Regulatory sequences involved in the transcriptional induction of the rat S14 gene in response to increased glucose metabolism in the hepatocyte were investigated and compared with those of the liver-type pyruvate kinase (L-PK) gene. The carbohydrate response element (ChoRE) of the S14 gene was found to consist of two motifs related to the consensus binding site for the c-myc family of transcription factors, CACGTG. These two motifs are separated by five base pairs, a similar arrangement to that found in the L-PK ChoRE. In its natural context, the S14 ChoRE requires a novel accessory factor to support the full response to glucose. This factor, as well as the factor hepatic nuclear factor-4, are both capable of binding to the L-PK gene to enhance its carbohydrate regulation. The need for an accessory factor for supporting the glucose response can be overcome in two ways. First, multimers of the ChoREs of either the L-PK or S14 genes can function independently to support the glucose response. Second, mutations in the S14 ChoRE that create a perfect match to the consensus CACGTG motif at each locus no longer require an accessory factor site. The spacing of the two CACGTG motifs, but not the nature of the bases within the spacer, are critical for control. These observations suggest that a carbohydrate responsive factor binds to both motifs in a highly specific spatial orientation to confer the response to increased carbohydrate metabolism.

Feeding of a high carbohydrate, fat-free diet to the rat induces the synthesis of a set of enzymes in the liver involved in the formation of triglycerides (1–3). Included in this set of lipogenic enzymes are pyruvate kinase, fatty acid synthase, malic enzyme, and many others required for conversion of glucose to triglycerides. In several cases, the induction of enzyme synthesis has been correlated with changes in transcription of the corresponding genes (4–6). Dietary effects of the high carbohydrate diet can be mimicked in cultured primary hepatocytes by changing the glucose concentrations in which cells are maintained (7–9). Using such systems, it has been shown that altered enzyme synthesis correlates with increased metabolism of glucose or other glycolytic intermediates (10). This observation led to the hypothesis that elevated carbohydrate metabolism is responsible through an unknown signaling pathway for increased gene transcription in the hepatocyte.

While insulin is necessary, at least in part due to its role in stimulating glucokinase, insulin does not appear to be the direct mediator of the transcriptional response for most of the lipogenic enzymes (11, 12).

We have been studying two genes induced by carbohydrate feeding in the rat: the liver-type pyruvate kinase (L-PK)7 and S14 genes. Both of these genes respond at the transcriptional level to increased glucose metabolism (4, 6). The DNA sequences responsible for the glucose response in primary hepatocytes, which we have designated as carbohydrate response elements (ChoREs), have been mapped. The ChoREs of the L-PK and S14 genes share a region with 9 out of 10 bp identity (13). This region is centered by a CACGTG motif, the core recognition site for the c-myc family of transcription factors. In liver, the predominant member of this family that is detected by in vitro binding studies is USF (also known as MLTF) (14–17). This factor was first identified by its ability to bind to the upstream stimulatory element of the adenosine virus major late promoter (18, 19). Two forms of USF are expressed widely in mammalian tissues (20) and have been implicated in the expression of several hepatic genes (21). The addition of the upstream stimulatory element from the adenosine virus major late promoter into either L-PK or S14 promoters in place of the normal ChoREs did not support the glucose response, indicating that USF binding alone is not sufficient for this control (15, 17).

We have found previously that a 21-bp segment of the S14 gene from −1448 to −1428 functions as a ChoRE (17). This segment contains a 5′ CACGTGNNNGCC motif that is sufficient to confer glucose induction to an unresponsive promoter construct in primary hepatocytes. Comparison of the S14 ChoRE with the corresponding regulatory region of the L-PK gene revealed two apparently distinct features. First, for the L-PK gene, two nuclear factor binding sites, the USF-binding site2 and an adjacent HNF-4 site, are required to establish a glucose response. In contrast, the S14 gene required only a single factor binding site related to the USF-binding site of the L-PK gene. Second, the USF-binding site of the L-PK gene consists of two imperfect CACGTG motifs separated by 5 bp (Fig. 1A), whereas only a single CACGTG motif was recognized in the S14 ChoRE. These apparent differences between the S14 and L-PK ChoREs perplexed us, as we anticipated that the control of these two genes was likely to be coordinated through a common mechanism. In this study, we have analyzed these differences through a closer examination of the S14 ChoRE and 1 The abbreviations used are: L-PK, liver-type pyruvate kinase; ChoRE, carbohydrate response element; bp, base pairs; EMSA, electrophoretic mobility shift assay; USF, upstream stimulatory factor; CAT, chloramphenicol acetyltransferase; HNF, hepatic nuclear factor.

2 We have previously referred to this site of the L-PK promoter as the MLTF-like site.
region. The results indicate that there is a great deal of similarity between the regulatory sequences of these two genes and that a unique arrangement of CACGTG motifs is responsible for determining the specificity of the carbohydrate response.

**EXPERIMENTAL PROCEDURES**

Primary Hepatocyte Culture and Transfection—The procedure described previously was followed (17). Primary hepatocytes were isolated from male Sprague-Dawley rats (180–240 g) using the collagenase perfusion method. After a 6-h attachment period, transfection was performed using Lipofectin reagent (Life Technologies, Inc.) in modified Williams E medium with 5.5 mM glucose for 12-14 h. Subsequently, cells were cultured for 48 h in medium containing either 5.5 or 27.5 mM glucose and harvested for chloramphenicol acetyltransferase (CAT) assay. Data are representative of three independent experiments.

**RESULTS**

A Second CACGTG Motif in the S14 Gene Is Required for Carbohydrate Regulation—S14 gene sequences from −1448 to −1428, which were previously shown to function as a ChoRE (17), contain a single perfect CACGTG motif. In contrast, the L-PK ChoRE (−171/−142) contains two imperfect CACGTG motifs. closer inspection of the oligonucleotide containing the S14 ChoRE suggested a possible explanation for this difference. In cloning the S14 ChoRE, an artificial Mulu site was inserted at the 3'-end of the oligonucleotide. Fortunately, this site introduced a sequence with a 5 out of 6 bp match to a CACGTG motif. This sequence is separated by 5 bp from the natural S14 CACGTG motif, thus resembling the L-PK ChoRE (Fig. 1A). Because mutations of either imperfect CACGTG motif in the L-PK promoter abolished the glucose response (24), the possibility arose that the imperfect CACGTG motif introduced in cloning the S14 ChoRE might have played a role in regulation. To test this possibility, an oligonucleotide containing a Xhol site in place of the Mulu site was synthesized and subcloned into a glucose-unresponsive construct. This construct included sequences from −4316 to −2111 of the S14 gene, which contain an uncharacterized enhancer, linked directly to an S14 promoter fragment from −290 to +18 (13). DNA constructs were transfected into primary hepatocytes by lipofection. Hepatocytes were cultured in media containing either 5.5 or 27.5 mM glucose for 48 h, and then CAT assays were performed on cell extracts. As shown previously, the S14 ChoRE oligonucleotide containing the Mulu site conferred a robust glucose response (23,9-fold) to the glucose-unresponsive S14 construct (Fig. 1B). Because mutations of either imperfect CACGTG motif in the L-PK promoter abolished the glucose response (24), the possibility arose that the imperfect CACGTG motif introduced in cloning the S14 ChoRE might have played a role in regulation. 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FIG. 3. Detection of a hepatic nuclear factor(s) that binds to the S\textsubscript{14} accessory site. Panel A, an oligonucleotide comprising S\textsubscript{14} sequences from $-1467$ to $-1440$ was radiolabeled and used to test for binding to hepatic nuclear factors by the EMSA. The ability of various unlabeled oligonucleotides to compete for binding when added in 50- or 100-fold molar excess was evaluated. TRE\textsubscript{pal} represents a control oligonucleotide containing the binding site for the thyroid hormone receptor (32). Arrows indicate the positions of two specific complexes formed.

Panel B, an oligonucleotide comprising L-PK sequences from $-146$ to $-124$ was radiolabeled and used to test for binding to hepatic nuclear factors by the EMSA. Various unlabeled competitors tested are shown. HNF-3 represents a control oligonucleotide containing the binding site for this nuclear factor (26). The thick arrow indicates the position of the HNF-4 complex. The two thin arrows indicate positions of two novel complexes that bind to this oligonucleotide and are competed by the S\textsubscript{14} accessory site.

A second CACGTG motif is required for activity of the S\textsubscript{14} ChoRE. This finding raised the question of what sequences in the natural S\textsubscript{14} gene might fulfill this role.

We have previously reported a 9 out of 10 bp identity between the S\textsubscript{14} and L-PK ChoREs (13). This comparison involved alignment of the CACGTG motif of the S\textsubscript{14} ChoRE with the downstream CCCGTG motif of the L-PK USF-binding site. If, instead, the CACGTG motif of the S\textsubscript{14} gene is aligned to the upstream CACGGG motif of the L-PK gene, as shown in Fig. 1A, the S\textsubscript{14} sequence from $-1428$ to $-1423$, CCTGTG, provides a 5 out of 6 bp match to the downstream motif of the L-PK ChoRE. Furthermore, the distance between the two motifs is identical to that found in the L-PK gene. This sequence similarity suggested that the $-1428$ to $-1423$ sequence in the S\textsubscript{14} gene might be involved in carbohydrate regulation. To test this possibility, an oligonucleotide containing the natural sequence of the S\textsubscript{14} gene with both potential CACGTG motifs ($-1448$ to $-1422$) was subcloned into the S\textsubscript{14} enhancer/promoter construct and assayed in transfected hepatocytes. Introduction of this oligonucleotide resulted in a response to elevated glucose (6.8-fold), although much weaker than that observed in the construct containing the artificial MluI site.

To verify the observation that the natural S\textsubscript{14} sequences from $-1448$ to $-1422$ are capable of mediating the glucose induction, we subcloned this segment upstream of the basal promoter of the L-PK gene containing sequences from $-96$ to $+12$. One copy of the S\textsubscript{14} ($-1448$ to $-1422$) segment was ineffective in rendering a response to glucose; however, two copies of this segment functioned effectively (Fig. 1C). These results were similar to observations made previously with the L-PK USF-binding site (15, 24) and suggest that S\textsubscript{14} and L-PK ChoREs are functioning in a similar fashion in mediating the carbohydrate regulation. In both cases, the USF-binding site likely serves as the recognition site for the factor(s) that is directly modulated by the carbohydrate signaling pathway.

An Accessory Factor Binding Site in the S\textsubscript{14} Gene—In its natural context, the L-PK ChoRE requires an adjacent binding site ($-146$ to $-124$) to support the glucose response. This site has been shown to bind the hepatic enriched factor HNF-4 (15, 16). Since the S\textsubscript{14} ChoRE functions weakly in a single copy, the possibility of an accessory factor for enhancing the glucose response was suggested for this gene as well. To test for such an accessory factor, a functional assay was performed. When a fragment from $-1601$ to $-1395$ was inserted into the glucose unresponsive S\textsubscript{14} construct, the ability to respond to glucose was comparable with that observed with constructs containing the intact $-4316$ to $+18$ S\textsubscript{14} gene fragment (Fig. 2). Thus, an accessory factor site appeared to be present in this 200-bp segment. Based on DNase I footprinting of this region (13), a binding site for a nuclear factor found in liver was known to exist immediately adjacent and upstream of the S\textsubscript{14} ChoRE. We therefore tested whether an oligonucleotide that included this additional footprinted site ($-1467$ to $-1395$) could support the glucose response. This turned out to be the case. Furthermore, a shorter segment from $-1467$ to $-1422$ was nearly as active. Thus, we surmised that the region directly upstream of the S\textsubscript{14} ChoRE was the binding site for an accessory factor capable of enhancing the glucose response of the S\textsubscript{14} promoter.

Since HNF-4 binds to the accessory factor site for the L-PK...
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Since both HNF-4 and the S14 accessory factor can bind to the accessory site (−146/−124) on the L-PK gene, it was of interest to determine whether both factors were capable of supporting the glucose response of this promoter. Previously we showed that an oligonucleotide containing the α1-antitrypsin HNF-4 site can substitute for the L-PK accessory site in supporting a glucose response (15). Since the α1-antitrypsin oligonucleotide does not bind the S14 accessory factor (Fig. 3A), HNF-4 is functioning as the accessory factor for the L-PK ChoRE in this case. To assess whether the S14 accessory factor can also function to support the L-PK ChoRE, mutations were made in the L-PK accessory site that differentially altered binding of the two factors. Comparison of the S14−1467/−1440 and L-PK−146/−124 segments showed two regions of sequence similarity (Fig. 5). These two regions were separated by 4 bp of dissimilar sequence. We reasoned that mutating these 4 bp should not disrupt binding of the S14 accessory factor to the L-PK oligonucleotide, but might disrupt HNF-4 binding. This prediction was supported by gel shift analysis (Fig. 5). A mutant L-PK oligonucleotide (m1PK) in which the 4 bp was changed to the sequence of the S14 gene was no longer capable of forming an HNF-4 complex. However, this mutant oligonucleotide still formed the two bands representing the S14 acces-

To confirm that the factor(s) binding to the S14 accessory factor site was distinct from HNF-4, gel shifts were performed in the presence of antibodies to HNF-4. These antibodies were capable of disrupting the major complex formed on the PK−146/−124 probe, resulting in the appearance of a novel supershifted complex (Fig. 4). However, they did not alter binding of nuclear factors to the S14 accessory site probe. Diaz Guerra et al. (16) have suggested that the related orphan receptor COUP-TFI or COUP-TFII (25) did not interact with the factors binding the S14 accessory factor. These antibodies also did not displace the additional bands seen with the L-PK probe. The COUP-TFI antibodies were shown to be effective by their ability to compete complexes formed on a consensus COUP-TF site (DR+1) using liver nuclear extract. Thus, the factor binding to the S14 accessory site is distinct from HNF-4 and the related factor COUP-TF.

ChoRE, we questioned whether this same factor might also serve the S14 ChoRE. Little sequence similarity is observed between −1467 to −1440 segment of the S14 gene and the known consensus binding site for HNF-4. When an oligonucleotide containing the S14 accessory factor site was used as a probe in a gel shift experiment with rat liver nuclear extract, two closely spaced bands were observed (Fig. 3A). In order to detect these bands effectively, a modified nuclear extraction buffer, which contains 1 M urea and 1% Nonidet P-40, was used (23). The two bands could be competed with the homologous S14 oligonucleotide or an oligonucleotide comprising the L-PK gene accessory site (−146/−124), but not an unrelated DNA sequence (TREpal). However, these two bands were not competed by an oligonucleotide from the α1-antitrypsin gene containing a well-characterized HNF-4 site. These two bands thus appear to be distinct from HNF-4 and will be referred to as the S14 accessory factor. This observation raised the question of whether the L-PK−146 to −124 segment might bind to the S14 accessory factor in addition to HNF-4. When this L-PK gene segment was radiolabeled and used as a probe, multiple bands were indeed observed from nuclear extracts prepared by the modified procedure (Fig. 3B). The major band was shown previously to represent HNF-4 (15, 16), and, as expected, this band could be competed by the α1-antitrypsin HNF-4 site. In addition, two less intense bands that migrated with similar mobilities to the bands formed on the S14−1467 to −1440 fragment were observed. These two bands were competed by an oligonucleotide containing the S14 accessory site. Thus, it appears that the L-PK gene segment from −146 to −124 is capable of recognizing two (or more) distinct factors, one of which is HNF-4 and one (or more) of which is shared with the S14 accessory site.

To confirm that the factor(s) binding to the S14 accessory factor site was distinct from HNF-4, gel shifts were performed in the presence of antibodies to HNF-4. These antibodies were capable of disrupting the major complex formed on the PK−146/−124 probe, resulting in the appearance of a novel supershifted complex (Fig. 4). However, they did not alter binding of nuclear factors to the S14 accessory site probe. Diaz Guerra et al. (16) have suggested that the related orphan receptor COUP-TF is also capable of binding to the L-PK−146/−124 segment. However, we found that antibodies recognizing either
The S$_{14}$ accessory factor can stimulate the glucose response of the L-PK ChoRE. Oligonucleotides containing the wild-type L-PK gene sequence between −146 and −124 or mutations that disrupt binding of nuclear factors to this sequence were inserted into constructs containing the L-PK ChoRE (−171/−147) and promoter. In these constructs, a 10-bp spacer is added between position −147 and −146 of the L-PK sequence, and L-PK sequences between −124 and −96 are deleted. The ability of these constructs to support a response to glucose was monitored as described in the legend to Fig. 1B. Data are representative of two separate experiments.

The Spacing between the Two CACGTG Motifs Is a Critical Determinant for Carbohydrate Regulation — The presence of two CACGTG motifs separated by 5 bp in the S$_{14}$ and L-PK ChoREs is distinct from the arrangement of such sites in other genes in which only a single CACGTG motif is generally found (21, 27–29). We next asked whether the sequence or spacing between the two motifs was critical for carbohydrate control. Oligonucleotides corresponding to the L-PK USF-binding site with single or double mutations within the spacer region were synthesized and inserted into an L-PK construct (Fig. 8A). As a positive control, a construct containing oligonucleotides with the wild-type sequences of the USF-binding and HNF-4 sites gave a 4.8-fold response to glucose. Mutation of any of the bases within the 5-bp region linking the CACGTG motifs gave similar -fold inductions (4.7–6.5-fold) to that seen with the wild-type sequence, although small reductions in absolute levels of CAT activity were seen. These findings suggest that the sequence of bases separating the two imperfect CACGTG motifs are not essential for determining carbohydrate regulation but may modulate the effect to some extent.

To test whether the distance between the two CACGTG motifs is critical for regulation, we assayed oligonucleotides containing the L-PK ChoRE with a single nucleotide either deleted or inserted between the two motifs. Remarkably, both mutations completely abolished the ability of this element to support the carbohydrate regulation (Fig. 8A), suggesting that the relative positioning of these two CACGTG motifs is a major determinant for glucose induction. To substantiate this observation, constructs containing variations in spacing between two perfect CACGTG motifs derived from the S$_{14}$ USF-binding site were also tested. As shown above, the oligonucleotide containing two perfect motifs separated by 5 bp conferred a strong glucose response. Similar to the L-PK USF-binding site, a spacing of 4 bp between the CACGTG motifs resulted in a complete loss of induction. On the other hand, spacing by either 6 or 7 bp gave a weak response to glucose; much less than the response seen with the 5-bp spacer (Fig. 8B). To see whether phasing might be important to establish control, a mutant with 15 bp of spacing was tested. This construct was also only weakly effective compared with the construct with the natural 5 bp of spacing. These results clearly indicate that spacing between the two CACGTG motifs is a critical determinant for carbohydrate regulation.

**DISCUSSION**

We have demonstrated that S$_{14}$ gene sequences from −1448 to −1422 function as a ChoRE. This DNA element, like the L-PK ChoRE, consists of two CACGTG motifs separated by 5 bp. The ability of either the S$_{14}$ or L-PK ChoRE by itself to support a response to glucose is weak (S$_{14}$) or absent (L-PK). However, multimers of either USF-binding element linked to a basal promoter render a response to glucose. In the context of
These studies suggested that the synergism of these two elements was not due to a direct interaction of factors binding to these two adjacent sites. On the other hand, the ability of the HNF-4 site to support the glucose response could not be substituted by insertion of either an HNF-3 or C/EBP binding site in its stead, suggesting that some level of specificity was involved in achieving the functional synergism. We now show that in addition to HNF-4, at least one other factor can functionally synergize with the ChoREs of either the S14 or L-PK genes. The nature of this factor is currently unknown.

Previously, we have shown that a CACGTGNNNGGCC motif of the S14 gene (−1448 to −1428) is sufficient for conferring carbohydrate regulation (17). The ability of this shorter sequence to respond resulted from an imperfect CACGTG motif introduced by an MluI site inserted at its 3’-end (underlined bases). Thus, this construct contained two CACGTG motifs separated by 5 bp, in which a natural C of the S14 gene (GCC) provided the first base of the downstream motif. In the natural S14 sequence, bases from −1428 to −1423 (CCTGGT) provide an imperfect CACGTG motif with a 4 out of 6 match to the perfect site. Converting the imperfect S14 motif to sequences with a 5 out of 6 or perfect match to CACGTG resulted in progressively enhanced responsiveness to glucose. We have previously reported that an element from the first intron of the fatty acid synthase gene at position +292 was capable of supporting a glucose response (17). However, in this case, the oligonucleotide was also cloned in the context of an MluI site that could provide a second CACGTG motif. In the natural context, the sequence corresponding to the downstream motif is 5’ CGCGCC, and an oligonucleotide containing this region of the fatty acid synthase gene was ineffective in supporting the glucose response. Thus, its role in regulation of this gene is questionable.

In addition to the presence of two CACGTG motifs, the distance between the two motifs is critical for determining carbohydrate regulation. When the two motifs were moved closer than 5 bp, a complete loss of glucose responsiveness was observed for either S14 or L-PK elements. At distances greater than 5 bp, the S14-derived element showed a significantly blunted response, whereas L-PK was unresponsive. This effect of relative positioning of the two CACGTG motifs argues for an interaction between factors bound at each of these motifs. This interaction could be a direct protein-protein interaction or mediated by a third component that simultaneously contacts both CACGTG-bound factors. It is worth noting that with a 5-bp spacing, the centers of the two CACGTG motifs are spaced by 11 bp, thus orienting proteins bound at these two sites on the same side of the DNA helix. The mutant with a spacing of 15 bp between the CACGTG motifs would again orient factors bound at each motif on the same side of the DNA helix. The fact that this construct showed only a marginal response suggests that a close proximity of the two factors is critical. This observation is most compatible with a direct protein-protein interaction between the two sites.

What is the nature of the carbohydrate responsive factor? Factors binding to the CACGTG motif have been shown to belong to the c-myc family. While many members of this family have been identified, USF is the predominant factor in hepatic extracts that binds to the ChoRE in vitro. However, the ubiquitous distribution of USF raises the question of how the specificity of carbohydrate regulation would be achieved through this factor. The observation that glucose responsiveness requires two specifically positioned motifs could provide an answer to this question. Perhaps, two USF molecules binding to the CACGTG motifs provide an interface for interaction with a

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3 Shih, H.-M., and Towle, H. C., unpublished results.
signaling molecule or for binding a third component that interacts directly with the signaling pathway. Members of the c-myc family are capable of heterodimerization in unique combinatorial fashions. It is also possible that USF may heterodimerize with another family member in binding to the ChoRE. The possibility that USF is involved in carbohydrate signaling was supported by the recent observations of Lefrancois-Martinez et al. (31). In this study, dominant negative forms of USF were shown to repress the ability of the L-PK ChoRE to respond in a hepatocyte-derived cell line. We have attempted to detect a novel carbohydrate responsive factor by comparing binding of liver nuclear extracts to responsive and unresponsive USF-binding oligonucleotides. No unique complexes were found (data not shown). The exact nature of the carbohydrate responsive factor thus remains an open question.

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