Identification of transcriptional targets for Six5: implication for the pathogenesis of myotonic dystrophy type 1

Shigeru Sato1, Miwa Nakamura1, Diane H. Cho2, Stephen J. Tapscott2, Hidenori Ozaki1 and Kiyoshi Kawakami1,

1Department of Biology, Jichi Medical School, Minamikawachi, Tochigi 329-0498, Japan and 2Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, Washington 98109-1024, USA

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Myotonic dystrophy 1 (DM1) is the most common inherited neuromuscular disease in adults. The disorder, characterized by myotonia, muscle wasting and weakness, cataract, insulin resistance, and mental impairment, is caused by the expansion of an unstable CTG repeat located in the 3' untranslated region of DMPK. The repeat expansion suppresses the expression of the homeobox gene SIX5. We describe here an experimental system to identify downstream transcriptional targets of mouse Six5 in order to elucidate the role of SIX5 in the pathogenesis of DM1 and development. By overexpressing a constitutively active Six5 (VP16–Six5wt) using adenovirus-mediated gene transfer in P19 cells and subsequent expression profiling using cDNA arrays, 21 genes, whose expression level increased by the treatment, were identified as potential target genes. Genes expressed in the somites, skeletal muscles, brain and meninges comprised the majority, suggesting the role of Six5 in the development and function of mesodermal tissues and brain. We provide evidence that Igfbp5 encoding a component of IGF signaling is a direct Six5-target. Moreover, the overall expression level of Igfbp5 was decreased in Six5-deficient mouse fibroblasts, and the response of human IGFBP5 to MyoD-induced muscle conversion was altered in cells of DM1 patients. Our results not only identify Six5 as an activator that directs Igfbp5 expression but also suggest that reduced SIX5 expression in DM1 might contribute to specific aspects of the DM1 phenotype.

INTRODUCTION

Myotonic dystrophy 1 (DM1, MIM160900) is the most common inherited neuromuscular disease in adults, characterized by myotonia, muscle wasting and weakness, cataract, insulin resistance, cardiac conduction defect, testicular atrophy, and mental impairment (1). DM1 is caused by the expansion of an unstable CTG repeat located in the 3' untranslated region (UTR) of DMPK on 19q13.3 (2–4). The consequence of the repeat expansion includes abnormal splicing and transport of DMPK transcripts, which result in a decrease in DMPK protein levels (5) and sequestration or induction of RNA-binding proteins by transcribed, expanded CUG repeats (6). In addition, the repeat lies immediately 5' to the regulatory region of the homeobox gene SIX5 (7–9), and repeat expansion causes the loss of a DNase I-hypersensitive site in the region and suppression of SIX5 expression (8,10,11).

SIX5 belongs to the Six class of homeobox genes (12). The gene is expressed in most of the tissues affected by DM1, such as skeletal and smooth muscles, central and peripheral nervous systems, and adult lens (7,9,13–18). As such, haploinsufficiency of SIX5 is thought to contribute to the development of some of the symptoms. Indeed, two independent lines of Six5-deficient mice have been produced and shown to develop cataract, suggesting that reduced SIX5 function underlies cataract phenotype in DM1 (15,17). While other phenotypes associated with DM1 are not apparent in those animal models, it is possible that SIX5 deficiency, in conjunction with other molecular defects resulting from the repeat expansion, is involved in the pathogenesis of DM1. For example, the fact that Six5 can activate transcription of the myogenin (Myog) gene encoding a myogenic basic helix–loop–helix (bHLH) protein through the MEF3 site, an essential promoter element required for the lineage-specific expression (19,20), may implicate SIX5 dysfunction in muscle phenotypes such as the fusion delay and fiber maturation defects seen in congenital DM1 cases (21,22). This notion is corroborated by the observation that mutations of D-Six4, the Drosophila counterpart of SIX5, cause a myoblast fusion defect and testicular/ovarian reduction (23). However, the phenotype of transgenic mice expressing an expanded CTG
repeat (24) and the molecular characterization of DM 2, which shows almost identical phenotype with DM 1 (25), cast some doubt on the extent of involvement of SIX5 in the pathogenesis of DM 1. This is partly due to insufficient characterization of SIX5, particularly of downstream transcriptional targets regulated by the SIX5 homeodomain. Identification of downstream targets for SIX5 is therefore essential to understand the involvement of SIX5 deficiency in DM 1.

Known downstream target genes for SIX5 include the aforementioned Myog identified by inference and Atpl1a1 encoding the α1 subunit of Na, K-ATPase, which was identified biochemically as a target gene for Six family proteins (13,26,27). A recent whole-genome PCR-based screening identified the 5’ flanking sequence of Drd5 encoding the dopamine SD receptor and several genomic DNA fragments as potential target sequences (28). The other way to screen SIX5-target genes is to overexpress SIX5 and detect genes whose expression levels change (increase or decrease) by overexpression. SIX5 is a transcription factor characterized by the presence of the Six domain (SD) and Six-type homeodomain (HD), both of which are required for specific DNA binding and interaction with Eya transcription coactivators (13,19). The SD is also known to interact with the Groucho family of corepressors (29), suggesting that SIX5 can act both as an activator and as a repressor.

In the present study, we sought to identify SIX5-target genes. A constitutively active form of mouse Six5 (VP16–Six5wt) was overexpressed in P19 embryonal carcinoma cells by adenovirus-mediated gene transfer and a group of genes induced by the treatment was detected by hybridization to cDNA arrays. To minimize background resulting from adenovirus infection and/or overexpression of the full-length Six5 and VP16 activation domain, both of which interact with various nuclear and cytoplasmic proteins, a HD mutant protein (VP16–Six5W241R) was overexpressed in the control set. Among 21 potential target genes, Igfbp5 and Igf2, which are involved in insulin-like growth factor (IGF) signaling, were subjected to further analyses in order to validate our approach. A Six5-binding site in the Igfbp5 promoter was determined by reporter gene assays and in vitro binding experiments. Expression levels of Igfbp5 and Igf2 were reduced in Six5+/− fibroblasts and expression of human IGFBP5 was altered in cells of DM 1 patients. These results not only identify Six5 as one of the activators directly involved in Igfbp5 expression but also suggest that reduced SIX5 expression in DM 1 might contribute to specific aspects of the DM 1 phenotype.

RESULTS

Production of recombinant adenovirus expressing a constitutively active Six5

To construct a constitutively active form of Six5, a strong transcription activation domain of herpes simplex virus VP16 was fused to full-length wild-type Six5 (Fig. 1A). As a control, a point mutation (W241R) was introduced in the HD. The site is conserved in the Six family as well as in many other HD proteins, and is thought to play a critical role in the structure and specific DNA binding of the proteins (30). When plasmids expressing VP16–Six5wt and VP16–Six5W241R were transiently transfected into COS7 cells, recombinant proteins were detected by anti-Six5 and anti-VP16 antibodies (Fig. 1B). The W241R mutation did not affect nuclear localization (data not shown). In gel retardation assays, nuclear extracts from COS7 cells expressing VP16–Six5wt contained proteins that bind to an oligonucleotide probe corresponding to the C3 element in the proximal promoter of rat Atpl1a1(27) (Fig. 1C, lane 3). The formation of the protein–DNA complex was interfered with by adding an excess amount of cold C3 oligonucleotide (lane 4) but not by a mutagenized oligonucleotide (lane 5). The protein–DNA complex was bound to anti-Six5 (lane 6) and anti-VP16 (data not shown) antibodies, resulting in the formation of a supershifted complex. Thus, VP16–Six5wt specifically binds to the C3 element. In contrast, VP16–Six5W241R retains only a residual activity to bind to the probe (lane 2).

We then constructed recombinant adenoviruses encoding VP16–Six5wt and VP16–Six5W241R in order to overexpress these two proteins. As shown in Figure 1D, recombinant proteins were readily detectable even in whole-cell extracts prepared from cells infected with two recombinant adenoviruses, AxCAwt VP16–Six5wt and AxCAwt VP16–Six5W241R.

To assess the effectiveness of our experimental design in detecting genes regulated by Six5, we analyzed Myog expression in C2C12 mouse myoblasts. As shown in Figure 1E, Myog expression levels were higher in myoblasts infected with AxCAwt VP16–Six5wt than in those infected with AxCAwt VP16–Six5W241R both at 14 and 38 h. In addition, VP16–Six5wt overexpression specifically activated luciferase reporters driven by the Myog promoter (pGL3M G-185) and a synthetic promoter (pTKW4FLF) (19) (data not shown).

Identification of potential downstream target genes for Six5

In our initial attempt to identify potential Six5-target genes, we chose P19 embryonal carcinoma cells to overexpress VP16–Six5wt because they express endogenous Six5 (7). Another reason for selecting these cells is their multipotent property to differentiate into various cell types, including skeletal and cardiac muscles and neurons (31,32).

A medium-scale expression profiling was performed using poly(A)+ RNA samples prepared from P19 cells that were infected with AxCAwt VP16–Six5wt and AxCAwt VP16–Six5W241R and cultured for 24 h. Hybridization of a pair of mouse cDNA macroarrays with probes synthesized using the two RNA samples allowed us to identify 21 genes (1.8%) out of 1176 genes spotted on the array as potential Six5-target genes whose expression levels were more than 2-fold higher in AxCAwt VP16–Six5W241R-infected P19 cells than in AxCAwt VP16–Six5W241R-infected cells (Table 1). The 21 potential target genes could be classified into the following categories according to the encoded proteins: transcription factors (Ebf2, Six2, Dermo1, Mesp2, Sim1, Six4, Mdfi, Foxd4 and Pua), signaling molecules and receptors (Igfbp5, Wnt4, Igf2, Sarp1, Fzd8 and Ptn), neurotransmitter receptor and transporter (Gatb3 and Htr3a) and those with other functions (Col9a2, Krt1-18, Pltp and Stit4). It is notable that four of nine transcription factors identified are bHLH proteins (Ebf2,
Dermo1, Mesp2 and Sim1) and Mef2 is known to interact with bHLH proteins. Remarkably, 10 genes are expressed in mesodermal tissues, including developing somites and skeletal muscle (Six2, Dermo1, Mesp2, Sim1, Six4, Mef2, Igfbp5, Igf2, Sarp1 and Fzd8) (33–42), and seven genes are expressed in neural tissues, including the neural tube, brain and meninges (Ebf2, Sim1, Foxd4, Wnt4, Fzd8, Galt3 and Htr3a) (36,43–47). Among the 21 potential targets, the expression levels of Igfbp5,
Igf2, Six2 and Mdfi were examined by northern blot analysis to confirm the array result (Fig. 2 and data not shown). Igfbp5 expression was almost undetectable in uninfected control and AxCAt VP16–Six5W241R–infected cells but markedly induced (>10-fold) in AxCAt VP16–Six5wt–infected cells. In contrast, although it was clear that Igf2 expression was induced after AxCAt VP16–Six5wt infection, the induction ratio was not very high (approximately 3-fold). The induction ratios for Six2 and Mdfi were 17.4- and 1.9-fold, respectively (data not shown).

**Direct activation of the Igfbp5 promoter by Six5**

We focused on Igfbp5 and Igf2 as two representative potential target genes regulated by Six5. Igfbp5 encodes IGF-binding protein 5 (IGFBP-5), which can either inhibit or potentiate IGF signaling by binding to IGF-I and IGF-II (48). Among the major sites of Igfbp5 expression during embryogenesis are somites and muscle precursor cells (39). Igf2 encoding IGF-II is abundantly expressed in the early embryonic mesoderm and its derivatives such as the developing somites and heart (40,49), whereas its expression in the adult is mostly confined to the choroid plexus and leptomeninges (50).

Regulation of Igfbp5 expression has been analyzed in some detail (51–55). Such studies showed that the proximal promoter is essential for the basal transcription and is also required for response to various signaling molecules. To determine whether the Igfbp5 promoter is directly regulated by Six5, we tested the response of luciferase reporter constructs driven by 5’flanking fragments of Igfbp5 to VP16–Six5wt and VP16–Six5W241R, which is defective in DNA binding (Fig. 1C). As shown in Figure 3A, VP16–Six5wt had a very strong inductive effect on
Characterization of a Six5-binding site in the Igfbp5 promoter

To explore the possibility that a Six5-binding site(s) lies within 105 bp of the 5' flanking region, we next performed gel retardation assays using a BseDI restriction fragment (positions −105 to −39, Fig. 3B, probe 1) and shorter oligonucleotide probes (Fig. 3B, probes 2–4). GST–Six5 bound to probe 1 as well as probe 3, which corresponds to positions −95 to −58 (Fig. 3C). Binding to probes 2 and 4 was not detectable. The results suggest that Six5 activates Igfbp5 expression directly through Six5-binding elements and that one of the elements is located in the region between positions −95 and −58.

We next sought to identify the Six5-binding sequence in the proximal promoter employing in vitro binding experiments (Fig. 4). DNase I footprinting and methylation interference assays revealed that GST–Six5 could bind to the sequence GCTCAATTGC located between −72 and −62 (Fig. 4A and B). The Six5-binding sequence was perfectly conserved in the corresponding rat and human genes (Fig. 4C). In addition, comparison with two previously characterized Six5-target sites, the C3 element in the Atp1a1 promoter and the MEF3 site in the Myog promoter (19,20), and the MEF3 consensus KSSTCAGGNNNY (56) allowed us to extract a consensus Six5-binding sequence, TCARRTTNC (Fig. 4C).

Expression of Six5-target genes in Six5−/− fibroblasts

To confirm that Six5 is involved in the regulation of downstream target genes identified in our screening, we examined the expression levels of Igfbp5 and Igf2 in fibroblasts prepared from homozygous Six5-deficient embryos (15). As shown in Fig. 5, overall expression levels of Igfbp5 and Igf2 in Six5−/− fibroblasts were decreased compared with wild-type fibroblasts. Interestingly, the responses to various stimuli such as serum starvation (Fig. 5, lanes 2 and 3), IGF-II (Fig. 5, lane 4) and IGF-I (data not shown) were essentially similar between the two types of fibroblasts. Both IGF-I and IGF-II have been shown to increase Igfbp5 mRNA level (57,58). These data suggest a positive regulatory role of Six5 in the basal transcription of Igfbp5 and Igf2, rather than the activated transcription in response to IGFs in cultured embryonic fibroblasts.

Cooperative activation of Igfbp5 expression by Six5 and Eya3

Although we have shown that a constitutively active Six5 (VP16–Six5wt) can activate reporter gene expression driven by the Igfbp5 promoter and that Igfbp5 expression level is decreased in cultured Six5−/− fibroblasts, the mechanism by which Six5 regulates Igfbp5 transcription is unclear. In this
regard, cooperative activation by Six5 and Eya3 has been demonstrated in Myog transcription (19). We examined whether Six5 acts cooperatively with Eya3 to activate transcription of Igfbp5. Transient transfection of the plasmid expressing FLAG–Six5 showed an activating effect on Igfbp5–160 Luc, one of the Six5-responsive reporter constructs (Fig. 3A) in COS7 cells (Fig. 6, column 2). Coexpression of HA-tagged Eya3 resulted in more than 4-fold induction relative to FLAG–Six5.
Figure 4. Identification of the Six5-binding site in the Igfbp5 promoter. (A) The result of DNase I footprinting and methylation interference assays. GST–Six5 and $^{32}P$-labeled Igfbp5 promoter fragments were used. The top and bottom strands indicate the strands that were end-labeled with $^{32}P$. Open square, a T residue protected weakly in DNase I footprinting; closed circles, methylation of these G residues strongly interfered Six5 binding; open circles, G residues that weakly interfered with Six5 binding; lane C, DNA-probes in the Six5-bound complexes; lane F, free DNA probes separated from the Six5–DNA complexes; lane G, G ladders of Maxam–Gilbert sequencing. (B) Summary of the DNase I footprinting and methylation interference assays shown in (A). The solid bar at the top indicates a predicted Six5-binding site. (C) A consensus Six5-binding site. Alignment of Six5-binding sites from the mouse Igfbp5 promoter (this study), corresponding sequences from human IGFBP5 (GenBank accession no. U20271) and rat Igfbp5 (L08275) promoters, the C3 element of rat Atplal1 promoter (X53233) and the MEF3 site of the mouse Myog promoter (M95800). Asterisks indicate identical residues in the aligned sequences. The MEF3 consensus sequence KSSTCAGGNNNY (56) and a proposed consensus Six5-binding sequence, TCARRTTNC, are shown below.
alone (columns 2 and 4). Taken together, these results suggest that Six5 is one of the activators that bind to the proximal promoter of Igfbp5 and that the activation mechanism by Six5 may involve interaction with the Eya3 coactivator. We also found that Six1, a member of the Six family proteins expressed in tissues of mesodermal origin, including the developing somites (33), is capable of activating the Igfbp5 promoter when expressed with Eya2 (Fig. 6, columns 5 and 7). This suggests that other Six family members in conjunction with coexpressed Eya proteins may also play a role in Igfbp5 expression in certain circumstances.

Expression of IGFBP5 in DM1 fibroblasts and skeletal muscles

In DM1 patients, a decrease in SIX5 expression caused by the CTG repeat expansion is likely to result in reduced expression of SIX5-target genes. If such is the case, genes identified in our screening, including IGFBP5, should exhibit low expression levels in cells of DM1 patients. However, considering that the repeat expansion also disturbs DMPK expression, RNA metabolism and intracellular signaling machinery, the effect on a given target gene may not be so simple. To examine whether IGFBP5 expression is affected by the repeat expansion, we isolated fibroblasts from three independent DM1 patients, each with an expansion of at least 1000 repeats, and examined IGFBP5 expression before and after MyoD-induced skeletal muscle conversion by northern blot analysis (Fig. 7). In two different DM1 fibroblast, the expression levels of IGFBP5 increased following skeletal muscle conversion but the...
assays, in vitro binding experiments and expression analysis using Six5 \(-/-\) cells. Finally, we showed that the expression of IGFBP5 was altered in cells of DM1 patients.

Identification of downstream target genes and binding elements for Six5

Recently, a whole genome PCR-based screening was performed to identify SIX5-target genes (28). The method, which utilized the DNA-binding domain of SIX5 fused to GST to survey genomic DNA fragments with potential Six5-binding sites in vitro, picked up several DNA fragments, leading to the identification of DRD5 as a potential target. In this study, we set up a different model system to screen potential target genes in a cellular context. We focused on genes induced by VP16–Six5wt overexpression because recombinant Six5 proteins fused to repression domains from Drosophila Engrailed and human AREB6 did not act as a repressor on various Six5-responsive reporters (data not shown). As to 21 potential Six5-target genes, we cannot exclude the possibility that VP16–Six5wt has the potential to regulate immediate early genes and thus overexpression of Six5 could lead to rapid changes in expression of genes that are not directly regulated by Six5. However, a consensus binding sequence for Six5, predicted using a binding site in the Igfbp5 promoter, was found in five of 10 other potential target genes, including Igf2, whose 5’ flanking and non-coding sequences are deposited in the data bank (Table 1), and in two of the SIX5-binding genomic DNA fragments, AF242570 and AF242573, isolated in the aforementioned study (28). When a less strict consensus, TCARRNNNY, was applied, the sequence was found in SIX5-binding DNA fragments at high-frequency except AF242572, suggesting that both methods could effectively detect Six5/SIX5 target genes.

In Table 2, we list positions of potential Six5-binding sites found in known Six5 targets (Atp1a1 and Mymog) and genes implicated in DM pathogenesis [Insr, Tnnt2, Clcn1 and Cnbp (the mouse orthologue of ZNF9)]. Among these, Mymog and Insr encoding the insulin receptor were present in the cDNA expression array used in our study. Induction ratios of Insr and Mymog by VP16–Six5wt overexpression in P19 cells were 1.1 and 0.88, respectively. Drd5 was not present on the array. It remains to be established whether any of the potential sites listed in Table 2 (except the C3 element in Atp1a1 and the MEF3 site in Mymog) are involved in Six5-mediated transcriptional regulation.

Igfbp5 as a direct target gene for Six5

We focused on Igfbp5 and Igf2 as two representative potential target genes. Changes in the expression of IGFBP5 to MyoD-induced muscle conversion in three independent DM1 fibroblasts may partly result from reduced SIX5 expression. As to Igf2, we did not test how Six5 activates the gene, since its expression is regulated not only by three different promoters (59) but also by multiple downstream enhancers that are shared with the closely linked H19 gene (60), making it difficult to assess the effect of Six5 on transcription using ordinary reporter gene assays. However, given an increase in Igf2 expression level after 12 h of Six5 overexpression in P19 cells,
Listed are genes known to be regulated by Six5 (known Six5 target genes) and those implicated in DM pathogenesis (DM-related genes). The positions of potential Six5-binding sites present in promoter regions, sequences Atp1a1 Igfbp5 binding sites may achieve sufficient levels of DM-related genes:

<table>
<thead>
<tr>
<th>Known Six5 target genes:</th>
<th>Symbol</th>
<th>Potential Six5-binding site</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATPase, Na+K+ transporting, alpha polypeptide myogenin</td>
<td>Atp1a1</td>
<td>736, 90(C3)</td>
<td>1291, 1236, 264</td>
</tr>
<tr>
<td>M yog</td>
<td>243, 87(MEF3)</td>
<td>1294*</td>
<td>M95800</td>
</tr>
<tr>
<td>DM-related genes:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cellular nucleic acid binding protein (ZNF9 orthologue)</td>
<td>Cnbp</td>
<td>2096</td>
<td>-4165*, -3255*, -2285</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-1584, -1480*, -1145</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-1078, -529</td>
</tr>
<tr>
<td>chloride channel 1</td>
<td>Clcn1</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>insulin receptor</td>
<td>Insr</td>
<td>No</td>
<td>-279*, -206*, 119</td>
</tr>
<tr>
<td>troponin T2, cardiac</td>
<td>Tnnt2</td>
<td>3427</td>
<td>-951*, -464*</td>
</tr>
</tbody>
</table>

Table 2. Six5-binding sites in genes implicated in DM

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Potential Six5-binding site</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TCARRTTC</td>
<td>TCARRNNNY</td>
</tr>
<tr>
<td>Known Six5 target genes:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATPase, Na+K+ transporting, alpha polypeptide myogenin</td>
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<td>736, 90(C3)</td>
</tr>
<tr>
<td>M yog</td>
<td>243, 87(MEF3)</td>
<td>1291, 1236, 264</td>
</tr>
<tr>
<td></td>
<td></td>
<td>X53233 (r)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M95800</td>
</tr>
<tr>
<td>DM-related genes:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cellular nucleic acid binding protein (ZNF9 orthologue)</td>
<td>Cnbp</td>
<td>2096</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-4165*, -3255*, -2285</td>
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<td>chloride channel 1</td>
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<td>No</td>
</tr>
<tr>
<td>insulin receptor</td>
<td>Insr</td>
<td>No</td>
</tr>
<tr>
<td>troponin T2, cardiac</td>
<td>Tnnt2</td>
<td>3427</td>
</tr>
</tbody>
</table>

a decrease in Igf2 expression in Six5 mutant fibroblasts and the presence of potential Six5-binding sites (Table 1), we believe the gene is also a direct target of Six5.

If Six5 directly (or indirectly) activates the expression of Igfbp5 and Igf2, the question arises as to why Six5 mutant mice do not show growth deficiency, a phenotype expected from reduced Igf2 expression (61), or defects in muscle or other mesodermal tissues that express Igf2 and Igfbp5. There are three potential explanations that account for the lack of a clear muscle phenotype in Six5 mutant mice. (i) Other members of the Six family of proteins, such as Six1 and Six4, may compensate for the loss of Six5 in Six5 mutant mice (15,37). We have shown that the Igfbp5 promoter can be activated not only by Six5 but also by Six1. Intriguingly, activation of the skeletal muscle differentiation program by overexpression of Pax3-FKHR results in the induction of Six1, Igf2 and Igfbp5 (62). (ii) Other transcription activators binding to the sequences flanking Six5-binding sites may achieve sufficient expression levels of Igf2 and Igfbp5 required for embryogenesis to proceed normally. It is possible that the effect of Six5 loss on overall expression levels of Igf2 and Igfbp5 became apparent because of the culture conditions used. (iii) A defect in Six5 mutant muscle may only appear during regeneration or after changes in functional loading (overloading and unloading).

Implications for insulin resistance and cataractogenesis

Insulin resistance is a common metabolic abnormality in DM1 patients. The defect was shown to be associated with aberrant regulation of insulin receptor pre-mRNA splicing and proposed to be caused by an increase in CUG-BP (63). While this seems to provide a plausible explanation, it is still possible that a decrease in SIX5 expression also contributes to insulin resistance. It has been reported that recombinant IGF-I can partially circumvent impaired insulin action in cultured DM1 myotubes (64) and improve metabolism and function in the muscle of DM1 patients in a clinical trial (65). Binding of IGFs to both IGF-I and insulin receptors and activation of intracellular targets common to the two receptors are thought to be the molecular basis of the beneficial effects of IGFs (66). As such, changes in the expression level of IGF signaling components such as IGF-II and IGFBP-5 would also modulate insulin action in DM1 patients.

A detergent cataract is the most characteristic eye phenotype in DM1. Six5 and SIX5 are expressed in most structures of the eye, including the lens epithelium (13–17), and cataract formation is recapitulated in two independent lines of Six5-deficient mice (15,17). In such mice, homo- and heterozygous losses of Six5 were considered to result in dysregulation of its downstream target genes in the lens and to cause cataracts. While expression of Igf2 and Igfbp5 is detected in the ciliary body, cornea, retina and sclera but not in the lens (67,68), dysregulation of IGF signaling is implicated in cataract formation (69,70). Our study may implicate Six5-target genes encoding proteins such as IGF-II and IGFBP-5 that are secreted from ocular tissues surrounding the lens in cataractogenesis in DM1 patients and in SIX5-deficient mice.

The role of Six5 in mesoderm development and brain function

Previous studies have demonstrated a broad expression of Six5 in various tissues. Of the 21 potential Six5-target genes identified, 10 are known to be expressed in the developing somites and skeletal muscles (Six2, Dermo1, Mesp2, Sim1, Six4, Mlf1, Igfbp5, Igf2, Sarp1 and Fzd8). Because it has been established that Six5 directly activates M yog expression through the MEF3 site (19,20), identification of additional genes expressed in muscle-forming mesodermal tissues further implicates a decrease in SIX5 expression in fusion delay and fiber maturation defects in congenital DM1 cases. Our observation that DM1 fibroblasts failed to upregulate IGFBP5...
expression to a normal level in response to MyoD-induced muscle conversion can be significant in this regard.

In addition to DRD5 described previously (28), we have found the 5-hydroxytryptamine (serotonin) receptor 3A (Htr3a) and Gabt3 as potential Six5 targets. Thus, Six5 may play roles in normal brain function, and dysregulation of genes encoding these might partly account for the psychological problems in DM1 patients.

Another interesting finding of the present study is that the expression levels of Six2 (> 17-fold) and Six4 (> 3-fold) were elevated by VP16–Six5wt overexpression in P19 cells. Expression analyses of Six genes in Six5- and Six4-deficient mice have previously shown that the loss of Six5 causes a slight increase in the expression of Six1 and Six4 in the skeletal muscle (15), while the loss of Six4 does not affect the expression of Six1, Six2 and Six5 when examined in whole embryos (37). This seems to suggest that Six family genes participate in complex feedback loops employing different members in different cell types.

MATERIALS AND METHODS

Plasmid construction

Plasmids expressing FLAG- and HA-tagged proteins, pSix5x, pHM 6 Eya2 and pHM 6 Eya3, were described previously (19), as was pSix1x (71). To construct pcS2+–VP16–Six5wt, full-length mouse Six5 cDNA, excised as an XbaI fragment from pSix5x, was cloned into the XbaI site of pcS2+–VP16-N (72). A point mutation, W241R, was introduced in the HD by cassette mutagenesis using the following primers: W241R–FP (mutagenized nucleotides are in lower case, 5'-AGGTCAAGACGAGGATGGG-3') and its complementary oligonucleotides W241R–RP. The W241R mutation was chosen based on a mutational study of Pit-1 (30).

The mouse Igfbp5 promoter was isolated using the LA PCR in vitro Cloning Kit (Takara). Downstream primers complementary to a published Igfbp5 exon 1 sequence (GenBank accession no. U02023; its transcription start site is numbered +1) were as follows: Igfbp5–RP2 (positions +123 to +147, 5'-TTTCTGAAAACCCGCGATTTGTCGT-3') and Igfbp5–RP3 (positions +97 to +121, flanked with a Sall restriction site, 5'-TAGTCAGAATGGGACAGCTCAGAGC-3'). BglII-digested genomic DNA (BALB/c, Clontech Laboratories) was ligated to a linker supplied in the Takara kit and subjected to two rounds of PCR amplification with primers corresponding to the linker sequence and the primers Igfbp5–RP2 and Igfbp5–RP3. The amplified 3043 bp BglII–Sall fragment was cloned into the BamHI–Sall sites of the pBluescript SK+ (Stratagene) and partially sequenced. The Igfbp5 promoter fragment was excised by digesting at the flanking NotI (blunted) and Xhol sites, and ligated to the Smal–Xhol sites of pGL3-basic (Promega) to generate pGL3 Igfbp5–2.9k. 5' deletion constructs were generated by cloning shorter Igfbp5 promoter fragments digested with various restriction enzymes at the 5' ends and with Xhol at the 3' end into pGL3-basic. The following restriction sites were used in the present study: EcoRI (−1.4 kb, blunted), Xbal (−160, blunted), Eco130I (−105, blunted), SacI (−69) and HaelII (−35).

Cells and transfection assays

P19 mouse embryonal carcinoma cells were grown in α-minimal essential medium (Gibco BRL) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin (P/S) at 37°C under 5% CO2. COS7 cells and human embryonic kidney 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and P/S at 37°C under 5% CO2. For C2C12 mouse myoblasts, DMEM containing 4.5 g/l glucose was used. Mice embryonic fibroblasts were prepared from 14.5 dpc embryos. Wild-type embryos were obtained from pregnant C57BL/6J females purchased fromCLEA Japan, Inc. (Tokyo). Six5-deficient embryos were obtained by crossing Six5−/−; Six4−/− mice (15,37). Embryo genotyping was performed on DNA samples extracted from dissected head by PCR with the following primers: 5'-ACATCAACGAGGAGATGGGGATGTTAG-3' (downstream, the sequence present in both wild-type and mutagenized Six4 alleles), 5'-CCGATTTACGGAAGCTGAGTCTGG-3' (upstream, specific to mutagenized Six4 allele), and 5'-AGAAATCGGGATGAGTCTGG/TTACC-3' (upstream, specific to wild-type Six4 allele). Cells at passages 5–7 were used in this study. Prior to RNA extraction, confluent cultures were serum-starved for 24 h in serum-free DMEM. Then, the cells were stimulated by replacing the serum-free medium with a fresh serum-free DMEM containing either 100 ng/ml IGF-II or 10% FCS and cultured for 12 h. Human fibroblast cultures were obtained from unaffected and DM1-affected adults as described previously (8). Normal (ST101) and DM1 fibroblasts (HFB-, HFC- and HDF-DM) were infected with pBABE M yoD–ER and pLXSN M yoD–ER, both of which expressed human M yoD fused to the ligand-binding domain of the estrogen receptor (ER) (73). Virus-infected fibroblasts were split in two, cultured to confluence and processed for RNA extraction before or after treatment with low-serum IT medium (DMEM, 2% heat-inactivated horse serum, 10 μg/ml transferrin, 10 μg/ml insulin) supplemented with 10−7 M β-estradiol for 72 h to convert to skeletal muscle. Transfections were performed as described previously (19). Transfected cells were harvested after 48 h and assayed for luciferase and β-galactosidase activities. β-Galactosidase activity of the cotransfected reporter pEFBOS/β-gal was used as an internal control to correct for the variability in transfection efficiency.

Protein preparation and western blotting

Nuclear extracts were prepared from cultured cells as described previously (74). For virus-infected cells, 1 × Laemmli sample buffer was added to the culture dish, and the whole-cell lysate was used as a source of protein. Proteins were separated by SDS polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech). VP16–Six5wt and VP16–Six5W241R expressed from plasmid or adenoviral vectors were detected using anti-VP16 (sc-7545 and sc-7546, Santa Cruz) and anti-Six5 (18) antibodies. For chemiluminescence detection, we used the ECL western blotting analysis system (Amersham Pharmacia Biotech).
Gel retardation assay

The gel retardation assay was carried out as described previously (75). Nuclear extracts prepared from transfected COS7 cells or GST-Six5, the SD and HD of Six5 fused to GST, purified from bacteria (13) were used in the assay. A Bse DI(−106)–Bse DI(−42) fragment of Igfbp5 promoter and the following double-stranded oligonucleotides were endlabeled with [α-32P]dCTP (Amersham Pharmacia Biotech) by Klenow fragment and used as probes: Igfbp5-105/79 (positions −105 to −79), 5′-CTCTGGCATCTTGATGGG-TGGGTGTG-3′, Igfbp5-95/58 (positions −95 to −58), 5′-TGCTTGTTGGAGCTCAATTGCAGCT-3′ and Igfbp5-64/39 (positions −64 to −39), 5′-TGCACTGATGCGCCAGCCAG-3′). C3 WT (which corresponds to the Six4-binding site in Atpra1 promoter) and C3 MUT were described previously (26). In some experiments, 20-fold molar excess of unlabeled oligonucleotides (C3 WT and C3 MUT) and anti-Six5 antiserum (0.2 μl) were added to the binding reaction.

Production and infection of recombinant adenovirus

We selected a replication-defective adenovirus vector A xCAwt (76), in which expression of a foreign gene is driven by a strong CAG promoter composed of the cytomegalovirus enhancer and chicken β-actin promoter. Recombinant adenoviruses were produced using the Adenovirus Expression Vector Kit (TaKaRa). DNA fragments encoding VP16–Six5 wt and VP16–Six5 WT241R were excised from pcS2+ VP16 constructs by digesting at the flanking BamHI and SnaBI sites, blunted and cloned into the Swai site of the cosmid vector pACXAwt. The recombinant cosmids was cotransfected with the Eco T22I-digested A XCAwt DNA-terminal protein complex supplied in the Takara kit into 293 cells. Recombinant viruses were isolated, propagated and checked for any contaminating E1-bearing parent virus, titer (plaque forming unit (PFU)) and transgene expression. Virus titration was carried out using 293 cells and the expression of recombinant proteins was assessed by western blotting. For virus-infection experiments, cells plated onto a 10 cm culture dish were overlaid with recombinant adenoviruses [at a multiplicity of infection (m.o.i.) of 100–200 PFU] in culture medium containing 5% FCS for 2–3 h. The virus-containing medium was replaced with normal culture medium, and cells were further incubated for 6–48 h.

RNA extraction and northern blot analysis

Total RNA was extracted from cultured cells with lso- gen reagent (Nippon Gene). Poly(A) + RNA was selected using Oligotex-dT30 ‘super’ latex beads (TaKaRa). For northern blot analysis, total RNA was separated by electrophoresis through a 1.3% agarose/formaldehyde gel, blotted onto a nylon membrane (Hybond N+, Amersham Pharmacia Biotech) and hybridized with 32P-labeled cDNA probes. Igfbp5 and Igfb2 cDNA fragments were isolated by PCR from a mouse skeletal muscle cDNA library (Clontech) using the following primers: Igfbp5-FP (5′-AGAATTCAATGGGATCGACGCCGTTGTCCT-3′) and Igfbp5-RP (5′-AGATACCTCAACGT-TACTGCTGTG-3′) for the 831 bp Igfbp5 cDNA fragment and Igf2-FP (5′-AGAATTCAATGGGATCGACGCCGTTGTCCT-3′) and Igf2-RP (5′-AGATACCTCAACGT-TACTGCTGTG-3′) for the 558 bp Igf2 cDNA fragment, respectively. A 317 bp human IgfBp5 cDNA fragment was obtained by digesting the plasmid pHBP5–501 (77) with SaeII (344) and Sall (660). Myogenin and β-actin cDNA fragments were prepared as described previously (37). Quantitation of the hybridization signals was carried out using the STORM system (Amersham Pharmacia Biotech) and ImageQuant software (Molecular Dynamics).

Expression profiling using a cDNA macroarray

Expression profiling was performed by hybridization of the Atlas Mouse 1.2 cDNA Expression Array containing 1176 known mouse cDNAs (Clontech) with 32P-labeled cDNA probes. P19 cells infected with A XCAwt VP16–Six5 wt and A XCAwt VP16–Six5 WT241R and cultured for 24 h were used as the source of poly(A) + RNA to prepare hybridization probes. The RNA samples were treated with RNase-free DNase I (Roche Diagnostics) before poly(A) + selection to remove any contaminating genomic DNA and subjected to PCR with the primers Six5–579F (5′-AGATACCTCGGGAGAGGCCCTC- TA-3′) and Six5–820R (5′-TCTCGAGTGGAACCTCAGC- CTCCGTAGTGTG-3′) to confirm that the samples produce no visible PCR products even after 35 cycles of amplification. Probe synthesis, hybridization and washing of the Atlas array were carried out as instructed by the manufacturer.

After a high-stringency wash instructed by the manufacturer, the hybridization pattern was quantified and analyzed using the STORM system and ArrayVision software (Imaging Research Inc.). The hybridization experiment was repeated using a set of RNAs prepared from P19 cells of a different passage number that were infected with the two recombinant adenoviruses. Genes whose expression levels were more than 2-fold higher in Six5wt-infected samples in both data sets were identified as differentially expressed genes.

DNase I footprinting and methylation interference assays

DNase I footprinting and methylation interference assays were carried out as described previously (75). Purified GST–Six5, which comprises a region encompassing only the SD and HD (13), was used in the assays. The probe was prepared as follows. A Bse DI(−106)–Bse DI(−42) fragment of the Igfbp5 promoter was blunted and cloned into the SmaI site of pBluescript SK +. The insert was excised by digesting at the flanking Sall and HindIII sites. The HindIII site was 5′-labeled with [γ-32P]dATP by T4 polynucleotide kinase or 3′-labeled with [α-32P]dCTP by Klenow fragment.

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REFERENCES


growth factor-I (IGF-I) regulates IGF-binding protein-5 gene expression through
the phosphatidylinositol 3-kinase, protein kinase B/Akt, and p70 S6 kinase
factor (IGF)-I regulates IGF-binding protein-5 gene expression through the
AP-2 promoter.

Kou, K., Mittanck, D.W., Fu, C. and Rotwein, P. (1995) Structure and
Expression of the insulin-like growth factor II gene in the choroid plexus,
slow/cardiac troponin C gene transcription in skeletal muscle.

Biol.

differentiation and regional specification in the central nervous system.
Dev. Dyn., 210, 191–205.

Kastner, K.H., Monaghan, A.P., Kern, H., Ang, S.L., Weitz, S., Lichter,

Boonyaratavanakit, V., Strong, D.D., Mohan, S., Baylins, D.J., Beck,

Duan, C., Liimatta, M.B. and Bottum, O.L. (1999) Insulin-like growth factor (IGF)-I regulates IGF-binding protein-5 gene expression through the phosphatidylinositol 3-kinase, protein kinase B/Akt, and p70 S6 kinase

Kou, K., Mitanka, D.W., Fu, C. and Rotwein, P. (1995) Structure and
function of the mouse insulin-like growth factor binding protein 5 gene

Parmar, M.S., Ip, H.S., Jung, F., Shen, T., Martin, J.F., Vora, A.J., Olson,

growth factor-I (IGF-I) regulates IGF-binding protein-5 synthesis through

Smink, J.J., Koster, J.G., Hendriks-Stegeman, B.I. and Van Buul-Offers,

Holthuizen, R.E., Cleutjens, C.B., Veenstra, G.J., van der Lee, F.M.,

skeletal muscle-specific mouse Igf2 repressor lies 40 kb downstream of the

DeChiara, T.M., Efstratiadis, A. and Robertson, E.J. (1999) A growth-
deficiency phenotype in heterozygous mice carrying an insulin-like growth

Khan, J., Bittner, M.L., Saal, L.H., Teichmann, U., Azorsa, D.O., Gooden,
detect activation of a myogenic transcription program by the PAX3-FKHR fusion oncogene. Proc. Natl Acad. Sci. USA, 96, 13264–13269.

Sawarkar, R.S., Phillips, A.V. and Cooper, T.A. (2001) A bivalent repressor of insulin receptor alternative splicing is associated with insulin resistance in

circumvents defective insulin action in human myotonic dystrophy skeletal

Vlachopapadopoulos, E., Zachwieja, J.J., Gertner, J.M., Manzione, D.,


Shirke, S., Faber, S.C., Hallerm, E., Makarenkova, H.P., Robinson, M.L.,

interactions between Eya1 harboring mutations found in patients with


Kawakami, K., Yanagisawa, K., Watanabe, Y., Tominaga, S. and Nagano,
K. (1993) Different factors bind to the regulatory region of the Na

Kawakami, K., Schiederet, C. and Roeder, R.G. (1988) Identification and purification of a human immunoglobulin-enhancer-binding (NF-

Kanegae, Y., Lee, G., Sato, Y., Tanaka, M., Nakai, M., Sakai, T., Sugano,