might contain additional elements capable of contributing to a glucose response. Second, ChoRF might have some ability to bind the SREBP site and mediate the glucose response. Third, SREBP itself might be stimulated by glucose. The latter explanation seems less likely because the glucose effect was not observed with the FAS construct that contains only SREBP sites (Fig. 6). Investigations are currently underway to explore these possibilities.

Because the induction of many or most lipogenic enzyme genes in cultured hepatocytes requires both glucose and insulin, we suggest that dual regulation by ChoRF and SREBP-1c might be a common mechanism for transcriptional control. We have found evidence for the fatty acid synthase gene to support this contention. In the FAS promoter, a pair of SREBP-binding sites is found overlapping an E box site at approximately −65 (38). This site has been shown to be critical for supporting a 3–6-fold induction of insulin in cultured cells or hepatocytes (17, 27, 42). However, the induction of FAS that occurs in the whole animal upon feeding carbohydrate diet is on the order of 25–30-fold, suggesting that additional regulatory sequences exist (43, 44). Recently, Rufo et al. (45) presented evidence for the role of an upstream enhancer of the fatty acid synthase gene that contributes to the overall nutritional response. We found that this region contains a site for binding of ChoRF and that this site is functional in supporting a response to glucose.

Thus, FAS may be regulated in a similar manner to S14 via a synergistic activation by both ChoRF and SREBP. That this may also be true for other lipogenic genes is suggested by the phenotype of SREBP-1 knockout mice. Although these mice are impaired with respect to their ability to respond to carbohydrate feeding, most of the lipogenic genes retain a significant level of induction even in the absence of SREBP-1 (22). For example, FAS mRNA is induced 10-fold in the knockout mice, whereas S14 mRNA levels increase 6-fold. These data imply that another factor in addition to SREBP-1 is capable of mediating nutritional responses to carbohydrate diet.

An alternative model proposed by Foretz et al. (19, 23) suggests that SREBP-1c is regulated by insulin at the level of transcription and by glucose post transcriptionally to mediate carbohydrate induction of lipogenic genes. This hypothesis was based on the observation that an adenoviral expression vector for the nuclear form of SREBP-1c enhanced expression of L-PK based on the observation that an adenoviral expression vector for the nuclear form of SREBP-1c enhanced expression of L-PK or FAS more effectively in cells cultured in high glucose than in cells in low glucose. However, no alterations in SREBP-1c mRNA or protein levels were found in hepatocytes exposed to varying glucose levels (23, 32). As shown in Fig. 1, glucose does not affect the transactivation potential of SREBP on cotransfected reporter constructs, further arguing against this model. A couple of explanations might account for the apparent discrepancy between these experiments. First, in a study by Foretz et al. (23), measurements were made on mRNA levels produced from endogenous genes. If SREBP and ChoRF can function synergistically, as suggested by our data, then the enhanced mRNA levels could be accounted for by the dual regulation of these gene products by exogenous SREBP and endogenous ChoRF stimulated by the high glucose conditions. A second explanation arises from the observation that glucose acts not only to stimulate transcription but also to stabilize mRNA levels for many lipogenic enzyme genes (2). Hence, in measuring mRNA levels in their study, Foretz et al. (23) could have been observing the effects of SREBP stimulation of transcription and glucose stabilization of mRNA. In the cotransfection experiments, effects at the level of mRNA stability would not be observed.

Mice bearing a homozygous deletion of the SREBP-1 gene display an impaired response to a carbohydrate diet in the induction of any of 10 lipogenic enzyme gene products examined, suggesting a broad role of SREBP in the nutritional induction (22). Although these results clearly demonstrate an

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essential role for SREBP in the nutritional induction pathway, they must be interpreted with some caution. Expression of glucokinasase is critical for the ability of the hepatocyte to increase its rate of glycolysis in response to glucose. If SREBP-1c is directly responsible for the induction of glucokinasase by insulin, as suggested by recent results (19), then the phenotype of the SREBP-1 null hepatocyte could arise from a defect in expression of this critical gene and indirectly affect other lipogenic enzyme genes. Similarly, studies in which SREBP-1 is overexpressed have shown that many or most lipogenic enzyme genes are induced (21). These data certainly support a broad role for SREBP in the nutritional stimulation of lipogenic enzyme genes. However, again the possibility that overexpression of SREBP could induce glucokinasase gene expression and turn on increased glucose metabolism must be considered. In fact, as shown in Fig. 6, overexpression of glucokinasase in hepatocytes does result in elevated promoter activity from a construct containing a ChoRE site even at fasting glucose levels. Thus, the demonstration of a direct role of SREBP in regulation of any lipogenic enzyme gene by carbohydrate feeding requires the detection of a functional SREBP-binding site that when mutated reduces or eliminates the ability of the promoter to respond to insulin and/or glucose.

If most of the lipogenic enzyme genes are indeed regulated by both ChoRF and SREBP similarly to S14 and FAS, what might be the physiological basis for this dual regulation? One possibility is that this control ensures that lipogenesis is not inappropriately turned on in the liver unless both anabolic signals from insulin and glucose metabolism are in agreement. An appropriately turned on in the liver unless both anabolic signals

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