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Characterization of the human skeletal muscle glycogen synthase gene (*GYS1*) promoter

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Abstract

Background Impaired activation of the human skeletal muscle glycogen synthase by insulin is typical for type 2 diabetic patients. Regulation of glycogen synthase occurs mainly by phosphorylation/dephoshorylation but little is known whether there also is transcriptional regulation. Therefore we studied transcriptional regulation of the human skeletal muscle glycogen synthase gene (*GYS1*) and evaluated the effects of insulin and forskolin on the promoter activity.

Methods Seven promoter fragments were expressed in C2C12 myoblasts and myotubes and in HEK293 cells, and the luciferase assay was used to determine transcriptional activity.

Results The highest luciferase activity, 350-fold of the promoterless vector, was obtained with nucleotides –692 to +59 in myotubes ($P < 0{\cdot}001$), while the nucleotides –250 to +59 provided the highest, 45-fold, activity in the HEK293 cells ($P < 0{\cdot}001$). Longer promoter constructs (nucleotides –971, –1707 and –2158 to +59, respectively) had low promoter activity in both cell types. Forskolin treatment for 24 h resulted in approximately 30% decreased promoter activity in myotubes ($P < 0{\cdot}05$). Insulin treatment for 0·5–3 h did not increase *GYS1* promoter activity; instead the activity was slightly but significantly decreased after 24 h in myotubes ($P < 0{\cdot}005$).

Conclusions From our results we conclude that basal *GYS1* promoter activity is obtained from the first 250 nucleotides of the promoter, while the nucleotides –692 to –544 seem to be responsible for muscle-specific expression, and nucleotides –971 to –692 for negative regulation. In myotubes, the *GYS1* promoter was sensitive to negative regulation by forskolin, whereas insulin did not increase *GYS1* transcription.

Keywords cAMP, glycogen synthase, insulin, promoter activity, transcription.
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Introduction

Glycogen synthase has a central role in glucose metabolism and exists in two isoforms. While the liver isoenzyme is exclusively expressed in the liver [1], the skeletal muscle isoenzyme is expressed in a variety of tissues besides skeletal muscle, including at least fat, heart, kidney and brain, but not in the liver [2]. The almost ubiquitous expression of the skeletal muscle glycogen synthase gene (*GYS1*) suggests that *GYS1* might exert vital housekeeping functions for most cells. Less is, however, known about the regulation of *GYS1* expression. The activity of glycogen synthase is under complex regulation. Glycogen synthase is inactivated by phosphorylation at several sites, and activation occurs by dephosphorylation by protein phosphatase 1 ($PP1_G$) [3]. A rise in the intramuscular glucose-6-phosphate concentration allows binding of this metabolite to glycogen synthase and facilitates dephosphorylation by allosteric mechanisms [4]. Activation of skeletal muscle glycogen synthase is triggered in response to insulin stimulation, whereas adrenaline promotes inactivation of the enzyme [4].

Activation of glycogen synthase by insulin is impaired in patients with type 2 diabetes [5–8] and in subjects with increased risk for developing the disease, i.e. first-degree relatives of type 2 diabetic patients [6], suggesting that impaired

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glycogen synthase activity might be an inherited feature. The muscle glycogen synthase gene (*GYS1*) has been considered a candidate gene for type 2 diabetes but also for the metabolic syndrome and hypertension. Associations between the *GYS1* gene and type 2 diabetes has been identified in several populations [9–14] but no common functional variations have been found to explain these associations [11,14–18]. Decreased *GYS1* mRNA levels in the skeletal muscle of type 2 diabetic patients compared with healthy control subjects has been reported in some [19–21] but not all studies [8,14,22]. Whether or not the expression of *GYS1* is influenced by insulin also remains to be established, as results from different studies are divergent [8,19–25].

A prerequisite for the study of transcriptional regulation of *GYS1* is better knowledge about the promoter structure. Our aim here was to characterize the promoter region of *GYS1* and to provide information about transcriptional regulation of *GYS1* in skeletal muscle.

Materials and methods

Sequencing of the *GYS1* 5′-flanking region

A chromosome 19 clone (AC008687) containing part of the *GYS1* gene and greater than 4 kb of the 5′-flanking region was used for primer design. The sequence was confirmed by automated sequencing of human genomic DNA using a Thermo Sequenase II dye terminator cycle sequencing premix kit (Amersham Pharmacia Biotech, Uppsala, Sweden) in an ABI 377 (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Sequences were analyzed using Sequencher 3·0 (Gene Codes Corporation, Ann Arbor, MI) software. Putative transcription factor binding sites were predicted using the MatInspector professional 3·5 program [26], and insulin responsive elements were predicted according to O'Brien *et al.* [27].

Rapid amplification of cDNA ends

The Marathon Ready cDNA kit (Clontech, Palo Alto, CA) containing cDNA from human skeletal muscle was used to confirm the previously determined transcription initiation site of *GYS1* [11,18]. The amplified PCR products were subcloned into a pGEM-T vector (Promega, Madison, WI) and 20 clones were sequenced with universal primers SP6 and T7.

Preparation of luciferase reporter constructs

Four fragments of the *GYS1* promoter region (containing nt –250, –995, –1707, and –2158 to nt +59) were amplified by PCR. Primers introduced *MluI* (at nt positions –995, –1707 and –2158) or *SacI* (at position –250) restriction sites in the 5′-end of the constructs, whereas an endogenous 3-prime *HindIII* site at nt position +59 defined the end of each promoter construct. By digestion of the fragment containing nt –995 to +59 with *XhoI* (MBI Fermentas, Vilnius, Lithuania), *BglII* (Amersham Pharmacia Biotech) or *SmaI* (Amersham Pharmacia Biotech); three shorter fragments were obtained containing nt –692, –544 and –121 to +59, respectively. Consequently, after digestion with *HindIII* (MBI Fermentas), each construct contained 59 bp of the 5′-untranslated region according to the previously published transcription start site [11,18]. Digested and gel-purified promoter fragments were then cloned into a similarly digested pGL3-Basic vector (Promega).

Cell culturing

Mouse C2C12 myoblasts, a cell line that differentiates and fuses into multinucleated myotubes and is sensitive to insulin [28], and human embryonic kidney HEK293 cells, a cell line chosen to represent nonmuscle *GYS1* expression [29], were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, ICN, Costa Mesa, CA), supplemented with 10% fetal bovine serum, 1% L-glutamine and 2% penicillin-streptomycin. The glucose concentration of the medium was 25 mM. To induce differentiation of C2C12 myoblasts into myotubes, medium was switched to DMEM containing 10% horse serum. Cells were cultured at 37 °C under 5% CO_2.

Transfection assays

The day before transfection, cells were seeded into six-well tissue culture plates at $2{\cdot}5 \times 10^5$ (HEK293), $2{\cdot}8 \times 10^5$ (C2C12 myoblasts) or $3{\cdot}8 \times 10^5$ (for C2C12 myotube differentiation) cells per well, respectively. Transfections were performed with LipofectAMINE PLUS reagent (Invitrogen, Lidingö, Sweden) according to the manufacturer's instructions. Each transfection was performed using 1 μg of luciferase reporter construct DNA and 100 ng of an internal control plasmid pRL-TK (Promega). Three hours after the start of transfection, fetal bovine serum was added to a final concentration of 10% and incubation was continued.

Luciferase assays

Luciferase assays (Dual Luciferase Reporter assay System, Promega) were carried out according to the manufacturer's instructions. Firefly luciferase activities were normalized by *Renilla* luciferase activities. At least three independent experiments including three transfections for each construct were performed.

Treatment with forskolin or insulin

After transfection the cells were cultured for 18–24 h (HEK293 cells) or 96 h (C2C12 differentiating myotubes). Cells were serum-starved for at least 2 h before the medium

was replaced with medium containing either 10 μM forskolin (Sigma-Aldrich, Stockholm, Sweden), 100 nM human insulin (a kind gift from Novo Nordisk, Bagsværd, Denmark), a frequently used concentration known to be sufficient to activate insulin signalling pathways in C2C12 cells [30], or vehicle, and incubation was continued for 4 h and 24 h with forskolin and 0·5 h, 1 h, 3 h and 24 h with insulin.

Statistical analysis

Data are expressed as mean ± SEM. The significance of differences between promoter activities of the constructs were determined by the Mann–Whitney independent rank sum test using the BMDP statistical software (Version 1·12, Biomedical Data Processing Statistical Software Inc., Los Angeles, CA). *P*-values less than 0·05 were considered statistically significant.

Results

GYS1 5′-region

DNA from a healthy subject was sequenced to confirm the sequence of *GYS1* obtained from the database. Two differences were found between the obtained sequence and the AC008687 sequence. At nucleotid position –1464 the database sequence lacked a t and had an extra t at position –1522 relative to the transcription initiation site. Promoter analysis revealed several putative transcription factor binding sites, including at least nine putative binding sites for the general transcription factor Sp1, or GC boxes, three activator protein 2 (Ap-2) sites, three cyclic AMP (cAMP) responsive elements (CRE), four sites for CAAT/enhancer binding proteins (CEBP), two muscle initiator sequences (MINI), five octamer binding protein 1 (Oct1) sites, three sterol responsive elements (SRE), 15 E-boxes of which two were putative sites for the myoblast determining factor MyoD, eight Ets-like motifs, two insulin response element A (IRE-A)-like motifs, and one site each for myocyte enhancer factor 2 (MEF2)-, transcriptional enhancer factor 1- and phosphoenolpyruvate carboxykinase (PEPCK)-like motifs (Fig. 1). No TATA or CAAT boxes could be identified, although a sequence related to a muscle TATA box was located three nucleotides downstream from the ATG start codon.

For determination of transcription initiation sites, 22 clones containing 5′-rapid amplification of cDNA ends (RACE) products were sequenced. A total of 13 putative transcription initiation sites were identified (Fig. 1). The site at position –180 relative to the ATG codon was indicated by sequences from seven different clones (Fig. 1).

Functional characterization of the *GYS1* promoter

Compared with the promoterless luciferase vector, the shortest (nt –121 to +59) and longest (nt –2158 to +59) *GYS1* promoter fragments had 4- and 10-fold higher activities in HEK293 cells, 19- and 4-fold higher activities in myoblasts and 28- and 45-fold higher activities in myotubes, respectively (Fig. 2).

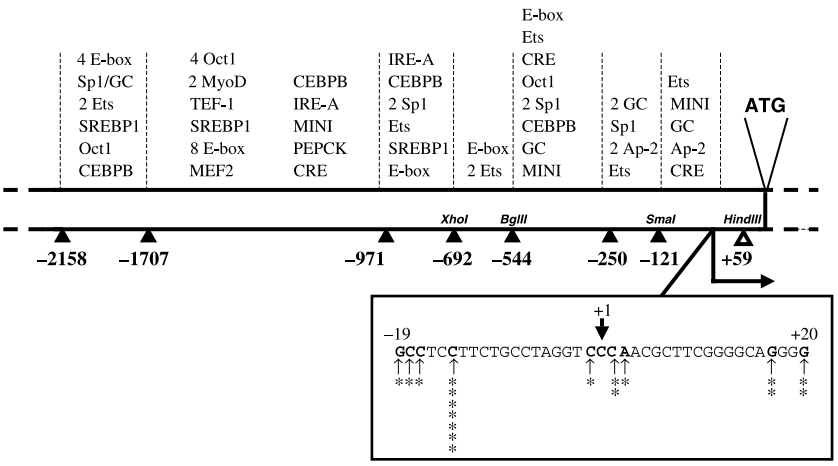

Figure 1 Glycogen synthase gene (*GYS1*) promoter constructs and putative transcription factor-binding sites within the constructs. Filled triangles (▲) indicate 5′-ends of the different constructs. The –250, –971, –1707 and –2158 constructs were made by introducing a *MluI* site in a linker primer, whereas the –692, –544, and –121 constructs were created using internal restriction sites. All constructs contained 59 bp of the 5′-untranslated region (△) numbered according to the previously determined transcription initiation site [11,18]. The major transcriptional initiation site, proposed by seven different clones in the present study, is indicated at position –14 relative to the previously determined single initiation site. Four sites, recognized by one clone each, are not shown in the figure. These sites were located at nt +92, +108, +125 and +168. Ap-2, activator protein 2; CEBPB, CAAT/enhancer-binding protein beta; CRE, cAMP responsive element; IRE-A, insulin response element A; MEF2, myocyte enhancer factor; MINI, muscle initiator; PEPCK, phosphoenolpyruvate carboxykinase; SREBP, sterol responsive element binding protein; TEF-1, transcriptional enhancer factor 1.

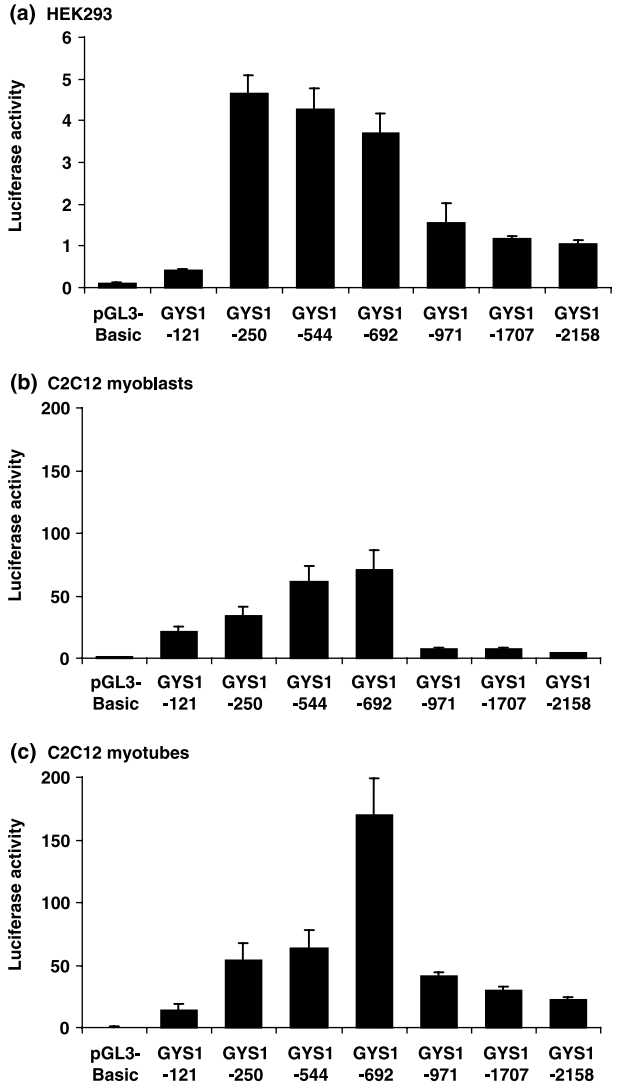

Figure 2 Transient expression of glycogen synthase gene (*GYS1*) promoter constructs. HEK293 cells (a), C2C12 myoblasts (b) or C2C12 myotubes (c) were cotransfected with the seven *GYS1* promoter-luciferase constructs or the promoterless pGL3-basic vector together with the pRL-TK Renilla vector. Firefly luciferase activities were corrected with the Renilla luciferase activities to adjust for transfection efficiency. Values are means ± SEM from three to eight independent experiments, each run in triplicate.

In HEK293 cells, the construct containing sequences from nt –250 to +59 had the highest promoter activity (45-fold of the promoterless vector) and was 12-fold more active compared with the construct containing nt –121 to +59 ($P < 0{\cdot}001$) (Fig. 2a). The promoter activities of the four longest promoter sequences (GYS1-692, GYS1-971, GYS1-1707, and GYS1-2158) were 0·8-, 0·3-, 0·3- and 0·2-fold compared with the activity of the GYS1-250 construct ($P < 0{\cdot}05$ for the GYS1-692 construct and $P < 0{\cdot}001$ for the GYS1-971, GYS1-1707, and GYS1-2158 constructs).

In C2C12 myoblasts, a gradual increase of the promoter activity was seen with increasing length of the promoter sequence, up to the construct containing the sequence corresponding to nt –692 to +59, which accounted for the highest, 65-fold, promoter activity (Fig. 2b). The sequence between nt –692 and –250 was responsible for a twofold increase in promoter activity compared with the sequence corresponding to nt –250 to +59 ($P < 0{\cdot}005$). In contrast, a decrease in promoter activity was associated with sequences between nt –971 and –692 (89% decrease, $P < 0{\cdot}001$) and even more between nt –2158 to –1707, leading to a total decrease of 94% from GYS1-692 to GYS1-2158 ($P < 0{\cdot}001$) (Fig. 2b).

Similar to results in C2C12 myoblasts, the sequence between nt –692 and +59 accounted for the highest promoter activity in C2C12 myotubes (340-fold compared with the promoterless vector) (Fig. 2c). The sequence between nt –692 and –544 accounted for a threefold increase in promoter activity (GYS1-544 vs. GYS1-692, $P = 0{\cdot}0014$), whereas, again, the sequence between nt –971 and –692 accounted for a 76% decrease (GYS1-971 vs. GYS1-692, $P < 0{\cdot}001$). The *GYS1* promoter was significantly more active in the differentiating C2C12 myotubes compared with the C2C12 myoblasts (twofold difference for the GYS1-692 construct, $P < 0{\cdot}005$).

Effect of forskolin

Treatment of C2C12-differentiating myotubes for 4 h with forskolin resulted in decreased activities of 18% and 22% for the constructs containing sequences corresponding to nt –692 to +59 and nt –1707 to +59, respectively ($P < 0{\cdot}05$ for GYS1-692 and $P < 0{\cdot}005$ for GYS1-1707) (Fig. 3a). After 24 h of forskolin treatment, down-regulation of the promoter activities was even more pronounced: 30% ($P < 0{\cdot}05$) and 36% ($P < 0{\cdot}005$) for GYS1-692 and GYS1-1707, respectively (Fig. 3b). In addition, forskolin caused a significant 46% decrease of the promoter activity carried by the sequence corresponding to nt –121 to +59 ($P < 0{\cdot}001$). However, as the nontreated promoter activity of the GYS1-121 construct was only 2% and 4% of that of GYS1-692 and GYS1-1707, respectively, the decrease in relative luciferase activity of GYS1-121 was only 3% and 5% of the decrease recorded for the two longer fragments. In contrast, forskolin treatment had no effect on *GYS1* promoter activity in HEK293 cells (Fig. 3c,d).

Effect of insulin

Insulin did not exert a significant effect on *GYS1* promoter activity after 0·5, 1 and 3 h of treatment, neither in HEK293 cells (Fig. 4) nor in C2C12-differentiating myotubes (Fig. 5). However, after insulin treatment for 24 h, a slight but significant decrease in promoter activity, was seen in the C2C12 myotubes (Fig. 5d) for the sequences corresponding to nt –250 to +59 (41% decrease, $P < 0{\cdot}005$) and nt –2158 to +59 (22% decrease, $P < 0{\cdot}05$). Although the largest

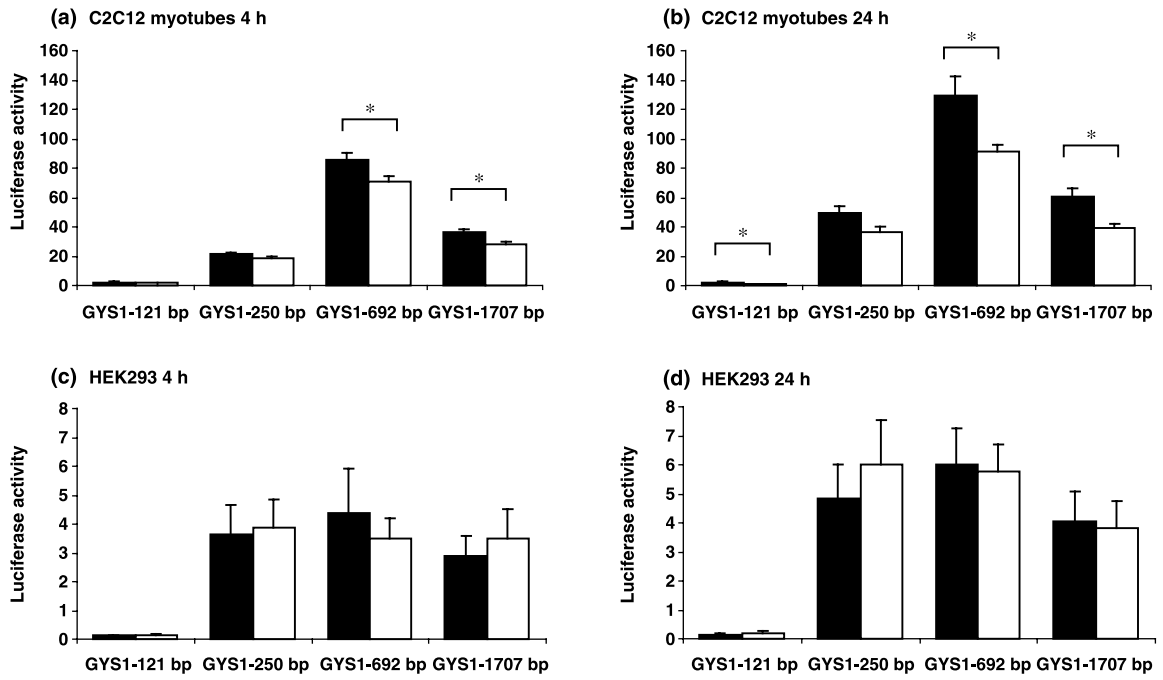

Figure 3 Glycogen synthase gene (*GYS1*) promoter activity after forskolin treatment of C2C12-differentiating myotubes (a,b) and HEK293 cells (c,d), transfected with GYS1-121, GYS1-250, GYS1-692 or GYS1-1707 constructs together with the pRL-TK Renilla vector. After 96 h (C2C12) or 24 h (HEK293) following the transfection, cells were serum starved and incubated with 10 μM forskolin for 4 h and 24 h. Filled bars represent control cells and open bars forskolin-treated cells. Values are expressed as relative luciferase activities (means ± SEM) and are from three independent transfections in triplicates. $^{*}P < 0{\cdot}05$.

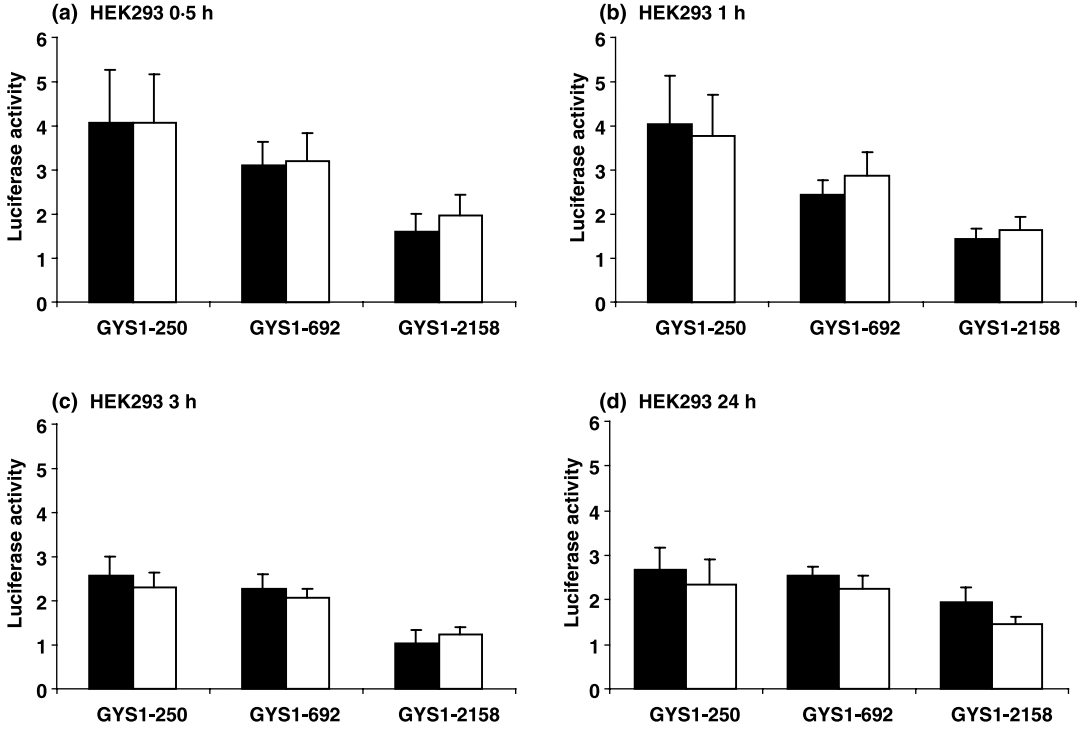

Figure 4 Glycogen synthase gene (*GYS1*) promoter activity after insulin treatment of HEK293 cells cotransfected with *GYS1* constructs GYS1-250, GYS1-692 or GYS1-2158 together with the pRL-TK vector. After 24 h following the transfection, cells were serum starved and treated with insulin for 0·5 h (a), 1 h (b), 3 h (c) or 24 h (d). Filled bars represent control cells and open bars insulin-treated cells. Values are expressed as relative luciferase activities (mean ± SEM) and are from three independent experiments in triplicates.

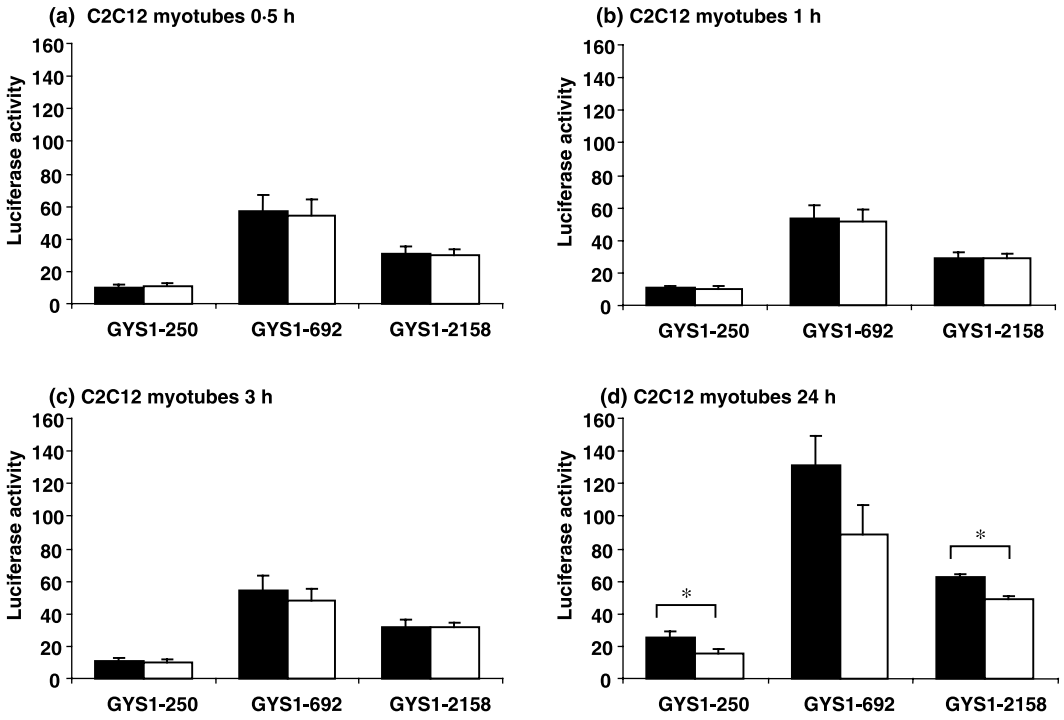

Figure 5 Glycogen synthase gene (*GYS1*) promoter activity after insulin treatment of C2C12 myotubes cotransfected with *GYS1* constructs GYS1-250, GYS1-692 or GYS1-2158 together with the pRL-TK vector. After 96 h following the transfection, cells were serum starved and treated with insulin for 0·5 h (a), 1 h (b), 3 h (c) or 24 h (d). Filled bars represent control cells and open bars insulin-treated cells. Values are expressed as relative luciferase activities (mean ± SEM) and the results are from three independent experiments in triplicates. $^{*}P < 0{\cdot}05$.

decrease in luciferase activity, calculated as relative luciferase units, was measured for the GYS1-692 construct, this decrease of 32% did not reach statistical significance ($P = 0{\cdot}10$).

Discussion

In this study we have functionally characterized the promoter of the human *GYS1* gene and evaluated the effect of cAMP as well as short- and long-term effects of insulin on the transcriptional activity of this promoter.

Fifty-four per cent of the clones in the 5′-RACE experiments proposed the nucleotide at position –180 relative to the translation start codon as the major transcription initiation site. Although some of the shorter clones could represent degraded mRNA products, instead of biological transcription initiation sites, the finding of rare clones extending upstream of position –180 indicates some variation in the transcription initiation of *GYS1*. The discrepancy with previous results suggesting a transciption initiation site at –166 [11,18] could be explained either by instability of the 5′-part of the *GYS1* mRNA, the quality of RNA used to create the cDNA kit, or by factors forcing reverse transcriptase to terminate such as extensive GC-rich regions or secondary structures. Further support for the nt –180 representing the major transcription initiation site is provided by the sequence surrounding the nt –180, $TCC_{+1}TTCT$, which shows conservation to the reported consensus transcription initiation sequence [31].

We performed characterization of the *GYS1* promoter in the embryonal kidney cell line HEK293 and in C2C12 myoblasts and myotubes to identify the minimal promoter of *GYS1* as well as regions responsible for muscle-specific up- or down-regulation of the gene. Our results indicate that the first 250 bp of the 5′-flanking region is responsible for the highest activity in HEK293 and thus accounts for the basal *GYS1* promoter activity. Lack of classical TATA- and CAAT-boxes within the *GYS1* promoter, increased GC content and CpG islands close to the transcription initiation site, as well as several Sp1 and Oct1 sites are all features of a housekeeping gene [32]. This is also in line with the fact that *GYS1* is ubiquitously expressed in many tissues. Despite this, *GYS1* has its major role in the skeletal muscle where glycogen is continuously synthesized and broken down. Correspondingly, several putative muscle-specific transcription factor binding sites were found within the *GYS1* promoter. Muscle-specific gene expression as well as myogenesis is regulated by association between myogenic basic helix-loop-helix (bHLH) proteins like MyoD and MEF2 factors [33]. Similar to *GYS1*, the glycogenin 1 promoter harbours several E-boxes as well as binding sites for MEF2, Ap-2, Oct1 and Sp1 [34]. To establish which of these sites are important for the regulation of *GYS1* transcription, mutational analysis would be required.

In the C2C12 cells, the highest *GYS1* promoter activity was observed for the sequence corresponding to nt –692 to +59, especially in differentiating myotubes where the activity of the GYS1-692 construct was threefold higher compared with the construct lacking this region (GYS1-544). These results suggest that the region between nt –692 and –544 could be responsible for myotube-specific expression. Sequence analysis did not provide any explanation for the high muscle-specific transcription from this region, as motifs in this region also are found throughout the *GYS1* promoter. The region upstream of nt –692 showed relatively weak promoter activity in all three cell types, suggesting that, in particular, the region from nt –971 to –692 could be responsible for negative regulation. However, no cAMP responsive elements or other obvious binding sites for negative regulation were found within this region.

Muscle glycogen synthase is subject to post-translational regulation in response to several hormones. Insulin is known to promote dephosphorylation and thereby activation of glycogen synthase by initiating a cascade of events leading to inactivation of glycogen synthase kinase 3 (GSK-3), as well as to activation of protein $PP1_G$ [3]. Adrenaline has a negative effect on glycogen synthase, as elevation of cAMP levels promotes both dissociation of glycogen synthase from the glycogen targeting subunit of PP1 (G_M) [35] and activation of the cAMP-dependent protein kinase, which is capable of inactivating glycogen synthase [36]. In HEK293 cells, forskolin treatment had no effect on *GYS1* promoter activity, whereas in C2C12 myoblasts an inhibitory effect was seen after both 4 h and 24 h. The negative effect of forskolin was expected, as the *GYS1* promoter contains several putative binding sites for transcription factors of the cAMP responsive element binding (CREB) family. In agreement with our results suggesting a role for cAMP as a negative regulator of glycogen synthesis at the level of *GYS1* transcription, the cAMP analogue db-cAMP has previously been shown to stimulate transcription of the muscle glycogen phosphorylase promoter [37]. In addition, raised cAMP levels down-regulated transcriptional activity of the glycogenin promoter [38]. It is therefore possible that the effect of enhanced lipolysis on glycogen synthesis also involves transcriptional steps.

It is well established that insulin plays a crucial role in the activation of glycogen synthase, while the effect of insulin on *GYS1* transcription is more unclear despite being addressed in several studies. Although insulin was found to stimulate *GYS1* mRNA expression in human skeletal muscle biopsies in one study [25], several other studies have not found an effect [8,19,20,22,23]. Neither could insulin significantly stimulate *GYS1* transcription in *in vitro* studies of cultured human nondiabetic myoblasts [24], while in cultured diabetic myoblasts hyperinsulinaemia increased and then normalized *GYS1* mRNA to levels seen in nondiabetic cultures in normal medium [21]. Our results are compatible with the view that short-term treatment with insulin does not increase *GYS1* transcription *in vitro*. On the contrary, we found a slightly but significantly decreased *GYS1* promoter activity after 24 h of treatment of cells with insulin. It remains, however, to be established whether this decreased transcriptional activity is a reflection of the situation seen in type 2 diabetes [19,20].

At least eight distinct consensus insulin-response sequences have been defined including the Ets- and E-box motifs, which mediate stimulatory effects, and PEPCK-like motifs, which mediate inhibitory effects of insulin [27]. In summary, we present evidence that basal *GYS1* promoter activity is obtained from the first 250 nt upstream of the *GYS1* transcription initiation site while a region between nt –692 and –544 is responsible for high muscle-specific expression and nt –971 and –692 for negative regulation. In differentiating myotubes, the *GYS1* promoter is sensitive to down-regulation by cAMP, indicating that epinephrine could regulate skeletal muscle glycogen metabolism also at the level of *GYS1* transcription. In contrast, insulin treatment did not increase *GYS1* promoter activity during these experimental conditions, suggesting that the effect of insulin is primarily mediated by phosphorylation/dephosphorylation and not by transcriptional regulation.

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