Identification of Sp1 and GC-boxes as transcriptional regulators of mouse Dag1 gene promoter

Alessandro Rettino,1 Francesca Rafanelli,1 Giannicola Genovese,1 Martina Goracci,1 Rosa Anna Cifarelli,3 Achille Cittadini,1,2 and Alessandro Sgambato1,2

1Centro di Ricerche Oncologiche “Giovanni XXIII”-Istituto di Patologia Generale, Università Cattolica del Sacro Cuore, Rome; 2Laboratorio di Oncologia Molecolare, Centro di Riferimento Oncologico di Basilicata-Istituto Di Ricovero e Cura a Carattere Scientifico, Rionero in Vulture (PZ); and 3X-life Lab, Ospedale Madonna delle Grazie, Matera, Italy

Submitted 30 April 2009; accepted in final form 4 August 2009

Rettino A, Rafanelli F, Genovese G, Goracci M, Cifarelli RA, Cittadini A, Sgambato A. Identification of Sp1 and GC-boxes as transcriptional regulators of mouse Dag1 gene promoter. Am J Physiol Cell Physiol 297: C1113–C1123, 2009. First published August 5, 2009; doi:10.1152/ajpcell.00189.2009.—Dystroglycan is a widely expressed adhesion complex that anchors cells to the basement membrane and is involved in embryonic development and differentiation. Dystroglycan expression is frequently reduced in human dystrophies and malignancies, and its molecular functions are not completely understood. Several posttranslational mechanisms have been identified that regulate dystroglycan expression and/or function, while little is known about how expression of the corresponding Dag1 gene is regulated. This study aimed to clone the Dag1 gene promoter and to characterize its regulatory elements. Analysis of the mouse Dag1 gene 5′-flanking region revealed a TATA and CAAT box-lacking promoter including a GC-rich region. Transfection studies with serially deleted promoter constructs allowed us to identify a minimal promoter region containing three Specificity protein 1 (Sp1) sites and an E-box. Sp1 binding was confirmed by chromatin immunoprecipitation assay, and Sp1 downregulation reduced dystroglycan expression in muscle cells. Treatment with 5-aza-2′-deoxycytidine and/or the histone deacetylase inhibitor trichostatin A increased Dag1 mRNA expression levels in myoblasts, and methylation decreased promoter activity in vitro. Furthermore, Dag1 gene promoter methylation was reduced while its expression increased during differentiation of C2C12 myoblast cells in myotubes. In conclusion, for the first time we have characterized the activity of the mouse Dag1 gene promoter, confirming a complex regulation by Sp1 transcription factor, DNA methylation, and histone acetylation, which might be relevant for a better understanding of the physiopathology of the dystroglycan complex.

dystroglycan; promoter; epigenetic modification; muscle differentiation; gene expression

Dystroglycan, a pivotal component of the dystrophin-glycoprotein complex, is a major nonintegrin adhesion molecule expressed in skeletal muscle and in a wide variety of tissues at the interface between the basement membrane and the cell membrane (2, 49). It is formed by two subunits that are encoded by a single gene (Dag1) and are formed as one precursor protein cleaved into two mature subunits, α (extracellular) and β (transmembrane), which bind to extracellular matrix (ECM) molecules and proteins involved in signal transduction and cytoskeleton organization, respectively. Thus dystroglycan forms a continuous link from the ECM to the actin cytoskeleton, providing structural integrity and perhaps transducing signals in a manner similar to integrins (2, 49).

The Dag1 gene was initially cloned in muscle, and interest has for a long time been limited to its role in muscle physiopathology, being involved in several dystrophies and neuromuscular disorders (2). However, given its widespread distribution and important role in cell-ECM interaction, dystroglycan was expected to play an important role outside skeletal muscle (11, 12). Indeed, it is important in early mouse development, and Dag1-knockout mice undergo premature death early in embryogenesis, before myogenesis has begun, mainly because of their inability to form and develop the Reichert’s basement membranes (48). Moreover, the dystroglycan complex has been shown to be essential for epithelial development in vivo (18) and for epithelial morphogenesis in vitro (13). Dystroglycan has been also implicated in cell adhesion, cell signaling, development and function of the central nervous system, myelination and nodal architecture of peripheral nerves, and synaptogenesis. Finally, dystroglycan is also the cellular receptor for arenaviruses and Mycobacterium leprae (2, 49). Recently, dystroglycan has been implicated in several epithelial cell functions such as growth control, cytoskeletal organization, cell polarization and differentiation, shape change, and movement. The mechanisms by which the dystroglycan complex exerts these effects remain unknown (27, 35, 49). However, it is of interest that these cellular functions are all relevant in the process of tumor development and metastasis, and, indeed, mounting evidence indicates that loss of dystroglycan expression is a frequent event in human malignancies and suggests that this molecule might play an important role in human tumor development, likely by altering the interactions between cells and the surrounding matrix (19, 32, 36).

In conclusion, although no mutations in the Dag1 gene have been identified in any human disorder, the importance of the dystroglycan complex in development and growth as well as in human diseases, such as dystrophies and cancer, is recognized, but little is known about the mechanisms regulating its expression (2). There is increasing evidence that dystroglycan expression is mainly regulated at a posttranscriptional level (2, 16, 32, 37). However, changes in the expression levels of Dag1 mRNA have been observed during cell proliferation and differentiation (26, 34, 35), thus suggesting regulation at a transcriptional level that remains largely unknown.

To further investigate this regulation, in this study we isolated and characterized the 5′-flanking region of the murine Dag1 gene and for the first time provide evidence that it includes several GC-rich sites and is susceptible of regulation.
Experimental Procedures

Bioinformatic analysis. Bioinformatic analysis of a 1,600-bp fragment of murine Dag1 gene comprising the 5′-flanking region and part of the first exon was performed by using Transcription Element Search Software (TESS) (http://www.ebi.ac.uk/Tess) for the presence of putative transcription factor binding sites (TFBS) and promoter modules. The skeletal muscle cell line C2C12 derived from adult mouse leg was purchased from SIGMA Proligo and transfected with the Code by binding to Specificity protein 1 (Sp1) as well as by epigenetic modifications.

Table 1. Oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5′→3′)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>FpromDag1</td>
<td>CTCGAGAACAGGTTATTGAGGTAAGGG</td>
<td>mDag1 promoter cloning</td>
</tr>
<tr>
<td>RpromDag1</td>
<td>AGGTTTACGGTAGAGATGGCCTCAAA</td>
<td>mDag1 promoter cloning</td>
</tr>
<tr>
<td>P1</td>
<td>AGATCTGGCTTGTATGAGGCGAGC</td>
<td>pGL3:peGFP −722/−1</td>
</tr>
<tr>
<td>P2</td>
<td>AGATCTCTAGCTGCGCGGGGCGGTTG</td>
<td>pGL3 −350/−1</td>
</tr>
<tr>
<td>P3</td>
<td>AGATCTCAATGGCGAGAGAAGAGGCGC</td>
<td>pGL3 −308/−1</td>
</tr>
<tr>
<td>R1</td>
<td>AAGCTTTACGGCGCGCGGCGCGCGCGC</td>
<td>All pGL3 constructs</td>
</tr>
<tr>
<td>ForSp1</td>
<td>GGAGCTGCTGTTGGTGTCTGCGG</td>
<td>Chip for Sp1</td>
</tr>
<tr>
<td>RevSp1</td>
<td>GCGGCCAACCAGGCGGCGC</td>
<td>Chip for Sp1</td>
</tr>
<tr>
<td>ForChIPCont</td>
<td>TGCTCTAGAGAGAGACCGG</td>
<td>Control for ChIP</td>
</tr>
<tr>
<td>RevChIPCont</td>
<td>CTAGCTCAAGCAAGCTAGT</td>
<td>Control for ChIP</td>
</tr>
<tr>
<td>ForMDag1</td>
<td>AGATCTACGAGATGGCCTCAAA</td>
<td>RT-qPCR mouse Dag1</td>
</tr>
<tr>
<td>RevMDag1</td>
<td>CTAGCTACGAGATGGCCTCAAA</td>
<td>RT-qPCR mouse Dag1</td>
</tr>
<tr>
<td>ForHDag1</td>
<td>GATATCTACGAGATGGCTAGCG</td>
<td>RT-qPCR human Dag1</td>
</tr>
<tr>
<td>RevHDag1</td>
<td>CTTACTGGTGGCTAGCTAGCCG</td>
<td>RT-qPCR human Dag1</td>
</tr>
<tr>
<td>For18S</td>
<td>CGCTCTAGAGAGCTAGCTAGG</td>
<td>RT-qPCR 18S</td>
</tr>
<tr>
<td>Rev18S</td>
<td>CGCTCTAGAGAGCTAGCTAGG</td>
<td>RT-qPCR 18S</td>
</tr>
<tr>
<td>ForCpG</td>
<td>TTTTTTTTTTTTTTGTTAGGGG</td>
<td>Promoter methylation assay</td>
</tr>
<tr>
<td>RevCpG</td>
<td>AAAAAGACGCTATTCCACCAACTTC</td>
<td>Promoter methylation analysis</td>
</tr>
<tr>
<td>For2CpG</td>
<td>GAAGTTTGGAGAAAGGTTGTTTTT</td>
<td>Promoter methylation analysis</td>
</tr>
<tr>
<td>Rev2CpG</td>
<td>CTTACTAGAGAGCTAGCTAGGCC</td>
<td>Promoter methylation analysis</td>
</tr>
</tbody>
</table>

For luciferase assays, cells were plated in 6-cm dishes the day before transfection. Each plate of cells was cotransfected with 1.5 μg each of pGL3-based constructs and 0.5 μg of pcDNA3.1/D/V5-His/ lacZ with FuGENE6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer’s instructions. For luciferase assay in myotubes, C2C12 cells were transfected at 80% confluence and the medium was replaced with differentiation medium after transfection.

At 48 h after transfection, cells were harvested and lysed with Reporter Lysis Buffer (Promega). Enzyme activities were determined with a luciferase assay kit and a β-galactosidase Enzyme Assay System assay kit (both from Promega). All luciferase activities were normalized to β-galactosidase activity and reported as fold increase over the pGL3 basic activity in C2C12 cells. The data shown represent mean ± SE of four independent experiments.

FuGENE6 transfection reagent was also used to transfect the peGFP-based constructs in C2C12 cells. After transfection, cells were selected by growth in 700 μg/ml of G418 (Boehringer Mannheim, Indianapolis, IN) for 15 days. G418-resistant colonies were then pooled, expanded, and used for further studies. Green fluorescent protein (GFP)-expressing cells were visualized by wide-field epifluorescence microscopy on a Nikon Eclipse TE-2000S inverted microscope (Nikon Instruments, Melville, NY).

Promoter methylation analysis was performed by bisulfite treatment as described above. methylation was followed by incubation with 250 ng trichostatin A (TSA) for 16 h. For TSA treatment, cells were incubated with 0.1 μM or 0.3 μM TSA for 16 or 32 h.

Sp1 small interfering RNA (siRNA) and small interfering RNA transfection.

The Sp1-specific small interfering RNA (siRNA) (target sequence: 5′-CCCTGGATGATGGCTAATA-3′) and scrambled siRNAs were purchased from SIGMA Proligo and transfected with the Code
Breaker siRNA transfection reagent (Promega) according to the manufacturer’s instructions. C2C12 cells were transfected with a 30 μM final concentration of Sp1 siRNA and were harvested after 48 h.

**Total protein extraction and Western blot analysis.** Protein extraction and Western blot analyses were performed as described previously with the monoclonal antibody to β-dystroglycan (clone 43DAG/SD5; Novocastra, Newcastle, UK) (33, 35).

**RNA extraction and real-time RT-PCR.** Total RNA was extracted from the cell lines with TRI Reagent (Sigma) according to the manufacturer’s instructions. First-strand cDNA was synthesized with 1 μg of total RNA, Moloney murine leukemia virus (MMLV) reverse transcriptase (Sigma), and random primers, as recommended by the manufacturer. Real-time quantitative PCR (RT-PCR) analysis was carried out with a Bio-Rad iCycler iQ Real-Time PCR System (Bio-Rad Laboratories, Hercules, CA). Reactions were prepared in triplicate with 2X SYBR Green Supermix (Bio-Rad) according to the manufacturer’s instructions to a final volume of 25 μl. The following conditions were used: 95°C for 3 min, followed by 40 cycles at 95°C for 15 s and 60°C for 30 s. Quality of PCR products was evaluated by generating a melting curve, which was also used to verify the absence of PCR artifacts (primer dimers) or nonspecific PCR products. Results were analyzed with Bio-Rad iCQ-5 software (Bio-Rad) and are expressed as mean Dag1 expression relative to mean 18S expression. The primers are listed in Table 1.

In vitro DNA methylation. SssI methylase (New England BioLabs, Beverly, MA) was used to methylate Dag1 promoter luciferase reporter constructs. Briefly, plasmid DNA was incubated without (mock methylated) or with (methylated) 1 unit of methylase per 1 μg of DNA in 20 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂, and 1 mM DTT, pH 7.9, supplemented with 160 μM S-adenosylmethionine every hour. Reactions were carried out at 37°C for 4 h. Complete methylation at CpG sites was confirmed by HpaII digestion of plasmid DNA. Methylated DNA was purified with the Wizard DNA Clean-Up System (Promega). For luciferase assays DNA was transfected into C2C12 cells in parallel with the corresponding unmethylated reporter constructs.

Methylation analysis of CpG islands. Genomic DNA was isolated with the Wizard Genomic DNA Purification Kit (Promega). For bisulfite genomic sequencing, genomic DNA (2 μg) was denatured in 0.3 M NaOH at 37°C for 10 min. After the addition of 3 M sodium bisulfite (Sigma) and 10 mM hydroquinone (Sigma), samples were incubated at 50°C for 16 h. The modified DNA was purified with the Wizard DNA Clean-Up system (Promega) and denatured by addition of 0.3 M NaOH at 37°C for 15 min. The bisulfite-reacted DNA was precipitated and resuspended in 1 mM Tris-HCl, pH 8, and used immediately or stored at −20°C. Bisulfite-modified DNA was amplified by PCR with two primer sets designed to amplify a 120-bp fragment. PCR products were cloned into the pGEM-Teasy vector (Promega) and sequenced by the manufacturer’s standard protocol. Ten clones were sequenced from each sample.

Chromatin immunoprecipitation assay. For chromatin immunoprecipitation (ChIP) assays, cells were fixed in a final concentration of 1% formaldehyde added directly to the culture dishes for 10 min at 37°C to cross-link protein complexes to the DNA. The reaction was stopped by the addition of glycine to a final concentration of 0.125 M for 5 min at room temperature. Cells were washed with cold PBS, scraped and lysed in SDS lysis buffer, and settled on ice for 10 min. Chromatin was sheared by sonication four times for 10 s, yielding DNA fragments of 200–1,000 bp. Samples were then preclared with salmon sperm DNA protein A-Sepharose beads and washed once with 1 ml of low-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris·HCl, pH 8.1), once with 1 ml of high-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris·HCl, pH 8.1), once with 1 ml of LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris·HCl, pH 8.1), and twice with 1 ml of Tris-EDTA (TE) buffer (10 mM Tris·HCl and 1 mM EDTA, pH 8.0). All washes were carried out with rotation for 5 min at 4°C. Bead precipitates were eluted with 1% SDS and 0.1 M NaHCO₃. Formaldehyde cross-linking was reversed by heating for 6 h at 65°C and incubating for 1 h at 37°C with RNase A. DNA was isolated by phenol-chloroform extraction and ethanol precipitation after proteinase K treatment for 1 h at 45°C. PCR amplifications were performed with specifically designed primers able to amplify a 120-bp promoter region, extending from −318 to −207 from TSS, including the Sp1 sites. The reaction was performed with an initial denaturation of 5 min at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C with a final extension at 72°C for 7 min. The exponential increase of PCR products at this number of cycles was confirmed in several preliminary experiments (data not shown). As an internal negative cis-control, a primer pair was chosen amplifying a 114-bp long fragment in the 5′-untranslated region (−111/+3) of the Dag1 gene. PCR products were separated on 2% agarose gels. Samples from at least three independent immunoprecipitations were analyzed and gave similar results. Primer sequences are listed in Table 1.

**RESULTS**

Dystroglycan expression is regulated at a transcriptional level during myoblast differentiation. To identify a useful in vitro model to study the transcriptional regulation of Dag1 gene, the expression level of Dag1 mRNA was analyzed during differentiation of C2C12 cells. These cells are murine myoblasts that can be induced to differentiate in myotubes and in which expression of Dag1 gene was previously shown to be modulated during differentiation (26). Analysis of mRNA expression levels by RT-PCR demonstrated an increase of Dag1 mRNA during muscle differentiation in vitro, which was already evident after 2 days (data not shown and Ref. 26), reached its peak after 4 days, and persisted after 7 days, although at a lower level (Fig. 1A). As shown in Fig. 1B, expression of the corresponding protein was also increased in the same cells.

Analysis and cloning of proximal 5′-flanking region of murine Dag1 gene. The mouse Dag1 gene comprises four exons, the first two of which are noncoding (Fig. 2A). The putative TSS was designated by the mRNA sequence available in GenBank (accession no. NM_010017). Sequence analysis of the 5′-flanking region of exon 1 showed a high GC content and lack of -acting regulatory elements found in the mouse promoter are well conserved between species. In fact, sequence alignment of the human, rhesus, mouse, and dog Dag1 gene promoters confirmed that not only the E-box and the Sp1 binding sites but also...
generated (pGL3 evaluated in C2C12 cells (Fig. 4). Results of the transactivation and pGL3/H11002 of the putative cloned in pGEM-Teasy vector. To investigate the functionality of genomic DNA fragment of the expected content (50%), and CpG dinucleotide frequency (observed/expected ≥0.6) (42) (Fig. 3A). Functional analysis of mouse Dag1 gene promoter region. A genomic DNA fragment of the Dag1 gene spanning positions −1187 to +404 relative to the TSS was amplified by PCR and cloned in pGEM-Teasy vector. To investigate the functionality of the putative Dag1 promoter region, four constructs of progressive deletion spanning positions −722 to −1 were generated (pGL3 −722/−1, pGL3 −368/−1, pGL3 −350/−1, and pGL3 −308/−1) and cloned into the promoterless pGL3-basic vector and their ability to drive luciferase expression was evaluated in C2C12 cells (Fig. 4). Results of the transactivation assays in C2C12 cells are shown in Fig. 4 as relative luciferase activity.

All constructs were able to drive luciferase transcription in C2C12 myoblast cells. The pGL3 −722/−1 construct, containing the three Sp1 sites and the E-box site, displayed the strongest activity. Luciferase activity decreased progressively by serial deletions eliminating the E-box (44%) and destroying the Sp1 clusters, removing one (12%) or two (6%) Sp1 sites (Fig. 4). These results suggest that sequences contained in the 368 bp proximal to the initiation of transcription are sufficient for promoter activity, and that a region up to 722 bp bears the cis-functional elements required for complete promoter function and for maximal Dag1 transcriptional activation.

The activity of the pGL3 −722/−1 construct, containing the three Sp1 and the E-box sites, and of the pGL3 −368/−1 construct, which contains the three Sp1 sites but lacks the E-box, was also evaluated in differentiated myotubes. As shown in Fig. 5A, both constructs displayed an increased (~4-fold) ability to drive luciferase expression in differentiated compared with undifferentiated cells. This increase in myotubes was higher for the E-box-containing construct, suggesting an involvement of this motif in the regulation of the Dag1 gene promoter during differentiation.

To further evaluate Dag1 promoter activity during myoblast differentiation, the −722/−1 and −368/−1 fragments were subcloned in the promoterless vector pEGFP and stably transfected in C2C12 cells that were then induced to differentiate, and transcriptional activity was monitored by analyzing GFP expression by fluorescence microscopy. This method enabled us to estimate the activity of each construct distinctively in myoblasts and myotubes. As shown in Fig. 5B, transcriptional activity of the pEGFP −722/−1 construct was more evident in differentiated cells (elongated and multinucleated thin myotubes), whereas the pEGFP −368/−1 construct showed the same level of activity in both undifferentiated myoblasts (small, round cells) and myotubes (elongated cells).

Sp1 transcription factor binds and activates mouse Dag1 gene promoter in vivo. To determine whether the Sp1 transcription factor is associated with the Dag1 promoter in vivo, ChIP analysis was performed on C2C12 myoblasts (Fig. 6A). A 120-bp DNA fragment covering all three Sp1 sites was amplified by chromatin immunoprecipitated with an anti-Sp1 specific antibody (Fig. 6A, Sp1). The same band was obtained with the input DNA as well as with mouse genomic DNA that served as positive controls (Fig. 6A, Input and Genomic, respectively), whereas the normal IgG control did not result in immunoprecipitation of DNA fragments detectable by PCR amplification (Fig. 6A, IgG). No bands were amplified by chromatin immunoprecipitated with the anti-Sp1 specific antibody using a primer encompassing a fragment in the 5′-untranslated region of Dag1 gene (Fig. 6A, cis-control), thus confirming the validity of the result obtained.

After having determined that Sp1 can bind to Dag1 gene promoter, we aimed to examine how and whether Sp1 affects Dag1 gene expression in the same cells. Since Sp1 was expressed at a high level in C2C12 myoblasts (data not shown), an anti-Sp1 siRNA was used to downregulate the expression of endogenous Sp1 protein. The Sp1 siRNA and a scrambled siRNA were transfected into C2C12 myoblasts (Fig. 6C), the Sp1-specific siRNA induced a significant reduction (~6-fold) in the expression of endogenous Dag1 mRNA compared with scrambled siRNA, thus suggesting that Sp1 plays a key role in the activation of Dag1 promoter.
Effect of trichostatin A and 5-aza-2'-deoxycytidine on activity of Dag1 promoter. Since changes in methylation at target promoter genes as well as chromatin remodeling due to changes in histone acetylation have been reported to play an important role in muscle differentiation (29, 44), it was of interest to investigate whether the activity of Dag1 gene promoter and its expression could be affected by changes in histone acetylation and/or DNA methylation.

To test the potential role of histone acetylation in the regulation of Dag1 expression, undifferentiated C2C12 cells were treated with the histone deacetylase (HDAC) inhibitor TSA, which affects chromatin condensation and can act as a transcriptional activator (39). C2C12 cells were incubated for 16 and 32 h with 0.1 or 0.3 μM TSA, and Dag1 mRNA levels were evaluated by RT-PCR. As shown in Fig. 7A, the treatment increased the transcription of Dag1 mRNA in a dose- and time-dependent fashion.

DNA methylation of 5'-regulatory regions harboring a higher than expected number of CpG dinucleotides is a key mechanism by which genes are silenced. As mentioned above, bioinformatic analysis of the 5'-flanking region of Dag1 gene confirmed the presence of a CpG island (Fig. 2B). To test the
potential role of DNA methylation in the regulation of Dag1 gene expression, demethylation was induced by treating undifferentiated C2C12 cells with the DNA methyltransferase inhibitor AZA. Cells were incubated for 96 h in the presence of 2.5 μM AZA, and Dag1 mRNA levels were evaluated by RT-PCR. As shown in Fig. 7B, the treatment increased the expression of Dag1 mRNA, and this effect was enhanced by subsequent treatment with TSA (0.1 μM for 16 h).

Dag1 promoter activity is repressed by in vitro and in vivo methylation. To further examine the effect of methylation on Dag1 promoter activity, pGL3−722/−1 and pGL3−368/−1 constructs were methylated in vitro with SssI methylase and assayed for their ability to drive luciferase expression in C2C12 cells compared with mock-methylated constructs. As shown in Fig. 8, methylation induced a significant reduction (>60%) of luciferase activity with both constructs.
Finally, to verify whether changes in the methylation pattern of Dag1 promoter are associated with C2C12 cell differentiation, genomic DNA from both undifferentiated and differentiated cells was subjected to bisulfite sequence analysis. Briefly, DNA was modified by bisulfite treatment, which converts unmethylated cytosine residues to uracil while leaving methylated cytosine residues unaffected, and was used as template to amplify a region of Dag1 promoter encompassing the E-box and the Sp1 consensus sequences. The PCR products were then cloned and sequenced to analyze the methylation state at several CpG dinucleotides on a single allele. Two sets of primers were designed in order to amplify a 369-bp-long region spanning from position 605 to 236 and covering 33 CpG dinucleotides within the E-box and the Sp1 binding sites (Fig. 3B). As shown in Fig. 3C, Dag1 promoter underwent a substantial change in methylation status during differentiation. While nearly all the clones sequenced were almost completely methylated in undifferentiated cells, which display a low level of Dag1 mRNA, 6 of 10 clones were not methylated 96 h after the shift to differentiation medium, when the Dag1 mRNA reaches its peak (Fig. 1A).

**DISCUSSION**

The dystroglycan complex has been involved in several human diseases, including muscle dystrophies and cancer (2, 32, 49). It is encoded by the Dag1 gene, which has been mapped to human chromosome 3p21 and mouse chromosome 9 (32). Mouse Dag1 gene comprises four exons (Fig. 2A), with only the last two coding and giving rise to a precursor propeptide that is proteolytically cleaved into two noncovalently associated proteins (2, 32, 49).

The present study was designed to investigate the structure, function, and regulation of the 5'-flanking region of the Dag1 gene. Since the Dag1 gene was initially cloned in muscle and is expressed at high levels in muscle cells, C2C12 murine myoblasts, which can be induced to differentiate in myotubes and in which expression of Dag1 gene is modulated during differentiation (Fig. 1 and Ref. 26), were chosen as an in vitro model for these studies. Sequence analysis of the region revealed that the putative promoter of the Dag1 gene lacks the TATA and CCAAT boxes, has a high GC content, and contains putative TFBS (Figs. 2 and 3).
Bioinformatic analysis revealed the presence of three close Sp1 sites and, more distally, an E-box element (Fig. 2B). It is noteworthy that the absence of a TATA box and a high content of GC-rich sequences with multiple Sp1 sites are typical properties of housekeeping genes, which is consistent with the ubiquitous expression of the dystroglycan complex and has been reported in other muscle genes such as the Duchenne muscular dystrophy gene (23) and mouse α-1- and β-syntrophin genes (1). The functionality of the putative Dag1 promoter region was tested with a luciferase-based assay in which all of the promoter constructs induced higher luciferase activity than the promoterless pGL3-Basic in C2C12 cells. The pH11002s construct encompassing the E-box and the three Sp1 binding sites displayed the highest activity (Fig. 4), and sequential 5′ deletion caused a progressive decrease of promoter activity, confirming the importance of the E-box site and the integrity of the cluster of the three Sp1 sites for maximum Dag1 gene expression (Fig. 4).

The Sp transcription factors are a family of zinc finger DNA-binding domain proteins that recognize the DNA-binding motifs GC-box (GGGCGGG) and GT-box (GGTGTGGGG) (40, 41). They are important for the expression of many different housekeeping genes as well as tissue-specific genes that generally do not contain TATA- or CAAT-boxes in their promoters (8). Ubiquitously expressed Sp1 was the first family member identified, followed by several others (Sp2–Sp8) (8, 41). Although the activity of Sp1 is believed to be constitutive, it has been shown to play a role in different cellular activities, including cell differentiation, cell cycle progression, apoptosis, chromatin remodeling, and development, and its function can be regulated through several different
mechanisms (8, 41). We showed that Sp1 directly binds to Sp1 sites in the Dag1 promoter and positively regulates the expression of the endogenous Dag1 mRNA (Fig. 6). These data are in agreement with previous evidence showing that Sp1 is a positive regulator of transcription in different cell types and that the presence of multiple Sp1 binding sites is a common feature and is important for the transcription of TATA-less promoters (7, 25, 38).

The E-box was essential for maximal transcriptional activity and exerted a profound effect on the activity of Dag1 promoter since its deletion by itself caused a 50% decrease of the activity (Figs. 4 and 5). The E-box is a specific DNA motif with a consensus sequence, CANNTG, that is recognized by the basic helix-loop-helix (bHLH) transcription factors involved in the development of various tissues including muscle (28, 44). The E-box is able to bind MyoD and other muscle-specific transcription factors and is found in the promoter of several muscle-specific genes such as troponin I (4), myosin heavy chain (MHC IIB) (47), MUSK (a muscle-specific tyrosine kinase receptor) (43), and the nicotinic acetylcholine receptor (nAChR) (17). MyoD, a member of the bHLH family of transcription factors, is the major regulator of muscle differentiation and promotes the transcription of several E-box-regulated genes (44). We did not directly analyze the interaction between MyoD and Dag1 gene promoter, but their interaction was previously demonstrated with a genomewide approach (50). This interaction could also explain the observed increased activity of the Dag1 promoter during muscle differentiation (Figs. 1 and 5), when, as mentioned, several E-box-regulated genes are activated, and would be in agreement with the involvement of dystroglycan in muscle regeneration (10).

Overall, the available data suggest a potential important role of the E-box element in the regulation of Dag1 promoter activity, which likely contributes to the increased expression of the dystroglycan complex during muscle differentiation by recruiting trans-acting elements activated at this stage in a specific fashion. MyoD is likely a binding partner of this element, but the involvement of other bHLH transcription factors regulating transcriptional activity during skeletal myogenesis (29) cannot be excluded at the moment.

We demonstrated that Dag1 gene promoter is functionally activated by binding to Sp1, and this interaction is relevant in dystroglycan physiology since it might affect other transcription factors involved in the regulation of basal and/or induced Dag1 promoter activity. Indeed, the interaction between Sp1 and other transcription factors, including MyoD, has been shown to be important for the regulation of various muscle-specific genes (3, 5, 6, 15, 22, 29, 44) and might well play a role also in the regulation of Dag1 gene expression (3, 22, 24, 30, 31, 46). Conflicting data have been reported on the expression and activity of Sp1 during muscle differentiation. Indeed, myogenesis has been reported to cause a downregulation of Sp1 expression and has been postulated also to cause alterations in the biological potency of Sp1 due to the presence of Sp1-binding inhibitory proteins (45). However, Sp1 downregulation is a variable entity in different cell types, is detectable later after the shift to differentiation medium, and has been related to the low-serum culture conditions to which cells are exposed to induce differentiation (14). Moreover, this downregulation has been shown not to correlate with Sp1 activity as a transcription factor during muscle differentiation due to a modulation of its activity by posttranslational modifications, interaction with other proteins, and/or higher affinity to the acetylated/demethylated promoters (5, 6, 14, 15, 30). For all these reasons we preferred to perform silencing of Sp1 in myoblasts, and the results obtained clearly demonstrate a role of Sp1 protein in regulating the transcriptional activity of Dag1 gene promoter (Fig. 6).

Another important finding of this study was the observation that DNA methylation can contribute to regulation of Dag1 gene expression. Indeed, the presence of a CpG island suggested the possibility that the Dag1 gene promoter might be regulated through changes in methylation status, and this hypothesis was confirmed by demonstrating that treatment with a demethylating agent, such as AZA, stimulates Dag1 expression (Fig. 7B). The reduction of luciferase reporter construct activity following SsII methylase treatment further supported a direct involvement of methylation in the regulation of Dag1 promoter activity (Fig. 8).

Finally, an inverse correlation between Dag1 gene promoter methylation and expression levels was demonstrated in muscle cells by bisulfite sequencing (Figs. 1A and 3C).

Overall, these results support the hypothesis that demethylation might be responsible for the observed increased expression of Dag1 gene during muscle differentiation. Indeed, differentiating muscle cells undergo a genomewide loss of DNA methylation followed by a gradual remethylation (20, 21), and several muscle-specific genes, including MyoD, undergo a general demethylation during development and differentiation (9). Muscle differentiation is also driven by chromatin remodeling, and methylated DNA binds methyl binding proteins that recruit HDACs (44). Thus we also investigated the effect of HDAC inhibitors on Dag1 expression and found that the HDAC inhibitor TSA synergistically enhanced the stimulatory effect of AZA (Fig. 7A) and was by itself able to promote Dag1 expression in undifferentiated C2C12 cells (Fig. 7B). These findings suggest a complex regulation of Dag1 promoter activity likely due to the same molecular events that play a pivotal role in skeletal myogenesis.

We do not have direct evidence of Dag1 promoter chromatin remodeling in response to TSA treatment, and it cannot be excluded that TSA’s effect might be indirect, for example, acting in trans on some general or specific transcription factors. If the effect of TSA is direct on Dag1 promoter, the consequence of chromatin opening could also be recruitment of Sp1. Regardless of the underlying molecular mechanisms, our results suggest that chromatin remodeling is sufficient to reactivate Dag1 expression and that histone acetylation might play an important role in the reactivation of Dag1 transcription. This finding might be important and could be therapeutically exploited to reactivate expression of dystroglycan in diseases, such as muscle dystrophies and malignancies in which expression of this protein is reduced or lost, if the involvement of a transcriptional mechanism as responsible of the lack of protein expression is demonstrated.

In conclusion, this is the first report characterizing the Dag1 gene promoter and demonstrating the role of Sp1 transcription factors and chromatin methylation, combined with histone acetylation state, in its transcriptional regulation. A regulated expression of Dag1 gene appears to be essential during embryonic development (48), and the high degree of conservation observed in its 5’-flanking region (Fig. 3) confirms the importance of this region and suggests that similar mechanisms...
might act in different species. The results of the present study support the hypothesis that the activation of Dag1 gene promoter during muscle differentiation is due to both trans-acting and cis-acting factors. Indeed, the higher luciferase activity shown by the Dag1 promoter in differentiated C2C12 cells (Fig. 5A) suggests the presence in these cells of transcription factors not present in myoblasts. On the other hand, epigenetic modifications of Dag1 gene promoter could modulate the binding of these factors, making its regulation even more flexible. To further screen the Dag1 gene promoter for evidence of endogenous methylation, we analyzed its methylation status by bisulfite sequencing in several human cell lines, and it is noteworthy that we found an inverse relationship between promoter methylation and expression of the corresponding mRNA, thus further confirming the role of epigenetic modifications in the regulation of Dag1 gene promoter activity (A. Rettino and A. Sgambato, unpublished data).

We believe these findings are relevant for a better understanding of the mechanisms regulating Dag1 gene expression, although further studies are warranted to identify other potential interacting trans-acting factor(s) and clarify the mechanisms underlying the regulation of Dag1 gene in a tissue-specific manner and during development as well as its alteration in human disease.

ACKNOWLEDGMENTS

We thank Giuseppe Lauria for excellent technical assistance in sequencing analyses.

GRANTS

This study was supported in part by a grant from the Università Cattolica del Sacro Cuore, Roma (Linea D1-2007).

REFERENCES


