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BREAKTHROUGHS AND VIEWS

Regulatory Elements in the Insulin-Responsive Glucose Transporter (GLUT4) Gene

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GLUT4, the insulin responsive-glucose transporter, mediates the rate limiting step of glucose metabolism in skeletal muscle and adipose tissue. GLUT4 expression is up-regulated by exercise training and thyroid hormone treatment and is down-regulated by fasting, streptozotocin-induced diabetes, obesity, high-fat diet, and denervation. Since overexpression of GLUT4 in insulin resistant db/db mice and high-fat diet-fed mice has been observed to dramatically improve glycemic control, increasing GLUT4 expression may be an effective strategy with which to alleviate insulin resistance. This review discusses recent findings on the regulation of the GLUT4 gene and on progress in the identification of regulatory elements in the promoter of the gene.

GLUT4, the insulin-responsive glucose transporter, plays an important role in whole body glucose homeostasis (1). It is expressed in heart, skeletal muscles, and adipose tissues, tissues originating from mesodermal somites. Expression of the GLUT4 gene is hormonally and metabolically regulated. For example, fasting (2-4), high-fat feeding (5,6), and obesity (7,8) lead to a decrease in the level of GLUT4 mRNA in white adipose tissues (WAT), while streptozotocin (STZ)-induced diabetes (9-11) results in a decrease of GLUT4 mRNA in both skeletal muscle and WAT, and denervation decreases the GLUT4 mRNA level in skeletal muscle (12). On the other hand, exercise training (13,14) and triiodo-L-thyronine (T3) administration (15,16) increase the GLUT4 mRNA level in skeletal muscles, while cold exposure increases GLUT4 mRNA levels in brown adipose tissue (BAT) (17). Since overexpression of GLUT4 in insulin resistant db/db mice and high-fat diet-fed mice has been observed to dramatically improve glycemic control (18, 19), an increase in GLUT4 expression may be a useful strategy with which to correct insulin resistance. Furthermore, disruption of the GLUT4 gene resulted in severely reduced glucose uptake by white fast-twitch skeletal muscle (20).

The mouse (21), human (22), and rat GLUT4 (23) genes and their putative 5′-flanking transcriptional regulatory regions have been isolated and characterized. The region of the mouse GLUT4 gene from −1000 bp to the transcription initiation site shows 63% sequence homology with the corresponding region of the human gene. Since the sequence homology between the rat and mouse genes in this region is very high (=86%), nucleotide sequences of the 5′-segment of the mouse and human GLUT4 gene have been compared and are presented in Fig. 1. Sequence data of the human gene was taken from the international nucleotide sequence database (Gene Bank Accession Number M61126). Sequence up to −780 of the mouse gene was taken from Kaestner et. al (21), and the remaining 220 bp was sequenced.

SKELETAL MUSCLE SPECIFIC GLUT4 EXPRESSION

Recent evidence suggests that myocyte enhancer factor 2 (MEF2) and myogenic basic-helix-loop-helix (bHLH) proteins such as MyoD function within a regulatory network for the establishment of the differentiated phenotype of skeletal muscle (24). Dimerized bHLH proteins bind to the E box consensus DNA sequence (CANNTG). Rat GLUT4/luciferase constructs were used to identify sequences conferring muscle-specific expression in C2C12 cells (25, 26). Transient transfection of progressive 5′ and 3′ deletions of the GLUT4-flanking DNA in C2C12 myoblasts and myotubes identified a 103-base pair region located between −522 and −420 of the rat GLUT4 gene which conferred myotube-specific expression. This region contains an E-box (CATTTG, −461/−456 of mouse GLUT4 in Fig. 1) and

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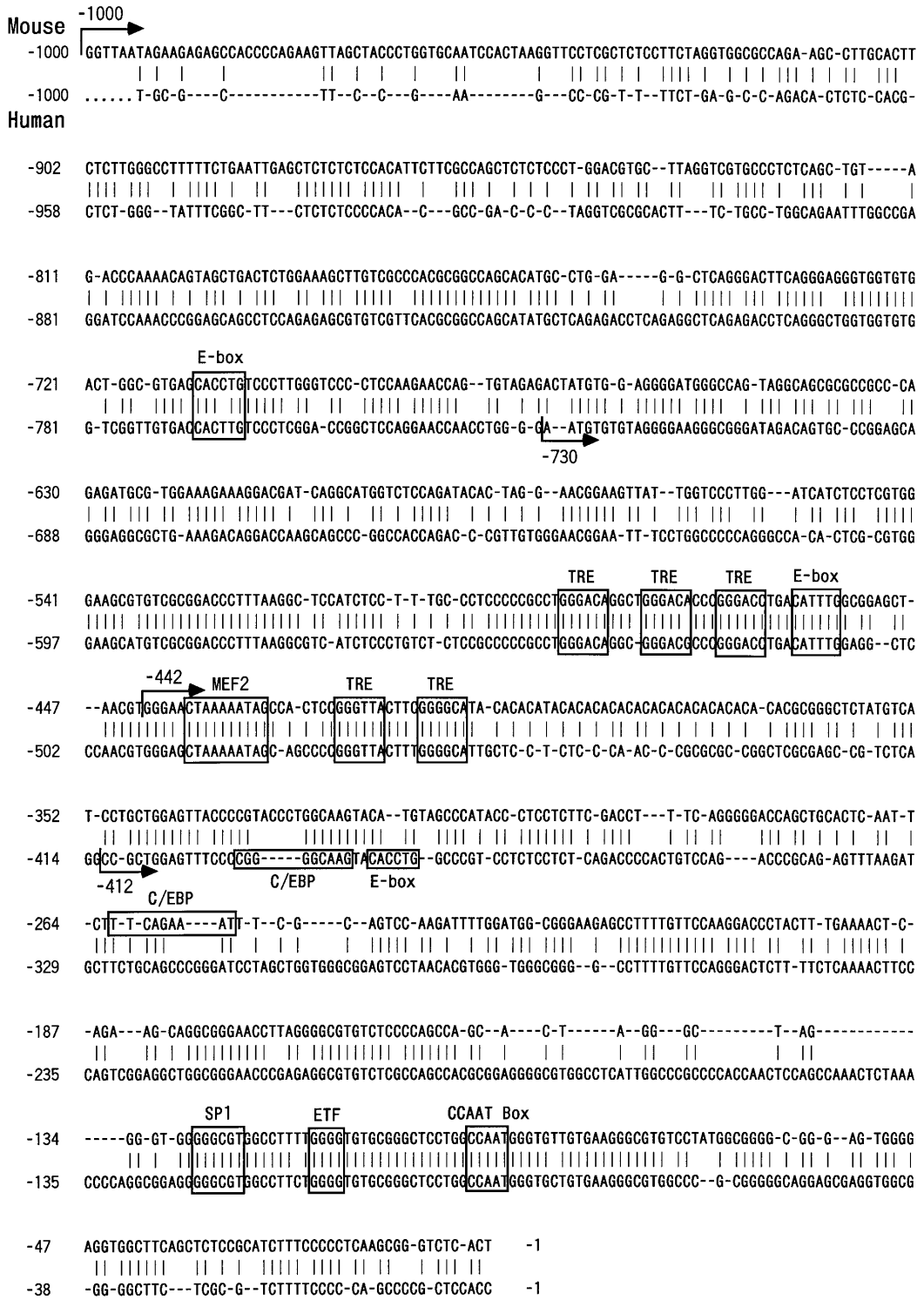

FIG. 1. Comparison of nucleotide sequence of the 5′-flanking region of the mouse and human GLUT4 gene. The numbers refer to the positions in the gene, where +1 is the transcription initiation site. Important cis-elements are boxed; TRE, thyroid hormone responsive element; MEF2, myocyte enhancer factor 2 binding site; C/EBP, CCAAT/enhancer binding protein; ETF, epidermal growth factor receptor-specific transcription factor binding site. The starting position of 5′-deleted GLUT4 constructs from mouse (27) and human (29) transgenic mice is shown by an arrow.

an MEF2 binding site (CTAAAAATAG, −437/−428). Mutations of this MEF2 binding site resulted in a nearly complete loss of reporter gene expression. These data strongly suggest that the MEF2 binding site of the rat GLUT4 gene is essential for myotube-specific expression of GLUT4. The importance of the MEF2 site is also supported by in vivo data showing that the −442 mouse GLUT4 minigene, which contains the MEF2 binding site (−437/−428), but not the E-box (−461/−456), is expressed substantially in skeletal muscles and heart in transgenic mice (27). However, the MEF2 binding sequence alone is insufficient to enhance GLUT4 expression in C2C12 myotubes, since expression of a 75-bp promoter fragment which contains the MEF2 binding site but is devoid of two downstream thyroid hormone responsive elements (TRE) resulted in a 60% loss of myotube-specific reporter gene expression (26).

WAT AND BAT SPECIFIC GLUT4 EXPRESSION

A 14 kb GLUT4 minigene (−7395 GLUT4) showed differentiation-induced expression in 3T3-L1 preadipocytes, suggesting that cis-elements required for differentiation-induced activation of transcription are located within the 14-kb GLUT4 minigene (28). In vivo studies in transgenic mice, using 5′-deletion analysis of the human GLUT4-CAT reporter gene revealed that a BAT enhancer is located between bases −1154 bp and −730 bp in the human GLUT4 5′-flanking region (29). Similarly, 5′-deletion analysis of the mouse GLUT4 minigene in a transgenic mouse model revealed that BAT and WAT enhancers are located between bases −1000 and −442 in the 5′-flanking region of the mouse GLUT4 gene (27).

Recent studies have identified two protein families that play important roles in the induction of the adipocyte phenotype: that is, the CCAAT/enhancer-binding protein (C/EBP) family and peroxisome proliferator-activateted receptors (PPARs) (30, 31). A computer search could not identify canonical PPAR (CCAGAGAGAAGG, ref. 32) or C/EBP binding sites (TT/GNNGC/TAAT/G in ref. 33) between −1000 bp and −442 bp of the mouse GLUT4 gene. However, a broad array of substitutions in the consensus binding site for transcription factors are tolerated, thus the presence of functional PPAR and/or C/EBP binding sites in this region have not been ruled out. There is at least some indirect evidence that C/EBP regulates GLUT4 expression. In 3T3-L1 cells, the down-regulation of GLUT4 mRNA by insulin treatment or TNF-treatment is paralleled by a down-regulation of C/EBP expression (34, 35). C/EBP was also shown to be able to transactivate the mouse GLUT4 promoter when a C/EBP expression vector was cotransfected into 3T3-L1 preadipocytes with a −469 GLUT4 CAT reporter gene (21). Footprint analysis in 3T3-L1 adipocyte has identified a potential C/EBP binding site (TTCAGAAAT, −262 and −254 of mouse GLUT4 in Fig. 1), but it is unlikely that this site plays a major role in adipocyte specific expression in vivo, as this sequence is not conserved in the human GLUT4 gene (Fig. 1), and the mouse −442 GLUT4 minigene showed very low expression levels in WAT (27). The role of PPAR in the regulation of GLUT4 expression is not yet clear. In 3T3-L1 cells, treatment with the PPAR ligands clofibrate and eicosatetraenoic acid resulted in down-regulation of GLUT4 mRNA (36). However, in an in vivo study, thiazolidinediones, a PPAR ligand, enhanced adipocyte differentiation and increased GLUT4 mRNA levels in WAT (37). One explanation of this difference may be that thiazolidinedione-mediated adipocyte differentiation leads to an increased number of small adipocytes, and since smaller adipocytes have more GLUT4 (38), GLUT4 mRNA is increased by thiazolidinediones treatment. In contrast, PPAR activation may decrease GLUT4 expression in fully-differentiated adipocytes, and this effect predominates in 3T3-L1 adipocytes, where no further differentiation can take place. Or simply, a PPAR/ligand complex may indirectly increase GLUT4 expression through induced metabolic and hormonal changes.

EXERCISE-INDUCED UP-REGULATION OF GLUT4

Exercise training is an important physiological stimulus to increase GLUT4 mRNA expression in skeletal muscles (13,14). 5′-deletion analysis of the mouse GLUT4 minigene has revealed exercise-responsive elements located between bases −1000 and −442 of the mouse GLUT4 5′-flanking region (27). Several factors which may be involved in exercise-induced up-regulation of GLUT4 have been studied. cAMP, insulin, and arachidonic acid have been shown to down-regulate expression of GLUT4 in 3T3-L1 adipocytes in culture (see following discussion). However, only a decline in the circulating arachidonate levels in vivo is correlated with the up-regulation of GLUT4 caused by exercise (39). In addition, the decrease of arachidonic acid levels was only modest (8-24%) and so it is not clear whether decreases of blood arachidonic acid levels play a role in the increase in GLUT4 expression with exercise.

COLD TEMPERATURE-INDUCED UP-REGULATION OF GLUT4

In rats, cold exposure (4°C) for 10 days increased GLUT4 mRNA in BAT, but not in skeletal muscle (17). Since cold exposure increases sympathetic nerve activity in BAT, the increased GLUT4 expression by cold exposure may be attributable to adrenergic stimulation. However, detailed GLUT4 promoter analysis of this effect has not yet been made.

THYROID HORMONE-INDUCED UP-REGULATION OF GLUT4

When 5′-deletion analysis of a rat GLUT4/reporter gene was performed in C2C12 myotubes that were treated with T3, a 281-bp region of rat GLUT4 was identified as a thyroid hormone responsive segment (26). In this region, five thyroid hormone responsive elements (TREs) consisting of hexameric half-sites (AGGT(C/A)A) were found (Fig. 1). Recently, a potential TRE, consisting of directly repeated TRE half-sites separated by 4 bp (DR+4 TRE) was identified downstream of the MEF2 site in the GLUT4 gene by an electrophoretic mobility shift assay (40). However, the GLUT4 TR-binding site has a significantly lower affinity compared to a consensus DR+4 TRE, and only binds TRs appreciably in the form of high affinity heterodimers; in this case with the cis-retinoic acid receptor. Furthermore, T3 treatment of diabetic KK mice increased both GLUT4 and MyoD mRNA (41), suggesting that T3 treatment may increase GLUT4 mRNA levels through increases in MyoD expression. Functional assays using transfected cells and transgenic mice will be necessary to clarify which elements actually mediate T3-induced up-regulation of GLUT4.

DOWN-REGULATION OF GLUT4 BY cAMP; DOWN REGULATION OF GLUT4 BY FASTING, STZ, AND DENERVATION CORRELATES WITH INCREASED cAMP

cAMP down-regulates expression of GLUT4 in 3T3-L1 cells (42) and in a muscle cell line, L6E9 myotubes (43). In addition, down-regulation of GLUT4 by arachidonic acid in 3T3-L1 cells might be attributable to an increase in cAMP (36). Furthermore, incubation of FRTL5 cells (a rat thyroid cell line) with TSH decreased GLUT4 mRNA, parallel with an increase of cAMP (44).

Correlation between changes of tissue cAMP levels with GLUT4 expression have also been observed in vivo. In fasted (48 hr) mice, substantial increases in cAMP levels were associated with decreased GLUT4 mRNA levels in BAT and WAT, while no changes of cAMP or GLUT4 levels were observed in gastrocnemius (Table 3 in ref. 39). WAT from STZ-induced diabetic rats also showed an increase of cAMP levels with a decrease of GLUT4 mRNA levels, and the decrease in the GLUT4 expression was prevented by subcutaneous injection of the adenosine receptor agonist, phenylisopropyl adenosine, which decreased cAMP levels (45). Denervation of gastrocnemius and soleus muscles by section of the sciatic nerve resulted in a decrease of GLUT4 mRNA levels (12) that paralleled a 1.5-2-fold increase of cAMP levels (46). These data indicate that fasting and STZ-induced GLUT4 down-regulation in WAT and denervation-induced GLUT4 mRNA decrease in skeletal muscles may be due to decrease of tissue cAMP.

To locate cis-element(s) responsible for cAMP-induced down-regulation of GLUT4, three studies have been conducted, but the results have not been consistent. A series of 5′-deleted mouse GLUT4-promoter/reporter CAT genes were stably transfected into 3T3-L1 preadipocytes, and expression was measured in differentiated adipocytes by an RT-PCR method. The cis-element(s) conferring cAMP mediated-down regulation of GLUT4 was located between −469 and −78 bp in the mouse GLUT4 5′-flanking region. (47). However, in rat skeletal muscle L6E9 myotubes, 8-bromo-cAMP treatment decreased endogenous GLUT4 mRNA but did not change CAT activity of −2212 rat GLUT4/CAT, suggesting that cAMP -induced GLUT4 down regulation was mediated by cis-elements located outside the 2212-bp fragment of the rat GLUT4 promoter (43). In transgenic mice harboring 5′-deleted human GLUT4/CAT constructs, it was shown that STZ-induced down-regulation of GLUT4 in skeletal muscles, WAT and BAT was mediated by DNA element(s) located between −1154 bp and −730 bp (29). The different conclusions may be due to differences between cells and animals, or metabolic changes other than cAMP may be involved in down-regulation of GLUT4 by STZ treatment.

The cAMP responsive element (CRE) has a consensus sequence "TGACGTCA" and usually acts as an enhancer, but sometimes acts as a repressor (48). A computer search identified the sequence "GTTCGTCA" at −1096 of the mouse GLUT4 gene. It is of interest that GLUT2 (49) and L-type pyruvate kinase gene (50) were down-regulated by cAMP in freshly isolated hepatocytes by unknown cAMP-responsive sequences that lack CRE elements.

HIGH-FAT DIET-INDUCED DOWN-REGULATION OF GLUT4 GENE IN WAT

In comparison with high-carbohydrate feeding, high-fat feeding for three-months resulted in a 50-70% decrease of endogenous GLUT4 mRNA levels in WAT in mice (19). 5′-deletion analysis of the mouse GLUT4 minigene revealed that high-fat diet-responsive elements are located between bases −1000 and −442 of the mouse GLUT4 5′-flanking region (27). In 3T3-L1 cells, arachidonate decreased GLUT4 expression, possibly by through an increase of cAMP or a PPAR ligand (36). However, we do not know whether the same mechanism(s) is involved in down-regulation of GLUT4 observed in vivo and in vitro.

INSULIN-INDUCED DOWN-REGULATION OF GLUT4

The effect of insulin on GLUT4 mRNA levels depends on the cell type. Insulin treatment decreased GLUT4

mRNA levels in 3T3-L1 cells (51, 52) and L6 muscle cells (53), but increased GLUT4 mRNA levels in primary ventricular cardiomyocytes (54) and fetal brown adipocyte primary cultures (55). The mechanisms of these differences and insulin-mediated GLUT4 transcriptional regulation have not yet been elucidated.

As described above, many physiological conditions affect GLUT4 expression and then alter glucose homeostasis in the whole body. The study of regulatory mechanisms of GLUT4 expression has just begun. It will be of interest to ascertain whether any of these responses is mediated through the same cis-elements and to ascertain how their trans-acting factors interact.

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