Dystrophin muscle enhancer 1 is implicated in the activation of non-muscle isoforms in the skeletal muscle of patients with X-linked dilated cardiomyopathy

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X-linked dilated cardiomyopathy (XLDC) is a dystrophinopathy characterized by severe cardiomyopathy with no skeletal muscle involvement. Several XLDC patients have been described with mutations that abolish dystrophin muscle (M) isoform expression. The absence of skeletal muscle degeneration normally associated with loss of dystrophin function was shown to be due to increased expression of brain (B) and cerebellar Purkinje (CP) isoforms of the gene exclusively in the skeletal muscle of these patients. This suggested that the B and CP promoters have an inherent capacity to function in skeletal muscle or that they are up-regulated by a skeletal muscle-specific enhancer unaffected by the mutations in these patients. In this work we have analyzed the deletion breakpoints of two XLDC patients with deletions removing the M promoter and exon 1, but not affecting the B and CP promoters. Despite the presence of several muscle-specific regulatory motifs, the B and CP promoters were found to be essentially inactive in muscle cell lines and primary cultures. As dystrophin muscle enhancer 1 (DME1), the only known muscle-specific enhancer within the dystrophin gene, is preserved in these patients, we tested its ability to up-regulate the B and CP promoters in muscle cells. B and CP promoter activity was significantly increased in the presence of DME1, and more importantly, activation was observed exclusively in cells presenting a skeletal muscle phenotype. These results point to a role for DME1 in the induction of B and CP isoform expression in the skeletal muscle of XLDC patients defective for M isoform expression.

INTRODUCTION

The Duchenne muscular dystrophy (DMD; dystrophin) gene is located on the X chromosome and contains 79 exons that are spliced into a 14 kb transcript in muscle tissues (1–3). Through alternative promoter usage, at least seven additional tissue-specific isoforms arise from this gene. However, only the muscle (M), brain (B) and cerebellar Purkinje (CP) cell isoforms encode the full-length 427 kDa dystrophin protein (3–11). Although they are generally considered to be functionally homologous, each of these transcripts contain a unique first exon and are expressed from different, tissue-specific promoters.

Mutations in the DMD gene that affect dystrophin function generally result in progressive skeletal muscle degeneration and cardiac insufficiency. Depending on the severity of the clinical picture, the disease is classified as either Duchenne (DMD) or Becker (BMD) muscular dystrophy (12). However, in recent years an increasing number of mutations in the DMD gene have been shown to result in severe dilated cardiomyopathy with no apparent skeletal muscle pathology. The term X-linked dilated cardiomyopathy (XLDC) has been assigned to this clinical phenotype (13,14).

The mechanism(s) through which specific mutations in the DMD gene selectively affect dystrophin function in cardiac muscle are not clearly understood. This is in part due to the extraordinary size and complexity of the gene and our relatively poor understanding of dystrophin gene regulation in cardiac...
and skeletal muscle. Several mutations resulting in XLDC have been described in different regions of the DMD gene, including a duplication of the region from exon 2 to 7, an insertion in intron 11, point mutations of exon 9 or 29, or deletions in the region from exon 48 to 51 (15). Interestingly, up to 25% of dystrophin gene mutations that cause XLDC specifically affect expression of the M isoform. These include an L1 insertion in M exon 1 (16,17), a point mutation in the 3′ splice site of M exon 1 (18) and a deletion that removes the M promoter, M exon 1 and part of M intron 1 (19). Recently, a second XLDC patient with a deletion removing the M promoter and exon 1 has been described (Broere et al., manuscript in preparation).

In each of these patients, the mutation results in the complete loss of expression of the M isoform and would be expected to produce a severe DMD phenotype. However, the loss of M isoform expression is thought to be offset by increased expression of the non-muscle B (7,8) and CP (9–11) isoforms of the dystrophin gene in the skeletal muscle of these patients (18,20,21). This phenomenon is not observed in cardiac muscle (18,22).

Regulation of DMD gene expression in muscle is complex and appears to involve several regulatory elements that are specific to skeletal and cardiac muscle. For example, the M promoter was shown to function in a muscle-specific manner in cell culture models (23,24), but transgenic mouse studies demonstrated that the M promoter alone is not active in mature skeletal muscle and directs reporter gene expression only to the right ventricle of the heart (25). A muscle-specific enhancer (dystrophin muscle enhancer 1; DME1), located 6.5 kb downstream of M exon 1 in the human dystrophin gene, was shown to increase M promoter activity in immature and mature skeletal muscle (26). Higher activity in cardiac muscle-derived H9C2(2-1) cells (27) as compared to skeletal muscle-derived C2C12 cells (28) pointed to a role for this enhancer in cardiac gene regulation (29). In contrast, recent studies have shown that homologous sequences in the mouse dystrophin gene are active in both skeletal and cardiac muscle-derived cell lines (30). The B isoform is expressed primarily in cortical and cerebellar tissues of the brain and low levels of this isoform have also been detected in the heart (8,10,21,31–33). B isoform expression has also been observed in fetal and adult skeletal muscle by some groups (21,33), but not by others (10,31). Expression of the CP isoform in muscle is somewhat controversial. Muntoni et al. (21) found no expression of the CP isoform in normal heart and skeletal muscle, whereas Abdulrazzak et al. (11) observed CP isoform transcripts in both tissues. Holder et al. (10) reported that up to 20% of mRNA transcripts in skeletal muscle correspond to the CP isoform. Torelli et al. (33) also observed CP expression in adult skeletal muscle, but not in fetal skeletal muscle or in fetal and adult heart.

Evidence for B and CP isoform expression in normal skeletal muscle has raised the possibility that these promoters are active in skeletal muscle and that this activity is sufficient to compensate for the loss of M isoform expression in a subset of XLDC patients. Alternatively, the B and CP promoters may fall under the influence of a muscle-specific enhancer within the dystrophin gene. This enhancer would be predicted to lie outside of the deleted region in the XLDC patients described by Muntoni et al. (19; Broere et al., manuscript in preparation), and in the absence of the M promoter would preferentially activate transcription from the B and CP promoters in skeletal muscle.

To explore these questions, we have determined the precise deletion breakpoints in these two XLDC patients. We have also cloned and analysed the B and CP promoters for inherent skeletal muscle activity, and for their responsiveness to the enhancer (DME1) within muscle intron 1. The results point to a role for DME1 in the induction of B and CP isoform expression in the skeletal muscle of XLDC patients with mutations that specifically abolish M isoform expression.

RESULTS

Localization of deletion breakpoints in XLDC patients lacking muscle exon 1

A PCR-based strategy was used to localize the deletion breakpoints in two patients [patient 1 (19); patient 2 (Broere et al., manuscript in preparation)] diagnosed with XLDC and having deletions that extend through muscle exon 1 of the dystrophin gene. The first exons of the B and CP isoforms flanking muscle exon 1 are preserved in both of these patients (Fig. 1A). To precisely define the deletion breakpoints, PCR primer sets were designed at regular intervals along the length of a 134 kb region (GenBank accession no. AL031643) from –113 to +21 kb relative to the transcription start site in muscle exon 1. All of the primer sets used in this study (Table 1) produced fragments of predicted size in PCR reactions containing DNA from a normal individual (Fig. 1B). Primer sets located at –20.4, –16.6, –14.2, +2.6 and +3.1 kb (as well as at –100 and –59 kb; data not shown) also generated PCR products of the predicted size using XLDC patient 1 DNA as template. However, no PCR products were generated by primer sets located at –13.0 and +1.8 kb (Fig. 1B). Predicted PCR fragments were also produced from XLDC patient 2 DNA using primers located at –20.4, –16.6, –14.2, –13.0, –1.4 and +6.4 kb (DME1). In this patient, primer sets located at +1.8, +2.6, +3.1 and +4.9 kb failed to produce PCR products (Fig. 1B). On the basis of these results, the 5′ and 3′ deletion breakpoints in patient 1 were predicted to lie between –14.2 and –13.0 kb, and between +1.8 and +2.6 kb, respectively. In patient 2, the deletion breakpoints were localized to between –1.4 and +0.3 kb, and between +4.9 and +6.4 kb, respectively (Fig. 1B). PCR primers flanking the predicted deletion breakpoints were subsequently used to amplify genomic fragments containing the deletion junctions in these two patients. As shown in Fig. 1C, PCR amplification of patient 1 DNA using primers positioned at –14 441 and +2829 bp generated a 2 kb fragment that was not observed in control reactions containing DNA from a normal individual where this primer pair is separated by 17 270 bp. Similarly, primers at –1505 and +6769 bp generated a 2.1 kb fragment using XLDC patient 2 DNA as template. No PCR product was obtained using DNA from a normal individual where these primers are separated by 8274 bp (Fig. 1C). Deletion junction fragments amplified from these two XLDC patients were cloned and sequenced (GenBank accession no. AF324932 and AF373844, respectively). Alignment with normal genomic DNA (GenBank accession no. AL031643) indicated that the deletion in XLDC patient 1 is 15 310 bp in length, spanning the region from –13 253 to +2057 bp relative to the transcription start site in muscle exon 1 (Fig. 1D). The deletion in XLDC patient 2 was found to be 6199 bp in length,
extending from –1199 to +5000 bp relative to muscle exon 1 (Fig. 1D).

Analysis of sequences on either side of these deletion breakpoints suggested that both mutations occurred through non-homologous recombination events. However, a 12 bp fragment with 100% homology to Alu sequences was found at the junction in patient 2 (GenBank accession no. AF373844), pointing to the possible involvement of Alu repeats in this recombination event. As shown schematically in Figure 1D, a comparison of the deletion breakpoints in these two XLDC patients identified a 3256 bp overlap between –1199 and +2057 bp which includes the promoter and first exon of the muscle isoform of the gene. This 3.3 kb region would be predicted to contain sequence elements necessary for dystrophin gene expression in cardiac muscle. Enhancer elements that might be responsible for regulating the B and CP isoforms of the gene in skeletal muscle would be predicted to lie outside of an 18 253 bp region defined by the 5′ deletion junction in patient 1 (–13 253 bp) and the 3′ deletion breakpoint in patient 2 (+5000 bp). The enhancer in intron 1 (DME1) is not directly affected by the deletion in either of these XLDC patients (Fig. 1D).

### Cloning and sequence analysis of the CP promoter

Evidence that B and CP isoform expression is activated in the skeletal muscle (but not the heart) of XLDC patient 1 (21,22) suggests that these promoters have intrinsic activity in skeletal muscle cells or that they are induced by a skeletal muscle-specific enhancer within the dystrophin gene. Functional analysis of the B and CP promoters in muscle cells could discriminate between these two possibilities. To address this question, a 1.8 kb fragment containing the promoter region of the B isoform was PCR-amplified, cloned and sequence-verified. As sequence information for the human CP promoter was not yet available, a 9.4 kb fragment (CP9.4) containing CP exon 1 was isolated by screening a genomic library with a CP exon 1-specific probe (Fig. 2A). A 1.8 kb HindIII fragment containing sequences upstream of the 5′ end of CP exon 1 was sub-cloned from CP9.4 and sequenced (GenBank accession no. AL049643). Sequence analysis indicated that this fragment contains 1568 bp upstream of CP exon 1 (Fig. 1D). Subsequent comparisons with the sequence of a PAC clone spanning this region (GenBank accession no. AL049643) revealed two differences: a T→A substitution at –1468 bp and the absence of a single T residue between –845 and –846 bp.

MatInspector Professional software (http://genomatix.gsf.de) was used to analyze the CP and B promoter regions for the presence of muscle-specific transcription factor-binding sites listed in the TRANSFAC database (34). As shown in Figure 2B, muscle-specific regulatory motifs identified within the CP promoter included nine E-box (35,36), five MEF2

### Table 1. Primers used to amplify regions at the 5′ end of the human dystrophin gene

<table>
<thead>
<tr>
<th>Region amplified</th>
<th>Forward primer sequence (5′–3′)</th>
<th>Reverse primer sequence (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP exon 1</td>
<td>TCAGGTAGATTGAGCCGGTGAGGGA</td>
<td>CTCGAGATTTTTCGAATTCGGGGA</td>
</tr>
<tr>
<td>DME1 (+6407 to +6769)*</td>
<td>GAGCGTATTCAAGGCTGGGAAGA</td>
<td>CTAGTGCTCCCAACACCAACAGA</td>
</tr>
<tr>
<td>+8476 to +5122</td>
<td>GGGATTCTTGCAGGCGGGGAA</td>
<td>ACTAAATGGCACTGGGAGGA</td>
</tr>
<tr>
<td>+3141 to +3433</td>
<td>GGGCCCTCCTGATGAAATGGCC</td>
<td>TTCGAAATTGGCCAGCCGCCC</td>
</tr>
<tr>
<td>+2626 to +2829</td>
<td>TACATTCTTTGAAACTGACGG</td>
<td>TGTTGTTTCTTUTGATGAAA</td>
</tr>
<tr>
<td>+1787 to +1967†</td>
<td>GTAAATCTTTTTTGGACATGCC</td>
<td>AAATGTTCAACATCAGAAGTACR</td>
</tr>
<tr>
<td>M exon 1 (–14 to +273)</td>
<td>GGGCCCTCTACAGGAATCCGGCC</td>
<td>CAGGTTCTACTCTCTCCACCC</td>
</tr>
<tr>
<td>–1665 to –1393</td>
<td>CATTGAGAACACCGGTGG</td>
<td>CAGATGTAAGTGCCACCTTG</td>
</tr>
<tr>
<td>–1505*</td>
<td>GTGATATTGATGGTTGTGGG</td>
<td>GCAGGCGTATTGAGCGGGGA</td>
</tr>
<tr>
<td>–13 280 to –13 016</td>
<td>TCCGCACTTACCCAGTTGCG</td>
<td>GGTGGGTCTACAGGAGGTGAAGA</td>
</tr>
<tr>
<td>–14 441 to –14 199†</td>
<td>TCCCTAAAGGCCAATGGCAC</td>
<td>GCCGTTCAAGGCTGGGAAGGA</td>
</tr>
<tr>
<td>–16 827 to –16 568</td>
<td>CCTATTTTGGCTACAGGGAGA</td>
<td>ACAACATTTAAACCTCAAAGCA</td>
</tr>
<tr>
<td>–20 604 to –20 357</td>
<td>AAATTCCCTCCACCTGTTTGG</td>
<td>CAGGAGACTCTGCGAGGA</td>
</tr>
<tr>
<td>–59 045 to –58 830</td>
<td>CTAGCTAGGGGCCTCCCCTGTA</td>
<td>GCCACACCTTGGACACCCCT</td>
</tr>
<tr>
<td>–100 844 to –100 630</td>
<td>CTAGCACTGACACGTGGATT</td>
<td>AAAACCCGGTTGGTGAATGC</td>
</tr>
<tr>
<td>B exon 1</td>
<td>CTGTTCTTCCAGCCAGACCGGGCAATGACATGCA</td>
<td>TCCATGGCCAGCTTCCTTCTTCTCCTCTGACTC</td>
</tr>
<tr>
<td>CP promoter (–1560 to +145)</td>
<td>TATTGAGAACGCAAGGCGGGACATCTAGTA</td>
<td>CAGCAGAAGCCACAGGAGA</td>
</tr>
<tr>
<td>B promoter (–1477 to +329)</td>
<td>TTTTGCCAGCGCTTCCAGACAGA</td>
<td>TGGTACCTCATCATCGCAAAT</td>
</tr>
</tbody>
</table>

Numbers in the upper 16 rows refer to the M exon 1 transcriptional start site, whereas numbers in the last two rows refer to the CP and B exon 1 transcriptional start sites, respectively. Primers in the last two rows were used to amplify the CP and B promoters. CP, cerebellar Purkinje; M, muscle; B, brain; DME1, dystrophin muscle enhancer 1.

*Primer used to amplify the 2.1 kb fragment containing the deletion breakpoints from genomic DNA of XLDC patient 2.
†Primer used to amplify the 2.1 kb fragment containing the deletion breakpoints from genomic DNA of XLDC patient 1.
motifs, were also identified within the B isoform promoter (45) (data not shown). The presence of multiple muscle-specific regulatory motifs within these promoters is consistent with the notion that these promoters are sufficiently active in skeletal muscle to compensate for the loss of muscle isoform expression in these XLDC patients.

**Functional analysis of CP and B promoter activity in muscle cells**

To determine whether the B and CP isoform promoters are transcriptionally active in skeletal (but not cardiac) muscle cells, each promoter was cloned into the enhancerless and promoterless pGL3basic luciferase expression plasmid and transfected into the C2 skeletal myoblast, H9C2(2-1) cardiac myoblast and 3T3 fibroblast cell lines, as well as primary human skeletal myoblasts (HSM) and primary neonatal rat cardiomyocytes (RMC). The pGL3-CP construct contains a 1.7 kb fragment corresponding to sequences from –1560 to +145 bp relative to the transcriptional start site in muscle exon 1. The pGL3-B contains a 1.8 kb fragment corresponding to sequences from –1447 to +329 bp relative to the transcriptional start site in B exon 1. Luciferase expression constructs containing no promoter (pGL3basic vector alone) or the SV40 promoter (pGL3promoter) were included in all experiments as negative and positive controls. Co-transfection with a plasmid (pCMVβgal) containing the *Escherichia coli* lacZ (β-galactosidase) gene under the control of the CMV promoter allowed for transfection efficiencies to be determined by measuring β-galactosidase activities in the same cell lysates. Luciferase activities were measured in cell lysates prepared after 72 h incubation in fusion medium [C2, H9C2(2-1) and HSM], differentiating medium (RMC) or normal growth medium (3T3). Promoter activities were expressed as relative luciferase activity (RLA) representing the ratio of total luciferase: β-galactosidase activity measured in each cell lysate. The results are shown in Figure 3. Little or no activity was observed from the B promoter following transfection of C2 (0.63 ± 0.07), HSM (4.73 ± 0.44), H9C2(2-1) (7.13 ± 0.53), RCM (0.59 ± 0.07) or 3T3 (0.54 ± 0.10) cultures. Similarly, CP promoter activity was very low or absent in all of the cell lines tested (C2, 0.12 ± 0.03; HSM, 1.13 ± 0.24; H9C2(2-1), 0.91 ± 0.07; RCM, 1.52 ± 0.06; 3T3, 0.14 ± 0.02). As expected, the SV40 promoter exhibited significant levels of activity in all cell lines C2, 27.29 ± 7.60; HSM, 51.07 ± 12.95; H9C2(2-1), 45.02 ± 8.03; RCM, 107.24 ± 27.42; 3T3, 30.11 ± 8.44. These results indicate that, despite the presence of multiple muscle-specific regulatory motifs, the B and the CP promoters display little or no activity in skeletal or cardiac muscle cell cultures. This would suggest that these promoters are not themselves responsible for the increased expression of these isoforms in the skeletal muscle of XLDC patients deleted for muscle exon 1.

**DME1 preferentially activates the B and the CP promoters in skeletal myoblasts**

Evidence that the deletions in XLDC patients 1 and 2 do not extend through DME1 in muscle intron 1, along with the observation that the B and CP promoters are inactive in skeletal muscle cells, raised the possibility that DME1 plays a role in the activation of these promoters in skeletal muscle. To

(37,38) and two CArG box (39–41) consensus elements. A number of regulatory elements associated with cardiac gene expression were also identified, including four S8 (42) and three Nkx2.5 (43,44) motifs. Several muscle-specific regulatory elements, including 10 MyoD, one CArG, 14 S8 and nine Nkx2.5 motifs, were also identified within the B isoform promoter position 1.52 of the human dystrophin gene in normal and XLDC patient DNA. Expected band sizes are 243 (–14.2), 265 (–13.0), 181 (+1.8), 204 (+2.6), 273 (–1.4), 287 (+0.3), 247 (+4.9) and 363 bp (+6.4). The deletions in XLDC patients deleted for muscle exon 1.
To examine this question, a 1.4 kb fragment containing DME1 (29) was cloned downstream of the luciferase gene in pGL3-B and pGL3-CP to generate pGL3-B-DME1 and pGL3-CP-DME1. Each of these constructs, together with the enhancerless pGL3-B and pGL3-CP vectors, were co-transfected with pCMVβgal into C2, HSM, H9C2(2-1), RCM and 3T3 cells. Luciferase and β-galactosidase activities were measured in cell lysates as described previously. The results are shown in Figure 3. In the presence of DME1, high levels of luciferase expression were observed from the B and CP promoters in differentiated C2 myoblasts (RLA fold increase: B, 124.6; CP, 44.3). Significant increases were also observed in HSM (RLA fold increase: B, 3.1; CP, 9.0) and H9C2(2-1) myotubes (RLA fold increase: B, 2.4; CP, 10.2). However, DME1 had no effect on B and CP promoter activities in RCM (RLA fold increase: B, 1.7; CP, 0.9) or 3T3 fibroblasts (RLA fold increase: B, 1.0; CP, 1.5). The absence of DME1 activity in these two cell lines is consistent with the muscle-specific nature of this enhancer (26,29,30) and, more importantly, the notion that DME1 functions to preferentially activate these promoters in skeletal muscle-derived cells. However, although lower than in C2 myoblasts, the presence of DME1 activity in H9C2(2-1) cells suggests that this enhancer does retain some activity in cardiomyocytes. To explore this important question further,
DISCUSSION

Dystrophin gene regulation in skeletal and cardiac muscle is complex, involving several different promoters and, presumably, one or more enhancers. Evidence of the complexity of dystrophin gene regulation in muscle has been provided by transgenic studies of muscle promoter activity (25) as well as by the identification of XLDC patients with mutations that specifically abolish expression of the M isoform of the gene (16-18-20). The absence of muscle weakness in these patients is thought to be due to compensatory increases in the expression of the non-muscle B and CP isoforms of the dystrophin gene in skeletal (but not cardiac) muscle (18,20,21). How these non-muscle promoters are specifically activated in the skeletal muscle of these patients is not understood. Evidence for the presence of B and CP isoform transcripts in the skeletal muscle of normal individuals has raised the possibility that these promoters contain skeletal muscle-specific regulatory elements and normally contribute to dystrophin gene transcription in skeletal muscle (10,11,21,33). To investigate this possibility, the human B and CP promoters were cloned, sequenced and tested for transcriptional activity in skeletal and cardiac muscle-derived cell lines and primary cultures. Sequence analysis revealed the presence of several muscle-specific regulatory motifs within the B and CP promoters. However, consistent with studies by Kimura et al. (32) and Abdulrazzak et al. (11), our functional studies demonstrated that these promoters exhibit little or no activity in either skeletal or cardiac muscle cells. The discrepancy between the sequence predictions and the functional data is consistent with the notion that muscle-specific transcriptional activity depends not only on the nature of transcription factor-binding sites within the promoter but also on the combination and arrangement of individual regulatory elements relative to each other (48).

The absence of B and CP promoter activity in skeletal muscle cells pointed to the involvement of a transcriptional enhancer in the activation of these promoters in XLDC patients. The large size of the dystrophin gene, particularly the introns surrounding muscle exon 1, presents a formidable challenge to the identification of enhancer elements within this gene. The identification of XLDC patients with deletion mutations that span muscle exon 1 (19; Broere et al., manuscript in preparation) provided us with a unique opportunity to delimit regions at the 5' end of the dystrophin gene that contain important cardiac and skeletal muscle-specific regulatory elements. Sequences necessary for dystrophin gene expression in cardiac muscle-specific promoter activities were examined in RCM, H9C2(2-1) and C2 myoblasts. As expected, high α-MHC promoter activities were observed in primary cardiomyocytes (RCM; RLA, 136.67 ± 43.42) but no activity was observed in skeletal muscle-derived C2 myoblasts (RLA, 0.73 ± 0.28). Although in H9C2(2-1) cells α-MHC promoter activity was 5-fold higher (RLA, 3.75 ± 0.64) than in C2 cells, but was 36-fold lower than in RCM cells. These results are consistent with previous studies showing that H9C2(2-1) cells support both cardiac and skeletal muscle-specific gene expression, but also demonstrate that they more closely resemble skeletal myoblasts than cardiomyocytes. This indicates that DME1-mediated activation of the B and CP promoters in H9C2(2-1) cells likely reflects the expression of skeletal muscle-specific transcription factors in this cell line, and provides further support for the notion that DME1 functions to enhance the B and CP promoters in a skeletal muscle-specific manner. Therefore, DME1 is implicated in the induction of these promoters in the skeletal muscle of normal individuals as well as in XLDC patients with mutations that specifically abolish muscle isoform expression.

Figure 3. Effect of DME1 on B and CP promoter activities in muscle cells. Genomic fragments corresponding to B and CP isoform promoters in the human dystrophin gene were cloned upstream of the luciferase reporter gene in the promoterless pGL3b basic vector to generate pGL3-B and pGL3-CP, respectively. pGL3-B-DME1 (DME1 + B) and pGL3-CP-DME1 (DME1 + CP) contain a 1.4 kb genomic DNA fragment including DME1 cloned downstream of the luciferase gene in pGL3-B and pGL3-CP, respectively. Luciferase expression constructs were transiently transfected into skeletal muscle-derived C2 myoblasts, HSM, cardiac muscle-derived H9C2(2-1) myoblasts, RCM or 3T3 fibroblasts. Cell extracts were prepared and analysed for luciferase activity as described in Material and Methods. The promoterless pGL3b vector (pGL3b) was included in each experiment as a negative control. Luciferase expression plasmids containing the SV40 promoter (SV40) or the cardiac-specific α-MHC were included as positive controls. Transfection efficiencies in different cell lines were normalized to β-galactosidase activities generated by a CMV-β-galactosidase expression plasmid co-transfected with luciferase constructs in each experiment. RLA corresponds to the ratio of total luciferase to β-galactosidase activity measured in each sample. Values represent the mean ± SE of at least three independent experiments. Asterisks denote a significant increase in mean RLA (P < 0.05) due to either promoter activity alone (relative to the promoterless pGL3b control) or the addition of DME1 (relative to the corresponding promoter alone).
muscle would be predicted to lie within the region deleted in these patients, whereas enhancer elements involved in B and CP promoter activation in skeletal muscle would be located outside of the deletion breakpoints. A comparison of sequences deleted in these patients identified a 3.2 kb overlap between –1199 and +2057 bp that spans the core M promoter (–850 to +1 bp) and M exon1 (+1 to +275 bp). This region contains transcriptional control elements essential for dystrophin gene expression in cardiac muscle. Transgenic studies have shown that sequences between –900 and +1 bp direct gene expression to the right ventricle of the heart (25), but the location of regulatory elements that direct dystrophin gene expression to other areas of the heart remains to be precisely determined. In a recent study, 7 kb of sequence upstream of muscle exon 1 was shown to direct reporter gene expression throughout the heart as well as in embryonic skeletal muscle, but was not sufficient for gene expression in adult skeletal muscle (49). This result indicates that sequences within 1199 bp upstream of muscle exon 1 are necessary but not sufficient for dystrophin gene expression in cardiac muscle. This result also predicts that regulatory elements required for the expression of the M isoform in adult skeletal muscle are located outside of a 14 kb region surrounding muscle exon 1 (49). Similarly, our study has delimited the position of skeletal muscle-specific elements involved in B and CP promoter regulation to a region outside of the 18.3 kb between the extreme 5' (–13 253 bp) and 3' (+5000 bp) deletion breakpoints in these two XLDC patients.

DME1 is the only known transcriptional enhancer within the dystrophin gene. Located 6.5 kb downstream of muscle exon 1, this enhancer is preserved in the two XLDC patients examined here and presumably functions normally in other XLDC patients with mutations in the M promoter region (16,18,20). Our functional studies demonstrated that DME1 can activate the B and CP promoters in mouse skeletal muscle-derived C2 myoblasts, primary HSM and rat cardiac muscle-derived H9C2(2-1) myoblasts but not in RCM or 3T3 fibroblasts. Furthermore, the cardiac-specific α-MHC promoter provided high levels of reporter gene expression in RCM but not in H9C2(2-1) myoblasts, pointing to an inverse relationship between the activity of α-MHC and the degree of DME1-mediated enhancement of the B and CP promoters in each of these muscle cell lines. These results suggest that DME1 can activate these non-muscle promoters in skeletal but not cardiac muscle-derived cells. This is in direct contrast to previous studies in our laboratory in which DME1 was seen to be preferentially active in H9C2(2-1) cells as compared to C2C12 and L6 skeletal myoblasts (29). These results pointed to a role for DME1 in cardiac-specific dystrophin gene regulation, a conclusion supported by recent transgenic studies (49). On the other hand, in vitro studies of the mouse homolog of DME1 suggested that this enhancer has greater activity in skeletal muscle-derived G8 myoblasts than in H9C2(2-1) cells (30). Differences in the apparent specificity of DME1 likely reflect the different promoter–enhancer combinations used in these experiments. For example, in our previous studies DME1 was shown to be less effective at inducing reporter gene expression from the dystrophin M promoter as compared to heterologous viral promoters (26). This was attributed to the fact that the M promoter itself is tightly regulated in muscle tissues, consistent with subsequent studies of the M promoter in transgenic mouse models (25,49). The B and CP promoters also appear to contain regulatory elements that tightly restrict their expression to specific cell types. The results of the present study suggest that the unique combination of regulatory elements within DME1 and the B and CP promoters results in their preferential activation in skeletal but not cardiac muscle. What specific regulatory elements are involved in skeletal muscle-specific activation, whether DME1 is the only enhancer involved in this process, and how mutations that affect M isoform expression influence this process are all important questions that remain to be answered. Knowledge of the transcriptional mechanisms governing dystrophin gene expression in skeletal and cardiac muscle can be applied to the design of more effective dystrophin minigenes as well as to the development of novel therapeutic interventions that activate the expression of non-muscle dystrophin isoforms in patients with mutations that specifically affect the M isoform of the gene.

MATERIALS AND METHODS

Deletion breakpoints analysis

Primer pairs were designed to amplify dystrophin gene sequences at 1–40 kb intervals between –100 kb and +10 kb relative to the transcription start site in muscle exon 1. Primer sequences were based on the sequence of a 134 kb PAC clone spanning muscle exon 1 (GenBank accession no. AL031643). Primers specific for the promoters and first exons of the B and CP isoforms were also synthesized. Sequences of all of the primers used in this study are presented in Table 1. PCRs were performed with an initial denaturation step at 94°C for 3 min, followed by 35 cycles with denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and extension at 68°C for 1 min, and a final 5 min extension at 68°C. PCR reaction products were fractionated on 1.5% agarose gels and visualized by ethidium bromide staining. It should be noted that extension was prolonged to 3 min for PCR amplification of the deletion junction fragments in the two XLDC patients. Deletion junction fragments were cloned into the pCR2.1-TOPO vector (Invitrogen) and both strands were sequenced commercially (ACGT Corp., Toronto, Ontario, Canada). Deletion breakpoints (GenBank accession no. patient 1, AF324932; patient 2, AF373844) were determined by comparison to the sequence of a 134 kb PAC clone spanning muscle exon 1 (GenBank accession no. AL031643). Sequences on either side of the deletion breakpoints were examined for the presence of repeats using the BLAST 2 Sequences program (http://www.ncbi.nlm.nih.gov).

Cloning and sequence analysis of the CP promoter

A 9.4 kb genomic fragment (CP9.4) containing CP exon 1 was isolated following hybridization of a genomic library prepared from a Sau3A partial digest of a PAC clone containing the first exons of both the M and CP isoforms of the dystrophin gene (PAC-ME1-CPE1; courtesy of Dr S.Ditta and Dr S.Sherar, MRC Genome Research Facility, The Hospital for Sick Children, Toronto, Ontario) with a radiolabeled probe corresponding to CP exon 1. Restriction enzyme digestion and Southern blot analysis of CP9.4 localized CP exon1 to a 1.8 kb HindIII fragment which was subsequently subcloned into pBluescript (Stratagene) and sequenced (ACGT Corp.). This
1.8 kb fragment contains 224 bp corresponding to CP exon 1 and 1568 bp of upstream sequence corresponding to the CP promoter (GenBank accession no. AF324931).

The CP promoter was analyzed for the presence of known transcription factor-binding site motifs using MatInspector Professional software (http://genomatix.gsf.de) and TRANSFAC 3.5 matrices.

**CP, B and α-MHC promoter reporter plasmids**

The CP promoter region from −1560 to +145 bp relative to the transcriptional start site in CP exon 1 was PCR-amplified from a PAC clone positive for both the M and CP isoforms of the dystrophin gene (PAC-ME1-CPE1). The B promoter region from −1477 to +329 bp relative to the transcriptional start site in brain exon 1 was PCR-amplified from normal human genomic DNA. Sequences of forward and reverse primers used to PCR amplify these promoters are shown in Table 1. Promoter fragments were amplified using the Expand High Fidelity PCR System (Boehringer), cloned into the pCR2.1-TOPO vector (Invitrogen) and sequenced commercially on both strands (ACGT Corp.). The CP and the B promoters were subsequently excised as KpnI–Xhol and Xhol–HindIII fragments, respectively, upstream of the luciferase gene in the promoterless and enhancerless pGL3basic reporter plasmid (Promega) to generate pGL3-CP and pGL3-B. pGL3-B-DME1 and pGL3-CP-DME1 were generated by first cloning a 1.4 kb genomic fragment containing the DME1 enhancer (26,29) into the SalI–BamHI sites downstream of the luciferase gene in pGL3basic to generate pGL3-DME1. The CP and B promoters were subsequently excised as KpnI–NarI and Xhol–NarI fragments from pGL3-CP and pGL3-B, respectively, and subcloned upstream of the luciferase gene in pGL3-DME1 to generate pGL3-CP-DME1 and pGL3-B-DME1. A cardiac-specific positive control vector (pGL3-αMHC) was also generated containing, upstream of the luciferase gene, the genomic region of the murine α-myosin heavy-chain promoter (47) from −4223 to +125 bp, relative to the transcriptional start site.

**Cell culture, transfection and reporter gene assays**

Murine skeletal muscle-derived C2 myoblasts (50), rat cardiac muscle-derived H9C2(2-1) myoblasts (27) and murine-derived 3T3 fibroblasts were obtained from the American Type Culture Collection (ATTC). Human skeletal muscle primary myoblasts were the same as previously reported by Tennyson et al. (51). These cell types were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) FBS and 2 mM glutamine (Growth Medium). Primary cardiomyocytes were isolated from 3-day-old rats using the protocol and medium described by Dostal et al. (52). DNA plasmids were purified using the Qiagen DNA Plasmid Maxi Kit. Transfections were carried out using the Effectene transfection reagent (Qiagen) according to the manufacturer’s protocol. Each transfection contained 0.25 µg of an experimental luciferase plasmid together with 0.25 µg of a CMV-β-galactosidase plasmid included as a control for transfection efficiency. Fusion medium [DMEM supplemented with 2% (v/v) heat-inactivated horse serum (HS) and 10 µg/ml insulin] was added to C2, H9C2(2-1) and HSM 24 h post-transfection. Cardiac myoblasts were differentiated using a medium containing 75% DMEM, 17% Medium 199, 5% heat-inactivated HS and 0.5% NBCS. Normal growth medium was added to 3T3 fibroblast cultures at this time. Cell lysates were prepared 72 h after the addition of fusion or differentiating medium (96 h post-transfection) and luciferase and β-galactosidase activities determined using the Dual-Light combined luciferase and β-galactosidase reporter gene assay system (Tropix) and a Lumat LB 9507 luminometer (EG&G Berthold) following the manufacturer’s protocol. Promoter activities are expressed as RLA representing the ratio of total luciferase (relative light units; RLU) to β-galactosidase (RLU) activity measured in each sample.

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**REFERENCES**


