FEBS 27006 FEBS Letters 537 (2003) 133–138

Increasing D4Z4 repeat copy number compromises C2C12 myoblast differentiation

Darren J. Yip[a,b], David J. Picketts[a,b,*]

[a] *Molecular Medicine Program, Ottawa Health Research Institute, 501 Smyth Road, Ottawa, ON, Canada K1H 8L6*
[b] *The University of Ottawa, Center for Neuromuscular Disease and Departments of Medicine and Biochemistry, Microbiology and Immunology, 451 Smyth Road, Ottawa, ON, Canada K1H 4M5*

Received 26 November 2002; revised 14 January 2003; accepted 16 January 2003

First published online 6 February 2003

Edited by Veli-Pekka Lehto

Abstract Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant myopathy associated with deletions of a subtelomeric repeat (D4Z4). A reduction in D4Z4 copy number coincides with increased expression of neighboring 4q35 genes, implying a normal repressive role for the repeats. Here we examine the effect of increasing D4Z4 repeat number on reporter gene activity in C2C12 cells. Repeat size had only a minor *cis*-effect on reporter gene activity but greatly compromised myotube formation. This latter *trans*-effect did not result from expression of a gene within the repeat (DUX4) but likely results from squelching of the D4Z4 recognition complex.

Key words: Facioscapulohumeral muscular dystrophy; D4Z4; DUX4; 4q35

1. Introduction

Facioscapulohumeral muscular dystrophy (FSHD), an autosomal dominant disorder with an incidence of 1 in 20 000, is characterized by facial and shoulder girdle muscle atrophy that often progresses to include muscles of the lower limb [1,2]. Genetic studies localized the FSHD gene to the subtelomeric region of chromosome 4 [3,4]. Molecular characterization of this region demonstrated that both familial and sporadic FSHD were associated with deletion of several copies of a 3.3 kb tandem repeat at 4q35, termed D4Z4 [5,6].

Each copy of the D4Z4 repeat contains several repetitive elements common to heterochromatin and an open reading frame encompassing a double homeodomain sequence motif, now called DUX4 [7,8]. However, the inability to identify a functional DUX4 transcript encouraged a position–effect hypothesis as the disease mechanism. As such, a reduced D4Z4 array would influence the expression of neighboring genes either by silencing, as a result of increased proximity to the telomere or, through inappropriate activation provided the D4Z4 repeats normally function as long-range repressors [7,9]. The region immediately proximal to the D4Z4 repeats then became a focal point for the identification of genes that may be subject to position effects. Sequence analysis demonstrated that the proximal 400 kb interval is relatively gene poor with a tubulin pseudogene (TUB4Q) and the FRG1 (FSHD region gene 1) gene residing closest to the repeats [10,11]. Also within 4q35 lie several attractive candidate genes encoding muscle-enriched transcripts (e.g. ALP, ANT1), yet none have proven to be causative of the disease [12–15].

A single nucleotide polymorphism was used to demonstrate that FRG1 expression from a D4Z4-deleted chromosome was slightly increased over the normal allele of an FSHD patient [11]. This suggested that a reduction in D4Z4 repeats relieves repression from nearby genes. Indeed, FRG1, FRG2 and ANT1 RNA levels were upregulated in FSHD muscle and this was correlated with decreasing repeat length [16]. Moreover, a 27 bp element (DBE) identified within the D4Z4 repeat was shown to be repressive when multiple copies were placed between a promoter and the corresponding reporter gene [16]. Further studies characterized the protein complex that binds to the DBE, named the D4Z4 recognition complex (DRC), comprising the proteins YY1, HMGB2, and nucleolin [16].

In this study, we used increasing copies of the D4Z4 repeats positioned directly upstream of a *LacZ* construct to determine the effect on reporter gene activity and muscle differentiation following transfection into C2C12 myoblasts. We demonstrate that increasing D4Z4 repeats have a significant *trans*-effect on myoblast differentiation with only a minor *cis*-effect on reporter gene activity.

2. Materials and methods

2.1. Generation of DNA constructs

D4Z4 repeats (3.3 kb) were excised by *Kpn*I digestion from cosmid cY13 [17]. Isolated repeats were concatamerized and ligated into pBluescript II KS (Stratagene, La Jolla, CA, USA) and sequenced for confirmation. Arrays containing one to four copies were cloned 93 bp upstream of the BOS-*LacZ* reporter of pRP2044 (gift of R. Parks, Ottawa, ON, Canada) to generate pDY01-pDY04 [18].

2.2. Cell culture, transfection and reporter assays

C2C12 myoblasts and stable clones were maintained as described [19]. Cells (2×10^5 cells/3 cm dish) were transiently transfected with equal molar ratios of plasmid using Lipofectamine Reagent (Invitrogen). After 48 h, replicate plates were either harvested for analysis, assayed for transfection efficiency using β-gal in situ analysis [20], or induced to differentiate by mitogen deprivation [21]. To establish stable clones, C2C12 cells were co-transfected with the neomycin selection plasmid pGTNeo (New England Biolabs) and grown in 0.8 mg/ml G418 (Invitrogen).

Positive clones were identified by PCR [50 μl reaction; 30 cycles of 94°C (30 s); 56°C (30 s); 72°C (60 s)] using primers for D4Z4 or *LacZ* (primer sequences listed in Section 2.4). Transgene copy number and insertion sites were determined by Southern analysis using a 2.3 kb

*Corresponding author. Fax: (1)-613-737 8803.
E-mail address: dpicketts@ohri.ca (D.J. Picketts).

0014-5793/03/$22.00 © 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00110-8

*Nde*I/*Nhe*I fragment of *LacZ* as a probe [22]. A fragment of the mouse *Snf*2h gene served as a loading control [23].

Cell lysates were prepared using Reporter Lysis Buffer (Promega, Madison, WI, USA). Following protein quantification (DC Protein Assay, Bio-Rad, Hercules, CA, USA), ~5 ng was used to assess β-gal activity with the *o*-nitrophenyl-β-D-galactopyranoside assay [18].

2.3. Immunocytochemistry and analysis of myotube fusion

Cells were stained with a monoclonal MF20 anti-myosin antibody (Developmental Studies Hybridoma Bank), detected using a goat anti-mouse IgG conjugated to horseradish peroxidase (Invitrogen), and counter stained with Harris modified Hematoxylin. Fusion competence was determined after 4 days of differentiation using the Myotube Fusion Index (MFI) following assessment of 20 fields from each of four to six plates [24]. The Deformed Myotube Index (DMI) was calculated as the proportion of myotubes with a deformed morphology and was determined similarly to the MFI.

2.4. RNA isolation and RT-PCR analysis

RNA was isolated from cells during growth or following differentiation by the guanidinium method [25]; 5 μg was reverse transcribed using Superscript RT (Invitrogen) and random hexamers (Invitrogen). PCR was for 30 cycles [94°C (30 s); 56°C (52°C for D4Z4; 30 s); 72°C (60 s)] using primers for D4Z4 (D4Z4f1905 – 5′-cgagcctgctttgaccg-3′; D4Z4r2385 – 5′-cgtggggtgcgggag-3′), *LacZ* (ras39 – 5′-ttggcctgaactgccagctggcgcagg-3′; ras40 – 5′-tcccgcagcgcagaccgttttcgctcg-3′), or β-actin (Ambion Inc.).

3. Results and discussion

To assess the silencing effect of D4Z4 repeats on reporter gene expression we generated plasmids containing one to four D4Z4 copies upstream of the BOS (EF1α) promoter and the *LacZ* reporter gene (Fig. 1A,B). The parental plasmid, pRP2044, which lacks D4Z4 served as a control. Plasmids were transient transfected into C2C12 myoblasts and analyzed for β-gal activity during growth conditions, or following myoblast differentiation. During growth we observed no statistical differences in reporter activity between the control and experimental plasmids pDY01, pDY02, and pDY04 (Fig. 1C). There was an increase in reporter activity between pDY03 and the control ($P = 0.018$), yet the significance of this result remains to be determined. Since we anticipated that D4Z4 would inhibit *LacZ* expression, these results suggested that repression might be specific to differentiating myoblasts. As such, transfected cells were cultured in differentiation media and then assessed for β-gal activity over a period of 96 h (Fig. 1D). We observed an initial decrease in β-gal activity for all samples at 12 h, coinciding with significant apoptosis, a common response to the stress of mitogen deprivation [24,26]. The control sample regained basal β-gal levels within 24 h (dark bars). Cells transfected with pDY02 and pDY04 (white and gray bars, respectively) appeared to demonstrate a lag in recovery but this was not statistically significant. However, β-gal activity was mildly compromised at 96 h for cells transfected with pDY04 ($P = 0.001$), suggesting that the repeats may subtly impede gene expression during differentiation. The subtle effects may be a reflection of the number of D4Z4 repeats contained within the constructs. Patient studies suggest there is a threshold number of repeats (>10) below which a phe-

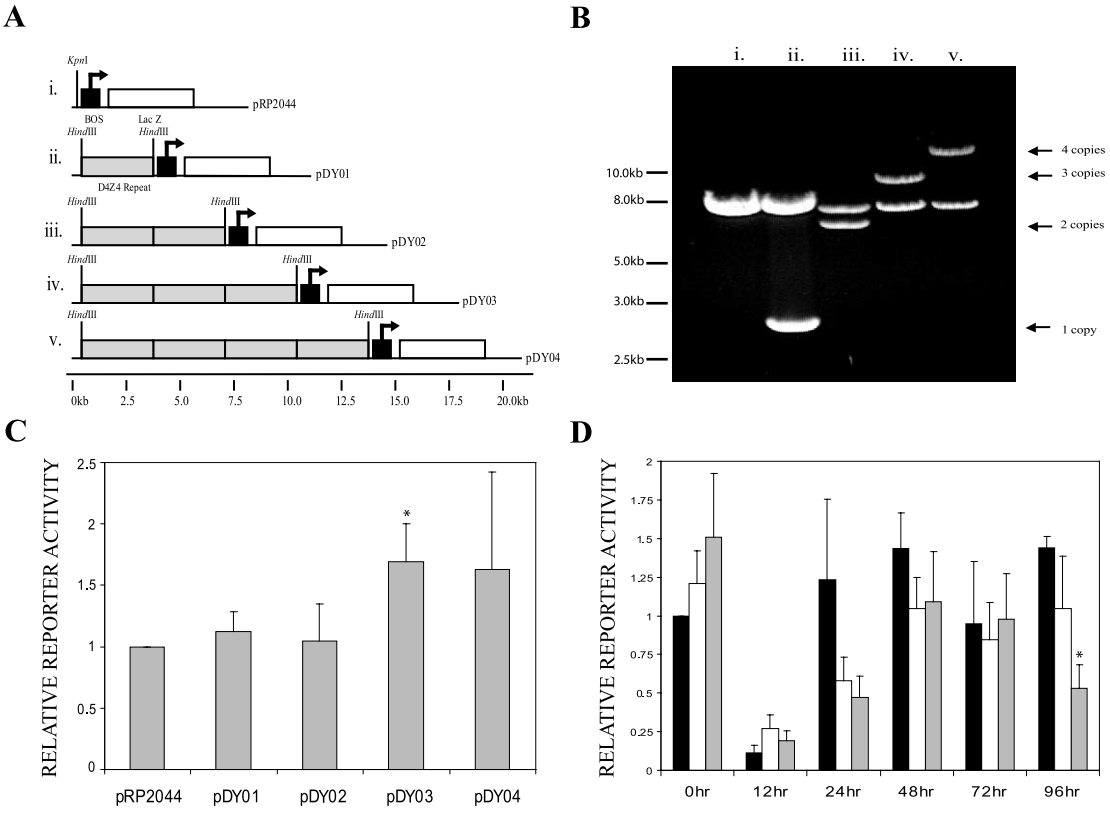

Fig. 1. D4Z4 repeats have a mild *cis*-effect on reporter gene activity. (A) Schematic diagram of pRP2044 and pDY01-pDY04 plasmids. (B) Agarose gel depicting the D4Z4 repeat array following digestion with *Hin*dIII. (C) β-Gal activity during growth normalized to pRP2044 ($n = 18$). No significant differences in reporter activity were observed for the constructs compared to the control (pRP2044) with the exception of pDY03 (*$P = 0.018$). (D) β-Gal activity plotted at 0, 12, 24, 48, 72 and 96 h after differentiation ($n = 3$). pRP2044 (black), pDY02 (white) and pDY04 (gray). After 96 h a significant decrease in β-gal activity was observed for pDY04 (*$P = 0.001$).

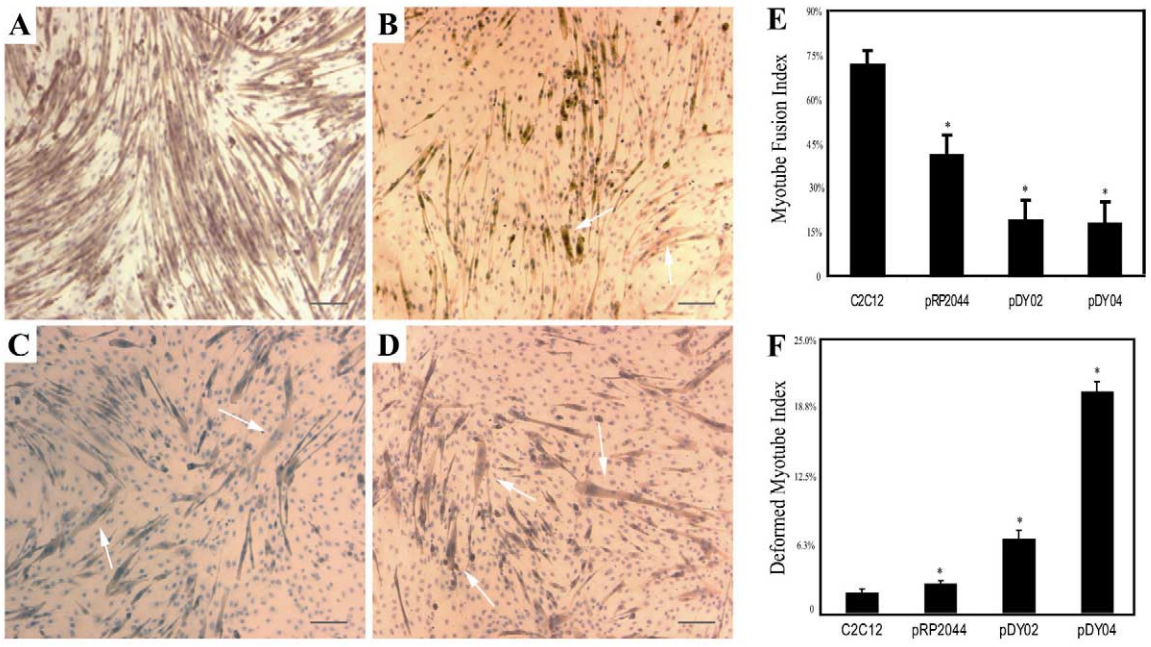

Fig. 2. Myotube fusion and morphology are effected by D4Z4 repeats. Following differentiation, cultures were stained for myosin heavy chain (MF20); C2C12 myotube cultures (A); pRP2044 (B); pDY02 (C); and pDY04 (D). Arrows denote specific myotubes with deformed morphology. Scale bars represent approximately 110 μm. (E) Plot of mean MFI ± S.E.M. for differentiated cultures ($n = 4$; 20 fields analyzed for each experiment). An asterisk denotes samples of statistical significance ($^*P < 0.00001$). (F) Plot of the mean DMI ± S.E.M. An asterisk denotes samples of statistical significance ($^*P < 0.03$).

notype is apparent [27]. In this regard, plasmids containing a greater number of repeats may be associated with enhanced reporter silencing.

Nonetheless, an abnormal morphology was apparent in the transfected cultures undergoing differentiation. Analysis of muscle differentiation by immunostaining (myosin heavy chain, MF20; Fig. 2A–D) was normal, suggesting that the defect was in myoblast fusion. To assess fusion competence we calculated the MFI, which is the ratio of nuclei present in myotubes to total nuclei in a field, where a myotube is defined as containing three or more nuclei. We observed typical MFI values of 70% for wild type C2C12 cells, but also noted that control transfections dropped the MFI to 40% (see Fig. 2E). Regardless, the D4Z4 constructs (pDY02 and pDY04) further reduced the MFI to 20% and the myotubes appeared thick and more rounded with the nuclei localized in the center, compared to the thin elongated structures of control cultures. To quantify this phenotype we developed the DMI, which is the proportion of myotubes in the culture with the abnormal phenotype. There is a low DMI in wild type C2C12 and pRP2044 transfected cells (1% and 2.6%, respectively) suggesting that non-D4Z4 plasmids have little effect in the formation of the deformed structures (Fig. 2F). However, we noted a very significant increase in the proportion of deformed myotubes upon pDY02 (6%) or pDY04 (21%) transfection suggesting that the repeats alter normal myotube fusion.

Transient transfections demonstrated that increasing D4Z4 repeat number has an escalating effect on myoblast fusion. To ensure that this was not an artifact of transfection we generated stable clones with pRP2044 and pDY04. We postulated

Table 1
Summary of transgene copy and insertion site number for stable clones

Clone	Copy number[a]	Insertions[a]	Homogeneity (%)[b]	Growth β-gal[c]	Differentiation β-gal[c]
cRP20442	3	ND	95	0.07 ± 0.008	0.02 ± 0.008
cRP20444	4	ND	100	0.08 ± 0.008	0.06 ± 0.001
cRP2044n5	2	1	100	0.77 ± 0.130	0.25 ± 0.068
cRP2044n7	3	1	95	0.36 ± 0.013	0.33 ± 0.064
cRP2044n15	ND	ND	93	0.07 ± 0.002	0.05 ± 0.014
cRP2044n24	4	1	100	0.35 ± 0.030	0.29 ± 0.031
cRP2044n31	1	1	100	0.63 ± 0.150	0.28 ± 0.012
cDY041	1	1	100	0.03 ± 0.006	0.06 ± 0.025
cDY042	3	ND	90	0.03 ± 0.001	0.05 ± 0.003
cDY04n2	12	1	95	0.16 ± 0.024	0.05 ± 0.009
cDY043	2	2	100	0.61 ± 0.190	0.07 ± 0.014
cDY044	2	2	85	0.02 ± 0.001	0.02 ± 0.006
cDY04n/3	2	1	100	0.01 ± 0.008	0.02 ± 0.007
cDY04n/23	1	1	95	0.03 ± 0.002	0.03 ± 0.004

[a]Copy and insertion numbers estimated from Southern blot analysis. Refer to Section 2 (data not shown). ND, not determined.
[b]Homogeneity reflects the percentage of cells in culture expressing β-gal.
[c]Data is represented as optical density (A_{420}) ± standard error of the mean, ($n = 8$).

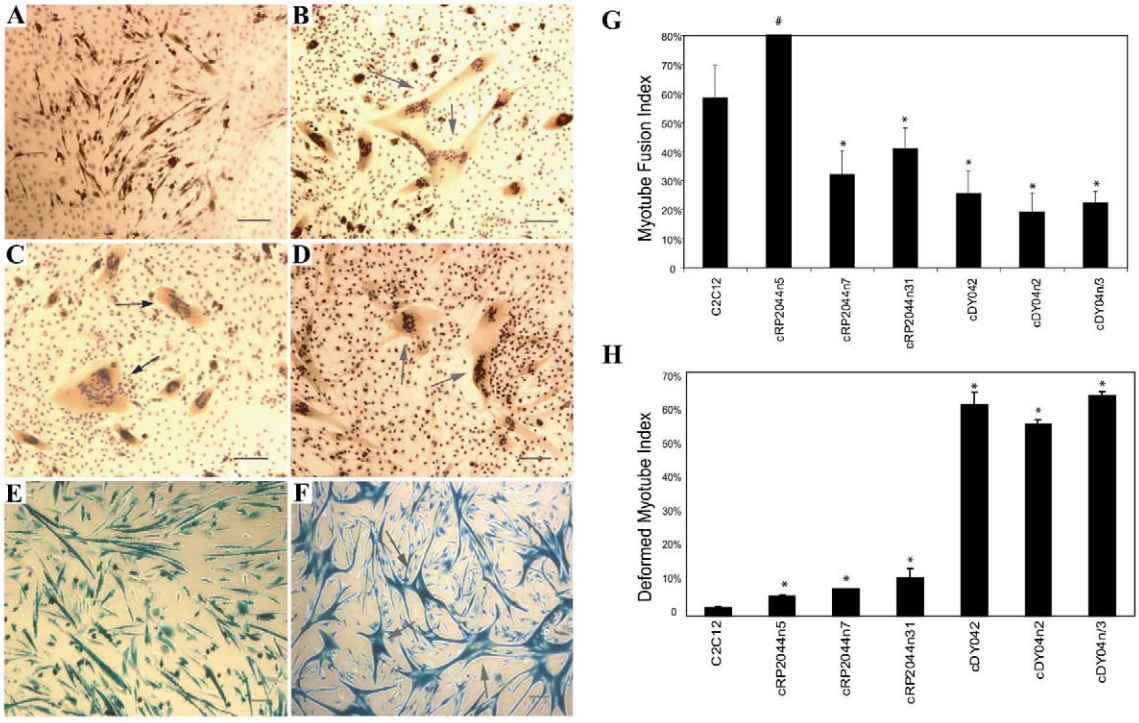

Fig. 3. Defective differentiation in stable clones is dependent on D4Z4 copy number. Differentiated cultures of stable clones cRP20444 (A), cDY042 (B), cDY043 (C), cDY04n3 (D), cRP2044n5 (E), and cDY04n2 (F) were stained with the MF20 antibody (A–D), or X-gal (E,F). The arrows denote specific myotubes with abnormal morphology. Scale bars represent approximately 93 μm. (G) Stable clones demonstrate a reduced MFI compared to wild type C2C12 cells. The mean MFI ± S.E.M. is plotted for each clone ($n = 6$; 30 fields analyzed for each experiment). Unincorporated myoblasts from clone cRP2044n5 (denoted by #) displayed an abnormally high level of apoptosis, such that MFI values were skewed. An asterisk denotes samples of statistical significance ($*P < 1 \times 10^{-8}$). (H) Plot of the mean DMI ± S.E.M. for each stable clone ($n = 6$; 30 fields analyzed per experiment). An asterisk denotes samples that were statistically significant ($*P < 0.001$).

that multiple insertions at a single integration site might enhance the repressive effect we observed in transient experiments simply by increasing repeat copy number or through repeat-induced heterochromatin formation. Since the neomycin gene can infer a negative effect on a promoter contained within the same plasmid, we generated stable clones by co-transfecting pRP2044 or pDY04 with the drug selectable plasmid pGTNeo [28]. In this way, we obtained seven clones for each construct that were characterized for *LacZ* expression (homogeneity), the D4Z4 fragment by PCR, and the number of integrations and transgene copies by Southern blot (Table 1 and data not shown). Each clone was 85–100% homogeneous for *LacZ* expressing cells and most clones contained a single insertion site. As expected, variable transgene copy number and position effects within our panel of clones contributed to provide a broad range of *LacZ* expression under growth conditions. Since the transient studies suggested that the D4Z4 repeat would repress reporter activity following differentiation, we compared β-gal activity during growth and following differentiation (Table 1). Decreased β-gal was detected for the D4Z4-clones cDY043 and cDY04n2. However, this trend was not consistent among all clones. These results demonstrate that position effects are a significant contributory factor to reporter gene activity that cannot be overcome by the presence of D4Z4 repeats.

However, we did observe a consistent defect in normal myoblast fusion among all clones that contained the D4Z4 repeats (Fig. 3B–D,F). Clones containing the D4Z4 repeat formed myotubes with a large central mass of nuclei and no structural uniformity (Fig. 3B–D, arrows). This phenotype was markedly enhanced from the morphology we observed for the transient assays. Indeed, DMI calculations showed an average level of 55% for pDY04 clones (Fig. 3H), whereas MFI values were similar to transient experiments. Moreover, cells containing D4Z4 repeats contribute to myotube formation as shown by in situ β-gal staining (Fig. 3E,F). Differentiation was normal as assessed by immunostaining (MF20) and immunoblot (MyoD, MEF2; data not shown). Pulse labeling with BrdU during differentiation demonstrated that the excess nuclei in the deformed myotubes were not caused by endoreduplication (data not shown). Thus, we conclude that, like the transient experiments the stable clones have a defect in fusion.

The consistent myoblast fusion defect observed between transient and stable assays suggests that the D4Z4 repeats might directly cause this impairment. Such a suggestion is not unprecedented. Sequence analysis of the D4Z4 repeat identified a putative gene, *DUX*4, encoding a double homeodomain protein while a subsequent study characterized a putative *DUX4* promoter that could drive reporter gene expression [8,29]. Taken together, this work suggested that FSHD patients may re-activate the *DUX*4 gene when copies are reduced below a threshold level [8]. Since a similar mechanism could explain our results, we designed primers to evaluate *DUX*4 gene expression by RT-PCR following differentiation of the stable clones (Fig. 4A–H). Expression of *LacZ* was confirmed by amplification of a 180 bp fragment in all stable clones but not in control samples (C2C12 or -RT samples).

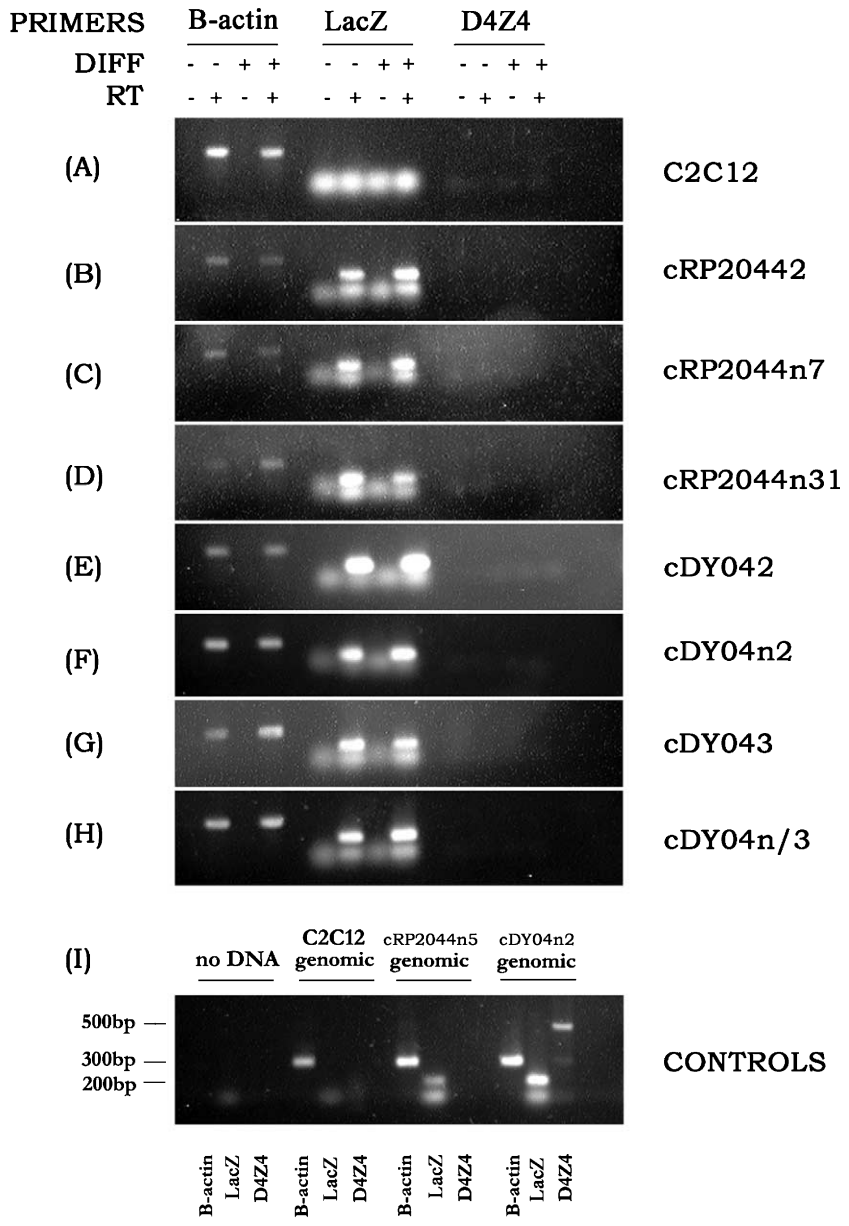

Fig. 4. Differentiation defects do not result from expression of D4Z4-specific sequences. Primers specific to amplify the D4Z4 repeat (480 bp), β-actin (300 bp), or *LacZ* (180 bp) were used for RT-PCR analysis of stable clones (panels A–H) using RNA isolated during growth (−DIFF) or following differentiation (+DIFF). -RT, samples prepared without reverse transcriptase. (I) PCR reactions using genomic DNA from C2C12, cRP2044n5, cDY04n2 or mock reactions with no DNA to test primer function.

RNA integrity was demonstrated by the amplification of β-actin (300 bp fragment) in all samples. However, we were unable to amplify the D4Z4-specific sequence (480 bp) from any clone. This was not the result of primer selection or technical difficulties as genomic DNA was amplified from the same clones (Fig. 4I). These results suggest that the effect of D4Z4 repeats on myotube differentiation is not due to expression from within the D4Z4 repeat but by some other mechanism.

The results of our study demonstrate that the D4Z4 repeats affect normal myotube formation, such that increasing the repeats resulted in a greater percentage of deformed myotubes. This is clearly a *trans*-effect as we observed minimal effects in *cis* on the BOS promoter and *LacZ* expression. Similar studies using the C2C12 culture system have been performed with the myotonic dystrophy protein kinase gene (DMPK) yet in this instance, the DM mutation had both *cis*- and *trans*-effects on myogenesis [21,30].

The *trans*-effect in our system is enhanced with increasing D4Z4 repeat number. This effect is opposite to that observed in FSHD patients where disease severity is inversely proportional to repeat number. One possible explanation for the discrepancy between our in vitro findings and those in patients is that the repeats may be squelching the DRC complex, thus allowing for inappropriate expression of genes normally silenced during muscle differentiation. The absence of D4Z4 repeats in the mouse genome [31] suggests that muscle-specific silencing of FRG1, FRG2, and ANT1 genes by the DRC complex occurs through promoter-specific elements. Thus, the presence of ectopic D4Z4 repeats in our C2C12 system

provide an alternative binding site for the DRC complex, thereby reducing the ability of this complex to silence its target genes. Indeed, such a squelching mechanism would be enhanced with increasing repeat number. To test this model, murine equivalents of the genes overexpressed in FSHD muscle, such as ANT1 and FRG1, should be examined for regulatory elements that interact with the DRC complex.

Acknowledgements: We would like to thank Drs. S. van der Maarel and R. Frants for providing the cosmid clone cY13 containing the D4Z4 repeats, R. Parks for plasmid pRP2044, R. Kothary and L. Megeney for critical comments on the manuscript, and R. Korneluk for introducing us to this fascinating disease. This work was supported by a Marjorie Bronfman Fellowship Grant from the FSH Society and grants from the MDA-USA. D.J.P. is a CIHR New Investigator.

References

[1] Lunt, P.W. and Harper, P.S. (1991) J. Med. Genet. 28, 655–664.
[2] Fisher, J. and Upadhyaya, M. (1997) Neuromuscul. Disord. 7, 55–62.
[3] Upadhyaya, M., Lunt, P.W., Sarfarazi, M., Broadhead, W., Daniels, J., Owen, M. and Harper, P.S. (1990) Lancet 336, 1320–1321.
[4] Wijmenga, C., Frants, R.R., Brouwer, O.F., Moerer, P., Weber, J.L. and Padberg, G.W. (1990) Lancet 336, 651–653.
[5] Wijmenga, C. et al. (1992) Nat. Genet. 2, 26–30.
[6] van Deutekom, J.C. et al. (1993) Hum. Mol. Genet. 2, 2037–2042.
[7] Hewitt, J.E. et al. (1994) Hum. Mol. Genet. 3, 1287–1295.
[8] Gabriels, J. et al. (1999) Gene 236, 25–32.
[9] Winokur, S.T. et al. (1994) Chromosome Res. 2, 225–234.
[10] van Geel, M., Heather, L.J., Lyle, R., Hewitt, J.E., Frants, R.R. and de Jong, P.J. (1999) Genomics 61, 55–65.
[11] van Deutekom, J.C. et al. (1996) Hum. Mol. Genet. 5, 581–590.
[12] Wijmenga, C. et al. (1993) Hum. Genet. 92, 198–203.
[13] Xia, H., Winokur, S.T., Kuo, W.L., Altherr, M.R. and Bredt, D.S. (1997) J. Cell. Biol. 139, 507–515.
[14] Haraguchi, Y. et al. (1993) Genomics 16, 479–485.
[15] Bouju, S. et al. (1999) Neuromuscul. Disord. 9, 3–10.
[16] Gabellini, D., Green, M.R. and Tupler, R. (2002) Cell 110, 339–348.
[17] Wright, T.J., Wijmenga, C., Clark, L.N., Frants, R.R., Williamson, R. and Hewitt, J.E. (1993) Hum. Mol. Genet. 2, 1673–1678.
[18] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Approach, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[19] Kolodziejczyk, S.M. et al. (2001) Curr. Biol. 11, 1278–1282.
[20] Guy, L.G., Kothary, R. and Wall, L. (1997) Nucleic Acids Res. 25, 4400–4407.
[21] Amack, J.D., Paguio, A.P. and Mahadevan, M.S. (1999) Hum. Mol. Genet. 8, 1975–1984.
[22] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1995) Short Protocols in Molecular Biology, 3rd edn., John Wiley and Sons, New York.
[23] Lazzaro, M.A. and Picketts, D.J. (2001) J. Neurochem. 77, 1145–1156.
[24] Langen, R.C., Schols, A.M., Kelders, M.C., Wouters, E.F. and Janssen-Heininger, Y.M. (2001) FASEB J. 15, 1169–1180.
[25] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[26] Gorza, L. and Vitadello, M. (2000) FASEB J. 14, 461–475.
[27] Lunt, P.W., Jardine, P.E., Koch, M.C., Maynard, J., Osborn, M., Williams, M., Harper, P.S. and Upadhyaya, M. (1995) Hum. Mol. Genet. 4, 951–958.
[28] Mei, Q., Kothary, R. and Wall, L. (2000) Exp. Cell. Res. 260, 304–312.
[29] Ding, H., Beckers, M.C., Plaisance, S., Marynen, P., Collen, D. and Belayew, A. (1998) Hum. Mol. Genet. 7, 1681–1694.
[30] Amack, J.D. and Mahadevan, M.S. (2001) Hum. Mol. Genet. 10, 1879–1887.
[31] Grewal, P.K., van Geel, M., Frants, R.R., de Jong, P. and Hewitt, J.E. (1999) Gene 227, 79–88.