The mammalian liver is primarily responsible for the conversion of excess dietary carbohydrate to triglycerides, a process known as lipogenesis. The first hepatic response to carbohydrate intake is the activation of key rate-limiting enzymes that convert carbohydrate to triglycerides. In the second phase, a longer term response is generated by the induction of a variety of enzymes involved in this process. These include enzymes of glycolysis, fatty acid biosynthesis, and triglyceride synthesis and maturation. Enzyme induction results from the increased levels of the respective mRNAs, and in a number of cases, this regulation occurs at the level of transcription (for review see Refs. 1–4). The effects of dietary carbohydrate in the animal can be mimicked in primary hepatocytes by increasing media glucose concentrations and consequently rates of glucose metabolism (5, 6). The glucose response in primary hepatocytes depends upon the presence of insulin, whose role is largely permissive through the activation of glucokinase gene expression (1, 7, 8). The intracellular mediator of the carbohydrate response has not conclusively been identified, although both glucose-6-phosphate and xylulose-5-phosphate have been proposed as candidates (9, 10).

Other effectors that coordinate regulate most lipogenic enzyme genes include glucagon and polyunsaturated fatty acids, which repress gene expression, and thyroid hormones, which induce expression (for review see Refs. 2 and 11). In several cases, thyroid hormones support the synergistic activation of lipogenic enzyme expression with carbohydrate both in whole animals and in cultured primary hepatocytes. The molecular mechanism of the functional synergism by thyroid hormones and carbohydrate is unknown but does occur at the pretranslational level (5, 6, 12, 13).

The rat $S_{14}$ gene was first investigated because of its rapid response to thyroid hormones in rat liver and primary hepatocytes (14–16). Subsequent studies showed that $S_{14}$ mRNA levels are controlled by stimuli that modulate fatty acid formation, including dietary carbohydrate (6, 12, 17). Together with its restricted distribution in tissues active in lipogenesis, these data suggested a role for $S_{14}$ in lipogenesis (17). This suggestion was supported by experiments using antisense $S_{14}$ oligonucleotides, which blocked the normal lipogenic response of hepatocytes (18, 19). The rapid induction of $S_{14}$ mRNA levels by carbohydrate occurs mostly at the transcriptional level (13). Transfection analysis in primary hepatocytes led to the identification of regulatory sequences responsible for the carbohydrate response (20–22). These sequences from −1467 to −1422 consist of two distinct sites. One site, designated the ChoRE or carbohydrate-response element, is found from −1448 to −1422 in the rat $S_{14}$ gene and consists of two motifs related to the E box sequence CACGTG separated by 5 bp. When coupled in tandem copies, the ChoRE confers a response to glucose by itself, suggesting it is the binding site for a factor directly regulated by glucose. However, in the context of the natural promoter, an adjacent site from −1467 to −1449 is required for maximal induction. This accessory site binds an unknown hepatic transcription factor to augment the glucose response (22). The ChoRE of the rat $S_{14}$ gene bears striking similarity to a glucose regulatory sequence mapped in the L-PK gene, another gene regulated by carbohydrate metabolism (23, 24). Based on the sequence of the ChoRE, it has been proposed that a member...
of the bHLH/LZ transcription factor family binds to the ChoRE and activates transcription in response to glucose. The nature of the carbohydrate-responsive factor, however, is controversial. Whereas the bHLH/LZ factor USF can bind to the ChoRE in vitro (21, 24–26) and has been proposed to be required for the glucose response (27–29), other data argue against its direct participation (30). Clearly, identification of the carbohydrate-responsive factor is critical to further studies on the mechanism governing carbohydrate regulation of lipogenic gene expression.

Recently, the mouse homologue of the S14 gene was cloned (31). Like its rat counterpart, the mouse S14 gene is regulated by dietary carbohydrate in vivo (28). In this report, we map the mouse S14 gene sequences critical for its control by carbohydrate. We demonstrate that the mouse S14 gene employs three adjacent regulatory sequences for supporting a glucose response in primary hepatocytes. One of these sequences functioned analogously with the rat S14 and L-PK ChoREs. Examination of this site in the mouse S14 gene led to the refinement of the ChoRE consensus sequence and identification of a novel liver nuclear factor that binds to the ChoRE.

**EXPERIMENTAL PROCEDURES**

**Primary Hepatocyte Culture and Transfection**—Primary hepatocytes were isolated from male Harlan Sprague-Dawley rats (180–260 g) using the collagenase perfusion method as described previously (30). After a 3–6-h attachment period, cells were transfected using either Lipofectin (Life Technologies, Inc.) or F1 reagent (Targeting Systems) in modified Williams’ E medium with 23 mM HEPES, 0.01 μM dexamethasone, 0.1 unit/ml insulin, 1 unit/ml penicillin, 1 μg/ml streptomycin, and 5 mM glucose for 12–14 h. Subsequently, cells were cultured in medium containing either 5.5 or 27.5 mM glucose, with or without 500 nM T3. For studies using T3, Matrigel (Collaborative Biomedical Products) was added to the plates at a concentration of 0.33 mg/ml after transfection. 

**Preparation of Nuclear Extracts**—Preparation of nuclear extracts was previously described (22). The crude liver nuclei were then fractionated by PEG (polyethylene glycol) precipitation. For the binding assays described, the PEG fraction from 0 to 6.7% was exclusively used.

**Oligonucleotide Synthesis**—Oligonucleotides containing sequences of the mouse S14 gene linked to the corresponding sequence of the mouse S14 gene were synthesized and used to amplify the mS14 gene sequence from mouse S14 genomic DNA using polymerase chain reaction as described previously (34). Briefly, each oligonucleotide creating either a unique Bgl II site (TRE mut) or Nsi I site (mut1-8) was synthesized and used to amplify the mS14 gene using polymerase chain reaction and digested at the introduced restriction enzyme site, purified by gel electrophoresis, ligated, and transformed into Escherichia coli. Mutant constructs were isolated, and mouse S14 sequences were excised with BamHI and HindIII and inserted into mS14 (−279/+18) CAT.

**Clustered point mutations were generated by inverse polymerase chain reaction as described previously (34).** Briefly, each oligonucleotide creating either a unique Bgl II site (TRE mut) or Nsi I site (mut1-8) was synthesized and used to amplify the mS14 gene using polymerase chain reaction and digested at the introduced restriction enzyme site, purified by gel electrophoresis, ligated, and transformed into Escherichia coli. Mutant constructs were isolated, and mouse S14 sequences were excised with BamHI and HindIII and inserted into mS14 (−279/+18) CAT construct.

**Results**

**The Sequence of the Mouse S14 Gene Corresponding to the Rat S14 ChoRE Does Not Support a Carbohydrate Response**—The S14 gene sequence from −1467 to −1422 contains two sites, the accessory factor site and ChoRE, which are required to support a transcriptional response to glucose (22). The mouse S14 gene sequence from −1411 to −1366 was exccised from the S14 gene sequence from −1411 to −1366 aligned to that region of the rat gene with an identity of 85%. Despite this high degree of similarity, the mouse sequence corresponding to the rat ChoRE contains a number of mismatches in the E box motifs at positions previously shown (30) to be essential for the glucose response (Fig. 1A). This observation raised the question of whether the rat ChoRE is functionally conserved in the mouse gene. To investigate this question, an oligonucleotide containing the mouse sequence from −1411 to −1366 was cloned into a glucose-unresponsive PK (−96/+12) CAT reporter construct, which we have previously used as a basal promoter construct (22, 30). As shown previously (22) and in Fig. 1B, a reporter

**Electrophoretic Mobility Shift Assay—** EMSA was performed as described previously (20). A typical reaction contained 100,000 cpm (10–30 fmol) of 32P-labeled oligonucleotide with 10–20 μg of nuclear protein. Nonspecific competitors were 0.1 μg of poly(dI-dC) and 1.9 μg of poly(dA-dT). Following incubation at room temperature for 30 min, samples were subjected to electrophoresis on a 4.5% nondenaturing polyacrylamide gel and subjected to phosphorimager analysis. Antibodies to USF1 (C-20) and USF2 (C-20) were from Santa Cruz Biotechnology and were added together to nuclear extract for 20 min at 4 °C prior to the addition of probe.

**FIG. 1.** The rat S14 gene ChoRE is not conserved in the corresponding region of the mouse S14 gene. A, the sequence of the rat S14 glucose regulatory region (−1467/−1422) was aligned with the sequence of the mouse S14 gene. The two E boxes of the rat S14 ChoRE are boxed and compared with the corresponding sequences of the mouse gene. Differences in sequence occur at bases that have previously been shown to be critical for glucose responsiveness (position 4 of the upstream E box and positions 2 and 3 of the downstream E box (30)). B, rat primary hepatocytes were transfected with CAT reporter constructs containing either the glucose-responsive sequence of the rat S14 gene or the corresponding sequence of the mouse S14 gene linked to the PK (−96) promoter. As a control, a CAT reporter construct containing the PK (−96) promoter alone was also transcribed. Cells were cultured in 5.5 (solid bars) or 27.5 (hatched bars) mM glucose for 48 h. CAT activity is shown as relative percentage conversion of chloramphenicol to its acetylated forms with the value for the rS14 (−1467/−1422)/PK (−96) CAT construct at 27.5 mM glucose as 100%. Values represent the mean (± S.E.) of three independent experiments, each with duplicate transfections.
Glucose Regulation of Mouse $S_{14}$ Gene Expression in Hepatocytes

A, CAT reporter constructs with varying segments of the 5'-flanking region of the mouse $S_{14}$ gene were tested for a response to glucose and T$_3$ in primary hepatocytes. Cells were cultured in four different conditions for 48 h as follows: 5.5 mM glucose (solid bars), 27.5 mM glucose (hatched bars), 5.5 mM glucose + 500 nM T$_3$ (stippled bars), or 27.5 mM glucose + 500 nM T$_3$ (crosshatched bars). CAT activity is shown as relative percentage conversion of chloramphenicol to its acetylated forms with the value of the mS$_{14}$(-5643/18) CAT construct at 27.5 mM glucose + 500 nM T$_3$ as 100%. Values represent the mean (± S.E.) of three to five independent experiments, each with duplicate transfections.

B, CAT reporter constructs with either deletions or mutations in the TRE of the 5'-flanking regions of the mouse $S_{14}$ gene were tested for the response to glucose and T$_3$ in primary hepatocytes as described above. The position of the TRE substitution mutation is shown in Fig. 3A. CAT activity is shown as relative percentage conversion of chloramphenicol to its acetylated forms with the value of the mS$_{14}$(-5643/18) CAT construct at 27.5 mM glucose + 500 nM T$_3$ as 100%. Values represent the mean (± S.E.) of three to five independent experiments, each with duplicate transfections.

The region of the mouse $S_{14}$ gene from −1540 to −1368 is sufficient to confer a glucose response in the presence of thyroid hormone. A, CAT reporter constructs with varying segments of the 5'-flanking region of the mouse $S_{14}$ gene were tested for a response to glucose and T$_3$ in primary hepatocytes. Cells were cultured in medium containing both high glucose and T$_3$. Activity of this construct was only slightly increased by either high glucose or T$_3$ alone compared with cells maintained in low glucose without T$_3$ (Fig. 2A). However, when transfected cells were cultured in medium containing both high glucose

and T$_3$, the mouse sequence conferred a strong induction, indicating a synergism between glucose and thyroid hormone in transactivating the gene. Thus, the mouse $S_{14}$ gene is capable of being regulated by factors in the rat hepatocyte.

To localize the regulatory sequences required for the effects of glucose and T$_3$, deletions of the 5'-end of the mouse $S_{14}$ genomic sequences were generated and tested in primary hepatocytes. Deletions from the 5'-end to −1540 still supported the synergistic effects of glucose and thyroid hormone, although the magnitude of the response diminished somewhat (Fig. 2A). Further deletion to −1147 completely abolished the response. These results suggest that the sequence from −1540 to −1148 contains a regulatory element or elements that supports the synergism between glucose and thyroid hormone. Further deletion analysis within this region demonstrated that the sequence from −1540 to −1148 was sufficient to confer the response to glucose and T$_3$ in primary hepatocytes (Fig. 2B). Promoter activity directed by sequences downstream from −1368 was unaffected by these treatments. Thus, regulatory sequences in the mouse $S_{14}$ gene are contained within the same DNA region as the rat gene but differ in requiring T$_3$ for their activity.

Sequence analysis revealed a putative thyroid hormone receptor binding site between −1522 to −1494 in the mouse $S_{14}$ gene (see Fig. 3A). This sequence contains two motifs related to the consensus TRE half-site (A/G)GGTCA in an inverted orientation with 7-bp spacing. Indeed, an oligonucleotide containing this sequence can bind to $in vitro$ translated thyroid hormone receptor/retinoid X receptor heterodimers by EMSA. To verify the functional significance of this sequence, a clustered

FIG. 3. Two regulatory regions of the mouse $S_{14}$ gene are responsible for the response to glucose. A, the positions of eight clustered point mutations with 8–10-bp substitutions within the mouse $S_{14}$ gene sequence from −1469 to −1368 are shown. In addition, the position of the TRE mutation tested in Fig. 2B is indicated. All mutations were tested in the context of mS$_{14}$(-1540/−1368/−279/+18) CAT reporter construct. B, each construct shown was tested for the response to glucose and thyroid hormone in primary hepatocytes. Cells were cultured in 5.5 mM glucose alone (solid bars) or 27.5 mM glucose + 500 nM T$_3$ (crosshatched bars) for 48 h. CAT activity is shown as percentage conversion of chloramphenicol to its acetylated forms. Values represent a representative set of data from three independent experiments, each with duplicate transfections.

S.-H. Koo, and H. C. Towle, unpublished results.
Glucose Regulation of Mouse S<sub>14</sub> Gene Expression in Hepatocytes

Fig. 4. A novel ChoRE and an accessory factor site from the mouse S<sub>14</sub> gene are identified. Oligonucleotides containing sequences of the mouse S<sub>14</sub> gene from -1450 to -1425 or from -1421 to -1392 were synthesized, ligated in three copies in a head-to-tail orientation, and inserted into the PK(-96)CAT reporter construct. Each construct was tested for a response to glucose in primary hepatocytes. Cells were cultured in 5.5 (solid bars) or 27.5 (hatched bars) mM glucose for 48 h. CAT activity is shown as relative percentage conversion of chloramphenicol to its acetylated forms with the value of the mS<sub>14</sub>(-1469/-1368)PK(-96)CAT construct at 27.5 mM glucose as 100%. Values represent the mean (± S.E.) of three independent experiments, each with duplicate transfections.

Two Separate Sequences, in Addition to the TRE, Are Essential for the Carbohydrate Induction—To identify additional regulatory sequences involved in the glucose response, further clustered point mutations were introduced into the mouse S<sub>14</sub> gene. For this purpose, we focused on the sequence from -1469 to -1368, as this segment was capable of supporting a glucose response independent of thyroid hormone when linked to the heterologous PK(-96/+12) promoter (see Fig. 4). Eight clustered point mutants were designed spanning this segment (Fig. 3A). Because the TRE present in -1522 to -1494 can augment the glucose-generated signal, the mutations were generated in the context of the mouse S<sub>14</sub> gene from -1540 to -1368 and linked to the mouse S<sub>14</sub>(-279/+18) promoter. Each mutant construct was transfected into primary hepatocytes and cultured in the presence of 5.5 mM glucose or 27.5 mM glucose plus 500 nM T₃ for 48 h. The ability of three of these mutant constructs (mut3, mut5, and mut6) to respond was largely or completely abolished (Fig. 3B). All other mutant constructs displayed responses that were comparable to or, in the case of mut7, greater than the wild type construct. It is worth noting that the mut8 mutation alters sequences corresponding to the ligand-bound thyroid hormone receptor works as an accessory factor to augment the glucose induction of the mouse S<sub>14</sub> gene, and an independent carbohydrate-response element is also present within this region.

Fig. 5. Two E box half sites are critical for a glucose response. A, the sequences of six point mutations within the mouse S<sub>14</sub> ChoRE are shown. B, oligonucleotides containing the indicated mutations were ligated in three copies in a head-to-tail orientation and inserted into the PK(-96)CAT reporter construct. Each construct shown was tested for a response to glucose in primary hepatocytes. Cells were cultured in 5.5 (solid bars) or 27.5 (hatched bars) mM glucose for 48 h. CAT activity is shown as percentage conversion of chloramphenicol to its acetylated forms with the value of the 3X(3-1)PK(-96)CAT construct at 27.5 mM glucose as 100%. Values represent the mean (± S.E.) of three to five independent experiments, each with duplicate transfections.

To investigate the role of the two regulatory sites predicted by mutagenesis in the mouse S<sub>14</sub> gene, two oligonucleotides containing these sequences were generated. Each oligonucleotide was ligated in three copies in a head-to-tail orientation and linked to a CAT construct containing the PK(-96) basal promoter. These constructs were tested in primary hepatocytes at low or high glucose concentrations. The construct containing the sequence from -1421 to -1392 (corresponding to sequences mutated in mut5 and mut6) did not respond to glucose but showed higher basal activity in low glucose, suggesting that a regulatory sequence for an accessory factor is present in this region (Fig. 4). Interestingly, the corresponding region of the rat S<sub>14</sub> gene also contains an accessory factor site. The construct containing the sequence from -1450 to -1425 of the mouse S<sub>14</sub> gene (corresponding to mut3) conferred a glucose response even in the absence of other regulatory sequences. This result indicates that this site functions as a ChoRE in the hepatocyte. Surprisingly, the mouse S<sub>14</sub> ChoRE did not contain two E box motifs separated by 5 bp as had been found in previously identified ChoREs from the rat S<sub>14</sub> and the L-PK genes (22).

Two E Box “Half Sites” Are Important for the Glucose Response—The lack of clearly distinguishable E box motifs in the mouse S<sub>14</sub> ChoRE led us to investigate which sequences within this region are critical for the glucose response. Consequently, five point mutations of 2 bp were introduced across the ChoRE sequence (Fig. 5A). In particular, we targeted CA (or TG) dinucleotides that might represent portions of degenerate E box sequences. Oligonucleotides containing these mutations were linked in three copies to the PK(-96)CAT construct to test for glucose responsiveness. Only two of the mutant constructs, 3-2 and 3-4, showed significant reductions in glucose-stimulated promoter activity (Fig. 5B). Examination of the sequences surrounding these inactivating mutations revealed
that each was part of a potential E box half site: CACG (for 3-2)
and CAGC (for 3-4). Further scrutiny of both the rat S14 and PK
ChoREs reveals that each also contains two potential E box
half sites related to CACG (see “Discussion”). To test if this E
box ‘half site’ might represent an essential element for the
response, an additional mutation was introduced into the
mouse S14 ChoRE to substitute GC in the second putative
E box half site with CG, generating a CACG sequence. This
mutated construct, 3-4, retained its ability to confer a strong
response to glucose. Thus, the presence of two E box half sites
related to the sequence CACG may be the critical determinants
for mediating the glucose response.

A Novel Factor That Binds to ChoREs Is Present in Liver
Nuclear Extract—To search for the specific nuclear factor that
is responsible for the glucose response of the mouse S14 ChoRE,
rat liver nuclear extracts were prepared, fractionated by PEG
precipitation, and used for EMSA with a mouse S14 ChoRE
probe. Using the fraction precipitated between 0 and 6.7%
PEG, three complexes, designated bands x, y, and z, were
observed (Fig. 6). The same three bands were detected with
unfractionated nuclear extract, although the intensities of bands x and y were much lower relative to band z prior to
fractionation. To determine whether any of these bands might
be a candidate for the carbohydrate-responsive complex, the six
mutant mouse S14 ChoREs tested above were used for EMSA.
Among the three bands detected with the wild type mouse S14
ChoRE, only the slowest migrating band (band x) was retained
in lanes with mutant ChoREs that were glucose-responsive but
not with glucose-unresponsive oligonucleotides. Note that the
weak band observed with the 3-4 oligonucleotide that migrates
similarly to band x on this gel showed distinctively slower
mobility with longer electrophoretic conditions and 3-4 could
not compete for band x formation with the wild type mouse S14
ChoRE. In contrast, band y was barely detectable with the
glucose-responsive 3-6 oligonucleotide, whereas band z was
observed with 3-3. Thus, band x contains a factor(s) that is a
candidate for the carbohydrate-responsive factor, and we have
designated this complex as ChoRF.

Fig. 6. Detection of a novel complex formed by mouse S14
ChoRE and ChoRE mutants that are glucose-responsive. EMSA
was performed as described under “Experimental Procedures” with rat
liver nuclear proteins and wild type (WT) or the six mutated mouse S14
ChoREs shown in Fig. 5A. The arrows indicate the positions of three
bands detected with the wild type mouse S14 ChoRE oligonucleotide.
Note that the band labeled “x” is detected with all oligonucleotides that
support a glucose response. Band “y” is not observed with the glucose-
responsive mutant 3-6, whereas band “z” (USF) is not observed with
the glucose-responsive mutant 3-3. All lanes contained 20 μg of nuclear
protein except lane 3–6, which contained 10 μg.

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and CAGC (for 3-4). Further scrutiny of both the rat S14 and PK
ChoREs reveals that each also contains two potential E box
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candidate for the carbohydrate-responsive factor, and we have
designated this complex as ChoRF.
Spacing mutations that modify glucose responsiveness of the ChoRE also alter formation of the ChoRF complex. EMSA was performed with 10 μg of rat liver nuclear protein and oligonucleotides derived from either the consensus CACGTG ChoRE (30) or L-PK ChoRE. Mutations that change the spacing between E box motifs from the natural spacing of 5 bp between the two E box motifs (N5) to either 4 bp (N4) or 6 bp (N6) were used (22). The thick arrow indicates the novel ChoRF complex, whereas the thin arrow indicates the position of the USF complex.

Although ChoRF migrates more slowly than USF, it is still possible that USF may be a component of the ChoRF band observed on EMSA. To investigate this possibility, we tested whether anti-USF antibody could disrupt the formation of ChoRF. As a control, the adenovirus major late promoter USF binding site was used. USF binding of the adenovirus USF binding site was effectively inhibited by adding increasing amounts of anti-USF antibody (Fig. 9). However, the intensity of the ChoRF complex on two different ChoRE probes was unchanged in the presence of anti-USF antibody. Thus, USF is not likely to be a component of the ChoRF complex.

DISCUSSION

The ability of the hepatocyte to respond to elevated metabolism of glucose and other glycolytic substrates by increasing transcription of genes involved in fatty acid production has provided a valuable model for exploring nutrient control of mammalian gene expression. In particular, the rat L-PK and S14 genes have been intensively analyzed by transfection studies and transgenic mice. The 5'-flanking regulatory sequences localized at different sites to respond to the same signal. Based on both functional and binding studies, the mouse ChoRE appears to be a weaker response element than the rat ChoRE. Thus, it might be expected that the mouse element requires the cooperation of two accessory factor sites, rather than just one, to effectively support a glucose response. In this case, the positioning of the mouse ChoRE between the TRE and an accessory factor site may allow it to effectively interact with factors binding at both of these sites. How this change occurred evolutionarily following the divergence of these two species is a matter for speculation. It would be
interesting to compare glucose regulatory sequences in other related species to explore which represents the primordial organization.

The organization of regulatory elements functioning coordinately with accessory factor sites is commonly found in genes encoding enzymes of metabolic importance. For example, genes encoding enzymes required for cholesterol synthesis and uptake are regulated by SREBP transcription factors in response to cellular levels of cholesterol (for review see Refs. 37 and 38). SREBPs bind to a common site, the SRE-1, found in those genes. However, in all cases tested, SREBPs are unable to transactivate without the presence of other transcription factors binding at sites adjacent or near to the SRE-1. Several different accessory factors, including Sp-1 and nuclear factor-Y, have been found to function with SREBPs to increase the transcriptional signal mediated in response to cholesterol depletion (39–41). In the case of Sp1 and nuclear factor-Y, the synergistic effect was due to cooperative binding to DNA through direct interaction of the two factors. In other cases, functional synergism between two factors does not involve direct interaction and presumably arises from interactions with additional components (36). The mechanism by which the accessory factor for the mouse and rat S14 gene functions to augment the glucose response is currently unknown.

Previous work on the rat S14 and L-PK genes suggested that two CACGTG-type E boxes are critical for the glucose response of these genes (22–24). Each E box was proposed to bind to a bHLH/LZ dimer and both dimers would presumably be required for glucose signaling. Based on the analysis of mouse S14 ChoRE, we postulate a new model for the ChoRE. In this model, the ChoRE consists of two E box half sites related to the CACG motif. Such a regulatory site could act by binding to a single dimer of the bHLH family. In the mouse S14 ChoRE, mutations that disrupted the CACG or CAGC sequences inhibited the glucose response, but mutations of bases immediately downstream of these sequences did not. Furthermore, formation of the ChoRF complex in the EMSA was not affected by the latter mutations. Because we detected the same complex in the EMSA with the rat S14 and L-PK ChoREs, it is possible that these ChoREs also function through two CACG-type E box half sites rather than the 6-bp E box motifs. Indeed, the rat S14 and L-PK ChoREs fit the new model (Fig. 10B). Mutations of the rat S14 or L-PK ChoREs that affect the first 4 bp of the CACGTG motif abolished the response to glucose, whereas mutations of the fifth and sixth positions did not disrupt the glucose response, suggesting that only the first 4 bp of the E box are critical (30). Similarly, mutations of the rat or mouse S14 ChoREs that give a better fit to the consensus CACG motif generally behave as up-mutations. Despite these similarities, there is one distinct difference in the structure of the previously characterized ChoREs and that of the mouse S14 gene. In the ChoREs of the rat S14 and L-PK genes, the two CACG motifs are separated by 9 bp in an inverted orientation, whereas the CACG motifs in the mouse S14 ChoRE are situated in a direct orientation separated by 7 bp. The ability of a single transcription factor to recognize different orientations of half site motifs is not unprecedented. For example, the thyroid hormone receptor and other members of the nuclear receptor superfamily recognize two AGGTCA motifs that can be organized with different arrangements. These include direct, inverted, and everted repeats with different spacing between the motifs (42). Similarly, SREBP is an example of an E box-binding protein than can recognize both direct repeats (SRE-1) and inverted repeats (CACGTG) of a half site motif (43). The carbohydrate-responsive factor also appears to display this flexibility in its binding requirements for different ChoREs.

USF first emerged as a candidate for the carbohydrate-responsive factor in hepatocytes. This possibility was based on the observation that USF forms a major complex with the rat S14 and L-PK ChoREs using liver nuclear extract in the EMSA (21, 24–26). Lefrancois-Martinez et al. (27) reported that the overexpression of USF can activate the transfected L-PK promoter in hepatocytes and hepatoma cell lines. In mice deleted for the USF2 gene, the induction of L-PK or S14 mRNAs in liver was delayed after feeding of high carbohydrate diet (28, 29). However, several lines of evidence argue against USF as a carbohydrate-responsive transcription factor. In the EMSA, USF lacks the ability to discern altered ChoREs that are glucose-responsive from glucose-unresponsive oligonucleotides either in binding experiments or transfected cells (30). Similarly, for the mouse S14 ChoRE mutant 3–3, USF binding (band z) is greatly reduced with no apparent reduction in functional activity. Furthermore, overexpression of a dominant negative form of USF in hepatocytes failed to block the glucose response of the rat S14 or L-PK promoters (30). Finally, the ChoRF activity detected by EMSA was found predominantly in a distinct PEG fraction than USF. ChoRF binding was not inhibited by adding USF antibody, indicating that USF is not present in this complex. Overall, these data indicate that USF is not likely to be directly involved in the transcriptional response of S14 and L-PK genes through the ChoRE. The abnormal carbohydrate response observed in USF2 knockout mice or cells treated with dominant negative forms of USF for several days, however, indicates that USF may play an indirect role in the process.
SREBP-1c is another bHLH/LZ factor that has been proposed as a potential carbohydrate-responsive factor. SREBP-1c has the ability to bind either the SRE-1 or E box motifs and is highly expressed in the mammalian liver (44). SREBP-1c expression in liver is up-regulated by feeding a carbohydrate diet to rodents and by insulin treatment of hepatocytes (45, 46). Overexpression of a constitutively active form of SREBP-1c in either cultured cells or transgenic mice can activate expression of several lipogenic enzyme genes, including fatty acid synthase (47–49). Finally, expression of a dominant negative form of SREBP-1c in primary hepatocytes resulted in a reduction of glucose-induced mRNA levels of the fatty acid synthase, S14, and L-PK genes (46). However, there are several inconsistencies with the binding and activation properties of SREBP-1c and the expected properties of ChoRF. For instance, nuclear SREBP-1 did not form a complex with several active ChoREs, including the mouse S14 and L-PK ChoREs, and a consensus SRE-1 oligonucleotide did not compete with ChoRF binding. In addition, cotransfected SREBP-1c showed little or no activation of L-PK ChoRE-containing constructs (50). Although SREBP appears to play a role in regulating some aspects of lipogenesis and perhaps coordinating cholesterol biosynthesis and lipogenesis, it does not appear to be the nuclear factor directly binding to the ChoRE of the S14 and L-PK genes.

The demonstration of the ChoRF complex by EMSA suggests the possibility of a novel carbohydrate-responsive factor(s) in liver. The ability of various oligonucleotides to form this complex correlates with their competence to support a glucose response in hepatocytes. Three natural ChoREs and seven mutants of these ChoREs with functional activity all yielded the ChoRF complex on EMSA. In contrast, ChoRE mutants without functional activity and unrelated oligonucleotides showed very little or no ChoRF complex formation. Of particular note is the spacing mutant N4, which differs by only 1 bp from the N5 oligonucleotide, and yet has no functional activity and unrelated oligonucleotides showed very little or no ChoRF complex formation. Hence, we suggest that the ChoRF complex contains the nuclear factor(s) that receives the glucose signal. The failure to detect the ChoRF complex in our previous work stemmed from two factors. First, the ChoRF complex is apparently of low abundance and not easily detected in crude nuclear extracts. Using the PEG fractionation, we were able to enrich this factor sufficiently to allow its routine detection. Second, the formation of the ChoRF complex is largely refractory to poly(dI–dC), which was used in much of our earlier work as a nonspecific competitor. The ChoRF complex migrates more slowly than either the USF dimer or the SREBP (1a, 1c, or 2) dimers. However, we cannot rule out the possibility that ChoRF might contain USF or SREBP as a component of a larger complex at this time. In addition, two recent reports have described distinct nuclear factors that bind to the L-PK ChoRE (51, 52). The relative migration of those bands was significantly faster than the ChoRF complex that we detected. Whereas we have not observed these faster migrating complexes in our experiments, these factors could also be components of the ChoRF complex. In preliminary experiments, we have not found a difference in the intensity of the ChoRF complex in extracts prepared from rats of varying dietary status. Further exploration of this pathway will require purification and characterization of the ChoRF complex.

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