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Ets, Ap-1 and GATA factor families regulate the utrophin B promoter: potential regulatory mechanisms for endothelial-specific expression

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Abstract Duchenne muscular dystrophy is caused by dystrophin deficiency, which can be prevented in the *mdx* mouse model by over-expression of an autosomal homologue, utrophin. Utrophin has two characterised full-length promoters, A and B. No data are available on the transcriptional regulation of B utrophin, which has been recently localised to the endothelium. Similar to characterised endothelial promoters, Ets and Ap-1 individually *trans*-activate the human B core promoter. Synergistic activation by GATA-2 and c-jun to the order of 20-fold was observed.

Key words: Utrophin; Endothelium; Promoter; Ets; GATA; Transcription

1. Introduction

Duchenne muscular dystrophy (DMD) is an X-linked muscle wasting disease caused by the absence of a cytoskeletal protein, dystrophin, from the cytoplasmic surface of the sarcolemma [1]. Treatment is palliative; patients die from cardiac or respiratory complications in their late teens or early twenties. Utrophin is the autosomal homologue of dystrophin [2] and binds similar protein complexes in muscle [3]. Therefore, utrophin up-regulation has an inherent attraction for a therapeutic strategy, as difficulties with immune responses associated with the introduction of dystrophin and gene delivery to muscle may be circumvented.

Two independently regulated promoters, A [4] and B [5], control expression of the utrophin transcript. Each promoter gives rise to a transcript with unique 5′ exons which splice into a common utrophin mRNA at exon 3 [4,5]. Promoter A lies within a CpG island at the 5′ end of the gene [4]; promoter B is located within the large second intron of the human and mouse utrophin genes, approximately 52 and 50 kb 3′ to exon 2 respectively [5]. The utrophin B transcript encodes a unique 31 amino acid first exon (1B), with human and mouse sequences showing 82% nucleotide and 77% translational identity.

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Abbreviations: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; Ap-1, activator protein-1; DMD, Duchenne muscular dystrophy; EMSA, electrophoretic mobility shift assay

In a recent study, the murine utrophin B protein was localised to vascular endothelia using an isoform-specific antibody [6], however, no data exist on transcriptional processes that regulate the B transcript. This study describes the delineation of factors from the activator protein-1 (Ap-1), Ets and GATA families that synergistically *trans*-activate the human utrophin B minimal promoter, and suggests a mechanism for endothelial-specific expression.

2. Materials and methods

2.1. Bioinformatics

Sequence data were assembled and analysed using the GCG Wisconsin package; transcription factor consensus sequences and databases were accessed using the TRANSFAC (http://transfac.gbf.de/TRANSFAC/) website [7].

2.2. Electrophoretic mobility shift assay (EMSA)

Synthetic oligonucleotides (Genosys) of cognate transcription factor binding sites were diluted to 1 μg/μl, with complementary primers annealed in 1×PNK buffer (NEB) by heating for 5 min at 85°c, and cooling to room temperature overnight. EMSA analysis was performed using the Gel Shift Assay System (Promega) with human recombinant Ap-1 (c-jun; 2 μl) and IN157 nuclear extracts, prepared as previously described [8]. Following electrophoresis (200 V for 90 min), gels were dried and exposed for 30 min at room temperature.

2.3. Construction of utrophin luciferase vectors

Mutant sequences (changes underlined): utroB-ap1 (ctgactaaa → cg-gacgaaa) and utroB-ets (taacttcctct → taactgaatct) were introduced into the defined human core 300 bp promoter fragment (region 1180–1503; previously named Δ5′*PvuII*-1199 [5]) in pBluescript KS$^{+/-}$ (Stratagene) using polymerase chain reaction mutagenesis (Fig. 1). Conditions were 95°c for 30 s, 12 cycles of 95°c for 30 s, 50°c for 1 min and 58°c for 2 min per kb amplified. Positive clones were identified by the abolition of restriction sites (with *Bst*YI and *Hin*fI, respectively), sequenced, excised by restriction digestion (*Kpn*I/*Sac*I) and directionally cloned into pGL3 Basic (Promega). Large-scale endotoxin free plasmid preparations were purified using a commercially available kit (Qiagen).

2.4. Tissue culture and transfection

Mammalian cell lines were maintained and transfected as previously described [8]. For co-transfection of GATA, Ap-1 and Ets expression vectors, 0.1–0.2 μg each of vector(s) and test plasmid was used. A stock solution (0.5 mg/ml) of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was diluted in Dulbecco's modified Eagle's medium, and added 24 h post-transfection of reporter plasmids. Cells were allowed to express the fusion genes for 12–24 h, and were harvested by scraping into 1×lysis buffer (Promega) after two washes in room temperature phosphate-buffered saline. Samples were freeze-thawed on dry ice, vortexed briefly and centrifuged (13 000 rpm for 30 s), with the supernatant assayed directly.

2.5. Luciferase reporter gene assays

Cell extracts were assayed for luciferase activity using a commercial

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reagent (Promega). Light output was read using a Turner-TD20e luminometer (delay 5 s, integrate 10 s). Assays were performed in triplicate from three separate cultures of transfected cells. The transfection/assay process was repeated in triplicate for all promoter studies.

3. Results

Characterised factor binding sites in a large number of characterised core promoter regions that contribute to endothelial-specific expression include interactions between Sp1, Ap-1 and the GATA and Ets transcription factor families [9–12]. Cognate binding sites (Ap-1, Ets and Sp1) were elucidated during the delineation of the minimal B promoter element ([5], illustrated in Fig. 1), and were selected for study.

3.1. EMSA and in vitro studies of promoter activity

Functional relevance of the cognate Ap-1 site was determined using EMSA studies, where formation of endogenous IN157 and recombinant human Ap-1 binding could both be specifically removed using an unlabelled Ap-1 consensus oligonucleotide. Binding of recombinant human Ap-1 was also disrupted by cognate site mutagenesis (Fig. 2). EMSA analysis discounted Sp1 binding to a cognate site (1345–55; Fig. 1) within the core promoter (data not shown).

Target nucleotides for mutagenesis of cognate binding sites in the utrophin B core promoter were sourced using a combination of commercial mutant oligonucleotide sequences and previous functional promoter studies of the endogenous binding affinity of Ap-1 and Ets. Promoter mutants were directionally cloned into a luciferase reporter vector and functionally assayed by measuring their ability to drive reporter gene expression in HeLa (cervical epithelial), IN157 (rhabdomyosarcoma) and Bend3 (murine brain primary endothelial) cultured cell lines in comparison to the wild-type promoter region (Fig. 3). Mutagenesis of the Ap-1 site resulted in a decrease in promoter activity in all cell lines studied, although the effect varied from a moderate 33% decrease in HeLa (±3.9) to 82% (±4.3) and 88% (±1.1) in IN157 and Bend3 cells respectively. Removal of the cognate Ets motif exhibited similar decreases in IN157 and Bend3 cells (87%±2.0 and 82%±8.8 respectively), with a slight increase in activity in HeLa cells (117%±15.8). These results indicate that core promoter binds members of the Ap-1 and Ets factor families through their cognate sequences, and that these sites are necessary for optimal transcription in IN157 and Bend3 cell lines.

3.2. Phorbol ester and Ap-1 trans-activation studies

As the binding of Ap-1 and Ets factors was implicated in optimal transcriptional activity of B-utrophin, the ability of core promoter to respond to stimulation with TPA was studied. TPA has the ability to *trans*-activate a number of characterised endothelial promoters, such as vascular endothelial growth factor [13], and human intracellular adhesion molecule-1 (ICAM-1), which results from potent up-regulation of c-jun and c-fos expression via the mitogen-activated protein kinase pathway [14]. The activation pathway of protein kinase C by TPA is reported to act on the transcriptional level through the dephosphorylation of the C-terminal region of c-jun and c-fos, leading to enhanced DNA binding [15,16]. Incubation with 50 and 500 ng/ml TPA caused increases in core promoter activity in all cell lines studied. For example, respective fold increases of 1.87 (±0.2) to 2.15 (±0.1) of core

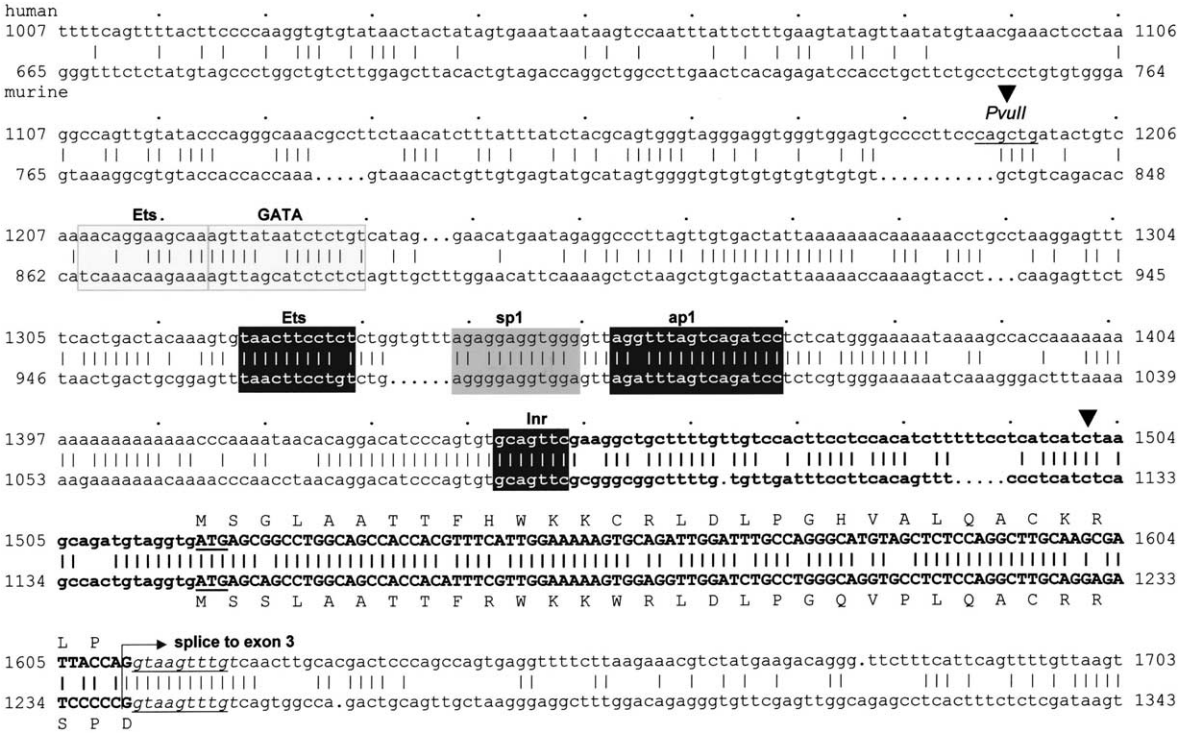

Fig. 1. Human and mouse alignment of the utrophin B core promoter. Sequence conservation of the human (top: GenBank accession number AJ250044) and mouse (bottom: accession number AJ250045) utrophin B region. The translated exon and conserved splice site is shown; triangles define the minimal promoter element, incorporating the single transcription start site (Inr) [5]. Transcription factor binding regions of interest are indicated (factor name above), Ap-1 and Ets cognate motifs (black); cognate region that did not bind Sp1 (grey); other predicted binding regions (light grey).

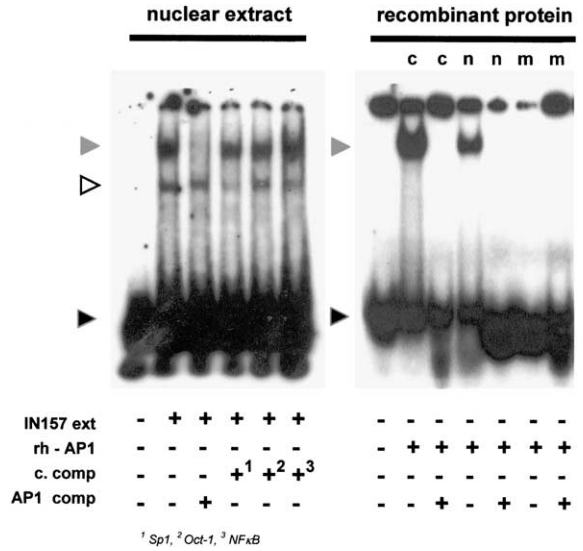

Fig. 2. Ap-1 binds to the utrophin B core promoter. 20 μg of IN157 nuclear extract was incubated with a labelled 22-mer oligonucleotide probe spanning the cognate Ap-1 site in the B promoter, and unlabelled competitor probes as indicated by plus (+) symbols. Two complexes were formed: Ap-1-specific (grey arrow) and a non-specific binding complex (white arrow). The specific complex was competed with Ap-1 competitor only (lane 3). For recombinant protein EMSA, 2 μg Ap-1 was added to the labelled B probe and an Ap-1 consensus oligonucleotide probe, indicated by plus (+) symbols. A specific complex (grey arrow) was competed by the addition of cold Ap-1 consensus oligonucleotide, or cognate site mutagenesis. Black arrows represent free radiolabelled probe. Legend: IN157 ext, IN157 nuclear extract; rh-AP1, recombinant human Ap-1 (c-jun); c.comp, cold consensus oligonucleotide; AP1 comp, cold consensus Ap-1 oligonucleotide; c, consensus probe; n, normal Ap-1 B promoter probe; m, Ap-1 mutant B promoter probe.

promoter activity were observed in the Bend3 cell line, which was hampered by mutagenesis of the Ap-1 site, which showed a 1.3 (±0.1) fold increase with 50 ng/ml TPA (Fig. 4A).

A more direct approach using c-jun and c-fos over-expression in HeLa and COS-7 cell lines was used to evaluate this response. Dosage-dependent increases in transcriptional activity were observed with c-jun (with fold increases of 5.1 ± 1.3 for 100 ng, and 9.5 ± 1.4 for 200 ng c-jun in HeLa cells) (Fig. 4B). Similar, albeit lower patterns of *trans*-activation were observed in COS-7 cells where the highest level of activation of 3.5-fold (±0.4) was observed with the co-transfection of 200 ng c-jun. Over-expression of c-fos had a positive effect on utrophin B, possibly as a consequence of complex formation with endogenous c-jun, as members of the Fos family cannot make stable homodimers and therefore cannot act as transcriptional activators by themselves [17]. This strongly suggests that the human utrophin B promoter functionally binds members of the Ap-1 family of transcription factors, and that c-jun homodimers are more potent in transcriptional activation of the core element than heterodimers of c-fos/jun.

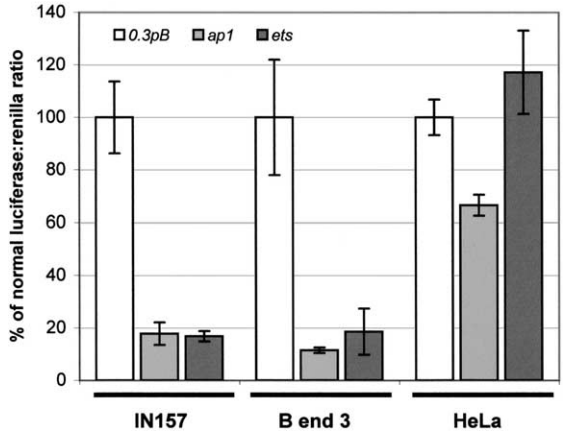

Fig. 3. In vitro activity of the core B promoter is affected by mutagenesis of Ap-1 and Ets sites. Activity of the 300 bp core promoter mutant constructs as compared to the wild-type construct. Abbreviations: 0.3B, wild-type core promoter region; others refer to the site mutated in the reporter construct. Cell lines used as indicated; wild-type promoter activity is represented by white columns (0.3B), with the mutants represented as a percentage of activity.

3.3. *Utrophin B is* trans*-activated by Ets family members*

Ets factors often function co-operatively with other nuclear factors, in particular Ap-1 [18]. Ets-1 and Ets-2 are expressed in endothelial cells and specifically *trans*-activate a large number of genes through interaction with c-jun/fos [19,20] and Sp1 [21]. PU.1 interacts via its Ets domain with the basic domain

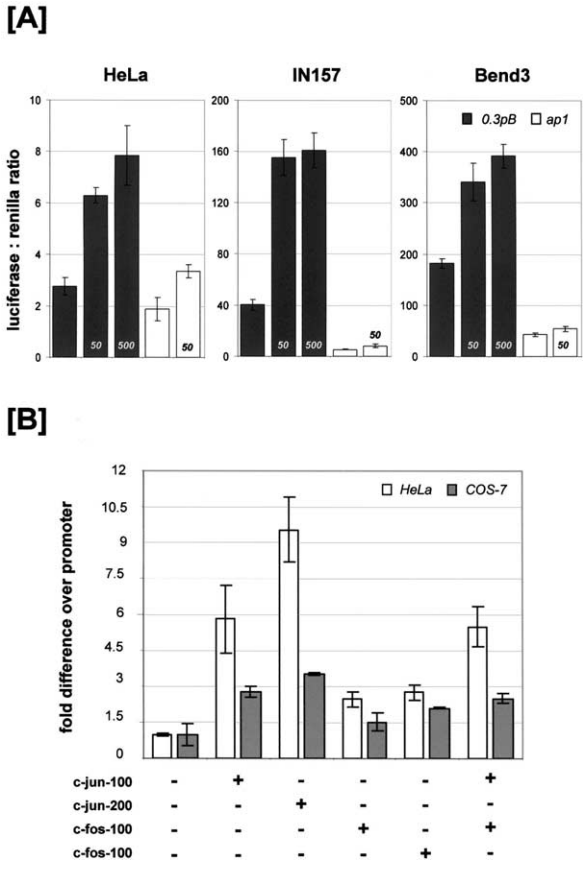

Fig. 4. Phorbol ester and Ap-1 stimulate the utrophin B promoter. A: The core promoter exhibits a dosage response to the addition of the phorbol ester TPA in HeLa, Bend3 and IN157 cells. Activities of the core (0.3pB) and the Ap-1 mutant (Ap-1) promoter regions are indicated, with addition of 50 ng and 500 ng/ml TPA represented by '50' and '500' on the respective columns. Activity is expressed as a ratio of luciferase activity relative to the *Renilla* transfection control. B: The utrophin B core promoter element exhibits a dosage response to raised levels of c-jun and c-fos in HeLa and COS-7 cell lines. Cells were transfected with 100 ng of the B promoter region, and 100–200 ng of c-jun and/or c-fos as indicated by plus (+) symbols in the figure legend. Activity is expressed as the fold difference over the normalised B promoter value.

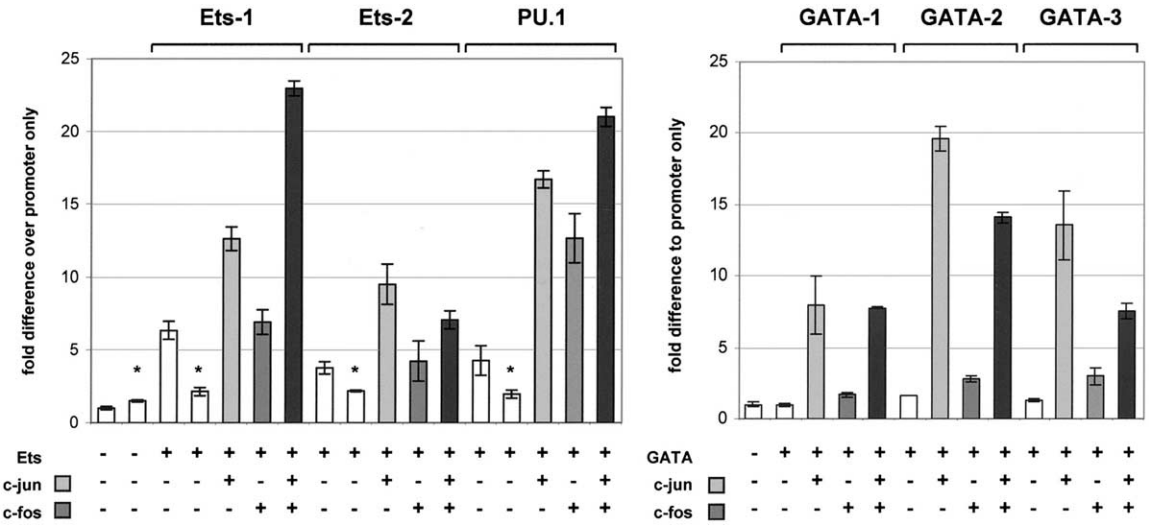

Fig. 5. Utrophin B is *trans*-activated by GATA, Ets and Ap-1 factors. The human utrophin B core promoter is activated by Ets factors and the synergistic co-operation of GATA and Ap-1. HeLa cells were transfected with 100 ng of the B promoter core construct, and 100 ng of Ets or GATA factors (indicated above), with 100 ng c-jun and/or c-fos indicated by plus (+) symbols in the figure legend and light grey (c-jun), dark grey (c-fos) and black (c-jun and c-fos) columns. Asterisks indicate values obtained for the Ets mutant. Activity is expressed as the fold difference over the normalised wild-type B promoter value.

of c-jun [22]. The B core promoter reporter construct was co-transfected in HeLa cells with Ets-1, Ets-2 and PU.1 expression vectors. Ets-1 exhibited the highest fold increase (6.3 ± 0.6), although PU.1 (4.3 ± 1.02) and Ets-2 (3.5 ± 0.41) also showed measurable effects on promoter activity. *Trans*-activation levels were hampered by the Ets mutant, of which the highest fold increase over wild-type promoter activity was observed with Ets-2 (2.18 ± 0.5). Co-transfection with c-jun or c-fos led to a further increase in core promoter activity (Fig. 5), with the highest level of *trans*-activation observed with PU.1 and c-jun (16.7 ± 0.59 fold). Both c-jun and c-fos resulted in a 23-fold increase in promoter activity (22.96 ± 0.5), with similar levels observed for PU.1 (21 ± 0.65). Levels of Ets-2-mediated *trans*-activation were comparatively lower (7 ± 0.6).

A limited degree of synergistic co-operation between Ap-1 and Ets factor families was noted, i.e. combined *trans*-activation of c-jun and PU.1 resulted in an almost two-fold increase over the expected value of the two factors individually (from an expected value of ~9.36- to almost 17-fold). Conversely, most Ets/Ap-1 combinations showed similar *trans*-activation values whether individually or co-transfected. For example, the sum of individual values obtained with transfection of c-jun (~5.1) and Ets-2 (~3.7) is similar to the co-transfected value of 9.5. Therefore, the degree of synergism (if any) between Ets and Ap-1 families to transcriptionally regulate the B core promoter appears complex and is likely to be reliant on specific combinations and/or individual factor availability. As exhibited by c-jun/fos over-expression (Section 3.2), co-transfection with c-jun appeared to be more potent in conferring a positive response from the core promoter, with reporter transcription levels generally two-fold higher than observed for c-fos.

3.4. GATA and Ap-1 factors synergistically activate utrophin B

GATA factors play a critical role in mediating transcriptional regulation of several endothelial-specific genes. This phenomenon is particular to GATA-2, which is abundantly expressed in endothelia and is critical for transcriptional regulation of ET-1 (endothelin-1 (ET-1), a potent vasoconstrictor and smooth muscle mitogen expressed in endothelial cells of the vascular wall) [23], von Willebrand factor [24], ICAM-2 and P-selectin (both adhesion receptors constitutively expressed in megakaryocytes and endothelial cells [9,25,26].

GATA factor transfection resulted in low levels of transcriptional activation from the utrophin B minimal element, i.e. 1.6 (± 0.1) and 1.3 (± 0.1) fold for GATA-2 and -3. However, co-transfection with c-jun (individually conferring a five- to seven-fold increase) showed a significant increase in utrophin B reporter activity (Fig. 5). Augmented *trans*-activation potential was most apparent with GATA-2 (19.56 ± 0.86), although GATA-1 (8.0 ± 1.8) and -3 (13.5 ± 2.4) were also responsive. Functional synergism between GATA and Ap-1 factor families was enhanced in comparison to Ap-1/Ets, and defines an additional/related regulatory mechanism residing within the utrophin B core promoter region.

4. Discussion

The utrophin B isoform has been recently localised to the endothelium [6]. This paper describes the analysis of signalling pathways and transcriptional mechanisms that may confer endothelial-specific transcription.

In addition to the functional analysis of a cognate Ap-1 site present within the minimal promoter region, mutagenesis of a cognate Ets site resulted in a severe attenuation of utrophin B reporter activity in gliomal IN157 and endothelial Bend3 cell lines. Three Ets factor family members are able to *trans*-activate the utrophin B promoter (of which Ets-1 and Ets-2 are expressed in endothelia). This suggests that B utrophin is subject to regulatory mechanisms observed for vascular bed-specific genes, such as platelet endothelial cell adhesion molecule-1 (PCAM-1) [27] and endothelial cell-specific molecule-1 [28], in which functional synergism between the Ets, Ap-1 or Sp1 factor families exists. Observed *trans*-activation levels are comparable to the von Willebrand factor core promoter (re-

stricted to endothelial cells and megakaryocytes) in which Ets-1, Ets-2 and Erg can *trans*-activate transcription approximately four-fold [29,30]. Co-transfection of Ets and Ap-1 factors resulted in a limited degree of co-operativity to up-regulate the B core element and suggested that alternative mechanisms may exist.

GATA factors (most notably GATA-2) showed co-operativity with Ap-1 to increase transcription in a greater than additive manner in a non-endothelial cell line; illustrated for other endothelial-specific promoters [31,32], such as the human ET-1 core promoter, where a GATA/Ap-1 site is capable of conferring on a heterologous promoter enhanced expression in endothelial cells [23]. Significant increases were only observed with co-transfection with members of the Ap-1 family and GATA-2 with c-jun/fos vastly increases transcription [32]. GATA *trans*-activation levels observed for utrophin B with or without the presence of Ap-1 are similar to those observed for the ET-1 core promoter by Kawana et al. [32] and strongly suggest that mechanisms governing the spatial expression of utrophin B may be compatible, in part, to those elucidated for ET-1.

The *cis*-acting GATA regulatory sequence responsible was not identified, although a putative site was identified at the most 5′ end of the core promoter region (Fig. 1), adjacent to an additional Ets site. Mutagenesis studies were not performed as a GATA binding motif may not be required, as GATA-2 can potentiate the action of Ap-1 with a GATA site deletion, and vice versa [32]. As GATA-2 and Jun/Fos family members are widely expressed, their sole action cannot be responsible for such cell-restricted behaviour, and may allow recruitment of additional proteins, such as the Ets and Sp transcription factor families. Sp1 co-operability with Ets and GATA factors is critical for core promoter transcription of several endothelial genes, such as Tie-2 [10], Flt-1 [33], ICAM-1 [25], PCAM-1 [27,34] and the endothelial nitric oxide synthase enhancer [35]. Although Sp1 did not bind a designated region in the B core promoter element, alternative sites may exist or may not be required, as this factor can interact directly with bound Ets-1 [36].

Selective formation of multiple protein complexes involving members of the Ets, GATA, Ap-1 and possibly Sp factor families may therefore be important for both spatial restriction and enhanced function of the utrophin B promoter in endothelium. This study therefore provides an insight into mechanisms directing cellular distribution of utrophin B, which is necessary for defining the potential of using alternative isoforms to utrophin A in an up-regulation-based therapy for DMD.

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